"STUDIES IN MULLER'S PHENOMENON WITH SPECIAL REFERENCE TO THE USE OF A MILK-AGAR SUBSTRATE."

T H E S I S

submitted by

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P R E F A C E.

The investigations recorded in this thesis, "Studies on Muller's Phenomenon with Special Reference to the use of a Milk-Agar Substrate" represents part of the work carried out by the author on coagulase-positive staphylococci in the Department of Bacteriology, Medical School, Newcastle upon Tyne during the last five years. For convenience this thesis has been presented in two volumes, Volume I containing Text, Figures and References and Volume II containing Experiments, Tables and Media, as Appendices.

Volume I is divided into eight parts. Part I gives a description of a number of exploratory experiments which resulted in the author undertaking these "Studies on Muller's Phenomenon". Part II is devoted to a historical review of the investigations carried out on Muller's phenomenon by a number of workers from 1927 to 1953. The ability of strains of coagulase-positive and coagulase-negative staphylococci and other organisms to produce Muller's phenomenon was examined and the results of this examination are given in Part III. The conditions which influence the production of Muller's phenomenon by staphylococci on different test proteins were examined and are described in Part IV. It was discovered in the course of these investigations that a staphylococcal factor free from

living staphylococcal cells could be prepared which, when tested under the appropriate conditions would produce Muller's phenomenon. In Part V, the results are given of the investigations carried out to ascertain the conditions in which the maximum yield of this staphylococcal factor could be obtained. The section Part VB contains work carried out in co-operation with the author's colleague, Miss B. A. Kirkhouse, B.Sc. In Part VI is presented a study of the conditions which influence the production of Muller's phenomenon by cell-free staphylococcal factor. The essential role of serum in the production of Muller's phenomenon was investigated and the results of this limited study are given in Part VII. A further investigation of a few of the conditions which affect Muller's phenomenon is presented in Part VIII. A general discussion of many of the points which have been investigated in the studies presented here, and their relationship to the work carried out previously by other investigators in this field is presented in Part IX. An indication of the direction which future work on this subject might take is also discussed.

ACKNOWLEDGMENTS.

I am grateful to Professor C. A. Green for providing me with strains of staphylococci isolated in the Department of Bacteriology, Royal Victoria Infirmary, Newcastle upon Tyne; to Dr. J. G. P. Hutchison for strains of staphylococci isolated during the survey conducted at the Princess Mary Maternity Hospital; and to the Department of Bacteriology, General Hospital for strains of staphylococci.

The concentration of the staphylococcal factor and its freeze-drying was carried out in the Department of Chemistry, King's College, Newcastle upon Tyne. I am indebted to Dr. J. McQuillan for his advice and assistance in this matter and to Miss Robinson for undertaking the concentration of the batches of staphylococcal factor.

PART I.

AN ACCOUNT OF EXPLORATORY EXPERIMENTS WHICH LED TO THIS INVESTIGATION OF MULLER'S PHENOMENON.

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III. SUMMARY.

PART I.

AN ACCOUNT OF EXPLORATORY EXPERIMENTS WHICH LED TO THIS INVESTIGATION OF MULLER'S PHENOMENON.

It would be desirable, I believe, if in this account of the exploratory experiments which led to my undertaking this investigation of Muller's phenomenon some indication were given of the circumstances which induced me to carry out these experiments. It happened that within the space of a few days I chanced to read two bacteriological papers which dealt with the proteolytic activities of two micro-organisms on different substrates. The activity of one organism was enhanced by the presence of human serum, while that of the other organism was inhibited by human serum.

The first of these papers was entitled "Digestion of Casein by Staphylococci on Milk Agar containing Serum" by Fisk and Mordvin (1943). The other paper was by Evans and Wardlaw (1952) "In vitro and in vivo properties of culture filtrates of Bacillus subtilis with high gelatinase activity". Fisk and Mordvin (1943) reported that on a milk-agar medium, which they employed for the determination of chromongenesis of staphylococci,

narrow indistinct zones of proteolysis were occasionally seen surrounding colonies of staphylococci. They observed a marked increase in the zone of digestion of casein if these milk-agar plates were enriched by the addition of human serum. This effect they investigated in 175 strains of staphylococci which they had isolated from human pathological material and from healthy individuals. They compared the ability of these 175 strains to produce caseinolysis on serum-milk-agar plates with their ability to produce coagulase and fibrinolysin. 102 of the strains were coagulase-positive; of these 91 were fibrinolytic and of these coagulase-positive fibrinolytic strains, 82 showed caseinolysis in serum-milk-agar plates. They established, therefore, that the majority of coagulase-positive staphylococci were able to produce caseinolysis in serum-milk-agar plates and they showed, in addition, that only 2 of the 73 strains of coagulase-negative staphylococci tested, produced this caseinolysis. The mechanism of casein digestion which occurs in such a medium, rich in serum was not determined.

The paper of Evans and Wardlaw (1952) reported that they had been able to prepare concentrated culture filtrates from a strain of Bacillus subtilis which had high gelatinase activity and

that they had been able to confirm the inhibitory action of normal serum, human as well as that of other species, (a finding originally noted by Bertiau in 1914), on the gelatinase activity of these filtrates. The nature of this inhibitory action of normal serum was not elucidated although it appeared that it was not due to naturally occurring antibody or to trypsin inhibitor in the serum.

The immediate result of having read these two papers and having accepted the facts presented in them, was that a number of questions were asked. If human serum enhances the caseinolytic activity of strains of coagulase-positive Staphylococcus aureus will it increase or decrease the caseinolytic activity of strains of Bacillus subtilis and other organisms which attack milk? If normal human serum inhibits the action of the gelatinase of strains of Bacillus subtilis will it have the same inhibitory action on the activity of the gelatinase of strains of Staphylococcus aureus and other organisms known to produce gelatinase?

It was in an attempt to answer these questions that the experiments described in this part were undertaken.

I. REPETITION OF THE EXPERIMENT OF FISK AND MORDVIN (1943) WHICH DEMONSTRATED INCREASED CASEINOLYSIS BY STRAINS OF STAPHYLOCOCCI IN MILK-AGAR ENRICHED WITH SERUM.

(a) Using the method given by Fisk and Mordvin (1943).

The serum-milk-agar medium of Fisk and Mordvin (1943) was prepared by the method recommended by these workers (App. 473). A milk-agar medium differing from this serum-milk-agar medium only in the fact that the human serum had been replaced by an equal volume of 1% "Lab-Lemco" broth, was also prepared. These media were dispensed in 15 ml. amounts in Petri dishes 9 cm. diameter.

A serum-milk-agar and milk-agar plate were each inoculated with a loopful of an 18 hour old culture in 1% "Lab-Lemco" broth of each organism to be tested for caseinolytic activity. In this experiment the organisms used were five strains of coagulase-positive Staphylococcus aureus and one strain of Bacillus subtilis, all laboratory strains. The inoculated plates were incubated at 37°C. for a period of 48 hours. Readings were taken at 24 hours and 48 hours. The results obtained are given in App. 1-2.

An area of lysis was observed on the serum-milk-agar plates around the growth of the staphylococci tested. With three of the strains the lysis was slight at 24 hours but it increased

during the following 24 hours and was then as great as that obtained at 48 hours with the other two strains of staphylococci. On the milk-agar plates there was no lysis around the growth of the staphylococci, although with three of the strains there was some precipitation of the medium near the growth. It appeared, therefore, from this experiment, that human serum added to a milk-agar plate resulted in a marked zone of caseinolysis around the strains of Staphylococcus aureus used, a finding which agreed with the report of Fisk and Mordvin. None of the strains produced in the milk-agar plates the narrow zone of indistinct lysis reported by Fisk and Mordvin with some of their strains. This narrow zone of indistinct lysis was found, however, in later experiments with other strains. On the other hand with Bacillus subtilis growing on the milk-agar plate there was a zone of lysis around the growth of the organism; on the serum-milk-agar plate there was no such zone. The fact that caseinolysis was absent around the growth of Bacillus subtilis in the serum-milk-agar plate indicated that the normal human serum inhibited the caseinolysis by this organism, a finding which suggested that the mechanism of caseinolysis by Bacillus subtilis was different from that by Staphylococcus aureus.

The serum-milk-agar plates of Fisk and Mordvin had

a large percentage of human serum incorporated in them - 50% human serum - a volume of 7.5 ml. in a serum-milk-agar plate of total volume 15 ml. If a large number of experiments were to be carried out using these plates containing 50% human serum it would involve procuring large volumes of serum. The next experiment was undertaken in an endeavour to answer the following questions. Is such a high percentage of serum necessary in these serum-milk-agar plates to demonstrate caseinolysis by staphylococci? Is 50% the optimal amount of serum required to produce the proteolysis of milk? Does caseinolysis occur when 10% or 30% of human serum is incorporated in a milk-agar plate?

(b) Modifications of the serum-milk-agar medium used by Fisk and Mordvin (1943).

(i) Reduction in the amount of human serum in the medium.

Serum-milk-agar plates were made according to the method of Fisk and Mordvin containing 50% human serum (App. 473). Other batches of medium were prepared in the same way except that to one batch 10 ml. and to another 30 ml. of pooled human serum was added to the milk-agar mixture in place of the usual 50 ml.

pooled human serum. The volume of the serum-milk-agar mixture was made up to the usual 100 ml. by the addition of 1% "Lab-Lemco" broth. Control plates in the form of milk-agar plates without serum were also prepared, 50 ml. of 1% "Lab-Lemco" broth replacing the serum omitted. The test organisms used included four strains of Staphylococcus aureus and strains of three other genera. After inoculation with a loopful of an 18 hour old 1% "Lab-Lemco" broth culture of each of the organisms used in this experiment the plates were incubated at 37°C. for 72 hours (App. 3-4).

The results obtained from this experiment were most interesting. On the milk-agar plate without serum, two of the strains of Staphylococcus aureus were without effect on the medium at 24 hours' but after 72 hours' incubation one had brought about some precipitation of the medium around its growth while the other had caused some indefinite lysis of the milk around it. The other two strains of Staphylococcus aureus showed slight indefinite lysis and precipitation of the medium around their growth on examination after 24 hours' and 72 hours' incubation at 37°C. The strain of Proteus vulgaris used, lysed completely the milk-agar plate and the strains of Pseudomonas pyocyanea, Serratia marcescens, Bacillus subtilis and Bacillus megatherium each produced a zone of lysis around its growth. There was no lysis around the growths of

Bacillus anthracoides or Bacillus mycoides after 24 hours' incubation, but a zone of lysis had developed around each after 72 hours' incubation.

On the serum-milk-agar plates (containing 10%, 30% and 50% human serum) the four strains of Staphylococcus aureus produced lysis and it was apparent in each case that the amount of lysis was associated with the amount of serum in the plates. Thus the zone of lysis was greater in the plate containing 50% serum than it was in the plate containing 30% serum, which was in turn greater than that in the plate containing 10% serum. The lysis in the milk-agar plates differed in appearance from that in these serum-milk-agar plates. In the milk-agar plates it was of an indefinite type, had no definite border, and faded imperceptibly into the unaltered medium. On the other hand the lysis around these same strains of staphylococci on the serum-milk-agar plates consisted of a zone of complete lysis which had a clear cut and definite border at its junction with the unaltered medium. The edge of the zone of lysis in the 50% serum-milk-agar plate was regular, so much so around isolated colonies as to suggest having been drawn by the aid of a pair of compasses.

This regular edge to the zones of lysis was, however, lacking in the plates containing 30% serum. The edge

of the lytic zone although clear cut was scalloped. It looked as if its geometric regularity had been destroyed by little circular areas of lysis developing outside the main zone of lysis and impinging on the edge of the latter. The clear cut edge of the lysis in these plates with 30% serum had thus the appearance of being made up of arcs of circles of different diameters.

In the serum-milk-agar plates containing 10% serum on which the four strains of Staphylococcus aureus were growing a narrow area of lysis was present around the colonies of the organisms - clear cut definite lysis similar to that described in the plates containing 50% serum and quite different from the indistinct indefinite lysis found around colonies of the same organisms in milk-agar plates without serum. The edge of this narrow zone of lysis was clear cut but its regularity broken by scalloping as in the plates containing 30% serum. In addition, however, in these 10% serum-milk-agar plates there were small circular areas of clearing 1.2 mm. to 1.5 mm. in diameter, scattered irregularly in a band about 5-8 mm. in depth measured outward into the normal serum-milk-agar medium from the scalloped edge. These circular areas showed as complete clearing of the medium as in the zones of lysis around colonies of staphylococci but were not directly associated with a colony. These small

circular areas of clearing were often present in close proximity to the lytic zone around a colony and were evidently responsible for the scalloping of the edge already referred to. With one of the strains of the staphylococci there was no lysis around the line of growth but beneath the growth small circular areas of lysis could be clearly seen.

The appearance of these small areas or "plaques" of clearing on the serum-milk-agar plates, well beyond the growth of the organism, and seemingly sterile was something which the writer had not before observed. It is unlikely that a possible explanation of this appearance would have been forthcoming very readily had it not been that a few weeks previously the paper by Elek (1953) "Studies on the Proteoclast ('Muller's Phenomenon')" had been read. Elek described appearances which he had observed on growing strains of staphylococci on a serum-haemoglobin-agar medium and which obviously corresponded with those of the plaques obtained by the writer with staphylococci growing on serum-milk-agar plates containing 10% and 30% human serum. The appearances described by Elek which had originally been reported by Leon Muller (1927) who, however, used a blood-agar substrate, have come to be known as 'Muller's Phenomenon'. Thus it appeared that Muller's phenomenon or something indistinguishable from it had been produced

by staphylococci growing on a substrate containing not blood but milk.

The results obtained with other organisms growing on serum-milk-agar plates containing 10%, 30% and 50% human serum are also of interest. The presence of human serum retarded caseinolysis by the strains of Proteus vulgaris, Bacillus subtilis and Bacillus megatherium used during the first 24 hours of their growth. This inhibition, however, was overcome by the organisms after 72 hours' incubation.

Such inhibition of caseinolysis by the other organisms used in this experiment could not be shown to occur in the presence of human serum.

(ii) Modification of the method of preparing the medium to permit easier preparation and alteration of constituents.

The method of preparation of serum-milk-agar plates as used by Fisk and Mordvin is satisfactory only if one wishes to prepare 50% serum-milk-agar plates. It is not, however, the easiest method which could have been devised for making these plates, involving as it does at one stage the mixing of 7.5% agar,

milk and serum. Moreover, this method of preparation leaves little scope for varying the constituents of the medium.

It was desired to test out two concentrations of milk with different concentrations of human serum, compensating for the loss of nutriment in those plates with less serum by the addition of 1% "Lab-Lemco" broth. A technique was devised using a 4% agar base. The method of making the plates and the results obtained in this experiment, in which three organisms were used, Staphylococcus aureus, strain 4, Bacillus subtilis and Bacillus anthracoides are given in App. 5-6. After incubation of the plates at 37°C. for 24 hours there was a broad zone of lysis around the growth of the Staphylococcus aureus, in the plates containing 20%, 30% and 40% serum while in the plates containing 10% serum there was a much narrower zone of lysis with a clear cut but irregular edge - a "rough edge" - and beyond this, small circular areas of clearing - plaques - in the otherwise unaltered medium. The milk-agar plates without serum showed only slight indefinite lysis around the growth of the Staphylococcus aureus and in addition there was some slight precipitation around the growth in the plate with the greater concentration of milk.

After 24 hours at 37°C. there was a broad band of

lysis around the growths of Bacillus subtilis and Bacillus anthracoides on the milk-agar plates but in the serum-milk-agar plates there was no caseinolysis. This inhibition of caseinolysis by serum was partially overcome in time, in the case of Bacillus subtilis for, after 48 hours' at 37°C., caseinolysis had developed around the growth of this organism in serum-milk-agar plates containing 10%, 20% and 30% serum but not in the plate containing 40% serum. In the case of Bacillus anthracoides there was no caseinolysis on any of the serum-milk-agar plates even after 48 hours at 37°C.

It was found that the plates with the greater concentration of milk were easier to read than those with the lesser concentration although there was no real difficulty in reading the results in the latter. The growths of the various organisms on the different media were comparable and the effects produced by them did not seem to be associated with any difference in the nutritive value of these media.

(iii) Alteration of milk and agar concentration.

In this next experiment a slight reduction in the concentration of milk in the plates to 25% and an increase in the concentration of the agar base to 4.5% was effected (App. 7-9).

It was found that the final concentration of agar in such plates, 1.35% gave a satisfactory gel. The concentrations of serum used were 10%, 20% and 40% pooled human serum. "Lab-Lemco" broth was included in the milk-agar plates without serum in a final concentration of 0.45% to ensure that there was sufficient nutrient to permit the organisms inoculated on them to grow well. "Lab-Lemco" broth, however, was omitted from all the serum-milk-agar plates.

Twelve strains of coagulase-positive staphylococci and one strain each of Bacillus mycoides, Serratia marcescens, Bacillus anthracoides, Bacillus megatherium, Bacillus subtilis, Proteus vulgaris and Pseudomonas pyocyanea were used.

The results obtained with eleven of the twelve staphylococcal strains were in keeping with observations previously made with staphylococci, viz. on milk-agar plates a slight indefinite lysis around the colonies or the medium entirely unaltered, while in serum-milk-agar plates a zone of definite clear-cut caseinolysis whose size depended on the concentration of serum in the medium. In addition, in the serum-milk-agar plates containing 10% or 20% serum, plaques of lysis as previously reported were also seen. No plaques were seen in the serum-milk-agar plate with 40% serum. The remaining staphylococcal strain, Staphylococcus aureus, Strain J appeared to have no caseinolytic activity although it grew as well

as the other strains.

Of the other organisms used the only one which gave a result which was of interest was Bacillus subtilis. This organism, although it produced caseinolysis on milk-agar plates, was unable to do so in the serum-milk-agar plates with the different concentrations of human serum in the period of 48 hours over which it was observed. Such inhibition of caseinolysis by Bacillus subtilis had previously been abolished in media containing the smaller concentrations of serum and added nutrient in the form of "Lab-Lemco" broth. In the present experiment "Lab-Lemco" broth was omitted.

(iv) The effect of substituting sheep serum for human serum in the medium.

So far the experiments carried out with a view to investigating the effect of the presence of serum on the proteolytic activities of various organisms, had involved the use of human serum. It was thought worthwhile to ascertain if sheep serum had an effect similar to that of human serum when added to milk-agar plates, particularly with regard to the production of caseinolysis by strains of staphylococci. Accordingly, the previous experiment

was repeated using the same strains of organisms, but differing only in the respect of the serum used, fresh sheep serum being substituted for the pooled human serum (App. 10-12).

All twelve strains of staphylococci showed a zone of lysis around their growths on sheep serum-milk-agar plates with any of the concentrations of serum used. Plaques were also observed in the plates with 10% and 20% sheep serum, occurring with all the strains. Plaques were also seen in the plates containing 40% sheep serum in the case of nine of the strains. This last finding had not been noted in the earlier experiment with 40% human serum in the plates.

In the case of the organisms other than staphylococci the findings were the same whether sheep serum or human serum was incorporated in the medium.

(v) <u>An increase in the total volume of medium used for each plate.</u>

The total volume of the serum-milk-agar media for the last three experiments had been limited to 10 ml. for each plate. It was realised that if the total volume were to be increased to 15 ml. much greater flexibility would be obtained and many more

Variations in the amounts of serum, milk and nutrients could be tried out. It was found by experiment that a plate of medium satisfactory in the consistency of the gel and varied in its content of milk, serum and nutrient could be prepared by using 4.5 ml. of melted 4.5% Oxoid agar in distilled water (pH 7.4) and making up to 15 ml. with whatever constituents had to be incorporated. The final concentration of agar in the gel was 1.3%. 1 ml. of 15% "Lab-Lemco" in distilled water was incorporated in each plate as it was found that this was sufficient to ensure good growth of the test organisms.

An experiment using this technique for preparing serum-milk-agar plates of varied composition in their contents of constituents is given in detail in App. 13-14. A set of three plates was prepared with 40% serum, 10% serum and without serum. A second set of plates corresponding in their serum contents to the former but differing only in that "Lab-Lemco" was omitted was also prepared.

Four strains of organisms were inoculated on to each plate, viz. one strain of each of the following:- Pseudomonas pyocyanea, Bacillus anthracoides, Staphylococcus aureus, Strain A, and Serratia marcescens. These plates were incubated at 37°C. for 24 hours.

FIG. I - Milk-agar plate containing 1% "Lab-Lemco" with single strokes of Pseudomonas pyocyanea, Staphylococcus aureus, Serratia marcescens and Bacillus anthracoides. (Incubated at 37°C. for 24 hours.)

FIG. II - Milk-agar plate without "Lab-Lemco" with single strokes of the same organisms as in Fig. I. (Incubated at 37°C. for 24 hours.)

Fig. I

Fig. II

FIG. III - Serum-milk-agar plate containing 40% serum and 1% "Lab-Lemco" with single strokes of Pseudomonas pyocyanea, Staphylococcus aureus, Serratia marcescens and Bacillus anthracoides. (Incubated at 37°C. for 24 hours.)

FIG. IV - Serum-milk-agar plate containing 40% serum but without "Lab-Lemco" with single strokes of the same organisms as in Fig. III. (Incubated at 37°C. for 24 hours.)

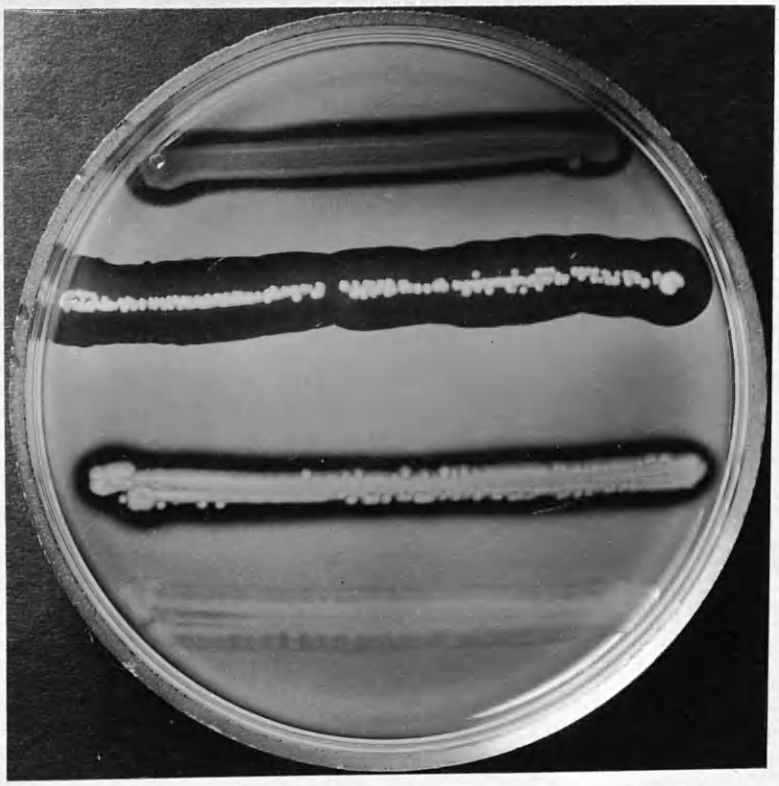

Fig. III

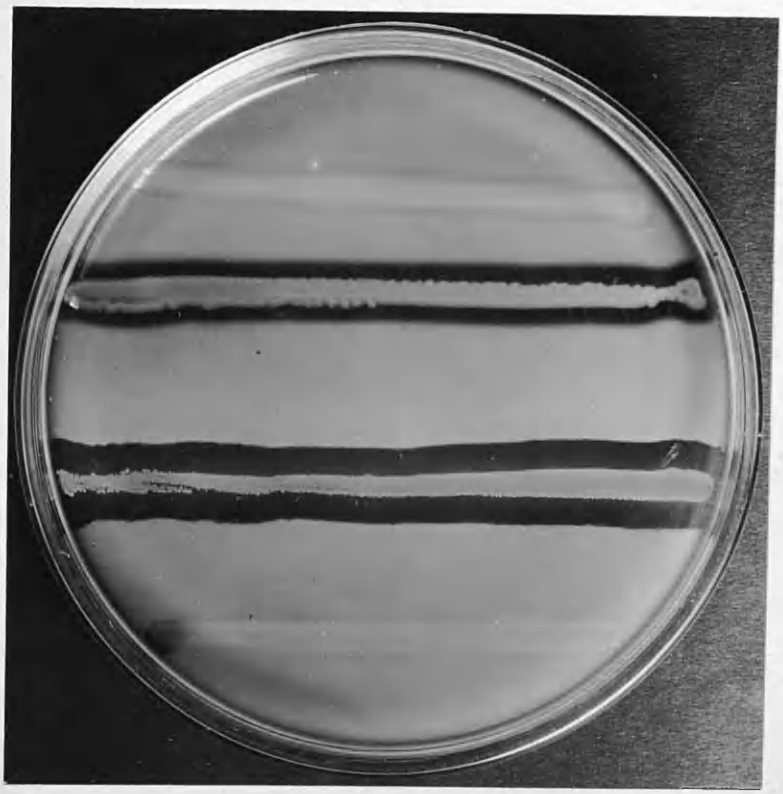

Fig. IV

FIG. V - Serum-milk-agar plate containing 10% serum and 1% "Lab-Lemco" with single strokes of Pseudomonas pyocyanea, Staphylococcus aureus, Serratia marcescens and Bacillus anthracoides. (Incubated at 37°C. for 24 hours.)

FIG. VI - Serum-milk-agar plate containing 10% serum but without "Lab-Lemco" with single strokes of the same organisms as in Fig. V. (Incubated at 37°C. for 24 hours.)

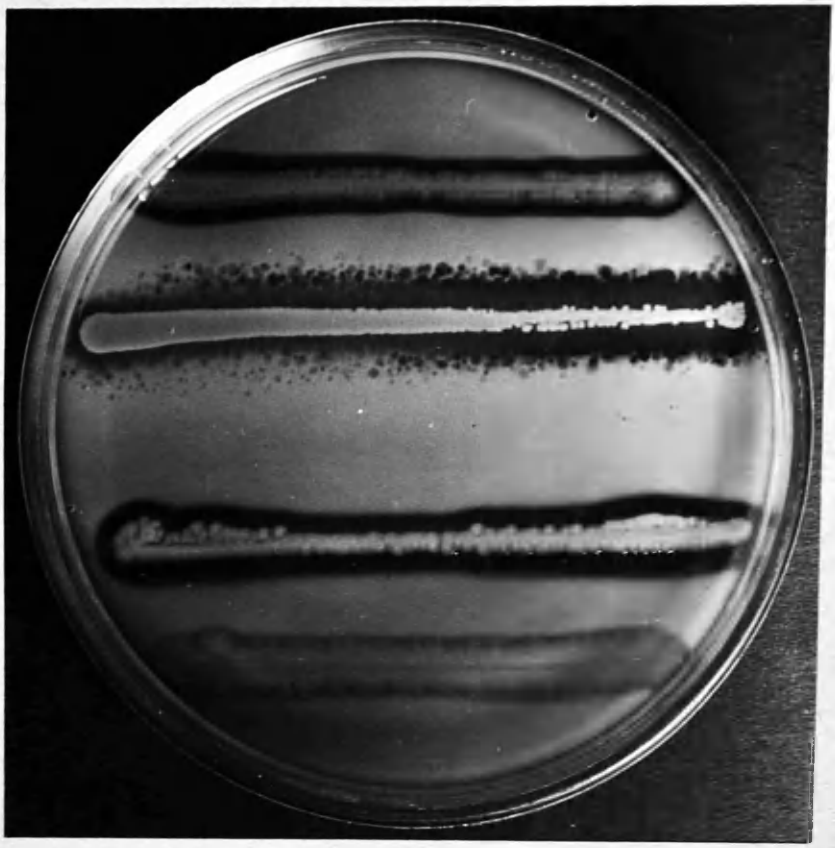

Fig. V

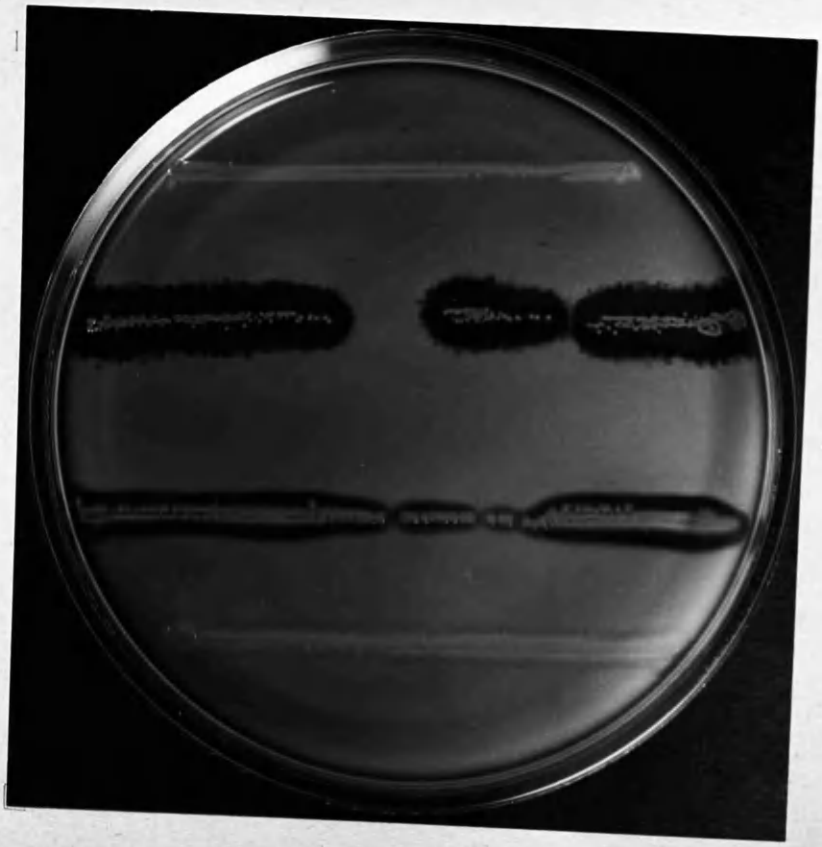

Fig. VI

The results obtained were in keeping with previous findings. The milk-agar plate without serum, but with "Lab-Lemco", gave much better growths of the organisms than the corresponding plate without "Lab-Lemco". On the latter, Staphylococcus aureus, Strain A, in particular did not grow well and did not produce the indefinite lysis which was present in the corresponding plate with "Lab-Lemco". In the serum-milk-agar plates both with and without "Lab-Lemco", the staphylococcus produced definite clear-cut lysis but plaques were present only in the plates with 10% serum.

As previously, Bacillus anthracoides had produced lysis only on the plates without serum and irrespective of whether "Lab-Lemco" was or was not present. Pseudomonas pyocyanea on the other hand produced lysis only in the plates containing "Lab-Lemco" and irrespective of whether serum was or was not present but the zone of lysis was narrower in the plates containing serum.

The six plates used in this experiment were photographed after incubation at 37°C. for 24 hours (Figs. I - VI).

II. THE EFFECT OF HUMAN SERUM ON THE GELATINASE ACTIVITY OF STAPHYLOCOCCUS AUREUS AND OTHER ORGANISMS.

While the experiments to provide information on the effect of human serum on caseinolysis were being carried out, other experiments were being undertaken to ascertain if the gelatinase activity of certain other organisms was affected in any way by the presence of human serum.

(a) An Experiment with living micro-organisms.

In the first experiment Staphylococcus aureus, Strain A and Bacillus anthracoides (a stock strain) were grown in tubes of 10% nutrient gelatin, with and without the addition of serum. The serum-gelatin tubes contained concentrations of serum of 10%, 20% and 40%. Inoculation of the gelatin and serum-gelatin tubes with the test organisms was made with a straight platinum wire and they were incubated at 20°C. Examination for the presence of liquefaction of gelatin was made after 24, 36, 48 and 72 hours' incubation (App. 15-16).

With the staphylococcus and Bacillus anthracoides,

it was found that liquefaction had occurred in all the gelatin tubes after 48 hours' incubation at 20°C.; but only in the 10% serum-gelatin tube inoculated with Bacillus anthracoides. All other serum-gelatin tubes were free from liquefaction at this stage but all showed liquefaction after 72 hours' incubation. It appeared therefore that human serum did delay the liquefaction of gelatin by these organisms for a short time.

There were obvious objections to the method employed in this experiment. The addition of varied amounts of human serum to the nutrient gelatin resulted in different quantities of nutrient being available for the organisms inoculated. Such differences in the amount of available nutrient might well be reflected in differences in the amounts of the resulting growths and consequently in the amounts of gelatinase produced. Moreover, the presence of human serum might influence the production of gelatinase by the bacterial cells. Thus, although it might appear, at first, that the slight delay in the liquefaction of the gelatin in the serum-gelatin tubes was due to the direct inhibition of the action of the enzyme, this was by no means proved by the experiment performed.

It was clear that this experiment involving as it did living and multiplying organisms could not provide an answer

to the question, "Does human serum have any effect on the action of the gelatinase of these organisms?" It did seem likely, however, that the answer could be obtained if cell-free gelatinase were available. With such a gelatinase preparation of known activity it would be possible to find out if human serum did, in fact, interfere with its action in liquefying gelatin.

It was decided, therefore, at this stage to examine the possibility of producing such a cell-free gelatinase.

(b) The production of cell-free gelatinase.

The production of cell-free gelatinase did not prove such a difficult task as might have been supposed. It was found possible to provide suitable conditions for gelatinase production by all three organisms chosen for this investigation, viz. Staphylococcus aureus, Strain A, Bacillus anthracoides and Bacillus subtilis and to prepare their gelatinases in a cell-free state.

A number of experiments were carried out with these organisms to find the optimum conditions for producing this enzyme. The type of medium, the nature of the inoculum and the period of incubation at 37°C. were some of the points to which

particular attention was paid. Attention was also given to the best method of rendering the culture containing the gelatinase free from living cells.

It is not proposed to give a detailed account of all the experiments but the procedure which was finally adopted and which had proved satisfactory on a number of occasions is given in detail in App. 17-18. This also records the results obtained with one of several batches of gelatinases prepared from the test organisms, when tested on 5% gelatin at 20°C.

It was found on the several occasions on which this method was employed that liquefaction of the 5% gelatin occurred in 24 hours with 1 ml. of the gelatinase produced by Bacillus subtilis or Bacillus anthracoides and within 48 hours with 1 ml. of gelatinase from Staphylococcus aureus, Strain A. Such preparations of gelatinase were deemed sufficiently active to permit investigation of the effects of human serum on its activity.

(c) The Effect of Fresh and Heated Human Serum on cell-free gelatinase produced by Staphylococcus aureus, Strain A, Bacillus anthracoides and Bacillus subtilis.

The effect of fresh human serum on the activity of

the cell-free gelatinases, prepared as already described from the three organisms, Staphylococcus aureus, Strain A, Bacillus anthracoides and Bacillus subtilis were investigated in a number of experiments. All three gelatinases were active in 1 ml. amounts, liquefying 5% gelatin in 24-48 hours. The serum employed was a batch of pooled human serum obtained by mixing six individual samples of serum.

The results obtained from these experiments (App. 19-20) showed that the activity of the gelatinase preparations from cultures of Staphylococcus aureus, Strain A, and Bacillus subtilis was inhibited by as small a quantity of serum as 0.25 ml. while that of the gelatinase prepared from Bacillus anthracoides was not inhibited by the serum in amounts of up to 2.0 ml.

This property of the serum in inhibiting the gelatinases of Staphylococcus aureus, Strain A and Bacillus subtilis and of not inhibiting the gelatinase of Bacillus anthracoides was not interfered with by preliminary heating of the serum at 65°C. for 30 minutes. (App. 19-20).

III. SUMMARY.

These preliminary experiments confirmed the finding of Fisk and Mordvin (1943) that a broader zone of lysis developed around colonies of pathogenic staphylococci growing on milk-agar enriched with human serum than on milk-agar without added serum. This enhancement by serum of the zone of staphylococcal caseinolysis was in marked contrast with the author's observation that serum had a more or less inhibitory action on the caseinolysis produced on the same two media by Bacillus subtilis. Similar inhibitory action by serum was demonstrated in the case of other aerobic spore-bearing bacilli, and in the cases of Proteus vulgaris and Pseudomonas pyocyanea. Only with staphylococci was increased caseinolysis determined by the presence of serum in the substrate. It thus appeared that normal human serum had two entirely different actions on the caseinolytic activity of different organisms, increasing that of staphylococci and diminishing that of others.

These preliminary experiments confirmed, too, the finding of Evans and Wardlaw (1952) that normal human serum inhibited the activity of the gelatinase of Bacillus subtilis, and

established that it also inhibited the activity of staphylococcal gelatinase. It had been intended to pursue the study of this gelatinase-inactivating property of serum, but an alteration in plan was occasioned by an arresting discovery in certain serum-milk-agar plates on which coagulase-positive staphylococci were growing. This was the appearance of numerous small circular areas of clearing of the medium, beyond and breaking into the normal zones of caseinolysis around the staphylococcal colonies, which developed in plates whose serum content had been reduced below the usual 50%. These small circular areas of clearing - "plaques" - were reminiscent of the appearance first recorded by Muller (1927), and observed by him on human blood-agar plates on which a strain of staphylococcus was growing. He described it as "L'hémophagie staphylococcique", but later workers have referred to it as "Muller's phenomenon".

Elek (1953) suggested the name "proteoclast" for Muller's phenomenon instead of the term "hémophagie" given to it by Muller; and did so because comparable appearances can be produced by staphylococci in the presence of proteins other than haemoglobin, and because he believed that the plaques were the result of proteolysis. There have not been any reports of the demonstration of the phenomenon with milk as the protein in the

substrate; and the appearances recorded in App. 7-8 were considered to be undoubtedly an example of it, occurring under conditions which satisfy the requirements stated by Elek - fresh serum, pathogenic staphylococci, and a protein substrate. The protein in this case was milk.

In view of the paucity of knowledge concerning Muller's phenomenon and the ease with which it can be demonstrated on serum-milk-agar plates, a detailed study of the phenomenon as it occurs on this substrate was undertaken and constitutes the remainder of this thesis.

PART II.

THE HISTORY OF MULLER'S PHENOMENON FROM 1927 to 1953.

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THE HISTORY OF MULLER'S PHENOMENON FROM 1927 to 1953.

I. THE WORK OF LEON MULLER AND HIS PUPILS.

II. THE OBSERVATIONS OF BURNET.

III. THE CONTRIBUTIONS BY CUCCO AND SEGRE.

IV. THE INDEPENDENT DISCOVERY OF THE PHENOMENON BY PACKALEN.

V. THE OBSERVATIONS MADE ON MULLER'S PHENOMENON BY OTHER WORKERS.

VI. THE EXPERIMENTS OF ELEK.

VII. DISCUSSION.

(a) A New Title for "Muller's Phenomenon"?

(b) The organisms in Muller's Phenomenon.

(c) The Staphylococcal Factor.

(d) Serum Factor in Muller's Phenomenon.

PART II.

THE HISTORY OF MULLER'S PHENOMENON FROM 1927 TO 1953.

When small circular areas of clearing were seen around colonies of Staphylococcus aureus in the course of the experiments which have been presented in Part I, it was recognised that this appearance was similar to that described by Muller and sometimes referred to in bacteriological literature as "Muller's phenomenon". Prior to undertaking more detailed investigation of this plaque-formation by staphylococci on a milk-substrate the literature bearing on "Muller's phenomenon" was studied and a review of this literature follows:

I. THE WORK OF LEON MULLER AND HIS PUPILS.

Leon Muller first brought to the notice of bacteriologists the phenomenon which now bears his name. Seven papers in all on this subject from his laboratory in Liége appeared in the journals between 1927 and 1930. Five of these were by Muller himself (1927a; b; 1928; 1929a; b.) and the remaining two by his pupils (Dormal 1927, Grégoire 1930).

In his first paper, Muller drew attention to an observation which he had made on human blood-agar plates on which was growing a strain of Staphylococcus aureus. He described small areas of clearing, 1.0 to 1.2 mm. in diameter in a sterile part of the plate (Muller 1927a). To this phenomenon he gave the name, "l'hémophagie staphylococcique" and carried out certain investigations on it in order that he might understand it more fully. He uncovered a number of facts and reached certain conclusions as a result of his experiments. He found that if human serum and red cells were added separately to agar to make a blood-agar plate the result obtained with regard to "l'hémophagie staphylococcique" was the same as when whole blood was used. He

also found that if washed red cells alone were incorporated in the agar without serum, the staphylococcal haemophagy did not appear on the plates; and that if serum or whole blood was heated at 54°C. for from 35 to 60 minutes (depending on the sample) and used for plates, haemophagy was again absent. The addition of fresh serum, however, to such inactivated blood or serum restored activity in relation to haemophagy. Similar inactivation of blood and serum specimens also followed incubation at 37°C., after a period of eleven days but did not occur on storage of specimens in an ice-box for up to four months.

The optimum pH of blood-agar plates prepared to demonstrate this haemophagic effect was pH 7.4. Alteration of the reaction of the medium had an important influence on the results obtained. The concentration of agar used in the plates, however, was not critical, 2.0%, 2.5% and 3.0% agar in these human blood-agar plates all gave the same result. The addition of 2.0% gelatin to a blood-agar plate had no effect on the appearance of the phenomenon in the plate.

Filtration of human serum through new used Chamberland filters of the L bis or L_2 type did not diminish its activity, although, if a sufficiently used L_2 filter was employed, serum filtered through it had reduced activity. The optimum

temperature for the production of these haemophagic spots was in the range 36.5°C. to 38.0°C. and there was no development of them at room temperature. Eighty-six specimens of serum in all were tested and all but six showed haemophagy.

Müller concluded that the haemophagic effect was not due to the action of the Staphylococcus aureus alone but that it required for its manifestation a filterable principle which was present in most specimens of human blood. For its haemodestructive activity the principle, which was thermolabile, required the presence of growing staphylococci.

Later in 1927 Muller and his pupil Dormal, each published a short paper on this staphylococcal haemophagy. Dormal (1927) investigated the action of various adsorbent substances on the active principle in human serum which plays an essential part in the production of haemophagy with Staphylococcus aureus. He found that wood charcoal removed the principle completely from specimens of serum and that infusorial earth and powdered porcelain dust reduced its activity by 80% to 90%. He found also that producing a calcium phosphate precipitate in serum brought down 90% of the active principle. When such a serum was tested after the removal of the precipitate it gave about a tenth of the number of haemophagic spots which an untreated specimen of

the same serum gave under the same experimental conditions.

Muller (1927b) extended the work of Dormal on the use of precipitates to remove the active principle from human serum. His aim was to precipitate the principle from the serum and subsequently to recover it from the precipitate. Precipitates of protein, prepared by adding saturated ammonium sulphate or saturated magnesium sulphate solutions to the serum and precipitates of calcium phosphate were used in this way. He also used precipitates of protein prepared by saturating diluted serum with carbon dioxide. The protein precipitates were ultimately dissolved in 0.5% sodium chloride solution and calcium phosphate precipitates in phosphate-citrate buffer.

When the redissolved precipitates were tested out under the appropriate conditions they were found to have different activities. The carbon dioxide precipitate and that formed by the saturated ammonium sulphate were as active as the original untreated serum whereas the saturated magnesium sulphate precipitate had an activity greater by 25% than that of the original untreated serum. The calcium phosphate precipitate was also active when redissolved and tested but the haemophagic manifestations which it gave were unusual. The average diameter of haemophagic spots obtained with serum was 2 mm.; the average diameter of the

haemophagic spots obtained with the calcium phosphate precipitate was 8 mm. Muller considered that this was due to the fact that although the active principle had been precipitated, it had not been possible to redissolve it completely and that it was not dispersed in the same manner as it had been in the original serum.

Muller (1928) presented the results of two years' work on l'hémophagie staphylococcique in an important contribution which contains much valuable information, and includes experiments by Dormal and Grégoire. This paper and its observations and conclusions are now summarised closely following the headings Muller used.

Variations in the appearance of haemophagic spots.

On the blood plates on which the haemophagy had been originally observed, the spots of clearing were seen to be cleanly cut, to have a punched-out appearance, and to average 2 mm. in diameter. Spots of this size, however, were later found not to be very common. Much more usual was the occurrence of smaller spots 1.0 to 1.5 mm. diameter. Larger blurred spots also occurred and might be so numerous as to be practically confluent, resulting in a discoloration of the plate as a whole. Most plates showed that the areas of clearing were polymorphous, consisting of mixtures

of large and small spots, the latter usually clearer than the former. It appeared that specimens of blood from different individuals were associated with variations in the overall picture.

Thermolability of the haemophagic property in blood.

The destruction by heat of the haemophagic property in blood was a constant finding, but its thermolability varied with different specimens of blood. Thus it was found that ten minutes at 55°C. inactivated the haemophagic property in blood from one individual but that fifty minutes at 55°C. was required for inactivation in case of blood from another individual. On the other hand, specimens of blood taken from one individual at intervals over a long period of time were found to be remarkably constant with regard to the thermolability of the haemophagic property.

In the case of separated serum Dormal found that it took a week at 37°C., 10 to 20 hours at 49°C., 3 hours at 52°C., and a few minutes at 55°C. to inactivate its haemophagic property. By subjecting sera to these various temperatures, Dormal also found that the factor in serum causing the large spots of clearing in blood-agar plates was less thermolabile than the factor which

caused small spots, and suggested that the large spots resulted from the floccules or aggregates in the serum to which the haemophagic principle became attached.

Sera deprived of their haemophagic property by heat were found to be also anti-haemophagic. This was shown by the inhibition of the haemophagic phenomenon when such inactivated serum was added to blood-agar and used for the demonstration of haemophagy. This property of heated serum in inhibiting haemophagy, however, was not constant.

Influence of certain factors affecting the blood used.

Muller reports contradictorarily that although citrated blood was found somewhat less satisfactory than defibrinated blood the incorporation of sodium citrate in blood-agar definitely favoured haemophagy.

The red cells of one individual and the serum of another were found suitable for haemophagy and sometimes a better result was obtained with a particular serum if the red cells from another individual were used.

Action of sterilising agents and antiseptics.

The addition of ether or chloroform to a mixture of blood and agar, in quantities insufficient to show any effect on the growth of Staphylococcus aureus had no inhibitory action on haemophagy. These chemicals, however, destroyed the haemophagic principle in blood if left in contact with it for 48 hours and their action was considered to be a sterilizing and not an inhibitory one.

The actions of sodium fluoride and phenol gave results similar to those of heat, viz. inhibition of the appearance of the smaller spots without any inhibition of the larger ones.

Filterability of the haemophagic principle.

Filtration through a Chamberland LIII filter reduced notably the activity of the majority of sera so treated; but in filtering certain samples of serum through L7 and L9 Chamberland filters there was little reduction of activity up to one third reduction in the number of spots. Centrifugation of specimens of sera at a speed of 5000 revolutions per minute for twelve hours made no difference to their ability to produce the haemophagic phenomenon.

Conditions of incubation of medium, reaction of medium and strains of staphylococci used.

The reaction of the blood-agar media used in the original experiments was alkaline, at pH 8.0 or more, but it was found subsequently that other staphylococci would only give the haemophagic effect within a pH range much lower than pH 8.0. Some strains worked only at pH 7.0 - 7.2, while others worked at pH 7.9 - 8.2.

With regard to the optimum temperature for the haemophagic effect it became apparent that with specimens of blood giving the polymorphous spots of clearing the large spots developed best at 36°C. and the small spots at 38°C. Increase in temperature beyond 38°C. resulted in a complete absence of the haemophagic effect.

Numerous experiments were carried out to determine the best medium for the demonstration of staphylococcal haemophagy. The conclusion was reached that a very highly nutritious medium was desirable, one which was without fermentable carbohydrate which if broken down, would be likely to alter the reaction of the medium.

Effect of adsorbent powders and precipitates formed in the serum.

The results obtained and discussed under this section correspond with those reported previously by Muller and Dormal (Muller 1927b; Dormal 1927), and referred to above.

Technique employed to demonstrate haemophagy.

Muller discusses the techniques used to demonstrate the haemophagic phenomenon under three headings, (1) Withdrawal of Blood, (2) Nutrient Medium, and (3) Staphylococci.

Most of the information given in this section is unimportant in relation to the work presented in the present thesis. There are, however, two statements which he makes to which attention should be drawn. He says that the appearance of haemophagic spots on human blood-agar plates was rare before the third day of incubation. He also states that two different criteria could be used for assessing the haemophagic activity of any sample of serum, the one based on the earliest appearance of the phenomenon, the other on the diffuseness of the final clearing; and found that for the former 0.25 ml. of serum in 40 ml. of his medium was usually the optimum and for the latter 1.0 to 1.2 ml.

Interpretation of these experiments.

He discusses at length the interpretation to be given to his experiments and concludes that the haemophagic phenomenon is the result of the action of a haemophagic principle which exists in the majority of human sera and which is thermolabile and chemicolabile. This principle is present in the form of filterable elements which are uniformly dispersed in blood, but which vary in number in different specimens and which cannot by themselves exert any haemo-destructive action. They are activated by particular secretions formed by certain strains of staphylococci and when thus activated lead to the characteristic appearance of haemophagy in blood agar.

He also discusses the possibility of these particulate filterable elements being living agents and notes the close resemblance of the haemophagic phenomenon to satellitism as shown by Pfeiffer's bacillus growing in association with staphylococcus.

Muller, (1929a), presented the results of the examination of the blood of different animals for the haemophagic property and found that rabbit blood usually possessed the property but that it was lacking in the blood of horse, ox, sheep, hen, goose and guinea-pig.

Muller, (1929b), found that the activating substance from staphylococci could pass through parchment but not through parchment and collodion together. He thought that the principle produced by the staphylococci and necessary to activate the haemophagic elements in the blood, was only generated if the medium on which these organisms were growing contained fresh or heated blood.

He referred to earlier experiments in which he had tried but failed to produce the haemophagic effect without the direct intervention of living staphylococci, by incorporating culture filtrates in the medium or by placing on the surface of the medium a collodion sac containing the culture. It is not surprising that the activating factor from staphylococci did not diffuse through the collodion sac since he had already found that although it would pass through parchment it would not pass through parchment and collodion together.

Grégoire (1930) one of Muller's pupils gave a detailed description of Muller's work and that of his fellow-worker, Dormal. He also gave the results of his own investigations and discussed the contribution of Burnet (vide infra). The following are Grégoire's results summarised under the headings which he used.

Microscopic Examination of Haemophagic Spots.

Small cubes of medium showing haemophagic spots were removed from blood-agar plates and fixed in alcohol or formalin for 24 hours. They were then embedded and sections, 5μ thick were cut vertically or horizontally. Microscopic examination of such sections even after staining failed to afford any useful information regarding the spots.

Spectroscopic examination and chemical analysis of the Haemophagic spots.

These examinations were undertaken with a view to finding if the haemoglobin was destroyed in the spots of clearing or if it passed into the surrounding medium or if it changed to a colourless form.

Neither urobilin nor bilirubin were detected in the haemophagic spots and this was not surprising since the form of haemoglobin destruction occurring in haemophagy is different from that which leads to the formation of these compounds.

The amount of iron present in the spots of clearing was significantly greater in the unaltered blood-agar, but no satisfactory explanation of this was reached.

Haemophagy and the blood of infants.

Only 45 of the 97 specimens of placental blood examined by Grégoire proved satisfactory for the demonstration of the haemophagic phenomenon and it was not possible to correlate the presence or absence of the haemophagic factor in these bloods with any condition of the mother or child.

Haemophagy with pathological fluids - transudates and exudates.

The haemophagic activity of transudates and exudates was investigated in a number of experiments. There was a clear difference in the ability of these two types of fluid to bring about the haemophagic spots of clearing when tested out with washed human red cells incorporated in a standard nutrient medium inoculated with staphylococci. Exudates were generally found to be very active in this respect whereas transudates and some exudates, obtained from conditions with minimal inflammation were inactive. Although the transudates had a much lower protein content than the exudates this was not thought by Grégoire to be the explanation of their differing haemophagic activity. He pointed out that in the production of an exudate, the lesion responsible brings about an alteration in the membranes so that the

passage of cells and of elements of defined dimensions is permitted. In contrast these cells and elements are held back by the intact membranes in the cases where the pathological process occasions a transudate. He stressed that plates made with these pathological fluids either manifested the phenomenon clearly and without doubt or failed completely to show it; the results were never equivocal.

The transudates included ascitic fluid from cases of cirrhosis of the liver and effusions from cardiac cases. Specimens of hydrococle fluid or from lymphangioma behaved like exudates showing marked haemophagic activity.

Grégoire regarded his results as in keeping with Muller's hypothesis that the haemophagic phenomenon is due to a particulate filterable element present in certain body fluids and capable of being activated by secretions of certain staphylococci.

II. THE OBSERVATIONS OF BURNET.

Burnet (1928) confirmed Muller's observation of the haemophagic effect produced by staphylococci and was the first to refer to this as "Muller's phenomenon". He describes it as distinct punctate haemolysis of blood-agar by staphylococci, regarding the spots of clearing as being essentially haemolytic in nature in contrast to Muller who believed that they were due to active destruction of haemoglobin.

The composition of the basic medium was considered by Burnet to be important particularly in relation to its peptone content since he found that peptone could have a definitely inhibitory action on the manifestation of the phenomenon. He recommended Huntoon's hormone agar.

He confirmed Muller's observations that haemophagy did not occur at room temperature or in plates incubated anaerobically at 37°C. He found, however, that a plate which had been incubated anaerobically at 37°C. without the development of haemophagy would show the characteristic spots of clearing if subsequently incubated aerobically for 2-3 days. He also found that the optimum temperature for the manifestation of haemophagy

was 38.5°C. for 3-4 days.

He found on testing the red blood cells from different animal species that only human cells were satisfactory for the demonstration of Muller's phenomenon. He also found that both guinea-pig and rabbit sera contained the serum factor as shown on testing appropriately with washed human red cells as the indicator. Seitz filtration did not diminish the activity of a serum in respect of its serum factor.

He examined a variety of organisms for their ability to produce Muller's phenomenon on human blood-agar, but found that only staphylococci were able to do so. Burnet wrote, "Amongst the staphylococci, it may be taken as a general rule that aureus strains, obtained from purulent lesions produced the phenomenon, while most air staphylococci and those of the epidermidis type do not manifest it".

Burnet regarded the discrete areas of haemolysis as the result of activation of what he called a haemolysinogen which diffused from growths of all or most pyogenic staphylococci and which was activated by a complex of a labile serum constituent (associated with the globulins) and some entity present in meat extract. Complement as the serum constituent of the complex could be excluded.

III. THE CONTRIBUTIONS BY CUCCO AND SEGRE.

During 1929 three papers appeared from the Italian workers, Cucco and Segre which confirmed Muller's findings although differing in their interpretation of these findings. Particular attention was paid to the serum factor required to demonstrate staphylococcal haemophagy and many individual specimens of human blood were examined.

Cucco (1929) examining over 100 specimens of blood from patients with different diseases found that haemophagy could not be demonstrated with blood from diabetics whether under insulin treatment or not; that only rarely could it be demonstrated with blood from nephritics; that it could always be demonstrated with blood from cases of tuberculosis, and very regularly with blood from cases of a variety of other diseases.

He did not agree with the hypothesis of Muller that the principle present in the serum necessary for the haemophagic effect was living and that it was this living principle which, when acted on by the staphylococcal factor developed a haemo-destructive property. To Cucco it was not necessary to postulate the presence of a living principle. He contended that it was only

necessary to have a certain quantity of serum globulins present in association with staphylococci, to produce the haemophagic effect.

He had noticed that the majority of specimens of foetal and neo-natal blood did not give rise to the haemophagic effect. This he believed was due to the small quantity of globulins present in these sera. The amount of globulins was greater in the adult blood and reached a maximum in inflammatory conditions acute and chronic, and in tumours but especially if malignant.

He confirmed Muller's opinion that only a few strains of staphylococci were able to bring about the haemophagic phenomenon and believed that the staphylococci were only active in this respect while alive. He also found that the addition of a filtrate of a broth culture of an active staphylococcus to a blood-agar plate did not produce Muller's phenomenon and like Burnet found that the serum factor for the haemophagy was present in rabbit blood.

Segre (1929a, b), like Cucco confirmed many of Muller's findings but did not agree with Muller's view that there was a living agent in human serum, which under the action of the staphylococcal factor became haemo-destructive. He was of the opinion that the staphylococcal factor activated some ill-defined but

non-living elements present in the serum globulins.

With human blood he noted that any signs of haemophagy rarely developed before the third or fourth day of incubation of the plates but with rabbit blood the characteristic plaques appeared much earlier and in greater profusion.

He found that not all strains of staphylococci, indeed only a few, were able to produce the haemophagic effect and that organisms other than staphylococci were quite inactive.

Pregnancy unless complicated by renal disease or albuminuria of pregnancy was not associated with any significant alteration in the haemophagic activity of human blood. During the puerperium or after abortion, maternal blood had normal haemophagic activity unless from cases with pyrexia. In neonatal blood the activity was either weak or absent.

He succeeded in precipitating from serum the serum factor necessary for haemophagy but failed to obtain the staphylococcal factor in filtrates from cultures. Like Burnet (1928) and others he confirmed the suitability of rabbit serum for the demonstration of haemophagy and also found that the incorporation in blood-agar plates of a staphylococcal antiserum prepared in the rabbit resulted in modification of haemophagy.

IV. INDEPENDENT DISCOVERY OF THE PHENOMENON BY PACKALEN.

The staphylococcal haemophagic phenomenon was first observed by Packalén in 1935 at a time when he was without knowledge of Muller's work. He designated the effect "secondary haemolysis" and had observed it when growing staphylococci on blood-agar. During the following years he investigated the effect in considerable detail and it was not until his work in manuscript form was ready for publication that he became aware that "secondary haemolysis" was none other than "l'hémophagie staphylococcique" of Muller.

Packalén (1938) recorded that minor variations in the composition of the medium did not greatly influence the appearance of the phenomenon and that an agar content of 1.7% to 2.5% was satisfactory.

He further found that 5% - 10% of blood gave better results than 20%; and that the phenomenon did not develop under anaerobic conditions. The optimum reaction of the medium for its production was between pH 6.9 and pH 7.5 but some effect was obtained even at pH 6.2

He found that Muller's phenomenon was a common

character of the twenty or so strains of Staphylococcus aureus which he had examined. Muller and others, however, had found that only very few of their strains showed the haemophagic effect. Packalén suggested that the failure of these workers to observe haemophagy with the majority of their strains might well have been due to the methods they employed.

The haemophagic property of Staphylococcus aureus appeared to Packalén to be a specific characteristic not shared with any of the other organisms which he had examined including Staphylococcus albus, Streptococcus β haemolyticus, Escherichia coli, vibrios and spore-bearing bacilli. He noticed, however, that there were slight differences in the size, number and clearness of the haemophagic spots as well as in the rapidity of their appearance in the case of different strains of Staphylococcus aureus.

In place of human blood, the blood of horse or rabbit - but not of sheep, ox or guinea pig - could be used satisfactorily for showing the haemophagic effect.

Packalén found it difficult to give an explanation of the irregular pattern of the haemophagic spots on a blood-agar plate. He accepted that blood was evenly distributed throughout the agar and that the staphylococcal substance produced by the colony diffused regularly and uniformly into the medium. Thus he

reckoned, the haemophagic spots of clearing should also be evenly distributed. He had observed, however, as Burnet had done previously, solid particles such as fragments of charred cotton in the centre of some of the haemophagic spots, and suggested that particles of fibrin, maybe not even visible to the naked-eye, might act as foci of adsorption for the haemophagic agent. The filtration of defibrinated blood through hard-packed cotton wool to remove any such particles had no effect on subsequent haemophagy. Moreover, as the addition of minute sterile particles of glass or coal to a blood-agar plate while it was setting had also no effect, it did not appear that the production of the spots depended on such particles in the medium.

Although Muller had suggested that the haemophagic effect was due to the activation of a corpuscular element in fresh serum by a staphylococcal substance and that this serum element might be a living filterable virus, Packalén was inclined to the view that the haemophagic areas of clearing were the visible manifestations of the activity of colonies of a virus derived from the staphylococcus. He compared the phenomenon with the colonies of ultra-microscopic viruses described by Ørskov and Dienes. The animate or inanimate nature of the virus he left open to discussion.

Packalén (1941) reported further observations and drew somewhat different conclusions from his earlier ones. He had discovered that intact red cells were not necessary for the demonstration of Muller's phenomenon since haemolysed blood in the culture medium proved equally satisfactory. He also found that a filtered solution of commercial horse haemoglobin together with fresh human serum could be used.

Of still greater importance was his finding that what was obviously Muller's phenomenon could be demonstrated in a medium which, although containing fresh serum did not contain blood cells or haemoglobin but the coagulated proteins of ascitic fluid. On this turbid medium a typical zone of circular areas of clearing of the turbidity developed around the growth of Staphylococcus aureus. This was in fact a demonstration of Muller's phenomenon on a new medium, with the lysis affecting a different protein.

Packalén also found that a cell-free filtrate from a staphylococcal broth culture when added to a blood-agar plate produced typical areas of haemophagic clearing and showed that the active principle in the filtrate was not identical with the α or β staphylococcal toxin or with the staphylococcal protease.

Further, he reproduced Muller's phenomenon on a

blood-tragacanth mixture, thus showing that the areas of haemophagic clearing were not dependent on structural irregularities in an agar colloid; and he found that Löffler's serum or plain heat-coagulated serum could serve as the basic medium.

This work of Packalén's established that the staphylococcal factor necessary for Muller's phenomenon could act in comparable fashion on proteins other than those of red blood cells. He suggested that its action was probably a proteolytic one with fresh serum providing an essential co-enzyme but was indeed undecided as to how the effects were produced.

V. OBSERVATIONS MADE ON MULLER'S PHENOMENON BY OTHER WORKERS.

The paper of Rhodes (1938) records another independent discovery of Muller's phenomenon and has no reference to the work of Muller, Dormal, Grégoire or Burnet which had been reported ten or more years previously. Rhodes' work in the main confirms that of these earlier workers and leads her to conclude that the essentials for the demonstration of what she termed "satellite zones of haemolysis" were a growth of staphylococci of appropriate type on a medium containing fresh serum derived from certain animal species and red blood cells from a more limited number of animal species. Appropriate staphylococci were haemolytic and mannitol fermenters. She also concluded that the staphylococci produced a diffusible substance concerned in the production of the satellite zones of haemolysis but did not succeed in isolating the factor.

What was undoubtedly a demonstration of Muller's phenomenon occurring in a medium whose indicator protein was not haemoglobin, were the observations of Fisk and Mordvin (1943), in the work which has already been cited. Using a substrate containing fibrin (heat-precipitated fibrinogen) and inoculated with staphylococci they noted the occurrence of "Plaque-like areas

of clearing which protruded from the periphery of the zone of fibrinolysis and which were sometimes entirely separate from this zone .....".

Christie, Graydon and Woods (1945) commented on their finding that the periphery of the zones of clearing around the colonies of staphylococci in heat-precipitated fibrinogen-agar plates were frequently irregular and that small discrete zones of lysis were occasionally found outside the main zones. They concluded that this was a demonstration of Muller's phenomenon and noted that Fisk and Mordvin had made a similar observation.

Christie, Graydon and Woods, however, found that only 37 of their 58 pathogenic strains of staphylococci gave the appearance and that these 37 strains were also fibrinolytic. The remaining 21 strains of pathogenic staphylococci were non-fibrinolytic and also failed to give the characteristic Muller phenomenon when tested on the particular lysed blood-agar which they used. Their 37 fibrinolytic and pathogenic strains gave the characteristic Muller phenomenon when appropriately tested.

They assumed that fibrinolysis which was responsible for the irregularities of the zones of clearing on their fibrinogen plates was also responsible for Muller's phenomenon. They regarded their assumption as receiving support from the fact that

a thermolabile substance in serum appeared to be necessary for the production of the irregularities which developed on their fibrinogen plates and that this thermolabile substance had a more or less inhibitory action on fibrinolysis. They were of the opinion too, that the explanation of the discreteness of the plaques of clearing was to be sought on a physical rather than on a chemical basis.

VI. THE EXPERIMENTS OF ELEK.

Elek (1953) contributes the most recent addition to the literature on Muller's phenomenon with a paper giving an excellent summary of earlier work on the subject and presenting many new observations including several on the action of the cell-free staphylococcal factor. He used serum-haemoglobin-agar as the indicator medium instead of blood-agar and as mentioned previously suggested the term 'proteoclast' for the phenomenon.

Only, but not all strains of coagulase-positive staphylococci, were found to be capable of producing the proteoclast effect. It was not produced by strains of coagulase-negative staphylococci or by the other organisms which he examined.

He failed to obtain the staphylococcal factor in filtrates from broth cultures of the organisms but succeeded in so doing in filtrates of extracts of cultures on solid media. He gives much useful information as to the conditions producing the best yields of the active staphylococcal factor, e.g. the time of harvesting, the sealing of plates with plasticine, the addition of 10% heated serum to the basal medium and investigated briefly its physical and chemical properties. It could not be correlated

with any of the hitherto defined diffusible products of staphylococci, appearing to be distinct from the growth factors which give rise to satellitism of Haemophilus influenzae and from staphylococcal coagulase, lipase, haemolysins and fibrinolysin.

He studied the role of fresh serum in the production of the haemophagic phenomenon ("proteoclast"), confirmed that fresh serum was essential and found that all of thirty specimens which he examined were active. The number of plaques produced under defined conditions showed a linear correlation with the amount of serum in the medium but not with the amount of staphylococcal factor. He concluded on the basis of his experimental work that two factors in fresh serum were concerned in the production of the "proteoclast", one being particulate, the other non-particulate.

In his discussion of the phenomenon he is of the opinion that the plaques in the gel are spheres of proteolysis of the haemoglobin, and that they develop around the particulate elements 20 - 70 mμ in diameter which constitute one of the two factors in fresh serum necessary for the proteolytic effect. The other factor, of course, in serum is the non-particulate one and the co-operation of these two serum factors with the staphylococcal factor results in proteolysis.

Elek in considering the focal nature of the

proteolysis and relating this, as had other workers to the likely presence of something particulate in the medium discusses four possible explanations:-

1. that the effect is the result of the action of an enzyme which is adsorbed to crystalline or other aggregates in the medium,
2. that it is due to the action of a living virus derived from staphylococci,
3. that it is due to the action of a living virus derived from the fresh serum which co-operates with a product of staphylococci,
4. that it is due to the activation by a product of staphylococci of a particulate precursor of a proteolytic enzyme present in the sera of animals.

The first of these possibilities is summarily dismissed by Elek, and the second possibility also is dismissed on the ground that the staphylococcal extract can withstand boiling, treatment that no known virus is able to resist. He is of the opinion that the third and fourth possibilities which he suggested would both fit the experimental findings and he summarises his views on this aspect of Muller's phenomenon in the following sentence:-

"It is suggested that the proteoclast effect is due either to a

non-pathogenic virus or to a hitherto unrecognised particulate enzyme precursor present in sera and activated by the staphylococcal factor".

VII. DISCUSSION.

It is not the intention to introduce at this stage a detailed discussion of the many aspects of Muller's phenomenon. Such a discussion will be included in Part IX, in which the findings made in the course of the present study will be viewed against the background of knowledge provided by those who have investigated the phenomenon during the last thirty years. The object meantime is to discuss briefly a few of the more important points arising from the work of these investigators and which influenced the planning of the experimental work incorporated in the present thesis.

(a) A NEW TITLE FOR "MULLER'S PHENOMENON"?

It is desirable first of all to consider the names which have been applied to Muller's phenomenon by different workers. Muller himself, (Muller, 1928) designated it "l'hémophagie staphylococcique", because there was destruction of haemoglobin in addition to lysis of red blood cells.

Burnet (1928) was the first to refer to it as "Muller's phenomenon", but also used the descriptive term "punctate

haemolysis". Grégoire (1930), in his paper on this subject referred to "l'hémophagie staphylococcique de L. Muller", while Rhodes (1938), who discovered the phenomenon independently termed it, "satellite haemolytic zones". Packalén (1938, 1941) wrote of "l'hémophagie staphylococcique de Muller" and also followed Rhodes in using "satellite zones".

It was Packalén who showed that intact red blood cells were not essential for the phenomenon and that haemoglobin or heated serum proteins could also be used as suitable substrates. Elek (1953) suggested that the term "the proteoclast" should be given to Muller's phenomenon rather than "haemophagie" as used by Muller, because, "This term seems preferable to "haemophage" (sic) since the lytic effect is not limited to haemoglobin".

Thus, it has come about that to the appearance originally described by Muller in 1927 the following terms have been applied - "l'hémophagie staphylococcique", "punctate haemolysis", "Muller's phenomenon", "satellite haemolytic zones", "l'hémophagie staphylococcique de Muller" and lastly "proteoclast". All these terms which contain hémophagie or its English equivalents haemophagy or haemophage, haemolysis or haemolytic should be avoided since the phenomenon can be demonstrated with proteins other than those of red blood cells. Is "proteoclast" a suitable term to be substituted?

Its use would certainly overcome any limitation of the Muller effect to some action on blood cell proteins but would give no indication of the essential role of the staphylococcus or of the very characteristic appearance of the small, discrete circular areas of lysis which develop.

The title given by Burnet (1928) viz. "Muller's phenomenon", has also a restricted meaning, if the phenomenon is regarded, as Muller originally described it, as the appearance of small circular areas of clearing on human blood-agar plates under the influence of growing staphylococci. In view of the fact that so little is known about this effect, its mechanism or its significance, would it not be preferable at the present stage to retain the term "Muller's phenomenon" and to redefine it taking cognisance of the facts discovered since the time of Muller's original description?

"Muller's phenomenon" could then be defined as "the appearance of small circular areas or plaques of clearing on a protein substrate in the presence of fresh human or other suitable mammalian serum and brought about by the action of a diffusible product from certain strains of staphylococci". The term "Muller's phenomenon" as thus defined will be used throughout the remainder of this thesis.

(b) THE ORGANISMS IN MULLER'S PHENOMENON.

All the workers in the field are agreed that the phenomenon requires for its demonstration the presence of living staphylococci and that of the many different organisms tested only staphylococci could bring about the effect.

With regard to the ability of different strains of staphylococci to produce Muller's phenomenon there does not appear to be the same measure of agreement. Muller was of the opinion that it was a characteristic of only a few strains, a view shared by Cucco (1929) and Segre (1929). Burnet (1928) believed that it could be demonstrated by aureus strains isolated from actual cases of infection. Packalén (1941) considered that it was a characteristic of Staphylococcus aureus while Elek (1953) ascribed it to coagulase-positive strains of Staphylococcus pyogenes. Elek noted, however, that some coagulase-positive strains were inactive in this respect.

It would appear that a survey of a large number of strains of staphylococci (aureus and albus, coagulase-positive and coagulase-negative), for their ability to produce Muller's phenomenon is still required. Only in this way will it be possible to ascertain how common a characteristic of staphylococci in general

is the ability to produce the phenomenon. More precise knowledge, for example, as to how frequently this ability is associated with pathogenicity or coagulase production and as to the consistency of its absence from non-pathogenic or coagulase-negative strains would be most valuable.

(c) THE STAPHYLOCOCCAL FACTOR.

It is reasonable to deduce that the role of living staphylococci in relation to Muller's phenomenon depends upon the production by the organisms of some product which diffuses from them into the culture medium and for convenience this will be referred to as the staphylococcal factor.

Staphylococcal filtrates tried by a number of workers in place of living staphylococci have generally proved incapable of producing Muller's phenomenon. Cucco (1929), Segre (1929) and Rhodes (1938) were all unsuccessful in their attempts with staphylococcal filtrates. Packalén (1941) was the first to obtain a staphylococcal filtrate which had some activity and gave rise to plaques of clearing when added to the surface of a blood-agar plate. Elek (1953) succeeded in preparing more active filtrates.

This success in preparing the staphylococcal factor

which plays such an essential role in Muller's phenomenon and obtaining it free from staphylococcal cells represents a notable advance, permitting the designing of much more satisfactory experiments for the study of the underlying mechanism of the phenomenon.

Having at one's disposal too, the cell-free factor should permit the study of the factor itself with a view to determining whether it is in any way related to any known staphylococcal product such as fibrinolysin which Christie, Graydon and Woods (1945) regarded as being responsible for the phenomenon.

(d) SERUM FACTOR IN MULLER'S PHENOMENON.

It is clear that the staphylococcal factor by itself does not cause Muller's phenomenon and that a factor provided by serum is also required. This serum factor is present in almost all adult human sera and in the sera of several species of lower animals such as rabbit or guinea pig.

It is generally held that the serum factor is activated in some fashion by the staphylococcal factor, though in

what way is unknown. It has been regarded as possibly a living virus; as a non-living entity associated with the globulin fraction of serum; or even as being dual in nature with one of the partners particulate and either a virus or an enzyme.

It is apparent that apart from the recognition of the essential role of a serum factor little or nothing is known of its nature and that much more study is still required.

Attention has been directed in this brief discussion to certain lines of investigation whose exploration might well yield data of value for the elucidation of the nature of Muller's phenomenon and reference was made earlier to the serviceability of serum-milk-agar instead of blood-agar for the demonstration of the phenomenon - using the term as defined on page 65. For the studies presented in the following parts of this thesis, serum-milk-agar has been employed as the regular substrate and the work has been directed particularly to:-

A survey of the activity of 556 strains of staphylococci in relation to Muller's phenomenon

and a study of the optimum conditions for its demonstration.

The preparation and testing of the staphylococcal factor.

A limited study of certain aspects of the serum factor.

P A R T III.

INVESTIGATIONS ON THE ABILITY OF STRAINS OF STAPHYLOCOCCI TO PRODUCE MULLER'S PHENOMENON.

PART III.

INVESTIGATIONS ON THE ABILITY OF STRAINS OF STAPHYLOCOCCI TO PRODUCE MULLER'S PHENOMENON.

It was decided at the beginning of this investigation of Muller's phenomenon that there were two aspects of the problem about which information should be obtained straight away if possible. One of these concerned the ability of strains of staphylococci to produce Muller's phenomenon, the other with the demonstration of Muller's phenomenon on serum-milk-agar plates.

Nowhere in the literature on Muller's phenomenon is there any reference to a large scale investigation of the ability of strains of staphylococci to produce it. Cucco (1929) and Segre (1929) both supported Muller's opinion that only a very few strains of staphylococci were capable of manifesting this effect. On the other hand Burnet (1928) stated that '<u>aureus</u>' strains of staphylococci isolated from purulent lesions did so while those from the air and those of the '<u>epidermidis</u>' type did not.

Packalén (1938) reported that the production of Muller's phenomenon was a common characteristic of strains of Staphylococcus aureus. He had examined about 10 strains in all. Elek (1953) found that 15 strains of coagulase-positive Staphylococcus pyogenes which he had tested, produced Muller's phenomenon. Rhodes (1938) found that all but 2 out of 30 strains of haemolytic Staphylococcus aureus produced "satellite zones of haemolysis" on human blood-agar plates. The 2 strains which failed to do so did not ferment mannitol.

It appeared desirable to examine a large number of strains of staphylococci, known pathogenic strains and others, to ascertain if this ability to produce Muller's phenomenon was a common characteristic of pathogenic as opposed to non-pathogenic strains. Was there any relationship between the ability of a strain of staphylococcus to produce Muller's phenomenon with its ability to produce staphylocoagulase or fibrinolysin? Only by examining a large number of strains of staphylococci would it be possible to answer this question with certainty.

It had been assumed, with good reason, that the appearance of plaques of clearing around colonies of staphylococci in the serum-milk-agar plates containing 10% human serum, was a

demonstration of Muller's phenomenon. The use of milk, however, as a suitable substrate for this effect had not been reported before, by those who had worked in this field. It was thought that if those strains of staphylococci which had produced the plaques of clearing in the serum-milk-agar plates were actually demonstrating Muller's phenomenon on this substrate, they would also be capable of doing so on a recognised substrate for this phenomenon. Thus, if there was correspondence in the abilities of a number of strains of staphylococci to produce plaques of clearing on serum-milk-agar plates with their production of Muller's phenomenon on (say) serum-haemoglobin-agar it could be concluded that this appearance on the serum-milk-agar plates regarded as Muller's phenomenon, was indeed almost certainly Muller's phenomenon.

The opportunity was also taken in this examination of a large number of strains of staphylococci to confirm what previous workers on this subject have claimed, viz. that serum is essential for the demonstration of Muller's phenomenon. This was achieved by testing every strain on the available substrates with and without added serum.

PART III.

INVESTIGATIONS ON THE ABILITY OF STRAINS OF STAPHYLOCOCCI TO PRODUCE MULLER'S PHENOMENON.

I. INVESTIGATION OF STRAINS OF STAPHYLOCOCCI PREVIOUSLY COLLECTED IN THE COURSE OF A SURVEY CONDUCTED AT THE PRINCESS MARY MATERNITY HOSPITAL AND FROM BACTERIOLOGICAL SPECIMENS EXAMINED AT THE ROYAL VICTORIA INFIRMARY AND AT THE GENERAL HOSPITAL, NEWCASTLE UPON TYNE.

II. INVESTIGATION OF 116 STRAINS OF STAPHYLOCOCCI OBTAINED AS PRIMARY SUBCULTURES FROM THE DEPARTMENT OF BACTERIOLOGY, ROYAL VICTORIA INFIRMARY.

III. INVESTIGATIONS OF STRAINS OF STAPHYLOCOCCUS AUREUS DERIVED IMMEDIATELY FROM SPECIMENS SUBMITTED FOR BACTERIOLOGICAL EXAMINATION.

IV. DISCUSSION.

I. INVESTIGATION OF STRAINS OF STAPHYLOCOCCI PREVIOUSLY COLLECTED IN THE COURSE OF A SURVEY CONDUCTED AT THE PRINCESS MARY MATERNITY HOSPITAL AND FROM BACTERIOLOGICAL SPECIMENS EXAMINED AT THE ROYAL VICTORIA INFIRMARY AND AT THE GENERAL HOSPITAL, NEWCASTLE UPON TYNE.

(a) THE STRAINS OF STAPHYLOCOCCI USED IN THIS INVESTIGATION.

(b) THE EXAMINATIONS CARRIED OUT ON THE 233 STRAINS OF STAPHYLOCOCCI AVAILABLE FOR THIS INVESTIGATION.

(i) COAGULASE PRODUCTION.

(ii) MULLER'S PHENOMENON.

(1) Media.

(2) Inoculation and Incubation of Plates of Different Media.

(c) THE RESULTS OBTAINED FROM THE EXAMINATION OF THE 233 STRAINS OF STAPHYLOCOCCI.

(d) THE COAGULASE-POSITIVE STRAINS WHICH INITIALLY AND REPEATEDLY FAILED TO PRODUCE MULLER'S PHENOMENON.

I. INVESTIGATION OF STRAINS OF STAPHYLOCOCCI PREVIOUSLY COLLECTED IN THE COURSE OF A SURVEY CONDUCTED AT THE PRINCESS MARY MATERNITY HOSPITAL AND FROM BACTERIOLOGICAL SPECIMENS EXAMINED AT THE ROYAL VICTORIA INFIRMARY AND AT THE GENERAL HOSPITAL, NEWCASTLE UPON TYNE.

The difficulty of obtaining a large number of strains of coagulase-positive and coagulase-negative staphylococci for the purpose of this investigation in a relatively short time was overcome by the help of Dr. J. G. P. Hutchison who had collected a number of such strains in the course of a survey at the Princess Mary Maternity Hospital and from bacteriological specimens submitted for examination to the Department of Bacteriology, Royal Victoria Infirmary. From him I received over 200 strains of staphylococci which had been maintained as stock cultures on nutrient agar slopes in bijou bottles. In addition, I obtained 29 strains of staphylococci from the Bacteriology Department, General Hospital, Newcastle upon Tyne. With these strains it was possible to begin this investigation of Muller's phenomenon.

(a) THE STRAINS OF STAPHYLOCOCCI USED IN THIS INVESTIGATION.

In all, 233 strains of staphylococci were used in this investigation. Of this number, 192 were coagulase-positive and 41 coagulase-negative. (App. 382). The sources of these strains are given in App. 383 and the nature of the pathological specimen from which they were isolated is included, where known, in App. 384-395.

The strains of staphylococci obtained from the General Hospital had all come from pathological conditions such as wounds and abscesses; 27 strains were coagulase-positive and the remaining 2 coagulase-negative. The cultures of these organisms which had been incubated at 37°C. for 24 hours were received on nutrient agar slopes. No information was available, however, about the number of subcultures on different media which these strains had had from the time of first isolation until received on the nutrient agar slopes as 24 hour old cultures.

The strains of staphylococci obtained from the stock cultures held by Dr. J. G. P. Hutchison fell into two groups. One group consisted of 74 strains isolated from nasal swabs and septic lesions in the course of a survey conducted at the Princess Mary Maternity Hospital. Of that number 62 were coagulase-positive and

12 coagulase-negative.

The other group was made up of 130 strains of staphylococci, 103 coagulase-positive and 27 coagulase-negative which had been isolated from specimens submitted for bacteriological examination to the Department of Bacteriology, Royal Victoria Infirmary.

All these strains were received on nutrient agar slopes in bijou bottles which had been stored for some months. No information was available about the number of times these strains had been subcultured from the time of their first isolation or the nature of the bacteriological media on which they had been grown.

(b) THE EXAMINATIONS CARRIED OUT ON THE 233 STRAINS OF STAPHYLOCOCCI AVAILABLE FOR THIS INVESTIGATION.

(i) Coagulase Production.

The coagulase activity of each strain was tested by the tube method (App. 492). It was found most convenient to do this with a 24 hour old culture of the organism to be tested. Each strain of staphylococcus was inoculated into a tube of 1% "Lab-Lemco" broth and incubated at 37°C. for a period of 6 hours. The 6 hour old cultures were used to inoculate the various media employed in this investigation of the ability of the strains to produce Muller's phenomenon. These broth cultures were then re-incubated at 37°C. until the following day when they were 24 hours old. They were then used in the tube coagulase test.

(ii) Muller's phenomenon.

(1) Media.

It was decided that the media used in this investigation of Muller's phenomenon should be milk-agar, with and without added serum. The haemoglobin-agar with added serum was as used by Elek (1953). The method of preparation of the various

media is given in App. 476 and 463.

(2) Inoculation and Incubation of Plates of Different Media.

In view of the large number of strains to be examined on these four different media it was found that the most convenient method of inoculating the plates was by means of the "stab" inoculum. This was made by touching the surface of the plate with the end of a straight platinum wire which had been charged from the 6 hour old "Lab-Lemco" broth culture of the organism to be tested. By using such an inoculum it was possible to test nine strains on one serum-milk-agar plate (10 cm. diameter) and seven strains on the smaller serum-haemoglobin-agar plates (9 cm. diameter). The control milk-agar and haemoglobin-agar plates were inoculated with nine and seven strains respectively.

The serum-milk-agar plates and the control milk-agar plates were incubated at 37°C. for 24 hours and thereafter at room temperature for 4 days. The serum-haemoglobin-agar plates and the control haemoglobin-agar plates were incubated at 37°C. for 2 days and thereafter at room temperature for 3 days.

(c) THE RESULTS OBTAINED FROM THE EXAMINATION OF THE 233 STRAINS OF STAPHYLOCOCCI.

The results of the examination of the 233 strains of staphylococci grown on the milk-agar and the haemoglobin-agar media are given in App. 384-395 and a summary of these results in App. 396. Forty-one of the strains were coagulase-negative and 192 coagulase-positive.

None of the 41 strains of coagulase-negative staphylococci produced plaques of clearing in either the serum-milk-agar, or in the serum-haemoglobin-agar media. They frequently produced a zone of precipitation on the serum-milk-agar plates and sometimes a narrow zone of lysis around the staphylococcal colony. This lysis, however, was completely different from that observed around colonies of coagulase-positive staphylococci, in that it was not clear cut and punched-out and lacked the sharp edge which the lysis around coagulase-positive strains consistently showed.

Of the 192 strains of coagulase-positive staphylococci, 185 produced plaques of clearing both in the serum-milk-agar and in the serum-haemoglobin-agar media. One other strain produced plaques only in the serum-haemoglobin-agar medium. The remaining 6 strains failed repeatedly to produce plaques in either of the media and their further investigation will be referred to in (d) below.

In serum-milk-agar most of the plaque-producing strains did so within 24 hours at 37°C.; and in the case of some, the plaques were visible as early as 18 hours. Few strains developed plaques for the first time after 2 days' incubation at 37°C., but those which had done so earlier showed an increase in the size and number of the plaques during the second day of incubation. Further development in the size and number of such plaques occurred subsequently when the plates were standing at room temperature, up to at least the fifth day after inoculation.

With some strains of staphylococci a zone of complete and sharply defined lysis of the casein developed around the colonies on the sera-containing medium and a halo of small plaques of further clearing developed subsequently at and beyond the edge of the completely lysed zone. With other strains the primary zone of complete lysis was absent and only a halo of small, more or less discrete plaques of clearing was visible around the colony. Sometimes this halo of plaques developed into a zone of complete lysis which tended to extend and around whose edge fresh crops of plaques appeared.

With still other strains neither a zone of complete lysis nor a halo of plaques but a broad band of precipitation (increased opacity of the serum-milk-agar) first developed around

the colonies. Later, complete lysis gradually occurred in this zone of precipitation with plaques of clearing appearing in and beyond its edge.

The development of plaques in serum-haemoglobin-agar plates was somewhat different. Clearing of the medium (diminution in the intensity of the red colour) began within 24 hours and increased up to 7 to 10 mm. or more in width by 48 hours. The majority of the 186 plaque-producing strains of coagulase-positive staphylococci had produced plaques after 48 hours' incubation, and these plaques increased in size and number up to at least the fifth day after inoculation. The remainder of the strains were rather slower in giving rise to plaque formation.

The 185 coagulase-positive strains of staphylococci which produced plaques both on* haemoglobin and on* milk-agar were retested on a number of occasions to determine if their ability to produce plaques was constant. The results were variable. In one such experiment in which all the strains were tested on both media, 42 failed to produce plaques on one or other of the media. After these 42 strains had been subcultured, however, in "Lab-Lemco" broth or on "Lab-Lemco" agar subsequent inoculation on to serum-milk-agar or serum-haemoglobin-agar resulted in plaque-formation.

The reason for this temporary loss or apparent loss

* i.e. serum-haemoglobin and serum-milk-agar.

of ability to produce plaques on a suitable substrate was not clear. It had been noted, however, that on occasions, when a plaque-producing strain of Staphylococcus aureus was plated out over the entire surface of a serum-milk-agar plate, so that single colonies of the organism were obtained, there were sometimes colonies which did not produce plaques or lysis although other colonies did so. It may be that in cultures of coagulase-positive staphylococci which invariably comprise a mixed population there are some cells which do not produce the staphylococcal factor necessary for Muller's phenomenon while other cells do so. Where a colony is grown from a stab inoculum, it may be that on occasions there is a predominance in the small inoculum of cells which do not produce the staphylococcal factor. Variation also with regard to the factor may occur in certain cultures. This question of variation is discussed more fully later on page 445.

When the coagulase-positive staphylococci were tested on control plates of milk-agar or haemoglobin-agar (without added serum), none of the strains ever showed plaque-formation. However, around the colonies of certain strains growing on milk-agar a narrow band of indefinite lysis with an indistinct edge developed. With still other strains the colonies were surrounded by a band of precipitation in the medium. The remainder of the strains had no

apparent effect on milk-agar. In the haemoglobin-agar medium the majority of the strains gave rise to a zone of lysis around the colonies. A number of strains produced an area of precipitation around the colonies while the remainder produced no alteration in the medium. It was thus confirmed that Muller's phenomenon could not be demonstrated on these two substrates except in the presence of added serum.

(d) THE COAGULASE-POSITIVE STRAINS WHICH INITIALLY AND REPEATEDLY FAILED TO PRODUCE MULLER'S PHENOMENON.

Six strains of coagulase-positive staphylococci which initially failed to produce Muller's phenomenon in serum-milk-agar or serum-haemoglobin-agar were further investigated. They had been isolated from the following pathological conditions:-

No. 33 - 34221 - Cataract.
No. 46 - 34471 - Ulcer.
No. 55 - 34772 - Burns.
No. 103 - 35035 - Meningitis.
No. 131 - 35240 - Burns.

Reference will also be made to one strain No. 59 - 34619 which had been isolated from an eye and which, although it failed to produce Muller's phenomenon on serum-milk-agar did so initially on serum-haemoglobin-agar.

A further strain No. 182 - P390 isolated during a survey at the Princess Mary Maternity Hospital will also be considered.

Fresh "Lab-Lemco" broth cultures of all eight strains were prepared from the original nutrient agar slopes on which they had been received. Tube coagulase tests carried out with these broth cultures confirmed that all eight strains were coagulase-positive. The broth cultures were then plated out on

serum-milk-agar and serum-haemoglobin-agar, one plate of each medium being used for each strain. No characteristic plaques of clearing developed on any of the plates after the customary incubation at 37°C. for 48 hours and subsequent holding at room temperature for five days or more.

Re-examination of these strains was carried out on at least six occasions during the following year and once or twice subsequently but never were they found to produce Muller's phenomenon on either serum-milk-agar or serum-haemoglobin-agar plates. The inocula used were "Lab-Lemco" broth cultures of different ages or suspensions in broth made from "Lab-Lemco" agar slopes or from the original nutrient agar slopes.

All the strains, in addition to being coagulase-positive, liquefied gelatin in 4-10 days, produced acid in mannitol, acid and clot in litmus milk, and haemolysis in 10% horse blood-agar. Strain No. 182 - P390, however, in addition to producing abundant haemolytic colonies on blood-agar also produced a few which were non-haemolytic. These latter were further investigated by subculturing from a single non-haemolytic colony into "Lab-Lemco" broth, incubating at 37°C. for 18 hours and then replating on blood-agar. This blood-agar plate yielded a pure growth of non-haemolytic staphylococci which proved to be coagulase-positive, and

which except for being non-haemolytic gave the same biochemical reactions as previously and as detailed above.

A wholly unexpected finding, however, was that this non-haemolytic strain in contrast to the haemolytic parent strain from which it had been derived gave rise to lysis and plaques when grown on serum-milk-agar. After many months it had thus been possible by selective subculturing to isolate from strain No. 182 - P390 which had consistently proved incapable of producing Muller's phenomenon, a variant capable of so doing.

Attempts to isolate similar variants from the other seven strains which initially and repeated failed to produce Muller's phenomenon in serum-milk-agar proved unsuccessful.

The strain No. 59 - 34619, which when first examined produced Muller's phenomenon only on serum-haemoglobin-agar was never again found to do so. It was replated on a number of occasions either directly from the original nutrient agar slope or from a subculture in "Lab-Lemco" broth, but consistently failed to produce plaques of clearing either on serum-milk-agar or on serum-haemoglobin-agar. The original serum-haemoglobin-agar culture showing Muller's phenomenon had been discarded and it was therefore impracticable to re-examine the actual growth which had given rise to the phenomenon.

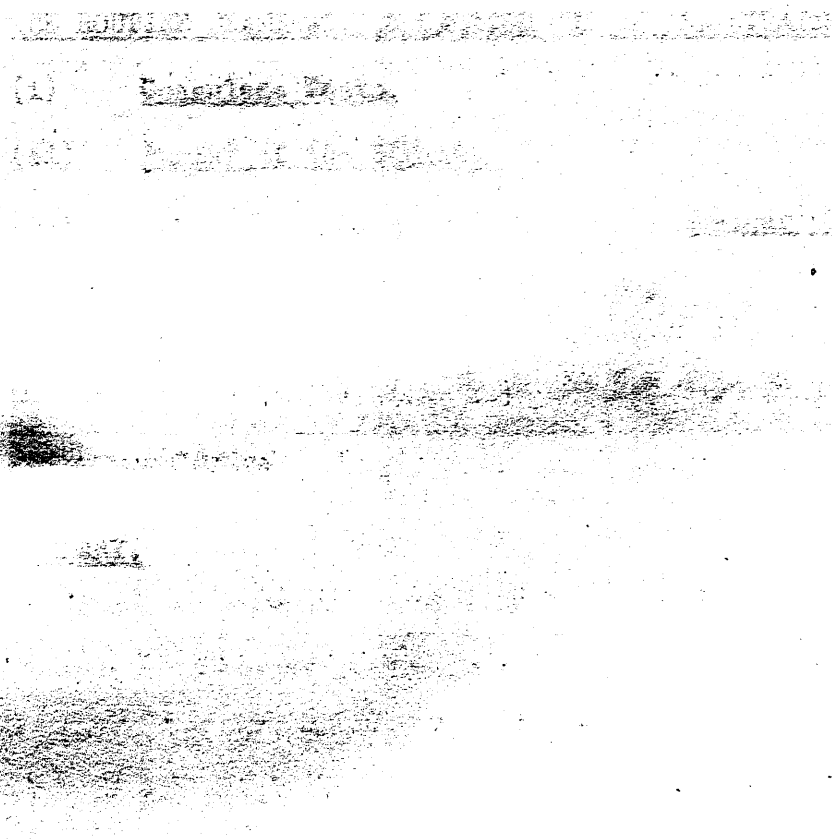
What is the explanation of this extraordinary set of findings? The original anomalous result might have been occasioned by a technical error of the type liable to occur when large numbers of strains are being handled. In view of the ease with which such calamities can occur, however, special care and attention had been paid to the accurate and adequate labelling of strains to prevent any confusion. The present worker therefore, while admitting that the occurrence of clerical or technical error might be the explanation of the anomalous result holds to the view that alternative explanations are more likely.

The fact that all staphylococcal cultures must be regarded as mixed populations has already been mentioned. It appears probable, therefore, that at any one time cultures have in them in varied proportions cells not capable of producing the factor necessary for Muller's phenomenon and other cells with this ability.

It is suggested that the cells in cultures of strain No. 59 - 34619 were regularly almost exclusively of "factor-negative" type and only very sparsely of the "factor-positive" type and that at the time of the original subculture into "Lab-Lemco" broth the platinum wire had picked up wholly by chance an inoculum in which factor-positive cells in inadequate numbers were present to ensure

their subsequent active growth.

The possibility too of variation accounting for the outcroping of "factor-positive" cells from an essentially "factor-negative" culture also merits consideration.



II. INVESTIGATION OF 116 STRAINS OF STAPHYLOCOCCI OBTAINED AS PRIMARY SUBCULTURES FROM THE DEPARTMENT OF BACTERIOLOGY, ROYAL VICTORIA INFIRMARY.

(a) THE STRAINS OF STAPHYLOCOCCI EXAMINED.

(b) THE ROUTINE EXAMINATIONS CARRIED OUT ON THE STRAINS.

- (i) Coagulase Tests.
- (ii) Purity of the Strains.
- (iii) The use of serum-milk-agar and Milk-agar plates.
- (iv) The results of the examinations.

(c) SPECIAL INVESTIGATION OF FIVE OF THE TEN STRAINS WHICH REPEATEDLY FAILED TO PRODUCE MULLER'S PHENOMENON ON SERUM-MILK-AGAR.

(d) SUMMARY.

II. INVESTIGATION OF 116 STRAINS OF STAPHYLOCOCCI OBTAINED AS PRIMARY SUBCULTURES FROM THE DEPARTMENT OF BACTERIOLOGY, ROYAL VICTORIA INFIRMARY.

None of the strains of staphylococci in the previous batch which had been examined for their ability to demonstrate Muller's phenomenon had been freshly isolated from pathological material, and the number of times they had been subcultured and the media used were unknown. Some of the strains had been collected in the course of the survey carried out at the Princess Mary Maternity Hospital and stored on nutrient agar for many months before being examined on serum-milk-agar plates.

The effect on the production of Muller's phenomenon by staphylococci which had been stored for varied periods on nutrient medium, or subcultured repeatedly on different media, being unknown, it appeared desirable to examine some freshly isolated strains of the organism.

Dr. C. A. Green, Department of Bacteriology, Royal Victoria Infirmary provided such freshly isolated strains of coagulase-positive staphylococci and thereby enabled this investigation of their ability to produce Muller's phenomenon to be carried out.

(a) THE STRAINS OF STAPHYLOCOCCI EXAMINED.

Specimens of pus from abscesses, infected wounds and the like submitted for bacteriological examination were inoculated on to 10% horse blood-agar plates. It was arranged that one colony from each strain of coagulase-positive staphylococci, developing on such plates would be picked into a tube of 1% "Lab-Lemco" broth which, after incubation at 37°C. for 18 hours, would be supplied for the purpose of investigating Muller's phenomenon on serum-milk-agar.

The total number of strains of coagulase positive staphylococci received in this way was 116. The origin of each strain, e.g. pus, sputum, is known and is recorded in App. 397-401.

(b) THE ROUTINE EXAMINATIONS CARRIED OUT ON THE STRAINS.

(i) Coagulase Tests.

A tube coagulase test was performed with the 18 hour old "Lab-Lemco" broth culture of each of the 116 strains supplied and all were found to be coagulase-positive.

One colony of each strain showing Muller's phenomenon on serum-milk-agar was also tested for coagulase production. In every instance the result was positive. Strains which did not produce Muller's phenomenon on serum-milk-agar were also retested and all were found to be coagulase-positive.

Confirmatory coagulase tests were carried out subsequently on all strains after they had been subcultured on to Dorset's egg medium for maintainance as stock cultures.

(ii) Purity of Strains.

The purity of each of the 116 strains as received was confirmed by plating out from the "Lab-Lemco" broth culture on to the serum-milk-agar and control plates used in studying Muller's phenomenon. All were found to be pure growths and

coagulase-positive and the overwhelming majority showed Muller's phenomenon.

The purity of the strains was subsequently re-checked by plating from stock cultures grown in bottles of Dorset's egg medium. These stock cultures were prepared in order to preserve the strains for any future investigation and each was a subculture from an isolated colony on the serum-milk-agar plate.

(iii) The use of Serum-milk-agar and Milk-agar plates.

The serum-milk-agar plates used in investigating Muller's phenomenon were made according to the method adopted as standard (App. 476) and contained 0.5 ml. of fresh serum and 2.0 ml. of milk, prepared from skim milk powder.

Each strain of staphylococci was inoculated onto one half of a serum-milk-agar plate by the method of a single continuous stroke; and in addition a stab inoculum of the strain was made into the medium at a place not involved by the stroke inoculum. In this way, it was possible to compare the production of Muller's phenomenon by any strain inoculated in two different ways on the same plate of medium.

Control plates consisting of milk-agar alone (without added serum) were inoculated at the same time but the stab inoculum was omitted in the case of these controls since Muller's phenomenon is not produced in the absence of serum.

The serum-milk-agar and the control plates were incubated at 37°C. for 24 hours when readings were made.

(iv) The results of the examinations.

The results of the examination of the 116 strains of coagulase-positive staphylococci grown on serum-milk-agar and milk-agar plates are given in App. 397-401 and summarised in App. 402.

In no case was Muller's phenomenon produced in the control plates of milk-agar although slight, indefinite lysis or a limited zone of precipitation usually developed.

One hundred and six of the 116 strains, i.e. 91.3% gave Muller's phenomenon on the serum-milk-agar plates with plaques of clearing, usually accompanied by a zone of definite lysis, around the growths. The phenomenon was equally apparent around the stroke and stab inoculations. In the case of 2 of these 106 strains, viz. Nos. 71060 and 72298 Muller's phenomenon was not

produced on the first occasion of their examination but was demonstrated when they were suitably retested.

The remaining 10 strains not only failed to produce Muller's phenomenon when first examined but consistently failed to do so throughout all subsequent investigation of them. One of the 10 caused slight indefinite lysis, without plaque-formation, but the remaining 9 brought about no apparent change in the test serum-milk-agar medium. Five of these 10 strains were subjected to special investigation as will now be recorded.

(c) SPECIAL INVESTIGATION OF FIVE OF THE TEN STRAINS OF STAPHYLOCOCCI WHICH REPEATEDLY FAILED TO PRODUCE MULLER'S PHENOMENON ON SERUM-MILK-AGAR.

Five of the ten strains of coagulase-positive staphylococci which had not produced Muller's phenomenon on the serum-milk-agar plates after incubation at 37°C. for 48 hours were subjected to a prolonged and more detailed investigation to discover if they would produce Muller's phenomenon under altered circumstances.

These five strains were Nos. 71090, 71273, 71706, 71709 and 71710. The original serum-milk-agar plates of these strains were closely examined after 48 hours' incubation at 37°C. and were found to have produced no change in the medium around their colonies. When, however, the plates were re-examined after they had been left at room temperature for a further 3 days, a slight degree of indefinite lysis was observed around the stab-inoculum of each strain. This indefinite lysis was not of the type associated with Muller's phenomenon but of the type found around the colonies of certain strains of coagulase-positive staphylococci growing on milk-agar without added serum. Such indefinite lysis which is usually only slight in degree is greatest next to the colony of the organism and merges almost imperceptibly

into the unaltered medium.

The original "Lab-Lemco" broth cultures of the 5 strains were now plated out on three different types of serum-milk-agar, but prior to this the coagulase test was repeated on each and found to be positive. One set of serum-milk-agar plates was prepared according to the standard method and a second and third set containing respectively 1.0 ml. and 1.5 ml. serum instead of the standard 0.5 ml. A strain of a coagulase-positive Staphylococcus aureus known to produce Muller's phenomenon was also used as a control and inoculated on these three types of serum-milk-agar which differed only in their serum content.

The control strain, as was to be expected, produced the characteristic plaques of Muller's phenomenon and a zone of sharply demarcated complete lysis around its growth in all three serum-milk-agar media but none of the 5 test strains gave plaque-formation on any of the media and what lysis occurred was again of the slight and indefinite type. Thus, increase in the serum content of the media had no influence in overcoming the inability of these 5 strains to produce Muller's phenomenon.

The Belgian workers Muller and Grégoire make it clear that some of their strains of staphylococci which did not produce plaques of clearing on human blood-agar plates at pH 7.2 - 7.4

would do so at pH 8.0 - 8.2. In view of this, it was decided to grow the 5 test strains on serum-milk-agar plates of different pH values.

The standard serum-milk-agar plate had a pH of 7.2. Three tubes of serum-milk-agar were prepared and just before they were poured into plates their pH values were adjusted to pH 7.0, 7.5 and 8.0 respectively by the addition of $\frac{N}{10}$ HCl or $\frac{N}{10}$ NaOH. The plates were dried at 37°C. and each inoculated from the original "Lab-Lemco" broth cultures of the 5 strains of staphylococci to be tested. In addition the same control strain as before of Staphylococcus aureus, was inoculated on to each of the plates.

The control strain gave lysis and plaques on all three plates but not one of the other 5 strains did so. Variation, therefore, of the hydrogen-ion concentration within the range used by the Belgian workers failed to enable any of the 5 test strains to produce Muller's phenomenon.

The original "Lab-Lemco" broth cultures of each of the 5 strains were next used to inoculate 10% horse blood-agar plates. Examination of the plates after 24 hours' incubation at 37°C. revealed that each of the strains had given rise to haemolytic and non-haemolytic colonies both of which were coagulase-positive.

Subcultures from each type of colony of each strain/in "Lab-Lemco" grown broth for 18 hours at 37°C. were used to inoculate serum-milk-agar plates by stab-inoculation. The growths from neither the haemolytic nor the non-haemolytic colonies of any of the 5 strains gave rise to Muller's phenomenon on the serum-milk-agar and none of the growths bred true when subsequently replated on horse blood-agar, i.e. both haemolytic and non-haemolytic colonies developed in all cases.

As it had been noticed that not only haemolytic and non-haemolytic colonies of these strains developed on horse blood-agar but also colonies which differed in colour - some being typically yellowish and others grey - those coloured-variant colonies were also subjected to examination comparable with that carried out on the haemolytic and non-haemolytic variants. All proved to be coagulase-positive but failed to give Muller's phenomenon on serum-milk-agar and failed to breed true. The fermentative characters of subcultures from the yellow and the grey colonies were also tested but no differences ever found.

In view of the failure of either the haemolytic and the non-haemolytic colonies or the yellowish and the grey colonies to breed true on subculture and in view of no subculture producing Muller's phenomenon on serum-milk-agar, this line of investigation was not pursued.

(d) SUMMARY.

In this series of 116 strains of coagulase-positive staphylococci 106 in all, i.e. 91.3% were found to produce Muller's phenomenon on a milk-agar substrate which contained human serum. The 10 strains which failed to produce the phenomenon were found to be unable to do so despite repeated re-examination carried out over a period of six months. Varied concentrations of serum in the serum-milk-agar plates exerted no influence on the inability of these 10 strains to produce the phenomenon, nor did alteration of the reaction of the medium. Colony variants - haemolytic and non-haemolytic, pigmented and non-pigmented - which arose from every one of these 10 strains also failed to produce the phenomenon.

III. INVESTIGATION OF STRAINS OF STAPHYLOCOCCUS AUREUS (COAGULASE-POSITIVE) DERIVED IMMEDIATELY FROM SPECIMENS SUBMITTED FOR BACTERIOLOGICAL EXAMINATION.

(a) ROUTINE EXAMINATIONS CARRIED OUT ON ALL THE SPECIMENS RECEIVED.

(i) Smears.

(ii) The use of serum-milk-agar and blood-agar plates.

(iii) Coagulase Tests.

(b) THE SPECIMENS EXAMINED.

(i) Number of specimens.

(ii) Nature of specimens.

(iii) Discarded specimens.

(c) THE RESULTS OF THE EXAMINATION OF 207 BACTERIOLOGICAL SPECIMENS CONTAINING STRAINS OF STAPHYLOCOCCUS AUREUS.

(d) CONSIDERATION OF CERTAIN DIFFICULTIES WHICH AROSE IN THE COURSE OF THE EXAMINATION OF THE 207 SPECIMENS.

(i) Paucity of colonies of Staphylococcus aureus.

(ii) The presence of other organisms.

(iii) Slide coagulase test.

(iv) Poor production of plaques by certain strains of Staphylococcus aureus.

(e) FIVE STRAINS OF STAPHYLOCOCCUS AUREUS WHICH FAILED TO PRODUCE MULLER'S PHENOMENON.

(f) THE ISOLATION AND EXAMINATION OF VARIANTS OF STRAINS OF STAPHYLOCOCCUS AUREUS.

(g) THE EXAMINATION OF 207 STRAINS OF STAPHYLOCOCCUS AUREUS ON DIFFERENT PROTEIN SUBSTRATES.

Milk-agar.

Heated-plasma-agar I.

Heated-plasma-agar II.

Heated-plasma-agar II with added fresh serum.

Heated-haemoglobin-agar.

Heated-haemoglobin-agar with added fresh serum.

(h) LIQUEFACTION OF GELATIN BY 202 STRAINS OF STAPHYLOCOCCUS AUREUS.

III. INVESTIGATION OF STRAINS OF STAPHYLOCOCCUS AUREUS (COAGULASE-POSITIVE) DERIVED IMMEDIATELY FROM SPECIMENS SUBMITTED FOR BACTERIOLOGICAL EXAMINATION.

The two previous batches of strains of staphylococci which had been examined with regard to their abilities to produce Muller's phenomenon had been obtained from the Bacteriological Department, Royal Victoria Infirmary, Newcastle upon Tyne. The first batch of strains of staphylococci had been received on slopes of a nutrient agar medium in bijou bottles in which they had been stored for some months. It was not known how often these organisms had been subcultured on bacteriological media during the period from their first isolation until the date of their storage. The second batch of staphylococci had been received in tubes of 1% "Lab-Lemco" broth into which they had been inoculated from a single colony of the strain growing on 10% horse blood-agar plates, which had been inoculated directly from the specimens. In the case of these two batches the strains had undergone at least two subcultures before their ability to produce Muller's phenomenon was tested and 96.3% and 91.3% respectively reacted positively. It was decided that it would be desirable to ascertain whether direct inoculation of serum-

milk-agar from pathological specimens, known to contain coagulase-positive staphylococci, would yield a comparable proportion of strains producing Muller's phenomenon since it seemed possible that repeated subculture of a strain might be associated with variation in its capacity to produce the phenomenon. Such direct inoculation of serum-milk-agar with specimens, some of which were from mixed infections, would be likely to yield information as to the value of the medium for differentiating between coagulase-positive staphylococci and other organisms.

The requisite specimens of pathological material were obtained through the co-operation of the Department of Bacteriology, Royal Victoria Infirmary, Newcastle upon Tyne, where their preliminary examination had been carried out. All specimens were known to have yielded growths of coagulase-positive staphylococci and the investigation of Muller's phenomenon was carried out on them within 4 days of their having been taken from the patients.

(a) ROUTINE EXAMINATIONS CARRIED OUT ON ALL THE SPECIMENS RECEIVED.

It was considered essential to standardise a procedure which could be carried out on all the specimens as they were received. On account of the fact that up to fifteen might arrive to be dealt with at any one time it was necessary that the procedure, although designed to give the maximum amount of information, should not be too complex or time consuming.

The hospital number of each specimen received was carefully noted in order that reference might be made to the hospital records should it be found desirable at a later date to obtain information about, say, the phage type or antibiotic sensitivity of the strain of Staphylococcus aureus isolated from it. The Infirmary laboratory recorded such information about all strains of staphylococci isolated by them.

The aim of this part of the investigation was as set out above, to ascertain the proportion of strains of coagulase-positive Staphylococcus aureus isolated directly from bacteriological specimens which gave rise to Muller's phenomenon and to obtain information about the value of serum-milk-agar as a medium for the isolation and identification of Staphylococcus aureus. With this

aim in view it was not difficult to devise a simple procedure which would be likely to afford the desired information.

(i) Smears.

Smears were made from each of the specimens and stained by Gram's method. Examination of the stained smears showed whether Gram positive cocci were abundant or otherwise and whether other organisms were also present and was found most helpful when the plates of media were being inoculated in that it gave some indication of whether a heavy or a light inoculum was required.

Smears from specimens other than swabs were made by spreading a loopful of the material on a glass slide. Smears from swabs were made on glass slides which had been sterilised by flaming and allowed to cool.

(ii) The use of serum-milk-agar and Blood-agar plates.

Each specimen was plated out on a serum-milk-agar and a 10% horse blood-agar plate. The preparation of these media is given in App. 476 and 456. A batch of pooled human serum had

been collected and suitably stored for the making of all the serum-milk-agar required for this part of the investigation. This ensured that all the strains of Staphylococcus aureus would be tested with the same serum for their ability to produce Muller's phenomenon.

The plates were incubated at 37°C. for 24 hours when readings were made.

On serum-milk-agar, a zone of lysis with in addition the characteristic halo of plaques of Muller's phenomenon developed around colonies of what were subsequently shown to be coagulase-positive staphylococci. In a small number of cases further incubation at 37°C. for a period of 24 hours was necessary before plaques were easily detected. In the great majority of cases colonies of staphylococci were sharply differentiated from those of other organisms on the plate by the appearance around the former of the halo of plaques. In a limited number of cases, plaques around colonies of staphylococci either developed only very scantily or were absent.

On horse blood-agar, colonies of what were subsequently shown to be coagulase-positive staphylococci did not produce the characteristic plaques of Muller's phenomenon. They were easily

distinguished from other organisms growing in the same plate such as coliforms, Pseudomonas pyocyanea and Proteus sp., but they did not produce changes in the blood medium which enabled them to be differentiated from colonies of coagulase-negative micrococci.

(iii) Coagulase Test.

It had initially been planned to carry out a slide coagulase test on colonies from both the serum-milk-agar and horse blood-agar plates that were suspected to be Staphylococcus aureus but this method was dropped and the tube coagulase test substituted as it was soon found that although Staphylococcus aureus growing on horse blood-agar invariably reacted positively to the slide coagulase test it sometimes failed to do so when growing on serum-milk-agar. The tube coagulase test which was carried out on all suspect colonies throughout this series had previously been found to give positive results with every strain of Staphylococcus aureus capable of producing Muller's phenomenon on serum-milk-agar.

A colony of the suspected Staphylococcus aureus was inoculated into a tube of 1% "Lab-Lemco" broth which was incubated

overnight at 37°C. The tube coagulase test was carried out with 0.5 ml. of this overnight "Lab-Lemco" broth culture by the method detailed in App. 492.

(b) THE SPECIMENS EXAMINED.

(i) Number of Specimens.

A total of 227 specimens were received over a period of some two months and from each of them a coagulase-positive Staphylococcus aureus, had been isolated in the Department of Bacteriology of the Infirmary. It was found, however, when the specimens were re-examined by me that coagulase-positive staphylococci were recoverable from only 207 of them. Twenty of the specimens were thus discarded leaving for the purpose of this investigation, which was concerned specifically with coagulase-positive staphylococci, a total of 207. The twenty discarded specimens are referred to below.

(ii) Nature of Specimens.

The nature of the 207 specimens is given in App. 403. More than half of them were swabs (128), while pus, sputum and urine accounted for 30, 21 and 27 respectively. One specimen was material obtained by gastric aspiration.

The origin of the 128 swabs is noted in App. 403. It will be seen that 38 of them were of "pus" from various sites;

the others were from "eye", "nose", "throat", "vagina" or "wound".

Although the specimens for examination had been kept in an ice-chest from the time they had been dealt with in the Infirmary laboratory until they were received by me it was noted that certain of them had dried up and were not in the best condition for the cultivation of whatever micro-organisms they had originally contained. It was found, however, that strains of Staphylococcus aureus could be isolated from the dried-up swabs without any great difficulty, if the swabs were moistened with 1% "Lab-Lemco" broth before they were used to inoculate plates.

The majority of the specimens yielded a good growth of Staphylococcus aureus, and such other viable organisms as they might contain, when inoculated on to serum-milk-agar and blood-agar; but a small number of specimens yielded only scanty growth on one or both of the media. Three specimens failed to give any growth on the blood-agar plates although so doing on the corresponding serum-milk-agar plates. Ten specimens which gave growth on both media yielded Staphylococcus aureus on the serum-milk-agar alone.

(iii) Discarded Specimens.

Twenty of the original 227 specimens were excluded from this series, for a number of reasons, the most important being that Staphylococcus aureus could not be isolated from them, when re-examined although it had been isolated previously in the Infirmary laboratory. These specimens are listed in App. 404.

Two of the specimens were from skin grafts and the plates of serum-milk-agar and blood-agar inoculated from each of them gave a profuse growth of Pseudomonas pyocyanea which spread the entire surface of the plates. What were almost certainly colonies of Staphylococcus aureus were seen on these overgrown plates but it was not considered worthwhile attempting to isolate pure cultures of the organism.

Two nasal swabs, one throat swab and a specimen of bloody purulent fluid failed to give growth on either the blood-agar or serum-milk-agar plates. Another throat swab yielded only streptococci, and micrococci which proved to be coagulase-negative. Three pus swabs and one wound swab, all of which were rather dried up as received, also gave mixed growths which did not include Staphylococcus aureus. It was noted in direct smears from these last four specimens that, although Gram positive cocci were present

in small numbers, they were "unhealthy" looking, suggesting that they were damaged or dead possibly as a result of chemotherapy.

Six specimens of sputum proved unsatisfactory and were discarded as, although they had been collected in waxed cartons, they had dried up almost completely. Three of them gave no growth of any kind and three yielded colonies of yeasts, micrococci, neisseriae and streptococci in addition to a heavy growth of coliforms. The failure to isolate Staphylococcus aureus from the last three specimens was probably due to the presence of the coliform organisms., since it has been found that recovery of staphylococci from mixtures containing coliforms becomes increasingly difficult with the passage of time. In the case of these three specimens, three days had elapsed between the isolation of staphylococci in the Infirmary laboratory and their re-examination by the author.

The one vaginal swab gave only scanty growth which consisted solely of organisms other than staphylococci and the remaining two specimens - one of urine and one of faeces -, although giving abundant growth, failed to yield any staphylococcal colonies.

(c) THE RESULT OF THE EXAMINATION OF 207 BACTERIOLOGICAL SPECIMENS CONTAINING STRAINS OF STAPHYLOCOCCUS AUREUS.

The 207 specimens, selected on account of the fact that they each contained a coagulase-positive Staphylococcus aureus were examined in the way indicated in (a) above. The results are given in detail in App. 405-415 and summarised in App. 416.

These findings may be epitomised as follows:- Staphylococcus aureus (coagulase-positive) grew on the serum-milk-agar plate from every one of the 207 specimens but on the horse blood-agar plate from only 194 of them. It was present alone in 104 of the 207 specimens and in the remaining 103, i.e. 50% it was admixed with organisms such as micrococci, coliforms, yeasts, Pseudomonas pyocyanea, Proteus sp., either singly or in combination. Twelve of the 13 specimens which failed to give growth of Staphylococcus aureus on horse blood-agar contained other organisms such as micrococci, coliforms, etc. which grew abundantly, not only on the horse blood-agar but also on the serum-milk-agar. The remaining specimen which contained only Staphylococcus aureus gave no growth of any kind on horse blood-agar although yielding several colonies of the staphylococcus (coagulase-positive) on serum-milk-agar. Thus in the case of these 13 specimens serum-milk-agar proved preferable to

horse blood-agar for the isolation of Staphylococcus aureus.

Muller's phenomenon which, as has already been mentioned does not develop on horse blood-agar, was given on the serum-milk-agar by the coagulase-positive staphylococci in 202 of the 207 specimens, i.e. in 97.6%. In the case of the remaining 5 specimens, whose coagulase-positive staphylococci failed to give the phenomenon, all except one were from mixed infections. The finding in the present series that 97.6% of strains of coagulase-positive Staphylococcus aureus growing on primary culture from bacteriological specimens, produced Muller's phenomenon, is of very great interest and will be further discussed later.

In addition to the main examination of the 207 specimens which was carried out by the standard method and whose results have just been referred to,46 of them were plated out on two special batches of serum-milk-agar. These special batches were prepared with pooled human sera ("serum 4" or "serum 7") and not with the serum ("test serum") of the standard medium which was used throughout the main examination. They were inoculated from the specimens at the same time as the standard medium. Three plates of serum-milk-agar were thus used for each of these 46 specimens (except No. 91364 for which a plate made with "serum 7"

was not available); and the plates differed only in respect of the sera used in their preparation. The results of this special examination are given in App. 417-418.

In the case of only 4 of the specimens was there any difference in the results obtained on the triplicate plates and these differences were in fact of no significance. Two of the four, Nos. 90912 and 90988 contained Staphylococcus aureus alone but only in very scanty numbers and in each instance the organism failed to grow on one of the three plates inoculated with the specimen. In the case of the other two, Nos. 90970 and 90974 the Staphylococcus aureus was heavily admixed with other organisms, including coliforms which are prone to interfere with the satisfactory growth of staphylococci. In each of these instances staphylococcal colonies were detected on only two of the three plates in the set. It would seem beyond reasonable doubt that in the case of each of these 4 specimens the failure of staphylococcal colonies to develop or to be detected on one of the three plates was the outcome of chance, relating to the size of inoculum or method of inoculation used.

In only one of the 46 specimens submitted to this additional examination did the infecting strain of staphylococcus fail to produce Muller's phenomenon and did so in all three plates.

Forty of the other 45 gave the phenomenon on all three plates; 3 gave the phenomenon on the two plates on which growth occurred; and 2 from cases of mixed infection gave the phenomenon around staphylococcal colonies on two of the plates, but staphylococcal colonies could not be detected amidst the heavy mixed growth on the third plate. Muller's phenomenon therefore occurred consistently with all 45 strains capable of producing it and was apparent on all three plates in the case of 40 of the specimens and on two plates in the case of each of the remaining 5. All these 45 strains are recorded with a "+" sign in the appropriate column of App. 417-418.

(d) CONSIDERATION OF CERTAIN DIFFICULTIES WHICH AROSE IN THE COURSE OF THE EXAMINATION OF THE 207 SPECIMENS.

There was not the slightest difficulty in recognising colonies of Staphylococcus aureus on serum-milk-agar plates from the majority of the 207 specimens. Around the colonies of these organisms a zone of plaques, or, more often, a zone of lysis with an outer ring of plaques of clearing, were easily visible. This was particularly true when the colonies were well dispersed over the surface of the plate. A well isolated colony of Staphylococcus aureus had the best opportunity of growing to its maximum size within the period of incubation of 24 hours and of bringing about this particular alteration in the serum-milk-agar medium. Thus, with specimens which contained only Staphylococcus aureus, and which, when plated out on this medium gave well isolated colonies, Muller's phenomenon was easily and strikingly recognisable.

Where on a plate of serum-milk-agar medium there was continuous growth, due to fusion of numerous colonies of Staphylococcus aureus, Muller's phenomenon could still be recognised although the appearance was not so striking as that seen around isolated colonies. At the edge of such growth there was usually to be seen a limited halo of plaques often associated with a narrow

zone of lysis but in certain instances plaques developed only in the medium underneath the growth and unassociated with any surrounding lysis. Such an appearance was reminiscent of Muller's original description of the phenomenon as observed by him on human blood-agar plates where the haemophagic spots of clearing lay beneath a "veil" of staphylococci.

With a small number of specimens, almost exclusively those with a mixed flora, Muller's phenomenon was not so easily observed on the serum-milk-agar. There were a number of reasons for this and these will now be discussed.

(i) Paucity of colonies of Staphylococcus aureus.

When plated out on serum-milk-agar a number of specimens gave rise to only a very few colonies of Staphylococcus aureus. If this organism was in pure culture no difficulty arose with regard to its recognition as its colonies developed to their full extent and were surrounded by easily seen plaques with or without a zone of lysis. Thus the appearance of one, five and eight colonies of Staphylococcus aureus on serum-milk-agar plates from three different specimens caused no difficulty with regard to

the recognition of the phenomenon and of the type of organism. When, however, similar small numbers of colonies of Staphylococcus aureus appeared on serum-milk-agar plates surrounded by many colonies of other organisms, recognition was not always so easy. Examination of such a plate by reflected and transmitted light would reveal colonies of Staphylococcus aureus if they were present. If the colonies were showing pigment, their recognition was greatly facilitated. All pigmented colonies were examined with particular care for the presence around them of lysis and plaques.

(ii) The presence of other organisms.

The presence on serum-milk-agar plates of organisms other than staphylococci did not usually hinder the recognition of these latter organisms. Only when the accompanying organisms were "spreaders" or when they produced lysis of the serum-milk-agar medium did any difficulty arise. On only one occasion when Pseudomonas pyocyanea had spread over the entire plate within the incubation period of 24 hours, was it necessary to replate a colony, suspected of being Staphylococcus aureus. On/this way was it possible to show that this strain of Staphylococcus aureus did in

in fact produce Muller's phenomenon.

In a few specimens, strains of Proteus vulgaris were present. They did not spread, or did not spread so rapidly, on the serum-milk-agar as on the blood-agar inoculated from the same specimen at the same time. These Proteus strains did not destroy the zones of lysis and plaques around colonies of Staphylococcus aureus which they had not overgrown showed Muller's phenomenon characteristically.

On a few occasions other organisms in mixed cultures produced an area of lysis around their colonies. This lysis was sometimes clear cut but at other times it was only partial and was never accompanied by plaque-formation. Except when the colonies of these lytic organisms were numerous and the colonies of Staphylococcus aureus few, was there any likelihood of missing the the appearance on the serum-milk-agar plate of Muller's phenomenon. No organism which produced lysis on a serum-milk-agar plate other than Staphylococcus aureus was ever found to produce the small clear-cut plaques of Muller's phenomenon. In addition, examination of the characters of the colony causing the lysis revealed whether or not it was in fact Staphylococcus aureus.

Certain organisms were found to inhibit the production of lysis and plaques by strains of Staphylococcus aureus. These

FIG. VII - The production of Muller's phenomenon in a standard serum-milk-agar plate inhibited by a strain of micrococcus. (Incubated at 37°C. for 24 hours.)

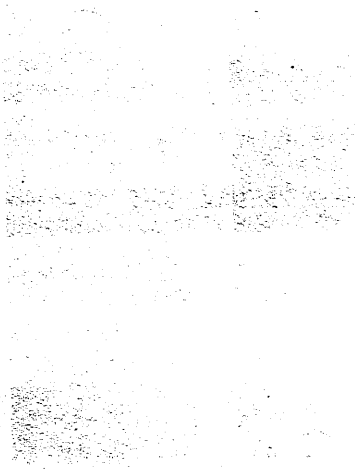


FIG. VIII - The production of Muller's phenomenon in a standard serum-milk-agar plate inhibited by a coliform strain. (Incubated at 37°C. for 24 hours.)

Fig. VII

Fig. VIII

organisms were strains of coliforms and of coagulase-negative micrococci. Specimen No. 90167, for example, a swab, when plated out on serum-milk-agar gave two undoubted colonies of Staphylococcus aureus, each of which showed a surrounding zone of lysis and plaques. In addition there were numerous unpigmented colonies of Gram positive cocci and a few of Gram negative bacilli none of which showed Muller's phenomenon. After incubation for a further period of 24 hours, however, some of these coccal colonies became pigmented, but plaque-formation remained totally in abeyance. On carrying out coagulase tests at this stage on representative pigmented and unpigmented coccal colonies, the former were found to be coagulase-positive and the latter coagulase-negative. Subcultures of the former on serum-milk-agar yielded pure growth of typical Staphylococcus aureus showing Muller's phenomenon. The non-pigmenting coagulase-negative Gram positive cocci were regarded as micrococci and it appeared that when they grew in close proximity to colonies of Staphylococcus aureus, they prevented the development of plaques around the latter. This inhibitory action of the Micrococcus on plaque-formation by the Staphylococcus was demonstrated by growing it at right-angles to the strain of Staphylococcus aureus on a serum-milk-agar plate and is shown in Fig. VII.

It was noted that when specimens containing coliforms in addition to Staphylococcus aureus were plated out on serum-milk-agar the latter rarely produced good areas of lysis and plaques around its colonies, especially when they were growing near colonies of the coliforms. Specimen No. 89228 for example, a swab inoculated on serum-milk-agar gave a heavy growth of coliforms and only three colonies of staphylococci. Around the staphylococcal colonies very poor plaque-production and only a trace of lysis developed. When this strain of staphylococcus was replated on serum-milk-agar a good demonstration of Muller's phenomenon was obtained with a distinct zone of plaques beyond a definite circle of lysis. The inhibitory action of the coliform organism on plaque-formation was demonstrated by making a stroke inoculation of it on a serum-milk-agar plate and at right-angles to this stroke making a stroke-inoculation of the Staphylococcus aureus. In addition a further stroke at right-angles to the coliform inoculation was made with another strain of Staphylococcus known to produce Muller's phenomenon particularly well. After incubation of the plate the growths of each of the staphylococci showed characteristic Muller's phenomenon except where they neared the growth of the coliform. This inhibitory effect of the coliform on Muller's phenomenon is illustrated in Fig. VIII.

Similar inhibitory action by micrococci and coliforms on plaque-formation by staphylococci has been encountered in the course of the present work on some six occasions.

(iii) Slide Coagulase Test.

Reference has already been made in (a) (iii) above to the tube coagulase test having been substituted for the slide coagulase test because the latter had proved unreliable in the case of growths from serum-milk-agar of certain strains of staphylococci. Some strains of staphylococci growing on this medium were found to be coagulase-positive when tested by the tube method but to give a negative or doubtful reaction by the slide method. These same strains from the corresponding horse blood-agar plates reacted positively in both methods of test. In some instances when with one colony from a serum-milk-agar plate the slide coagulase test gave a negative or doubtful result the testing of another colony from the same plate gave a positive result. With colonies from three coagulase-positive (tube method) staphylococcal strains among the first twenty-two specimens examined

the slide test was negative and consistently negative with different colonies taken from the same serum-milk-agar plate.

The explanation of these consistently negative results in the slide-test with staphylococci which reacted positively in the tube test was not ascertained but in view of what is known regarding the different natures of the factors determining slide and tube positivity - "bound coagulase" and "free coagulase" - it might be that serum-milk-agar was unfavourable for the production of "bound coagulase" by certain strains of staphylococci. This possibility gains support from the observation that these same strains which were slide coagulase-negative from serum-milk-agar reacted positively in this test from horse blood-agar when tested at the same time and with the same sample of human plasma. Apart altogether from the differences in the basic nutrient constituents of the two media they differed in that one contained human serum and the other oxalated horse blood.

May it not be that the failure of the slide coagulase test with certain strains of staphylococci known to be positive by the tube method, or even with certain colonies of a particular strain, can be explained on a quantitative basis, the production of "bound-coagulase" at a low level being inherent in certain strains

or resulting in others from variation occurring within a strain? Such a low level production might well be further decreased by the particular nature of the medium on which the organisms were growing, and for instance the human serum in the standard serum-milk-agar might of itself be responsible for modifying the production of "bound coagulase" in a way in which the horse blood in the blood-agar medium did not.

(iv) Poor production of plaques by certain strains of Staphylococcus aureus.

Plaque-production by the staphylococci growing on serum-milk-agar from the great majority of the 207 specimens was so striking as to be noted on the most casual inspection of the plates and in only about a dozen instances was more careful examination of the plates necessary in order to detect it. In three cases rather critical examination was needed to determine whether the minor alteration in the medium around the staphylococcal colonies was or was not attributable to the presence of plaque-formation.

The specimens concerned in these three cases were Nos. 89277, 89278 and 89407.

Specimens No. 89277 and No. 89278 were swabs from

the right and left nostril of the same patient. Each was inoculated on a serum-milk-agar plate which after 24 hours' incubation at 37°C. yielded a few colonies of Staphylococcus aureus. There were no areas of lysis around the colonies and only certain of them showed anything suggestive of plaque-formation. After a period of 24 hours at room temperature there was some slight increase in the size and number of the previously doubtful plaques.

One colony from each plate was subcultured in a 1% "Lab-Lemco" broth, which after incubation at 37°C. for 24 hours was plated on serum-milk-agar. This procedure was carried out in series five times and on each of the ten plates most of the colonies showed a few, but poorly developed surrounding plaques, but others failed completely to do so. On subsequent standing at room temperature some increase in the number or size of plaques occurred. When a colony not showing plaques was replated on serum-milk-agar, after growing in 1% "Lab-Lemco" broth it did not breed true. Some of the colonies showed limited plaque-formation, others did not.

These two strains throughout the period of their special observation thus proved to be consistently poor in their ability to give Muller's phenomenon on the standard serum-milk-agar. Great improvement, however, in plaque formation with or without accompanying lysis by these two strains was subsequently obtained

by growing them on a serum-milk-agar plate containing 1.5 ml. of the same serum as regularly used, instead of the standard amount of 0.5 ml.

Specimen No. 89407 like the preceding two yielded a growth on serum-milk-agar of Staphylococcus aureus whose colonies showed only limited and very poor plaque-formation. In contrast, however, to the preceding specimens subculturing from a colony into 1% "Lab-Lemco" broth and plating from the subculture on to serum-milk-agar gave a growth all of whose colonies showed characteristic Muller's phenomenon. In this case a change from poor to typically good plaque-formation resulted from simple subculturing. This strain of Staphylococcus aureus was also interesting in that it gave some minute colonies as well as those of normal size. These minute colonies which were coagulase-positive behaved comparably to the normal ones in their production of Muller's phenomenon.

There was one other specimen No. 90928 whose staphylococcus although not to be classed as a poor plaque-producer behaved in an unusual way. This strain growing on the primary serum-milk-agar plate yielded not only numerous colonies showing characteristic lysis and plaque formation but also some around which there was no detectable change in the medium. On removing

one of the latter from the plate it was found that there was lysis in the medium underneath the site of the colony. Replating on serum-milk-agar from a subculture of such a colony gave a mixture of colonies the majority showing typical Muller's phenomenon, but some producing no change except in the medium underneath the colonies.

Three strains of Staphylococcus aureus were thus encountered in this series which produced Muller's phenomenon on primary culture on serum-milk-agar either erratically or only very poorly. The poor plaque-production of one of these was overcome after subculture, but in the case of the other two serial subcultures proved ineffective although increasing the serum content of the medium enabled them to give typical Muller's phenomenon.

In addition to 5 of the 207 of Staphylococcus aureus in the present series which failed to produce Muller's phenomenon in primary culture and which are being referred to separately there were only 3 others which produced the phenomenon poorly. Each of the remaining 199 strains produced it characteristically. The reason for the poor production of Muller's phenomenon by the 3 strains in the primary cultures is not known. Since it proved possible to overcome this atypical behaviour of theirs either by repeated subculture or by increasing the serum content of the

substrate, it is obvious that the initial poor plaque production was not an inherent trait of the strains. Here again what seems probable is that variation and selection occurring in the mixed population of which any strain of Staphylococcus is composed accounts for the varied behaviour of these 3 strains.

This question of variation and selection will be considered in Part IX.

(e) FIVE STRAINS OF STAPHYLOCOCCUS AUREUS WHICH FAILED TO PRODUCE MULLER'S PHENOMENON.

Of the 207 strains of Staphylococcus aureus isolated from the bacteriological specimens available in this series, only 5 strains consistently failed to produce Muller's phenomenon on serum-milk-agar. These were from specimens No. 89182 (a pus swab), No. 89371 (a cough swab), No. 89466 (pus), No. 90389 (a pus swab), and No. 90942 (a vaginal swab). All 5 strains were Gram positive micrococci possessing all the usual attributes of Staphylococcus aureus - they produced coagulase, they fermented mannitol with the production of acid, they produced haemolysis on horse - or human - blood-agar plates and they liquefied gelatin. They did not, however, produce Muller's phenomenon on serum-milk-agar plates on any of the many occasions when as pure cultures they were inoculated on to this substrate. They were also inoculated, as pure cultures, on to serum-milk-agar plates, after repeated subculture in a number of different media, but on not one occasion did they produce any colonies which showed surrounding plaque formation. Three of the strains, however, did show around their colonies an area of faint indefinite clearing of the medium. This is the type of caseinolysis which as will be referred to later, is associated with the action of the protease of Staphylococcus aureus, an action which, in contrast to

that involved in producing Muller's phenomenon does not require the presence of human serum and is thus shown on milk-agar (no added serum).

Replating on serum-milk-agar from each of the original specimens, although again giving abundant growths of Staphylococcus aureus, either alone or mixed with other organisms did not yield a single colony showing Muller's phenomenon.

The 5 strains were submitted to phage typing by the Infirmary laboratory, from which the original specimens had been received, in order to ascertain if they were all of the same phage type or not. The result of this examination are given in App. 419. It was evident from the routine phage typing records of the Infirmary laboratory that the phage types of the 5 strains did not differ significantly from those of the staphylococci which were being regularly met with.

Thus these 5 strains differed from the commonly occurring coagulase-positive staphylococci only with regard to their inability to produce Muller's phenomenon on serum-milk-agar.

One point remains which seems to call for special mention. Four of these strains of Staphylococcus aureus which did not produce Muller's phenomenon had come from mixed cultures in which either micrococci or coliforms were present. It has

already been pointed out above ((d) (ii)) that micrococci or coliforms are sometimes able to inhibit the production of Muller's phenomenon by staphylococci when growing on the same plate close to the latter organisms. It may be that in certain circumstances a strain of a micrococcus or a coliform growing alone with a staphylococcus can cause some more or less permanent alteration in the biological activities of the latter so that it fails to produce the staphylococcal factor necessary for Muller's phenomenon.

(f) THE ISOLATION AND EXAMINATION OF VARIANTS OF STRAINS OF STAPHYLOCOCCUS AUREUS.

In the course of the present series, a number of staphylococcal variants were observed on the serum-milk-agar medium and some of these were subjected to further examination. One strain was found to yield yellow and white colonies both of which showed surrounding plaque formation while 3 strains gave only yellow colonies not all of which, however, produced Muller's phenomenon. These variants in pigment formation or in ability to produce Muller's phenomenon were found to be stable and to breed true on repeated replating over a long period and were to be distinguished from a number of other variants met with which, when replated always gave rise to a mixture of two types and which accordingly were not investigated further.

Specimen No. 89065 was a swab which when plated on serum-milk-agar gave rise to yellow and to white colonies. Both types of colony were coagulase-positive and both produced Muller's phenomenon. Replating of these two types on many subsequent occasions always resulted in the appearance of colonies which resembled the parent colonies in every way; a yellow colony being produced from a yellow parent, a white from a white parent and both

FIG. IX - Staphylococcus aureus, Strain 88592 plated on a standard serum-milk-agar plate. Many colonies show Muller's phenomenon (plaque-formation) and lysis. A few colonies show no alteration of the surrounding medium. (Incubated at 37°C. for 24 hours.)

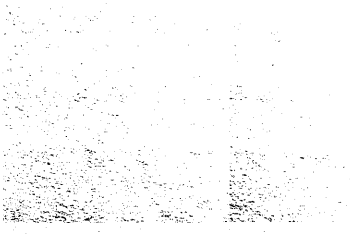


FIG. X - A specimen of pus plated on a standard serum-milk-agar plate. A pure culture of Staphylococcus aureus has been obtained and all colonies show Muller's phenomenon (plaque-formation) and lysis. (Incubated at 37°C. for 24 hours.)

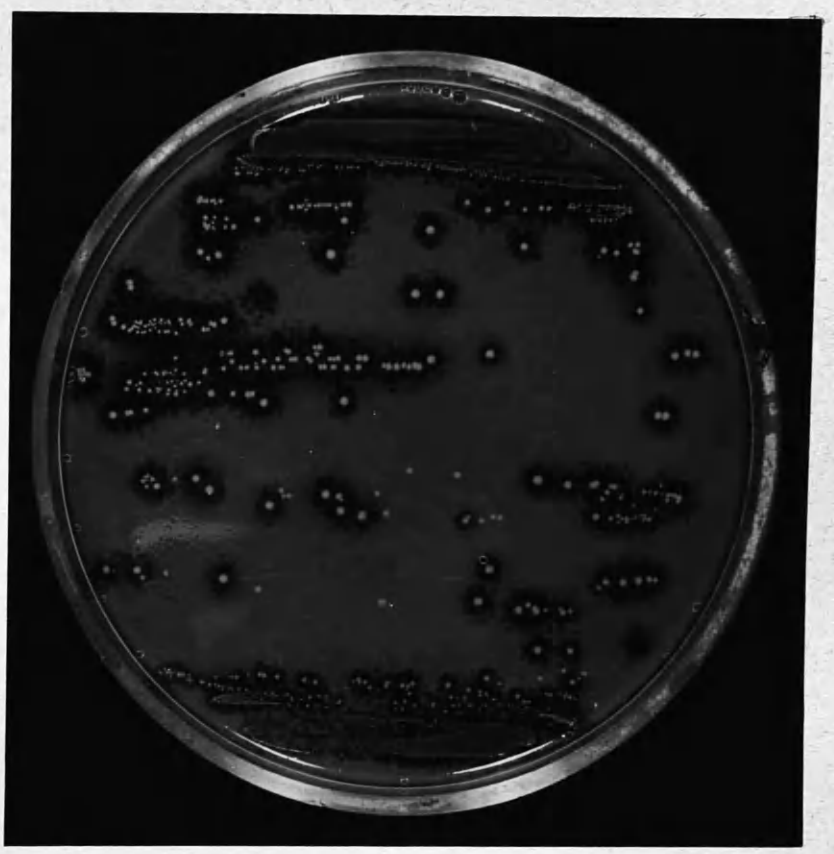


Fig. IX

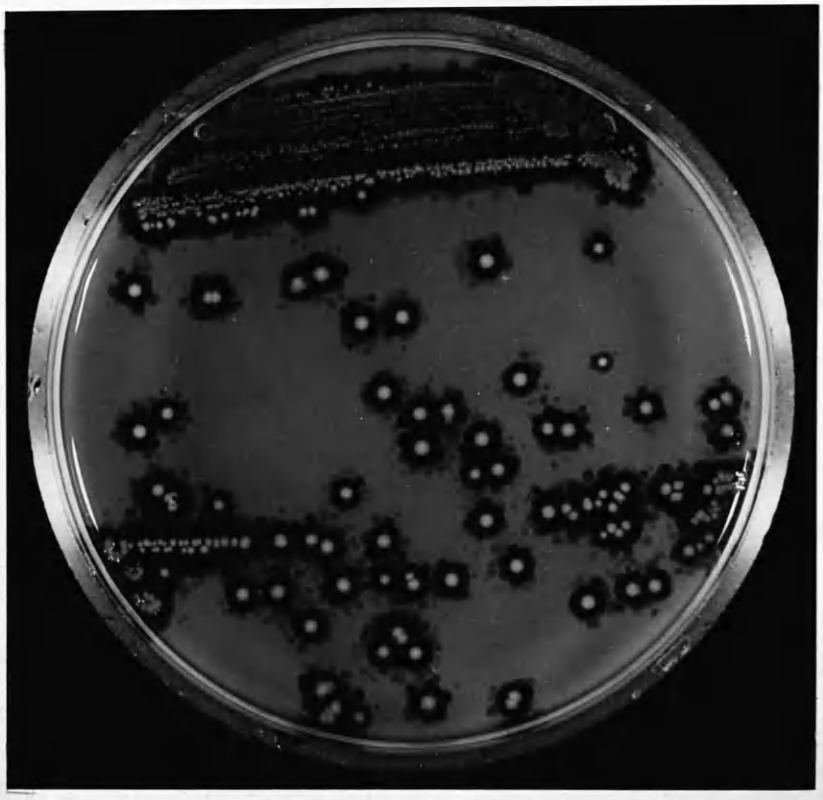


Fig. X

types giving Muller's phenomenon. No differences were found in the biochemical activities of the two types (App. 420).

Specimen No. 88592 was pus from an abscess and when plated on serum-milk-agar gave rise to colonies of Staphylococcus aureus, which, although they were similar in appearance, differed in respect of their effect on the medium immediately surrounding them (Fig. IX). Most of the colonies produced Muller's phenomenon - plaques of clearing accompanied by a zone of lysis. The medium around others was completely unchanged. Both types of colony were coagulase-positive and gave the same biochemical reactions (App. 420). In this instance again, each type of colony bred true over a lengthy period of observation, the one consistently failing to cause any change in the medium, the other uniformly producing Muller's phenomenon.

Specimen No. 91343 was a pus swab and when plated out on three plates of serum-milk-agar, each of which had been prepared with a different batch of serum, gave rise on each plate to two types of colony of Staphylococcus aureus, one showing Muller's phenomenon, the other producing no change in the medium. Both types gave the same biochemical reactions (App. 420) and bred true on numerous subsequent replatings.

Specimen No. 91587 was a wound swab which when plated

out on serum-milk-agar gave rise to two types of coagulase-positive staphylococci, one of which showed Muller's phenomenon, the other showed only slight indefinite lysis with a zone of precipitation around its colonies. Again the biochemical activities of these two types were the same (App. 420) and both bred true over a lengthy period of observation.

In the case of all of the above specimens, the antibiotic sensitivity patterns - and in the case of three of the specimens, the phage pattern - of the two types of colony which each had yielded, were investigated, and compared with the results obtained by the Infirmary laboratory with the strain which they had originally isolated from the corresponding specimen. This comparison is given in App. 421 and 422 from which it can be seen that the antibiotic sensitivity patterns and the phage patterns of the original strains isolated by the Infirmary laboratory do not differ significantly from those obtained with their variants isolated in the course of the present study.

The actions of the two variant strains isolated from each of the specimens Nos. 88592, 91343 and 91587 were tested out on protein substrates other than serum-milk-agar with a view to comparing their ability to produce lysis or plaques or both on these special media. The results are shown in App. 423. The

special substrates were heated-plasma-agar I; heated-plasma agar II; heated-plasma-agar II with added fresh serum; heated-haemoglobin-agar; and heated-haemoglobin-agar with added fresh serum.

On heated-plasma-agar I, the 3 strains which gave lysis and plaques on serum-milk-agar gave only lysis; while the 3 strains which on serum-milk-agar which produced no apparent change behaved similarly on the plasma-agar or produced only slight precipitation. On heated-plasma-agar II none of the strains produced either lysis or plaques; but on this medium with fresh serum incorporated lysis and plaques were produced by the 3 strains which did so on serum-milk-agar. The addition of fresh serum to heated-plasma-agar II was without effect on the action of the other 3 strains. On heated-haemoglobin-agar the 2 strains from specimen No. 91587 each caused slight lysis while the strains from the other specimens were without effect on this medium. The addition of fresh serum to haemoglobin-agar medium resulted in plaque-formation with or without lysis by the 3 strains which behaved similarly on serum-milk-agar, but was without such effect in the case of the remaining 3 strains. They produced no change in the serum containing medium.

The important finding from these results is the confirmation that plaque-formation (Muller's phenomenon) does not occur on any of the substrates used - milk, heated plasma or heated haemoglobin - in the absence of fresh serum. A further finding was that the variants which did not produce Muller's phenomenon on serum-milk-agar were also incapable of so doing on the other serum - containing substrates used.

(g) THE EXAMINATION OF 207 STRAINS OF STAPHYLOCOCCUS AUREUS ON DIFFERENT PROTEIN SUBSTRATES.

The whole of the 207 strains of coagulase-positive staphylococci of the present series were subsequently tested out on the same special protein substrates as have just been referred to and also on milk-agar, i.e. serum-milk-agar without added serum. Plates of each of these special media were inoculated directly from the stock cultures of the staphylococci on Dorset's egg medium, incubated at 37°C. for 48 hours and examined after 24 hours' and 48 hours' incubation. The readings after 48 hours' incubation are shown in App. 424-434 and may be summarised as follows:-

Milk-Agar:- The 207 strains differed in no way in their action on milk-agar from that observed in the case of other staphylococci and already recorded in Part III, I (c) and II (b) (iv) above. Plaque-formation (Muller's phenomenon) never occurred, and lysis, if present, was of the slight and indefinite type. Many of the strains produced no change in the medium and a few caused some slight precipitation around their colonies.

These results confirmed that Muller's phenomenon does not develop on milk-agar in the absence of added serum.

Heated-Plasma-Agar I:- Only 202 of the 207 strains

grew when subcultures on to this medium (App. 479), the stock cultures of the other 5 having died out. The medium is opaque owing to the "heat-precipitated fibrinogen" which it contains and around the colonies of 197 of the 202 strains some clearing of the opacity developed. Such clearing is usually referred to as fibrinolysis. These strains were the ones which gave Muller's phenomenon - on serum-milk-agar. The remaining 5 strains which not only failed to produce any clearing of the opacity but actually caused increased precipitation were the strains which had not given Muller's phenomenon on serum-milk-agar (App. 435).

These findings are in keeping with the observation of Christie, Graydon and Woods (1945) viz. that 37 fibrinolytic strains of staphylococci produced Muller's phenomenon while none of their 42 non-fibrinolytic strains were able to do so.

The question of the relationship of the ability of coagulase-positive strains of staphylococci to produce fibrinolysis with that to produce Muller's phenomenon is a most important one and will be discussed in Part IX. On heated-plasma-agar I fibrinolysis occurred with every one of the strains known to be able to give Muller's phenomenon.

Heated-Plasma-Agar II:- This medium differs from the

preceding one only in that its content of plasma is 25% as opposed to 15% and that the temperature of heating is 65°C. as opposed to 56°C. It is more opaque than heated-plasma-agar I, thus enabling any clearing which might develop around staphylococcal growth to be more easily seen and its mode of preparation is given in App. 479.

Only 204 of the 207 strains grew when subcultured on to this medium, the stock cultures of the other 3 having died out. Around the colonies of only 36 of the 204 strains which grew, a narrow zone of lysis developed and a further 5 strains caused some slight precipitation in the medium, the remaining 163 strains produced no detectable change. One hundred and ninety-nine of the 204 strains were known to give Muller's phenomenon on the appropriate substrate (serum-milk-agar); but, in contrast to what had been observed with their growths on heated-plasma-agar I, only 37 of them produced any change on heated-plasma-agar II. Thirty-six showed a narrow zone of lysis around their colonies and one produced slight precipitation. Four of the 5 strains known not to give Muller's phenomenon also caused some change (slight precipitation) in the latter medium. (See abridged Table - App. 436.)

With regard to the possible relationship of

fibrinolytic activity on the part of staphylococci with their ability to give Muller's phenomenon it is to be noted that on heated-plasma-agar II only a minority of the strains known to give Muller's phenomenon showed any fibrinolysis.

Heated-Plasma-Agar II with added fresh serum:-

This medium is the same as the preceding but with the addition of fresh human serum and was used in parallel with it and with the same 204 strains of staphylococci (App. 480).

Every one of the 199 strains known to be able to give Muller's phenomenon produced an area of lysis around its colonies, (as they had done when grown previously on heated-plasma-agar I), and 75 of them showed in addition plaque-formation, i.e. only 37.5% of the strains giving Muller's phenomenon on serum-milk-agar did so on this heated-plasma medium. Neither lysis nor plaque-formation was produced by any of the 5 strains known to be incapable of giving Muller's phenomenon. (See abridged Table - App. 437).

A noteworthy observation in the case of the use of this medium is that the incorporation of fresh human serum in heated-plasma-agar II results in fibrinolysis occurring with every one of the 199 strains of staphylococcus known to give Muller's phenomenon, although only 36 of them caused any lysis on the same

basic medium without added serum.

Heated-Haemoglobin-Agar:- Only 201 strains of this series were grown on this medium, the stock-cultures of the other 6 having died out.

A narrow zone of lysis developed around colonies of 115 of the strains, and no apparent change in the medium was produced by the remaining 86.

There was no correlation between the lytic activities of the staphylococci on this medium and their lytic activities on the others.

The results are included in App. 424-434 and the preparation of the medium is given in App. 465.

Heated-Haemoglobin-Agar with Added Fresh Serum:- This medium is the same as the preceding but with the addition of fresh human serum and was used in parallel with it and with the same 201 strains of staphylococci (App. 466).

Every one of the 196 strains known to be able to give Muller's phenomenon produced plaques and the plaque-formation was associated in the case of some of them with confluent lysis. The remaining 5 strains were known to be incapable of giving Muller's phenomenon but one of them produced a very narrow zone of partial

lysis around its growth. This partial lysis was quite different in its appearance from the lysis sometimes associated with plaque-formation. The remaining 4 strains produced no apparent change in the medium.

The results are included in App. 424-434 and in abridged form in App. 438.

Media containing heated haemoglobin do not appear to have been used before for the demonstration or study of Muller's phenomenon and the heated-haemoglobin-agar employed during the present work has been found to be fully as satisfactory as serum-milk-agar for this purpose and more satisfactory than media containing unheated haemoglobin.

(h) LIQUEFACTION OF GELATIN BY 202 STRAINS OF STAPHYLOCOCCUS AUREUS.

The liquefaction of gelatin by 202 of the 207 strains of Staphylococcus aureus constituting the present series was also investigated, by way of completing as far as possible the data relating to the strains and also in case there might be any relation between ability to liquefy gelatin and to produce Muller's phenomenon.

Stab-inoculations of each of the strains were made in 15% nutrient gelatin (App. 459) from the stock-cultures on Dorset's egg medium. The gelatin cultures together with a number of uninoculated tubes of gelatin, to serve as controls, were incubated at 37°C. for up to 14 days. The tubes were removed from the incubator after 4, 7 and 10 days and on each occasion were placed in the ice-chest for 3 hours, after which any which failed to gel were recorded as positive (+) and discarded. The others, with the controls were returned to the incubator for further incubation. The final examination was thus made on tubes which had been incubated in all for 14 days and which had not shown liquefaction by the tenth day.

Since uninoculated but incubated tubes of gelatin (controls) set to a gel within 30 minutes in the ice-chest a note

was made on each occasion of any cultures which were still fluid at this stage. Some subcultures were found to have gelled by the end of the period of observation (3 hours); while others remained fluid. Only the latter were discarded. The former were regarded, provided that they behaved in this way when examined after the full 14 days of incubation as cultures of strains which although not liquefiers, had some action on gelatin.

The results are shown in App. 439-447. One hundred and forty-six of the strains were gelatin liquefiers; 52 showed the modified action referred to; and only 4 strains proved to be non-liquefiers.

There was no correlation between these findings and the ability of the strains to produce Muller's phenomenon.

The method of testing for gelatin-liquefaction used in this investigation had been investigated previously on a number of occasions and had been found satisfactory. One experiment in which twelve strains of coagulase-positive staphylococci had been examined in 15% nutrient gelatin and in which tubes of this medium, 2% to 15% were used as controls, is given in App. 23-29.

IV. DISCUSSION.

The present discussion is concerned with the investigations carried out with the 556 strains of staphylococci, included in the preceding three sections of Part III of this thesis.

One of the two main objects of the investigation was to ascertain how commonly the ability to produce Muller's phenomenon is to be found in strains of staphylococci. Muller himself and some of the early workers believed that only very few strains possessed the ability, although Burnet (1928) was of the opinion that "aureus" strains isolated from actual cases of infection could generally produce this effect. More recently Elek (1953) reported that 15 coagulase-positive strains examined by him produced Muller's phenomenon but that some coagulase-positive strains failed to do so. He also found that coagulase-negative strains failed to do so. It did appear important therefore to find out how general was this ability of strains of staphylococci to produce Muller's phenomenon and how intimately it

was associated with the production of staphylocoagulase.

The other main object of this investigation was to confirm the author's belief that the small circular areas or plaques of clearing which developed around colonies of staphylococci growing on serum-milk-agar plates, as described in the experiments in Part I, were a manifestation of Muller's phenomenon. Accordingly a large number of strains of staphylococci were grown in parallel on a medium recognised as a substrate on which Muller's phenomenon could be demonstrated and on serum-milk-agar. If the strains of staphylococci which gave Muller's phenomenon on an orthodox substrate also produced the characteristic areas of clearing on serum-milk-agar there could be little doubt that serum-milk-agar was a suitable substrate for the demonstration of the phenomenon.

Five hundred and fifteen of the 556 strains examined were coagulase-positive, and 41 coagulase-negative.

The sources and nature of the strains, including their ages and the media on which they had been maintained, are detailed in the appropriate sections and the results summarised.

Only one of the 186 strains of coagulase-positive staphylococci which gave Muller's phenomenon on an orthodox substrate on the first occasion of examination failed to do so in the parallel culture on serum-milk-agar. On all subsequent

examinations this strain failed to give the phenomenon on either substrate. The explanation of this isolated instance of any discrepancy between the results obtained on the two substrates remains obscure but there would appear to be no need to regard the finding as other than quite exceptional, and possibly though not probably due to technical error. It certainly does not detract from the reliability of serum-milk-agar as a suitable substrate for the demonstration of Muller's phenomenon.

One hundred and eighty-five of the 186 strains showed the phenomenon on each of the media and indeed rather better on the serum-milk-agar; while a further 6 strains, although coagulase-positive, failed to give the phenomenon on either medium. Moreover with the 41 strains of coagulase-negative staphylococci tested in parallel on the same two media Muller's phenomenon was absent in all instances.

In view therefore of the proven suitability of serum-milk-agar for the special purpose, the study of the production of Muller's phenomenon by a further 323 strains of coagulase-positive staphylococci was carried out on this medium. Thus the total number of coagulase-positive staphylococci investigated was 515. Only 21 of these failed to produce Muller's phenomenon, i.e. 4.1%. Thus, although not every strain of coagulase-positive staphylococcus

gives the phenomenon, the overwhelming majority do, and no strain of coagulase-negative staphylococcus has yet been met with which can do so.

Attention may also be directed here to the fact that Muller's phenomenon was not produced by any of the organisms other than staphylococci which were encountered in the course of the work. These other organisms included streptococci, coliforms, Pseudomonas pyocyanea, Proteus vulgaris, anthracoids, diphtheroids and yeasts.

Full confirmation was obtained, under controlled conditions and using four different media, of the observation by earlier workers that the presence of fresh serum in the substrate is essential for the production of Muller's phenomenon. The four media used were milk-agar, haemoglobin-agar, heated-haemoglobin-agar and heated plasma-agar.

As regards the 21 strains of coagulase-positive staphylococci which proved consistently incapable of producing Muller's phenomenon (Muller - negative), the question arises as to why these strains lacked this ability. May it be that Muller negativity is to be regarded as a result of variation having occurred in a character of staphylococcus comparable with the occasional variation in other characters of the organism, e.g.

pigment formation, fibrinolytic activity, haemolysin production, which is also usually of stable type?

A final point relates to the serviceability of serum-milk-agar as a routine medium for the isolation of Staphylococcus aureus from specimens which may contain this organism. It has been found that the staphylococcus, whether present alone or associated with other organisms, grows particularly well on this medium, pigment production being easily detectable against the whitish substrate and the colonies being surrounded by the characteristic plaques of Muller's phenomenon (Fig. X). Only 4% of strains fail to show the Muller effect and might be missed unless submitted to coagulase-testing but the 96% which show the effect can be identified at once as Staphylococcus aureus by direct inspection of the plate and without having recourse to any coagulase test, since they would be bound to react positively in it.

The preparation of serum-milk-agar is no more complicated than that of blood-agar and the growth on the former of staphylococci, streptococci and other common pyogenic organisms is not surpassed by that obtained on the latter. It is not suggested that serum-milk-agar can replace blood-agar for all purposes but it has the advantage over blood-agar that in the

identification of Staphylococcus aureus the coagulase-test can be dispensed with.

Serum-milk-agar, moreover, keeps well and plates prepared 7-10 days previously and stored at 4°C. have proved satisfactory for purposes of Muller's phenomenon.

PART IV.

INVESTIGATION OF THE CONDITIONS INFLUENCING THE PRODUCTION OF MULLER'S PHENOMENON BY STAPHYLOCOCCI.

I. MILK IN SUBSTRATE.

(a) AMOUNT AND TYPE OF MILK.

(b) METHODS OF INOCULATION OF PLATES.

(c) THE INFLUENCE OF SERUM.

(d) THE EFFECTS OF VARYING THE TEMPERATURE OF INCUBATION, THE GASEOUS ENVIRONMENT AND OF ALTERING THE CHARACTERS OF THE GEL.

(e) THE EFFECTS OF VARIOUS NUTRIENTS.

(f) SOME MISCELLANEOUS EXPERIMENTS.

[illegible] ml. in a total volume [illegible]

[illegible] ml. of 1% "Lab-lem[illegible]

[illegible] the mixture [illegible]

I. MILK IN SUBSTRATE.

(a) AMOUNT AND TYPE OF MILK.

The concentration of milk in the serum-milk-agar plates used by Fisk and Mordvin (1943) was 30% and this concentration was employed in some of the preliminary experiments reported in Part I. Included in these experiments was one in which a concentration of milk of the order of 10% was used and found to be satisfactory for the demonstration of Muller's phenomenon. 20% and 25% milk had been incorporated in the medium in the course of other experiments and had been found to be equally satisfactory. It did seem, however, to be worthwhile to ascertain the optimal concentration of milk for the demonstration of Muller's phenomenon in order that the preparation of serum-milk-agar might be standardised.

Concentrations of milk from 3.3% to 40%, i.e. 0.5 ml. to 6.0 ml. in a total volume of 15 ml. with 1.0 ml. human serum and 1.0 ml. of 15% "Lab-Lemco" were incorporated in a plain agar base and the mixtures poured as plates. This series of plates was

stab-inoculated with six strains of staphylococci and incubated at 37°C. for 24 hours. Readings were made at this time and again after the plates had been at room temperature for a further 48 hours (App. 30-31).

Muller's phenomenon was shown by all six strains with all the concentrations of milk used. Except in the plate containing 3.3% milk the characteristic plaques were discrete, distinct and well-developed. The plate containing 3.3% milk, had a zone of complete lysis around each colony and the edge of this zone of lysis had its regularity broken by plaques impinging on it - a "rough edge" - but no discrete plaques of clearing could be seen in the medium beyond this irregular edge.

The decision as to the optimal concentration of milk for the demonstration of the phenomenon was influenced by two considerations viz. the development of isolated plaques and the ease with which they could be seen. Concentrations of milk below 13.3%, although associated with good plaque formation, gave media which were deficient in opacity and provided little contrast between unaltered medium and areas which were lysed as a result of plaque-formation. Concentrations of milk above 25% gave a medium in which any lysis of the milk was not so apparent as in plates with lower concentrations. The concentrations of milk from 13.3% to 25%

gave good and easily visible demonstrations of Muller's phenomenon and this range 13.3% to 25% was accepted as optimal.

As a routine it was decided to use the lowest concentration of milk in the optimal range, viz. 13.3%. Not only did this permit good development of easily detected plaques but it also allowed greater latitude for varying the amounts or nature of the other constituents of the serum-milk-agar mixtures whose volume was restricted to 15 ml. for each plate.

Cow's milk had been used in earlier experiments on Muller's phenomenon. This milk bought by the pint was skimmed and distributed in 20 ml. amounts in Universal containers and sterilised by autoclaving at 15 lbs. pressure/sq. in. for 15 minutes. Such milk had proved satisfactory for the production of the phenomenon. It did seem, however, that if the available commercial powdered skimmed milk should prove equally satisfactory a number of advantages would accrue from its use. For example, even minor differences in different samples of liquid milk might possibly have some effect on Muller's phenomenon, and it was not practicable to lay in and conserve unchanged, an adequate supply of one and the same batch of bottled liquid milk to serve for the whole of the projected experiments. On the other hand there was no difficulty in obtaining an ample supply of a particular makers, Skim Milk Powder,

all of the same manufacturing batch and to reconstitute from this at any time in amounts as required.

Accordingly several pounds of one batch of Oxoid Skim Milk Powder was purchased and the suitability of milk reconstituted from this powder as a substitute for raw cow's milk (skimmed) was investigated. A batch of serum-milk-agar was prepared with liquid cow's milk and this served as the standard for comparison. Two batches of serum-milk-agar were prepared with milk reconstituted from skim milk powder, the reconstituted milk for one having been sterilised by autoclaving at 15 lbs./sq. in for 15 minutes, for the other by steaming it at 100°C. for 20 minutes on each of three successive days.

Seven strains of coagulase-positive staphylococci were stab-inoculated into plates of each of the three media. The plates were incubated at 37°C. for 24 hours when the results were read (App. 32-34). The whole experiment was carried out in triplicate. There was no significant difference in the results obtained with the three media. Accordingly the use of raw cow's milk (skimmed) in the preparation of serum-milk-agar was discontinued.

(b) METHODS OF INOCULATION OF PLATES.

"L'hémophagie staphylococcique" was obtained by Muller and his associates by covering the entire surface of a blood-agar plate with a veil of growth of staphylococci. The plaques of clearing could be seen in the medium beneath this veil. In many of the early experiments carried out by the present worker single stroke inoculations resulting in confluent growth were used while in others single stab-inoculations were made. This latter method of inoculation permits the testing of up to 9 strains in a Petri-plate, 9 cm. in diameter, and yields a single colony of growth at each site. Although both these methods had been employed successfully it was thought desirable to carry out a controlled comparison of them and of other methods of inoculation in order to standardise future work.

Two sets of serum-milk-agar plates were prepared the one containing 0.5 ml., the other 1.0 ml. of serum per plate. Six strains of coagulase-positive staphylococci were used and each of these was inoculated from a "Lab-Lemco" broth culture on to a plate of each of the sets. The methods of inoculation were (1) a single-stroke-inoculation, (2) stab-inoculation, and (3) a loopful of culture deposited on the surface of the medium. In the case of

FIG. XI - Demonstration of Muller's phenomenon. Different methods of inoculation - a stab, a streak and a loop inoculum of Staphylococcus aureus, Strain A on a serum-milk-agar plate containing 0.5 ml. of serum. (After 24 hours' incubation at 37°C.)

FIG. XII - Demonstration of Muller's phenomenon. A loop-ful of a 6 hour old "Lab-Lemco" broth culture of Staphylococcus aureus, Strain A spread over the surface of a serum-milk-agar plate containing 0.5 ml. of serum. (After 24 hours' incubation at 37°C.)

Fig. XI

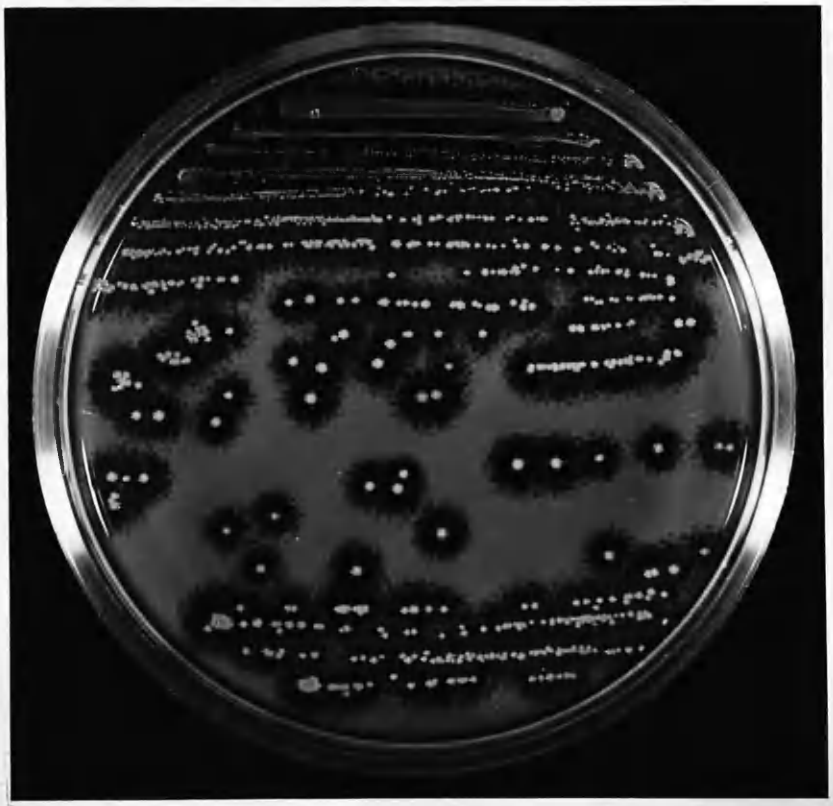


Fig. XII

Strain A, two additional methods of inoculation were used - the method of successive strokes and the spreading of one drop of culture over the entire surface of the plate with a glass spreader.

By way of control, plates of milk-agar(without added serum) were included. The plates were incubated at 37°C. for 24 hours and then left at room temperature for 24 hours. Readings were taken at the end of each of these 24 hour periods and the results are shown in App. 35-37.

Muller's phenomenon, of course, was absent from the control plates (milk-agar without added serum). The phenomenon was produced by all the strains of staphylococci on each of the sets of serum-milk-agar and in the case of any one strain there was no significant difference in the results yielded irrespective of the concentration of serum in the medium or of the method of inoculation. Examples of the results obtained by different methods of inoculation are illustrated in Figs. XI and XII.

With the particular strains of staphylococci used in this experiment the method of stab-inoculation proved in no way inferior for the purpose of eliciting Muller's phenomenon to any of the other methods of inoculation which were carried out in parallel. Since stab-inoculation has many advantages it was adopted as the

method of choice. It permits a good demonstration of the phenomenon, it is easily made and its use enables many strains to be tested on one plate instead of requiring a separate plate for each strain as is necessary if successive strokes are to be made or the inoculum spread with a glass-spreader in order to obtain isolated colonies. If single-stroke-inoculations are used there is again limitation of the number of strains which can be tested per plate; and if inoculation by spotting a loopful of culture on the surface of the medium is employed, it is the author's experience that this method is technically less simple than stab-inoculation.

It must be noted, however, that the potential of any strain at any one time to produce Muller's phenomenon can be assessed only when isolated colonies can be examined which have arisen presumably from single, or at least very few, cells. Attention has already been drawn in Part III to the fact that certain cultures of staphylococci comprise mixtures of cells, some of which can, while others cannot give Muller's phenomenon.

Fortunately the overwhelming majority of strains of coagulase-positive staphylococci, although inevitably mixed populations, include a sufficiently high proportion of cells capable of giving Muller's phenomenon to ensure that stab-inoculation will

succeed in revealing this ability. Strains of undoubted Staphylococcus aureus, however, are very occasionally met with which on first examination or re-examination, if stab-inoculation is used fail to give Muller's phenomenon and any such strain must of course be re-examined using a method of inoculation which will yield isolated colonies before accepting it as Muller-negative. The usual result of such re-examination is the finding that only a limited number of colonies show the phenomenon, others failing completely to do so.

(c) THE INFLUENCE OF SERUM.

Although the role of serum in relation to Muller's phenomenon is discussed in Part VII and the chief investigations on its importance are also presented there, some of the early experiments will now be mentioned.

It was by reducing the amount of serum in the serum-milk-agar used by Fisk and Mordvin (1943), that it was first revealed that plaques of clearing would be produced on this medium and this observation was the origin of these investigations on Muller's phenomenon. To answer the question, "What is the optimal concentration of human serum to incorporate in serum-milk-agar for Muller's phenomenon?", the following experiment was carried out.

A series of serum-milk-agar plates containing amounts of serum from 0.25 ml. up to 5.0 ml. (concentrations of 1.6% up to 33.3%) were prepared, stab-inoculated from 6 hour old "Lab-Lemco" broth cultures of seven strains of staphylococci and incubated at 37°C. for 24 hours (App. 38-39). Examination of the plates after this period of incubation revealed that all the strains of staphylococci but one showed Muller's phenomenon around their colonies in all the plates containing amounts of serum from 0.25 ml. up to 5.0 ml. The remaining strain showed Muller's phenomenon

FIG. XIII - The growth of seven strains of coagulase-positive staphylococci on a milk-agar plate without added serum. (Incubated at 37°C. for 24 hours.)

FIG. XIV - Enlargement (x5) of one of the colonies in Fig. XIII. (Incubated at 37°C. for 24 hours.)

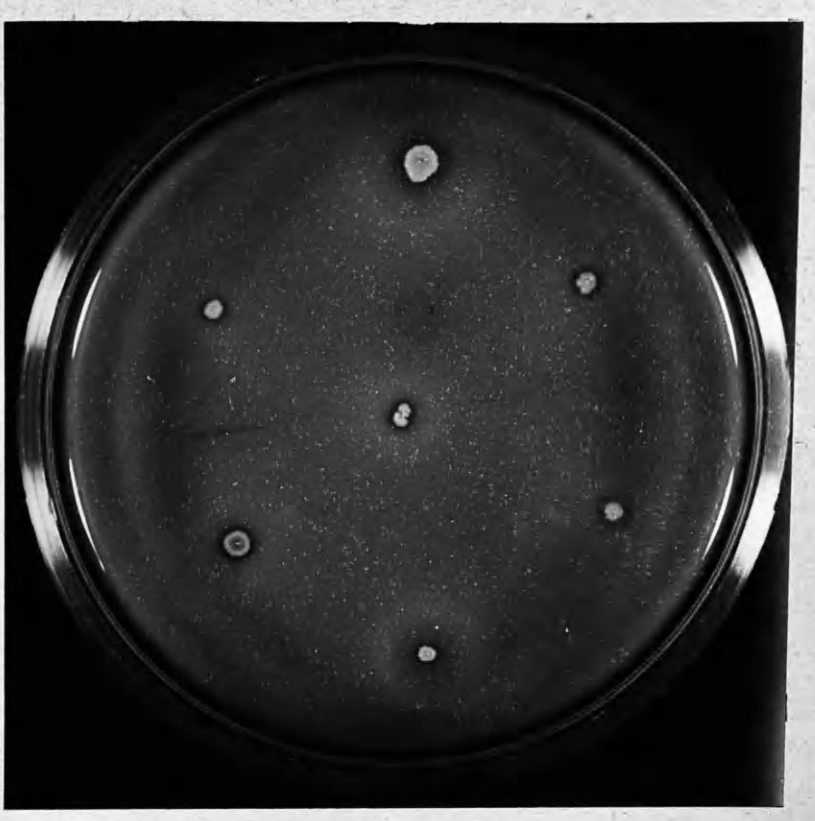


Fig. XIII

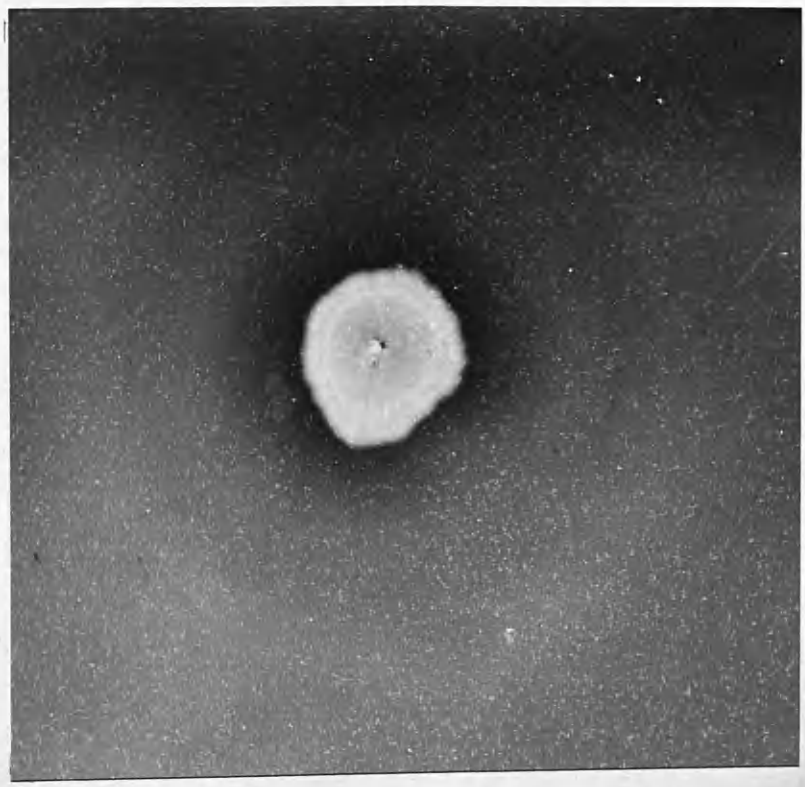


Fig. XIV

FIG. XV - The demonstration of Muller's phenomenon by seven strains of coagulase-positive staphylococci on a serum-milk-agar plate containing 1.0 ml. of serum. (Incubated at 37°C. for 24 hours.)

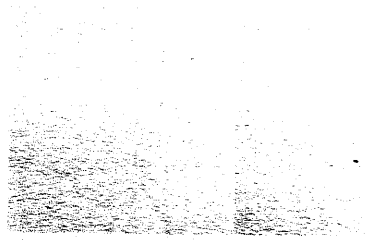


FIG. XVI - Enlargement (x5) of one of the colonies in Fig. XV. (Incubated at 37°C. for 24 hours.)

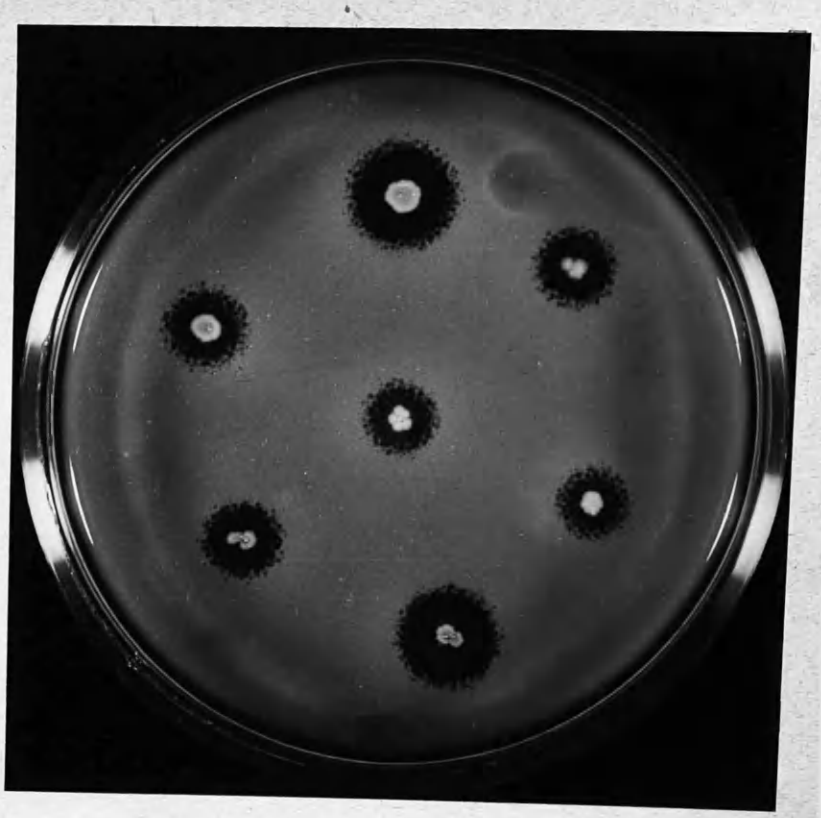


Fig. XV

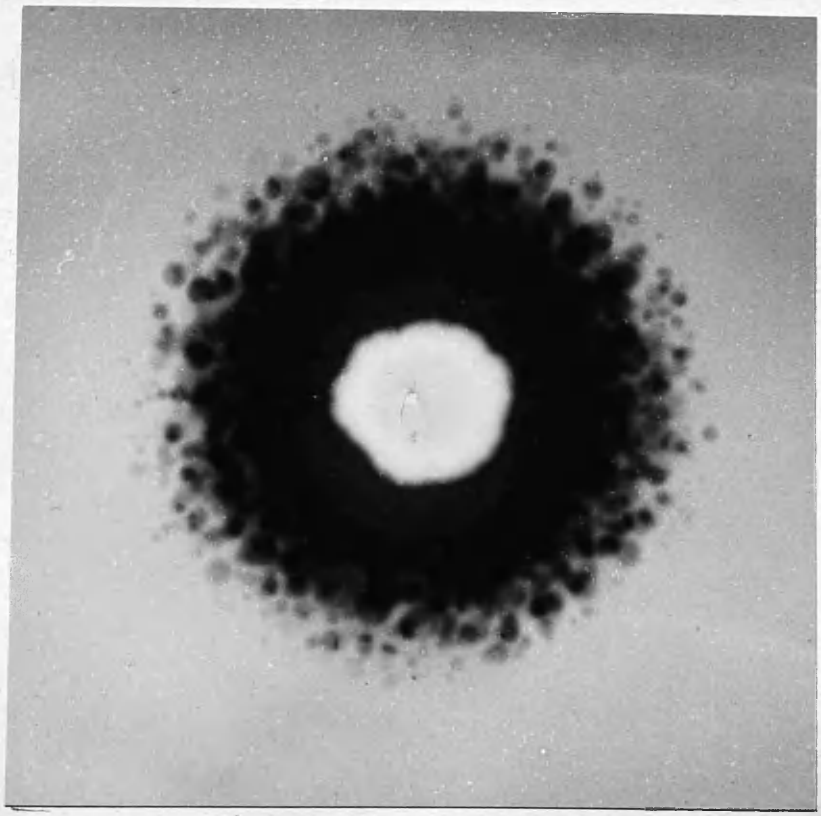


Fig. XVI

FIG. XVII - The growth of seven strains of coagulase-positive staphylococci on a serum-milk-agar plate containing 4.0 ml. of serum and showing a "rough-edge" to the zone of lysis around the colonies. (Incubated at 37°C. for 24 hours.)

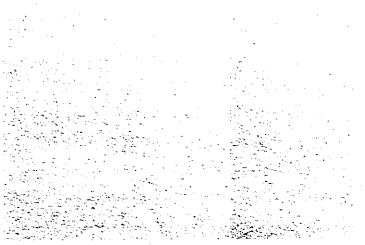


FIG. XVIII - Enlargement (x5) of one of the colonies in Fig. XVII. (Incubated at 37°C. for 24 hours.)

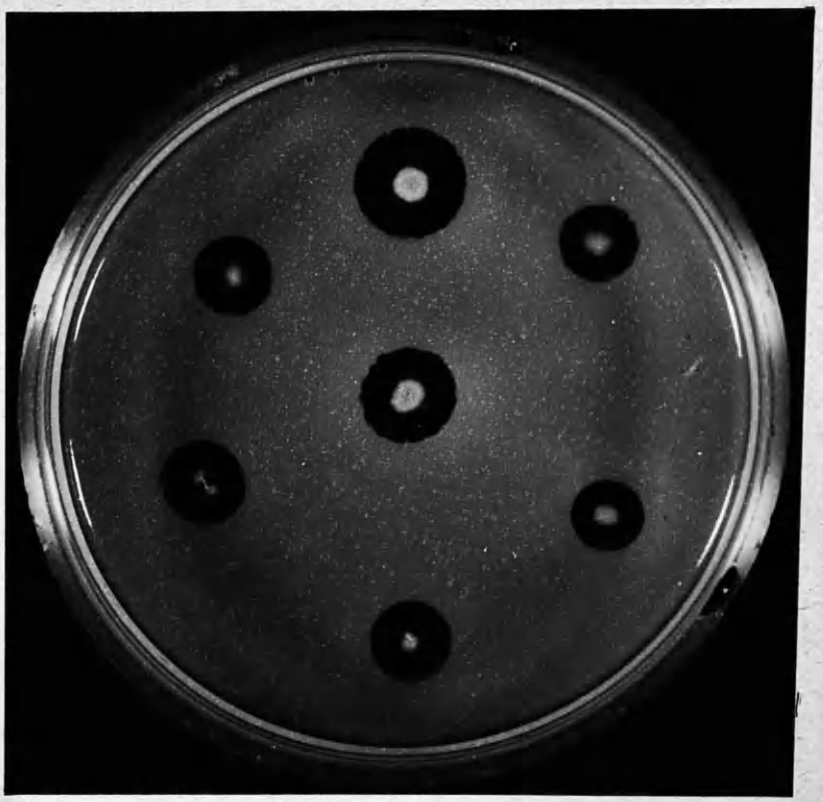


Fig. XVII

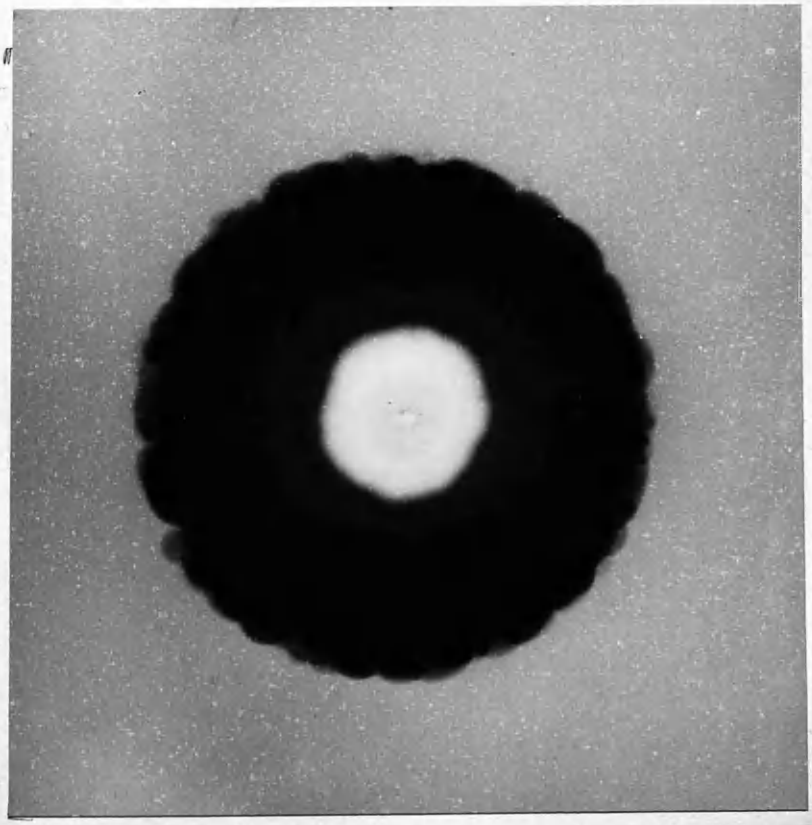


Fig. XVIII

FIG. XIX - The growth of seven strains of coagulase-positive staphylococci on a serum-milk-agar plate containing 4.0 ml. of serum. Note the absence of plaques and "rough-edge", associated with the zone of lysis. (Incubated at 37°C. for 24 hours and then left at room temperature for 48 hours.)

FIG. XX - Enlargement (x5) of one of the colonies in Fig. XIX. (Incubated at 37°C. for 24 hours and then left at room temperature for 48 hours.)

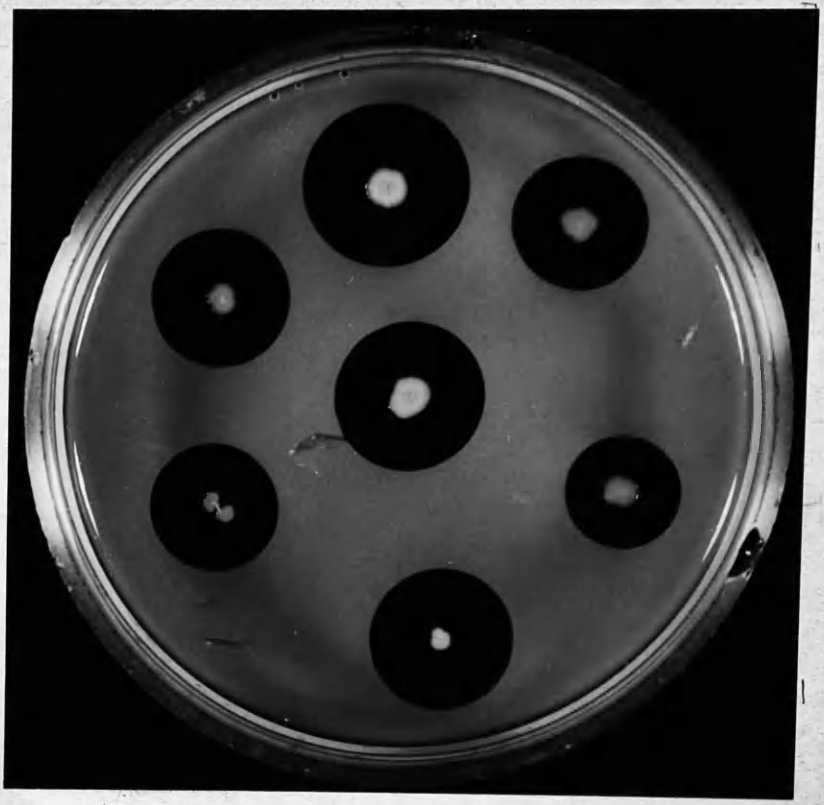


Fig. XIX

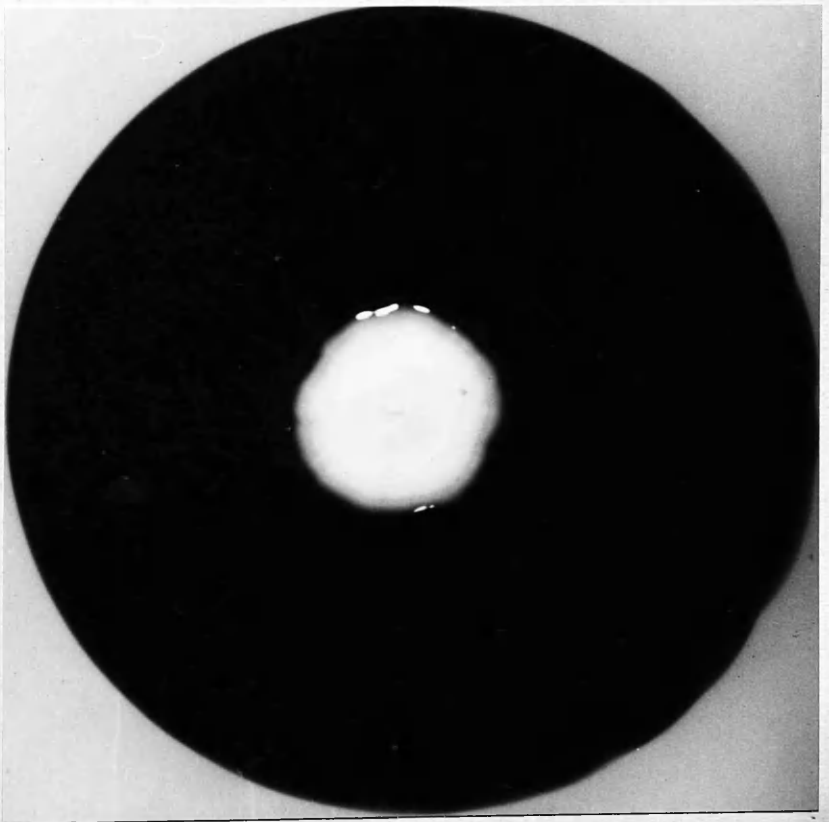


Fig. XX

in the plates containing amounts of serum from 0.5 ml. up to 3.0 ml.; in the plates with 4.0 ml. and 5.0 ml. of serum, no plaques were visible but only a broad zone of complete and definite lysis. A control plate, milk-agar without serum, did not show the phenomenon, but around the colonies of the staphylococci there was the indefinite incomplete lysis which is not associated in any way with Muller's phenomenon.

Thus with the strains of staphylococci and the particular sample of serum which were used there was an upper limit to the amount of serum which could be incorporated in a plate if Muller's phenomenon was to be produced. This upper limit was 3.0 ml. Within the range of concentrations, 0.5 ml. to 3.0 ml. per plate, every one of the seven strains gave the phenomenon and gave it best in the plates containing 0.5 ml. and 1.0 ml. of serum. It may be noted in passing that the standard serum-milk-agar used as a routine throughout these studies contained 0.5 ml. per plate, i.e. the optimum as confirmed by the above experiment. Photographs of the control plate and the serum-milk-agar plates containing 1.0 ml. and 4.0 ml. of serum and photographic enlargements of the growth of one and the same strain on each of the three plates are shown (Figs. XIII to XVIII).

After a further period of 48 hours at room temperature

the appearance of the control plate and of all the serum-milk-agar plates containing from 0.25 ml. up to 3.0 ml. of serum was essentially unchanged. Within this range, however, increasing serum concentration was associated with a progressive tendency for plaques to coalesce and give a broadening of the zone of complete lysis with or without fresh plaque-formation at and beyond its periphery. In the plates containing 4.0 ml. of serum or more plaques had wholly disappeared, almost certainly as a result of fusion of the original plaques and of any subsequently developed. A photograph of the plate containing 4.0 ml. of serum is shown at this stage (Fig. XIX) and an enlargement of the growth of one of the strains on it (Fig. XX).

Since Muller (1927a) had reported that the phenomenon now associated with his name was not produced if the blood used in the preparation of blood-agar had been heated for one hour at 54°C. and that its serviceability for the purpose was diminished by heating at this temperature for shorter periods, the effect of heating serum to be used in the preparation of serum-milk-agar was investigated.

A sample of fresh human serum was divided into seven portions. One of these was not subjected to any heating, and the others were heated as follows - 55°C. for 15 minutes, 55°C. for 30

minutes, 60°C. for 15 minutes, 60°C. for 30 minutes, 65°C. for 15 minutes and 65°C. for 30 minutes. A set of six serum-milk-agar plates was prepared from each of the portions of serum in from amounts of 1.0 ml. to 6.0 ml. per plate. The control set was the one containing the unheated serum.

Six strains of coagulase-positive staphylococci were stab-inoculated on to each of these plates from 6 hour old "Lab-Lemco" broth cultures and the plates then incubated at 37°C. for 48 hours. Readings were made at 24 hours and again at 48 hours (App. 43-47).

The experiment revealed that heating the serum for 15 minutes at either 60°C. or 65°C. deprived it wholly of its activity in relation to Muller's phenomenon and that the effect of heating it at 55°C. was dependent on the concentration in which it was used and also of course on the particular strain of staphylococcus being tested. For example, in the serum-milk-agar plate containing 1.0 ml. of serum heated at 55°C., only two of the six strains of staphylococci showed the phenomenon although all six did so in the control plate; but on the serum-milk-agar containing 4.0 ml. of the heated serum all six strains behaved as in the control.

It would appear that the factor in serum which is

necessary for Muller's phenomenon is thermolabile and that at a temperature of 55°C. for 30 minutes only partial inactivation of this factor occurs. In consequence if sufficient of the heated serum is incorporated in the medium Muller's phenomenon can develop.

The above type of experiment was repeated later using six of the same seven strains of staphylococci but with a different sample of human serum. The range of concentrations of serum incorporated in the serum-milk-agar plates was from 0.2 ml. to 6.0 ml. Since Burnet (1928) had stated that the presence of meat extract or other meat preparation in the substrate was necessary for the production of Muller's phenomenon opportunity was taken to check this observation. To this end a parallel set of plates was prepared with a milk-agar base without the usual added "Lab-Lemco" and incorporated in them the same range of concentration of the same serum as in the main set.

The various plates of each set were stab-inoculated with the staphylococcal strains, incubated at 37°C. for 24 hours and readings were then taken (App. 40-42).

In the main set of plates, i.e. those made with milk-agar containing "Lab-Lemco", all six strains of staphylococci showed Muller's phenomenon in the plates containing concentrations of serum from 0.5 ml. up to 5.5 ml. Outside this range of serum

concentrations some of the strains failed to show the phenomenon. The finding in the earlier experiment that the optimal concentration of serum was 0.5 ml. to 1.0 ml. per plate was confirmed. In the earlier experiment Strain 061 failed to give Muller's phenomenon in the presence of serum concentrations above 3.0 ml. per plate but in the present experiment did so up to a concentration of 6.0 ml. The only essential differences in the set-up of the two experiments were that a different sample of serum was used in the second and that different subcultures of the staphylococcal strains were inoculated. The altered behaviour of Strain 061 may thus be related either to the particular sample of serum in use or to some variation having occurred in the strain.

After a further period of 24 hours at room temperature the changes in the appearance of the plates were comparable with those already described as having occurred in the plates of the earlier experiment after 48 hours at room temperature, viz. a progressive tendency with increasing serum concentration for plaques to coalesce and give a broadening of the zone of complete lysis. Whereas, however, in the earlier experiment plaques were not wholly merged in the zones of lysis until the serum concentration exceeded 3.0 ml., in this repetition they were not so merged until the serum

concentration exceeded 4.5 ml. In this connection, of course, the fact that a different sample of serum was used in the repeat experiment probably accounts for this quantitative modification of the findings. The actual readings after the 24 hours at room temperature are shown in App. 42 under "At 48 hours - Plates with nutrient".

In the case of the parallel set of plates containing the same range of serum concentrations as above but having no "Lab-Lemco" in the substrate, Muller's phenomenon was shown after 24 hours' incubation at 37°C. by all six strains of staphylococci, in the presence of serum concentrations 1.0 ml. to 3.0 ml. thus contraverting Burnet's contention that the presence of a meat preparation in the substrate was necessary for the development of the phenomenon. These plates on standing at room temperature for a further 24 hours showed changes similar to those which occurred in the corresponding plates containing "Lab-Lemco". The readings of the plates from which "Lab-Lemco" had been withheld ("Without added nutrient") are shown in App. 41 and 42.

It can be noted that Muller's phenomenon developed with smaller amounts of serum when "Lab-Lemco" was present in the medium than when it was absent.

(d) THE EFFECTS OF VARYING THE TEMPERATURE OF INCUBATION, THE GASEOUS ENVIRONMENT AND OF ALTERING THE CHARACTERS OF THE GEL.

Muller's phenomenon, according to the earlier workers, will develop in a suitable medium only at 37°C. or thereabouts. This, however, was not found to be true in the case of serum-milk-agar as can be seen from the results of the following experiment (App. 48-52).

Four sets of three plates, each consisting of serum-milk-agar containing 1.0 ml. and 1.5 ml. serum and a milk-agar control plate without serum, were prepared and every plate was stab-inoculated with seven strains of coagulase-positive staphylococci from 6 hour old "Lab-Lemco" broth cultures. The four sets of plates were incubated at 37°C., at 30°C., at 26°C. and at 22°C. respectively. After 20 hours' incubation all seven strains of staphylococci were found to have produced Muller's phenomenon on the plates at 37°C., at 30°C. and at 26°C. but not on the plates at 22°C. The growths at 22°C. were very slight at this stage.

Incubation of the sets of plates was continued at their respective temperatures up till the fourth or seventh day after inoculation. Muller's phenomenon, of course, was absent

from the control plates throughout. By the third day of incubation at 22°C. the set of plates at this temperature showed the phenomenon with all the strains.

The best and most rapid demonstration of the phenomenon was obtained on the plates incubated at 37°C., the optimum temperature for the growth of the staphylococci; but equally good demonstrations of the phenomenon developed in the other sets of plates by prolonging their incubation periods. This observation indicates clearly that the development of the phenomenon is intimately associated with the actual growth of the staphylococci and therefore of the production of the essential staphylococcal factor.

Since blood-agar had been the medium used by the earlier workers who had reported that Muller's phenomenon was not produced at temperatures other than approximately 37°C. it was deemed essential to repeat the foregoing type of experiment using blood-agar or its variant serum-haemoglobin-agar which in the author's hands has been found to be even better than blood-agar for purposes of Muller's phenomenon. In this repeat experiment parallel sets of serum-haemoglobin-agar and serum-milk-agar were inoculated with seven strains of coagulase-positive staphylococci. One inoculated plate of each medium was incubated at 42°C., another

pair at 37°C., another pair at 22°C. and the last pair at 4°C. Readings were made at 24 hours, 2 days and 3 days (App. 53-55).

As was to be expected no growth occurred on the plates at 4°C. On serum-haemoglobin-agar incubated at 22°C. the phenomenon did not develop within the 3 day-period although it appeared later after 4 days' incubation, but did so with all seven strains on the plates of this medium incubated at 37°C. and at 42°C. On serum-milk-agar all seven strains grown at 37°C. and 42°C. showed the phenomenon but only six of the seven strains grown at 22°C. did so within the 3 days. The strain at 22°C. which on this occasion failed to give the phenomenon had given it in the earlier experiment by this time.

It is noteworthy that as regards the relative suitability of the two media in the case of the plates at 22°C. at which the rate of growth was slowed down, the phenomenon developed much more quickly on serum-milk-agar than on serum-haemoglobin-agar. Further, since the author has found that development of the phenomenon on blood-agar is slower than on serum-haemoglobin-agar, may it be that the observation of earlier workers that the phenomenon could not be elicited on blood-agar except at temperatures around 37°C. is linked with the relative inferiority of the former medium for the purpose?

Muller and other workers reported that haemophagy was not manifested under anaerobic conditions but when the seven strains of coagulase-positive staphylococci used routinely in these experiments were grown anaerobically, plaques and zones of lysis were produced on both serum-haemoglobin-agar and serum-milk-agar plates fully as satisfactorily as on duplicate plates incubated aerobically (App. 56-57).

The anaerobic cultivation was carried out by two different methods in parallel. In the one a McIntosh and Fildes jar with the customary indicator was employed and at the end of the period of incubation the colourless nature of the indicator confirmed that anaerobic conditions had been obtained. In the other, Fortner's plate was used in which, in a sealed Petri dish, the test organism is grown on appropriate medium. In the lid of the plate Pseudomonas pyocyanea is grown on nutrient agar within an aluminium ring in the base of the plate. The Pseudomonas pyocyanea in its growth rapidly uses up all the oxygen inside the plasticine-sealed plate and also serves as an indicator that the conditions are anaerobic since the characteristic colour of the pigment pyocyanin does not develop in the absence of free oxygen.

A physical condition which would be thought to play an important part in the production of Muller's phenomenon is the

nature of the gel in which it occurs. Previous workers, however, have reported that Muller's phenomenon occurs in gels of different kinds - agar, gelatin and agar - and of different strengths. Experiments carried out with serum-milk-agar made with varied concentrations of agar and serum confirmed that Muller's phenomenon can be demonstrated in plates with agar concentrations of 1.0% to 5.0% and serum concentrations of 6.6% to 26.6% (App. 58-61).

Only four strains of coagulase-positive staphylococci were used for this experiment and the stab-inoculated plates were incubated at 37°C. for 24 hours and then allowed to stand at room temperature for a further 48 hours. Increase of the concentration of agar in the gel did have an effect on the development of the phenomenon and resulted in the plaques being uniformly of very much smaller size but this effect was evident only in the presence of the lower serum concentrations. In the presence of the higher serum concentrations the development of the phenomenon was unaffected by the consistence of the gel and the plaques were of normal size and appearance.

Experiments of similar type which were carried out later confirmed this observation that increase in the concentration of agar in a plate, resulting in a stiffer gel, led to the development

of smaller finer plaques than in plates with the standard agar concentration. They also confirmed that this effect on the size of the plaques could be overcome by increasing the amount of serum in plates with a higher agar concentration.

A further experiment was also carried out using milk and varied concentrations of serum in a gel containing 20% gelatin and 1.0% agar. Seven strains of coagulase-positive staphylococci were used for test purposes and all produced Muller's phenomenon in this gelatin-agar gel in the presence of all concentrations of serum. (App. 62-63). There was some slight liquefaction of the gel around each of the stab-inoculations. Thus, it can be concluded that the early workers were correct in saying that Muller's phenomenon could be demonstrated in different gels. One point, however, which they did not record and which is of importance is the fact that alteration of the gel strength is associated with change in the size and appearance of plaques.

(e) THE EFFECTS OF VARIOUS NUTRIENTS.

It appeared to be desirable to provide in serum-milk-agar an amount of additional nutrient, sufficient to permit abundant growth of staphylococci in order to favour the production of the staphylococcal factor necessary for Muller's phenomenon. It has already been shown that staphylococci will grow well on serum-milk-agar without additional nutrient and produce the phenomenon although in such a medium, if only very small amounts of serum are present, growth is sometimes not as good as usual and plaque-formation is commonly absent. If a constant amount of additional nutrient is incorporated in all serum-milk-agar plates, irrespective of varied serum content, differences in plaque-production are still evident although the growths of staphylococci are equally good in all of them, and such differences must inevitably be attributable to the varied serum content of the plates.

A large number of nutrients commonly used in bacteriological culture media were investigated with a view to ascertaining their effect on the production of Muller's phenomenon. In one experiment varied amounts of "Lab-Lemco" broth or meat broth or peptone water (Oxoid peptone) or whey-broth or casein-digest broth were incorporated in serum-milk-agar plates which were stab-inoculated

from 6 hour old "Lab-Lemco" broth cultures of five strains of coagulase-positive staphylococci. A control plate of the same serum-milk-agar without added nutrient was also inoculated and all the plates were incubated at 37°C. for 48 hours. Readings were taken after 24 and 48 hours' incubation (App. 64-69).

In addition in the case of each of the nutrients a plate of milk-agar without added serum but containing the smallest amount of the particular nutrient used in the main test was prepared, stab-inoculated with the staphylococci and incubated at 37°C. These plates were included to afford further confirmation that Muller's phenomenon is not produced in milk-agar in the absence of serum even if additional nutrient to ensure abundant growth of the staphylococci is incorporated in the medium. The phenomenon did not develop on any of them.

Using the control serum-milk-agar plate to which no additional nutrient had been added as the standard for comparison, and on which all five strains of staphylococci yielded a good demonstration of Muller's phenomenon it was apparent that the presence of casein-digest broth in the medium (App. 69), inhibited the phenomenon and that whey broth (App. 68) could interfere markedly with its production. "Lab-Lemco" broth (App. 65), meat broth (App. 66) or peptone water (App. 67) in the medium had no

effect in the development of the phenomenon.

In the foregoing experiment in which "Lab-Lemco" broth was incorporated in serum-milk-agar the maximum concentration of "Lab-Lemco" itself which was used was of the order of 0.3% and the effect of the addition of larger amounts of this meat extract was further investigated (App. 70-71). Concentrations of "Lab-Lemco" from 0.5% up to 5.0% were used. Concentrations of "Lab-Lemco" in excess of 2.0% inhibited the production of Muller's phenomenon while concentrations of 0.5% and 1.0% gave better demonstrations of the phenomenon than in the control plates without "Lab-Lemco", or than in the earlier experiment in which lower concentrations of "Lab-Lemco" had been used. This finding which was confirmed in other experiments was the basis of the routine addition of 1.0 ml. of 15% "Lab-Lemco" to the standard serum-milk-agar.

In comparable fashion a further experiment was carried out to determine the inhibitory effect, if any, on Muller's phenomenon of concentrations of peptone in serum-milk-agar in excess of those already used (App. 72-73). It was found that the presence of as little as 0.5% peptone (Oxoid) in the medium inhibited the phenomenon. In view of this inhibitory property of peptone and of the poor nutritive value of concentrations below 0.5%, the inclusion of peptone in serum-milk-agar was avoided.

The effect on Muller's phenomenon of the addition of sodium chloride to serum-milk-agar was also examined (App. 74-75). Concentrations of sodium chloride in excess of 0.5% in serum-milk-agar inhibited plaque-production and reduced the extent of lysis around the staphylococcal colonies of all the seven strains used. Concentrations of 0.25% and 0.5% reduced markedly the number of plaques which developed around each staphylococcal colony and the few plaques which did appear were abnormally large having a diameter of 3-5 mm. as opposed to the normal average diameter of approximately 1-2 mm.

In another experiment, in which a series of serum-milk-agar plates containing varied amounts of serum had added to them sodium chloride to give concentrations of from 0.5% to 3.0%, progressive inhibition of plaque-formation was again obtained with all six strains of staphylococci grown on these plates (App. 76-79). Plaques were obtained in all the plates containing 0.5% sodium chloride, irrespective of the serum concentration, although they were fewer in number than in the control plates without added sodium chloride, a finding noted in the previous experiment.

In contrast, however, to what had been noted in the previous experiment in which concentrations of sodium chloride exceeding 0.5% inhibited plaque-formation it was now found that

FIG. XXI - Production of Muller's phenomenon by five strains of coagulase-positive staphylococci on a serum-milk-agar plate containing 0.5 ml. of serum. (Incubated at 37°C. for 24 hours and then left at room temperature for a further 24 hours.)

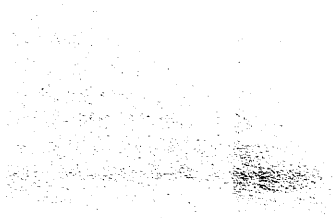


FIG. XXII - Production of indefinite lysis by the same five strains of coagulase-positive staphylococci as used in Fig. XXI, on a milk-agar plate without serum. (Incubated at 37°C. for 24 hours and then left at room temperature for a further 24 hours.)

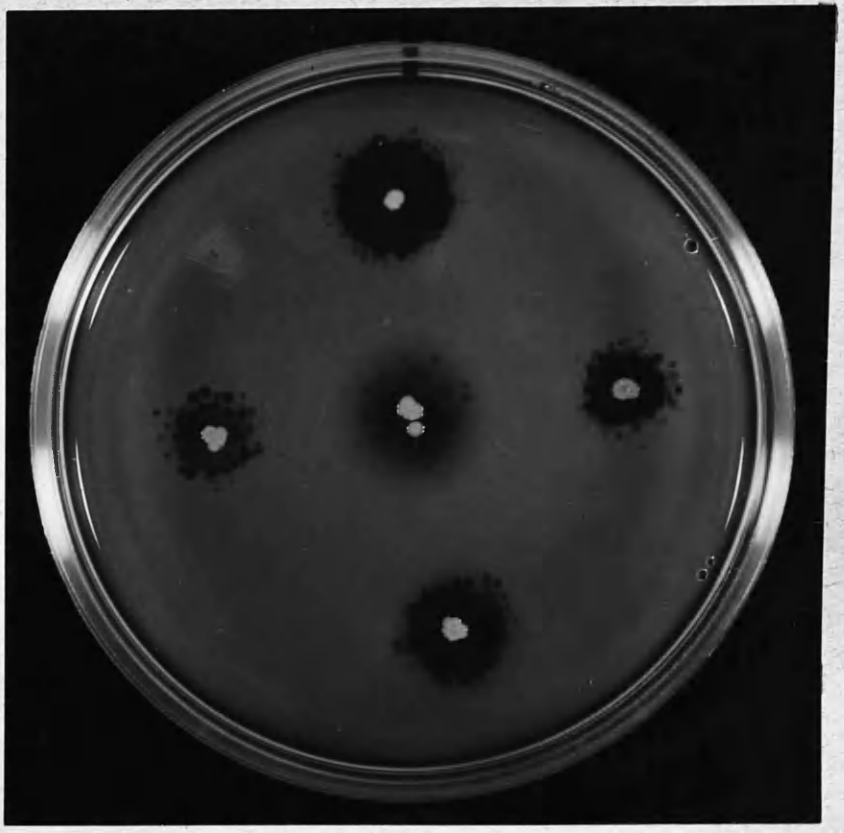


Fig. XXI

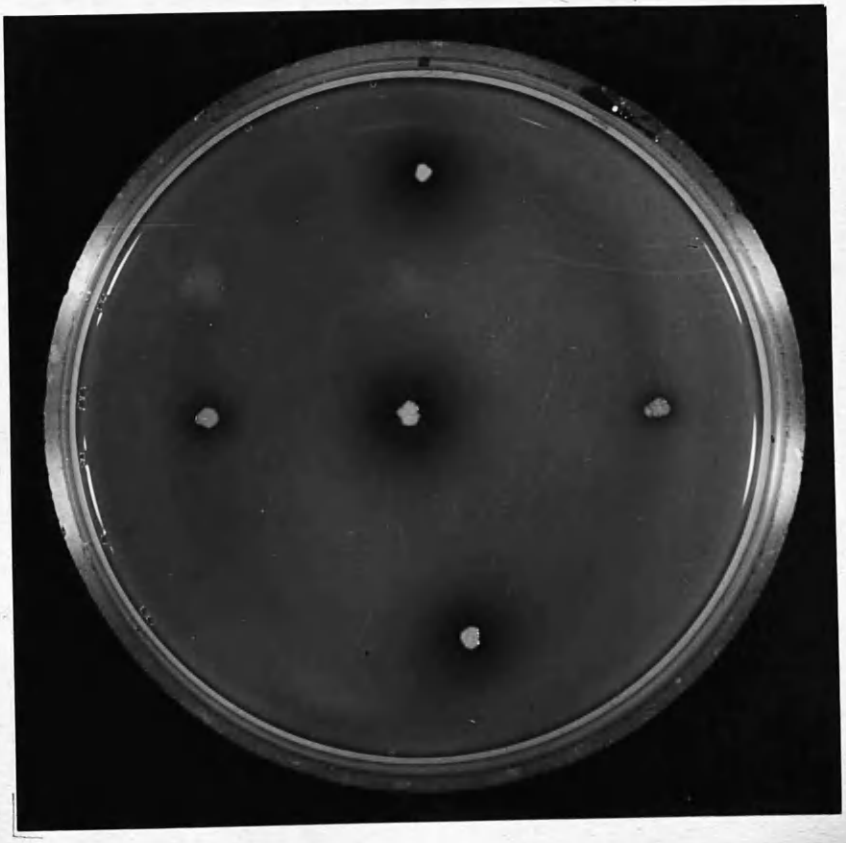


Fig. XXII

FIG. XXIII - Production of Muller's phenomenon by five strains of coagulase-positive staphylococci on a serum-milk-agar plate containing 0.5 ml. of serum, partially inhibited by the addition of 0.5% sodium chloride to the plate. (Incubated at 37°C. for 24 hours and then left at room temperature for a further 24 hours.)

FIG. XXIV - Production of indefinite lysis by the same five strains of staphylococci as used in Fig. XXIII on a milk-agar plate without serum, uninhibited by the addition of 0.5% sodium chloride. (Incubated at 37°C. for 24 hours and then left at room temperature for a further 24 hours.)

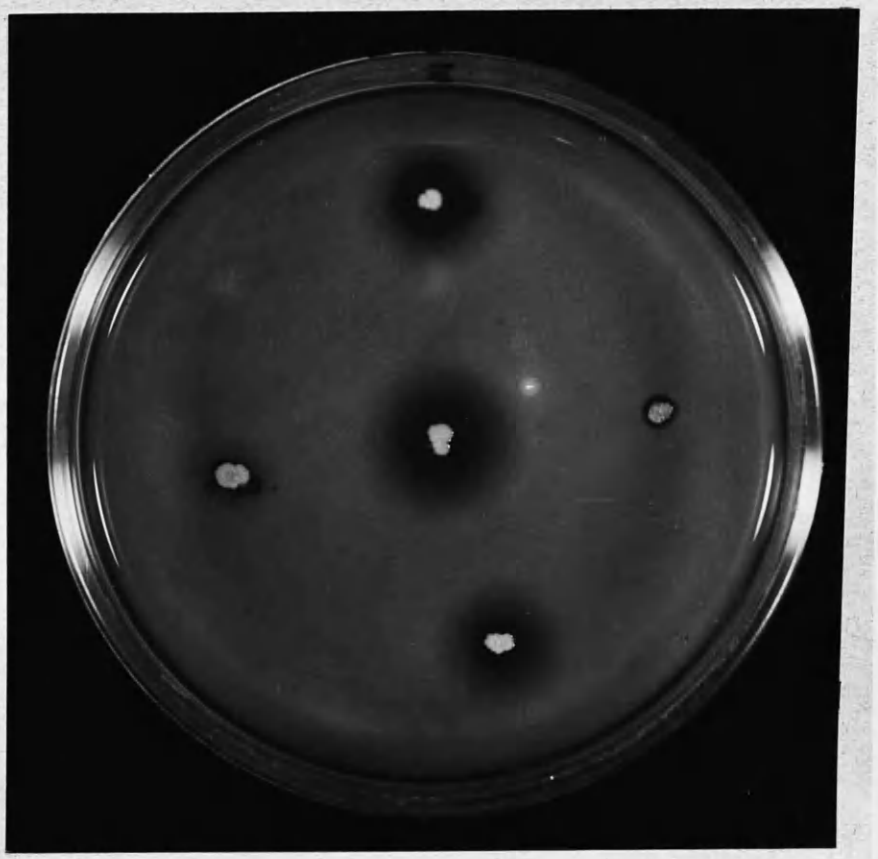


Fig. XXIII

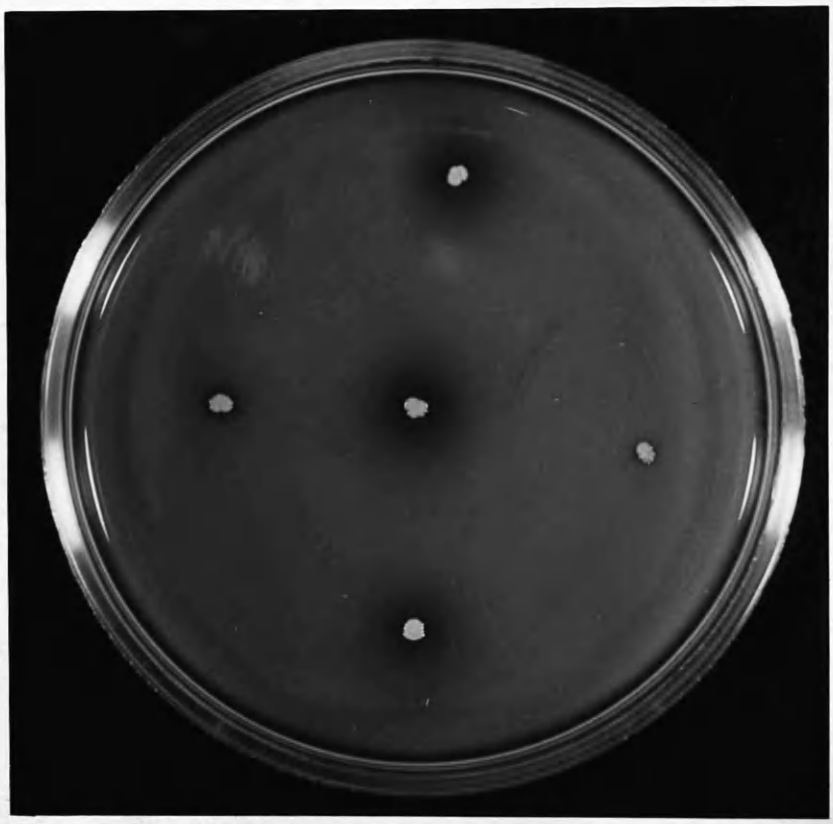


Fig. XXIV

FIG. XXV - Production of Muller's phenomenon by five strains of coagulase-positive staphylococci on a serum-milk-agar plate containing 0.5 ml. of serum completely inhibited by the addition of 1.0% sodium chloride. (Incubated at 37°C. for 24 hours and then left at room temperature for a further 24 hours.)

FIG. XXVI - Production of indefinite lysis by the same five strains of staphylococci as used in Fig. XXV on a milk-agar plate without serum uninhibited by the addition of 1.0% sodium chloride. (Incubated at 37°C. for 24 hours and then left at room temperature for a further 24 hours.)

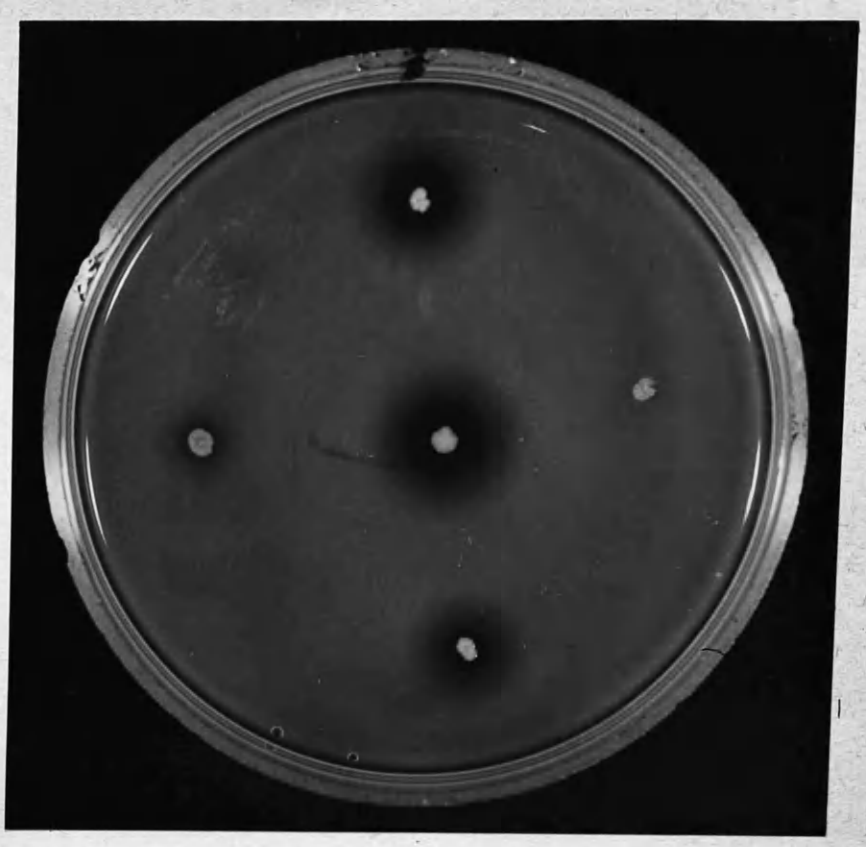


Fig. XXV

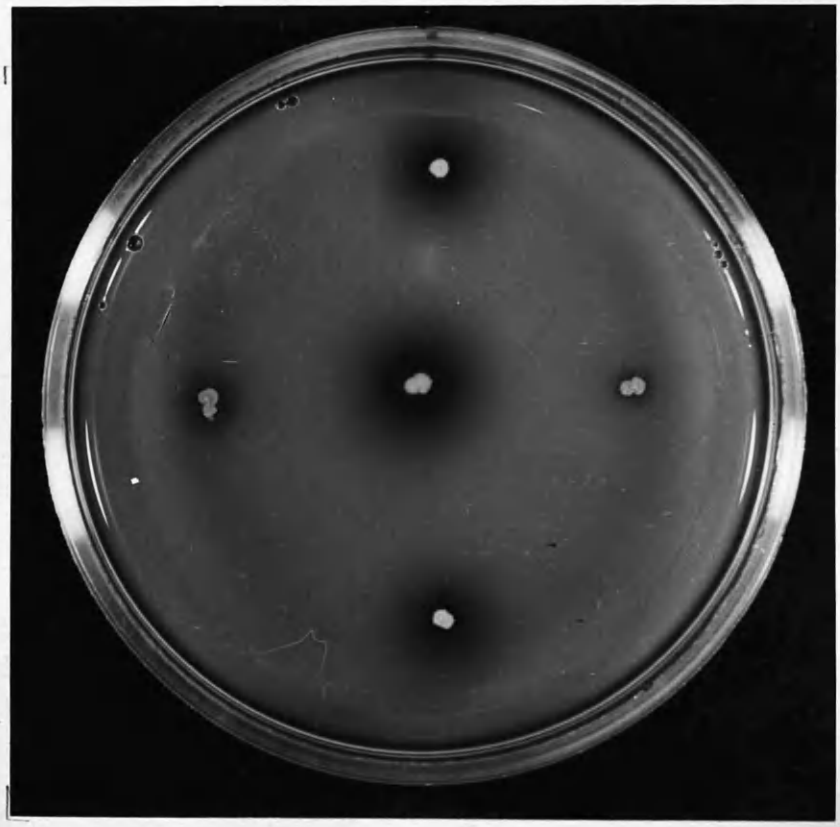


Fig. XXVI

with increased serum concentration plaques were produced by four of the six strains of staphylococci in the presence of 1.0% sodium chloride, although not in the presence of higher concentrations.

The zones of lysis which developed around colonies could only be recognised with certainty as being of the type associated with Muller's phenomenon in the plates containing 0.5% and 1.0% sodium chloride. In all other plates it was essentially of the type which has already been described as occurring with certain staphylococci growing on milk-agar without added serum and which has no relationship with Muller's phenomenon. The type of lysis associated with Muller's phenomenon is well illustrated in Fig. XXI and the indefinite type of lysis which has no relationship with the phenomenon in Fig. XXII. These Figures relate to the following experiment which was carried out later and which afforded further confirmation of the inhibitory effect of sodium chloride on Muller's phenomenon.

Increasing concentrations of sodium chloride were added to a series of serum-milk-agar plates and to a series of milk-agar plates without serum. In each series a control plate without sodium chloride was included. All the plates were stab-inoculated with five strains of coagulase-positive staphylococci and incubated at 37°C. for 24 hours and then left at room temperature

for a further 24 hours when readings were made (App. 80-81). Plaque-formation was totally inhibited in serum-milk-agar plates containing more than 0.5% sodium chloride and even at this concentration only two of the five strains showed any plaques. All strains showed plaque-formation in the plates with 0.25% sodium chloride and in the control. In the case of the milk-agar plates (without added serum) there was, of course, no plaque-formation but four of the five strains showed the indefinite lysis around their colonies which has already been referred to and this indefinite type of lysis was uninfluenced significantly by the presence of sodium chloride in any of the concentrations used. The results of this experiment are illustrated in Figs. XXI to XXVI.

(f) SOME MISCELLANEOUS EXPERIMENTS.

It is well established that for the production of Muller's phenomenon the co-operation of two factors are required, of which one is supplied by the staphylococcus - the staphylococcal factor, the other by fresh serum from one of a number of suitable animal species - the serum factor (Elek, 1953). Three experiments are now presented which have a bearing on the staphylococcal factor.

The first of these experiments relates to the inability of the staphylococcal factor to diffuse through cellophane. Serum-milk-agar plates were prepared in duplicate and the surface of one was covered with a layer of sterile cellophane. Both plates were inoculated with a loopful of a 6 hour old "Lab-Lemco" broth culture to give lines of growth, the plate with the cellophane being inoculated on the upper side of the cellophane, care being taken not to tear it. Comparable growth of staphylococci was obtained on these two plates of serum-milk-agar, but, whereas Muller's phenomenon appeared on the plate without the cellophane, it did not develop on the other plate with the layer of cellophane.

It is possible that the cellophane prevented the

acquisition, by the staphylococci growing on its surface, of the nutrients necessary for the production of the staphylococcal factor, but it seems more likely that the factor was produced and failed to pass through the cellophane to the serum-milk-agar below. This latter view was confirmed later when it was found that cell-free staphylococcal factor would not pass through a cellophane sac or through "Visking" dialysis tubing.

Although the development of Muller's phenomenon depends in part on a factor derived from staphylococci it does not require the presence of living staphylococcal cells for its manifestation. This is shown in the following experiment (App. 82-84).

Three sets of plates were prepared, each set consisting of a serum-milk-agar plate containing 1.0 ml. of serum, a serum-milk-agar plate containing 4.0 ml. of serum and a milk-agar control plate without serum. All the plates were stab-inoculated with seven strains of coagulase-positive staphylococci and placed in the incubator at 37°C. One set of plates was withdrawn from the incubator after 4½ hours, the organisms killed by exposure to chloroform vapour for 15 minutes and the plates returned to the incubator for the remainder of a 24 hour period. The second set of plates was withdrawn from the incubator after 9 hours' incubation, similarly treated with chloroform vapour and returned to the

incubator. The third set of plates was not treated with chloroform and remained in the incubator for 24 hours when readings of all the plates were made.

Muller's phenomenon was produced by all seven strains of staphylococci on the serum-containing plates of the third set. In the second set of plates, the phenomenon was shown by all seven strains but only in the plate containing 1.0 ml. of serum. In the first set of plates, the phenomenon was shown by only four of the seven strains and again only in the plate containing 1.0 ml. of serum. Except in the three control plates without serum lysis when present was of the type associated with Muller's phenomenon. In these control plates any lysis was of the indefinite type which has no relationship with Muller's phenomenon.

After 48 hours at room temperature in the serum-containing plates of the third set, i.e. those whose growths had not been killed, marked development of plaques had occurred associated as usual with considerable extension of the zones of lysis. In the corresponding plates of the other two sets whose growth had been killed, any such development and extension was slight especially in the first set killed at $4\frac{1}{2}$ hours.

It is obvious from these observations that killing

the organisms in the first and second sets after 4½ and 9 hours growth limited the amount of staphylococcal factor available for diffusion into the medium and thus resulted in poorer demonstrations of Muller's phenomenon than in the case of the corresponding plates of the third set whose growths had not been killed.

Lack (1948, 1954) had reported that the fibrinolytic action of staphylokinase-activated plasmin was inhibited by soya-bean trypsin inhibitor. The third experiment was carried out to determine whether this agent had any action on the production of Muller's phenomenon. The results of this experiment are shown in App. 85-87.

Five strains of coagulase-positive staphylococci were used and stab-inoculated into plates of serum-milk-agar containing varied amounts of serum and varied amounts of soya-bean trypsin inhibitor. Control plates without the inhibitor were similarly inoculated and also control plates without serum. Muller's phenomenon was shown by all five strains in the control plates containing serum but was totally inhibited in the corresponding plates containing soya-bean trypsin inhibitor. A very striking demonstration was obtained of this inhibitory action of the soya-bean preparation on the phenomenon in a special set of plates in which the inhibitor was not incorporated in the media but placed in

porous cups on the surface of the agar and the stab-inoculations made around these cups at a distance of 1.5 cm. from their centres. After inoculation the serum-containing plates of this set showed Muller's phenomenon only on the sides of the stab-inoculations remote from the cups.

The indefinite lysis which is produced by some staphylococci growing on milk-agar, i.e. without added serum was in no way affected by the presence of soya-bean trypsin inhibitor, and indeed, this type of lysis was seen on serum-milk-agar plates in which the development of Muller's phenomenon had been inhibited by the presence of the soya-bean preparation.

It seems probable therefore that the characteristic plaque-formation and clear-cut lysis of Muller's phenomenon both of which are inhibited by the soya-bean preparation are of quite a different nature from the indefinite type of lysis which some staphylococci can produce even on milk-agar (without serum) and which has been suspected to be a manifestation of the activity of a protease.

II. HAEMOGLOBIN IN SUBSTRATE.

(a) USE OF BLOOD-AGAR.

(b) EXPERIMENTS WITH HAEMOGLOBIN SOLUTION.

(c) "CHOCOLATE"-AGAR (MODIFIED) WITH ADDED FRESH SERUM.

(d) HEATED HAEMOGLOBIN-AGAR WITH ADDED FRESH SERUM.

II. HAEMOGLOBIN IN SUBSTRATE.

Although serum-milk-agar was the substrate used in these studies of Muller's phenomenon, other substrates were also employed. Blood-agar was the medium on which Muller (1927a) had observed the small circular areas of clearing and from which his original description of "l'hémophagie staphylococcique" was taken. A haemoglobin solution in agar to which fresh serum was added was the medium favoured by Elek (1953). Thus, both blood-agar, and haemoglobin-agar with added serum, were used in a number of experiments to be described in this part of the thesis. In addition, haemoglobin was used in a way not hitherto described in investigations of Muller's phenomenon, viz. as a heated haemoglobin substrate with added serum.

(a) USE OF BLOOD-AGAR.

It seemed desirable, if not essential, to compare the production and appearance of Muller's phenomenon on blood-agar, the medium originally used, with that on serum-milk-agar.

An experiment in which blood-agar was employed is given in detail in App. 88-89. Two pairs of blood-agar plates containing 5% and 10% human blood (citrated) and 1% "Lab-Lemco" were prepared. Inoculations were made from a 6 hour old "Lab-Lemco" broth culture of Staphylococcus aureus, Strain A to give isolated colonies of the organism on one pair of plates and a confluent growth on the second. After incubation at 37°C. Muller's phenomenon was produced on all the plates but it required 3-4 days before it was manifested on the media compared with the 20-24 hours required for its production by the same strain of Staphylococcus aureus on serum-milk-agar. The appearances of the phenomenon on the two media were strictly comparable and it was thus confirmed that although Muller's phenomenon was originally observed on blood-agar this is not the best medium for its speedy demonstration.

(b) EXPERIMENTS WITH HAEMOGLOBIN SOLUTION.

It has already been noted (p. 80) that serum-haemoglobin-agar made according to the method recommended by Elek (1953), was satisfactory for the demonstration of Muller's phenomenon, the results being identical with those obtained with the same strains of staphylococci growing on serum-milk-agar. Serum-haemoglobin-agar was made with the crude haemoglobin solution and the nutrient agar base detailed by Elek (App. 463). It was thought desirable, however, to bring this medium into line with serum-milk-agar and use 4.5 ml. of 4.5% agar with 1% concentration of "Lab-Lemco" as the nutrient for each plate (App. 463a). At the same time the filtered haemoglobin preparation referred to by Elek (1953) was substituted for his crude haemoglobin solution. The results obtained with this modified serum-haemoglobin-agar medium were in no way inferior to those obtained with the original type. The modified medium had the advantage of being more comparable in its composition with serum-milk-agar than Elek's serum-haemoglobin-agar and in the modified form was used consistently in experimental work as occasion demanded.

It remained to be determined, however, whether there was an optimal concentration of haemoglobin for incorporation in

the modified medium to ensure the best demonstration of Muller's phenomenon, and to this end the experiment shown in App. 90-91 was carried out.

Varied amounts of filtered haemoglobin solution were incorporated in a series of agar - "Lab-Lemco" - serum mixtures to provide a set of serum-haemoglobin-agar plates which differed only in their haemoglobin content. Each plate was stab-inoculated with seven strains of coagulase-positive staphylococci from 6 hour old "Lab-Lemco" broth cultures. The plates were incubated at 37°C. for 48 hours when readings were made and preliminary readings were also recorded at 24 hours. At 48 hours, all seven strains showed Muller's phenomenon on all the plates with haemoglobin solution content of from 0.5 ml. to 3.0 ml. and the best demonstrations of the phenomenon were in the plate containing 1.5 ml. At the preliminary readings of this plate only four of the seven strains showed the phenomenon, which thus developed more slowly on this occasion in the case of the remaining three. It may be noted here that these same three strains were not consistently slow in giving Muller's phenomenon as on other occasions they showed the characteristic plaque-formation on serum-haemoglobin-agar after 24 hours' incubation. Comparable variability in the capacity of strains to produce Muller's

phenomenon has already been referred to earlier in this thesis.

Since in an earlier experiment (App. 53-55) it had been found that the effect of the incubation temperature on the development of Muller's phenomenon on serum-haemoglobin-agar had not the importance attributed to it by earlier workers it was deemed desirable to confirm this earlier observation by the following experiment (App. 92-93).

Five plates of serum-haemoglobin-agar were each stab-inoculated with seven strains of coagulase-positive staphylococci; the plates were incubated respectively at 4°C., 22°C., 37°C., 42°C. and 46°C. and readings were made at 24 hours, 48 hours, 72 hours and 96 hours. Except at temperatures of 4°C. and 46°C. Muller's phenomenon was produced by all the strains. No growth occurred on the plate kept at 4°C. for 96 hours but on subsequent incubation at 37°C. Muller's phenomenon developed with all strains. These findings confirmed completely the results of the earlier experiment and established that an incubation temperature of 37°C. is not essential for Muller's phenomenon when serum-haemoglobin-agar is the medium used. This parallels what has already been established in the case of serum-milk-agar.

One further experiment was carried out in view of the observations of Burnet (1928) and Elek (1953) that Muller's

phenomenon fails to develop on blood-agar or serum-haemoglobin-agar under anaerobic conditions. It has already been shown (App. 56-57) that the phenomenon does develop on serum-milk-agar under these conditions. A series of serum-haemoglobin-agar plates containing varied amounts of serum (App. 94-95) was prepared and each plate stab-inoculated with seven strains of coagulase-positive staphylococci. The plates were incubated at 37°C. for 48 hours by two different anaerobic methods and readings were then made.

Muller's phenomenon was produced under the anaerobic conditions by all seven strains although in the case of the plates with the lowest amounts of serum there was minor variation in the behaviour of three of these strains as between anaerobiosis in McIntosh and Fildes jar and in Fortner's plate.

Thus serum-haemoglobin-agar has proved as satisfactory as serum-milk-agar for purposes of Muller's phenomenon which develops both aerobically and anaerobically on either of the media and on whose development the temperature of incubation is not of prime importance. In actual practice, however, serum-haemoglobin-agar is not so simple to prepare as serum-milk-agar and on the latter demonstrations of Muller's phenomenon are much more striking and easily observed.

(c) "CHOCOLATE"-AGAR (MODIFIED) WITH ADDED FRESH SERUM.

An attempt to obtain Muller's phenomenon on a modified "chocolate"-agar to which fresh serum had been added proved successful. Horse red-cells, thrice washed with saline and finally made up with saline to the original volume of the blood were mixed in appropriate proportions with 4.5% agar, "Lab-Lemco" and distilled water, and heated at 75°C. for 10 minutes. Some of this medium was used for pouring plates; the remainder was cooled to 50°C. and 0.5 ml. of fresh human serum was added for every 14.5 ml. of volume and the mixture was immediately poured as plates, each of 15 ml. (App. 96-97).

Three strains of coagulase-positive staphylococci were streaked on each type of medium, i.e. with and without serum, and incubated at 37°C. for 24 hours. Muller's phenomenon was produced by all three strains in the medium to which serum had been added but not in the medium without added serum.

Duplicate plates which had been prepared at the same time but which had a layer of sterile cellophane placed on their surface prior to inoculation were also incubated at 37°C. for 48 hours. Although all three strains of staphylococci grew well on the cellophane interposed between them and the surface of the medium

no change could be detected in either of the media, neither clearing indicative of proteolytic action nor anything suggestive of plaque-formation, even after a further period at room temperature.

In another experiment in which washed human red cells were substituted for horse red cells in the preparation of a modified "chocolate"-agar medium, the effect of adding varied amounts of human serum to this medium was investigated (App. 98-99). Nine strains of coagulase-positive staphylococci were stab-inoculated on to a series of plates containing amounts of serum from 0.1 ml. up to 2.0 ml. After incubation at 37°C. for 24 hours zones of small very fine plaques were observed around all the staphylococcal colonies. If such plates were incubated for a further period or left at room temperature the breadth of the zone of plaques increased. In addition, especially in the plates with the larger amounts of serum, lysis occurred around the colonies where previously there had been plaques only. A serum-milk-agar plate prepared with the same human serum and inoculated at the same time with the same strains showed plaques and marked zones of lysis around the colonies within 24 hours. This serum-milk-agar plate contained 0.5 ml. serum and when its pattern of plaques associated with lysis around the colonies was compared with that around the colonies on the

corresponding "chocolate"-agar plate with 0.5 ml. of serum the two patterns were seen to be quite different. In the former the plaques were larger, easily detected and fewer in number; in the latter they were small and very fine, not quite so easily detected but very numerous. In the former there was a halo of definite lysis within the zone of plaques surrounding each colony; in the latter there were only plaques without any lysis. Thus it was observed that Muller's phenomenon gave different patterns of plaques and lysis on different protein substrates, which contained, however, the same proportions of the same fresh serum.

In a further experiment a human red cell-suspension heated at 75°C. for 10 minutes was used in amounts of 0.25 ml., 0.5 ml. and 1.0 ml. to prepare three sets of plates in which fresh human serum was incorporated in amounts of from 0.5 ml. to 5.0 ml. (App. 100-102). Muller's phenomenon was produced after 18 hours' incubation at 37°C. by all five strains of coagulase-positive staphylococci used to inoculate these plates. The phenomenon was shown in the plates with the smallest quantities of serum (0.5 ml. and 1.0 ml.) by the presence of fine plaques alone, in the plates with 2.0 ml. and 3.0 ml. of serum by the presence of definite lysis in addition to plaques, and in the plates with 4.0 ml. and 5.0 ml. serum only by a "rough edge" to the zone of lysis brought about by

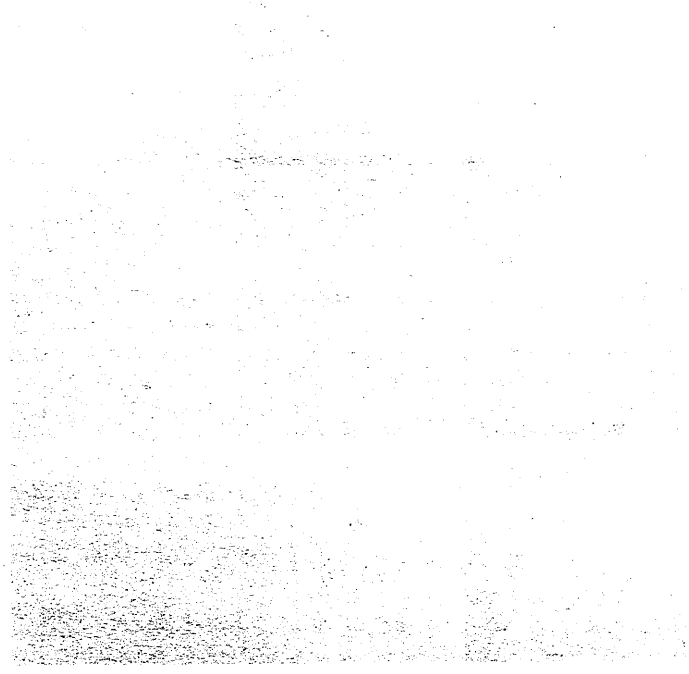
a narrow rim of plaques impinging on this edge. After the plates had been left at room temperature for a further period of 48 hours, however, plaques alone were still present in the plates with 0.5 ml. and 1.0 ml. of serum; in the plates with 2.0 ml. of serum plaques were only present when that amount of serum was associated with the largest amount of red cell suspension (1.0 ml.). In all the other plates plaques had virtually vanished and only zones of definite lysis were present.

(d) HEATED HAEMOGLOBIN-AGAR WITH ADDED FRESH SERUM.

In the preparation of the foregoing modified "chocolate"-agars red cells had been used but it was found that filtered haemoglobin solution could be substituted for them to provide an excellent medium, after appropriate heating, for the demonstration of Muller's phenomenon. In one experiment varied amounts of filtered haemoglobin solution were added to three separate batches of the same basic "Lab-Lemco" agar. The mixtures were heated at 75°C. for 10 minutes and then cooled to 50°C. Each of the batches was divided into three portions, each portion being sufficient for one plate, and to the portions were added respectively the following volumes of fresh human serum - 0.5 ml., 1.0 ml., 1.5 ml. The nine mixtures were immediately poured as plates, varied in their haemoglobin and serum content. Every plate was stab-inoculated with seven strains of coagulase-positive staphylococci and incubated at 37°C. for 24 hours (App. 103-104).

The characteristic plaques of Muller's phenomenon developed around the colonies of every strain, irrespective of the haemoglobin or serum content of the plate but the plaques were small and very fine. Apart from the plate with the highest

FIG. XXVII - The production of Muller's phenomenon by Staphylococcus aureus, Strain A on a heated-haemoglobin-agar plate containing 0.5 ml. added serum. (Incubated at 37°C. for 24 hours.)



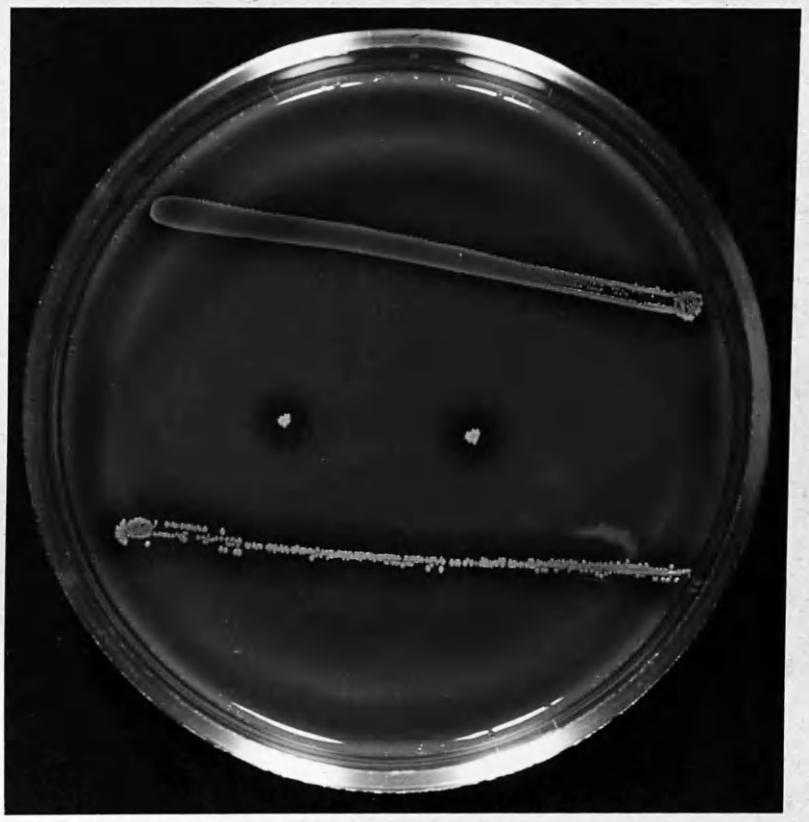


Fig. XXVII

haemoglobin content and the lowest serum content some degree of lysis also was present almost without exception around the colonies of all the strains. Figure XXVII illustrates the appearances in a heated haemoglobin-agar plate containing 0.5 ml. of serum.

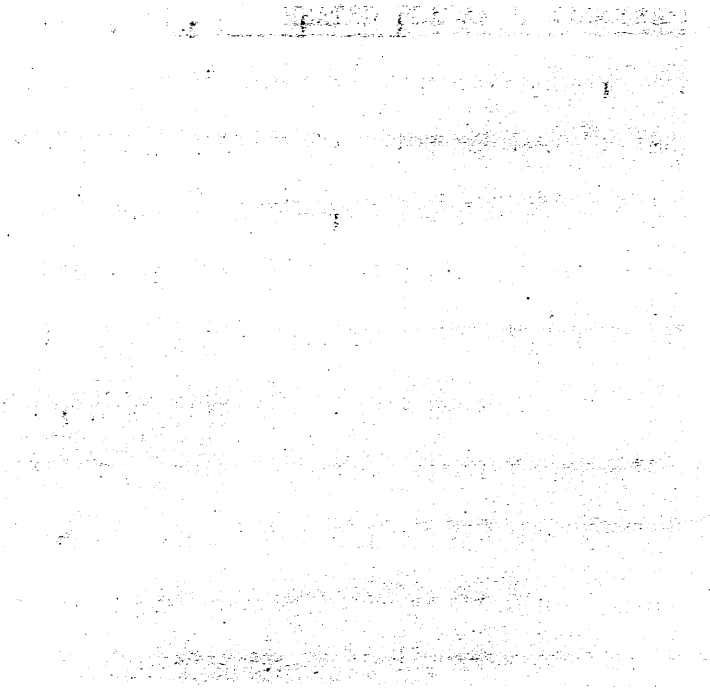
It seemed worthwhile to determine whether the temperature at which the haemoglobin solution was heated had any effect on the subsequent development of Muller's phenomenon and experiments, of which the one recorded in App. 105-106 is an example were carried out.

Aliquots of filtered haemoglobin solution were heated at 65°C. and 75°C. respectively for 10 minutes and then used to prepare two sets of "chocolate"-agar plates, varied in both their haemoglobin and fresh serum content. Each plate of each set was stab-inoculated with five strains of coagulase-positive staphylococci and incubated at 37°C. for 24 hours. All five strains produced Muller's phenomenon on all the plates containing fresh serum. The plates, however, containing haemoglobin heated at 65°C. gave a broad zone of clear-cut lysis around the colonies and a narrow border of plaques beyond this, whereas the plates containing haemoglobin heated at 75°C. showed no lysis around the colonies but only a broad zone of plaques. The control plate of each set to which fresh serum had not been added, showed only a narrow zone of indefinite

lysis around the colonies but, of course, no plaque formation.

The filtered haemoglobin solution used in this and the preceding experiment was prepared according to the method of Elek (1953) but other preparations of haemoglobin were also employed successfully, viz. haemoglobin from cells lysed by saponin, and haemoglobin from cells lysed by freezing and thawing. App. 107-108 is the record of an experiment in which haemoglobin solution prepared by the latter method was used in amounts of 0.25 ml., 0.5 ml. and 0.75 ml. for heated haemoglobin ("chocolate") agar plates to which fresh serum was added. Muller's phenomenon was produced by all five strains of coagulase-positive staphylococci inoculated on this medium. Control plates without the added serum showed only indefinite lysis with four of the strains, and no change in the medium with the remaining strain; and, as was to be expected, failed to show any characteristic plaques. Thus it was found that staphylococci produced Muller's phenomenon not only in blood-agar containing whole blood as originally described by Muller and confirmed by others, but also in an agar medium containing unheated haemoglobin, prepared in a variety of ways from horse or human red cells or in an agar medium containing haemoglobin heated at 65°C. - 75°C., provided always that fresh serum was present. It was noted that the size and pattern of

plaques, and the breadth of the zone of plaques and of any clear-cut lysis were affected by the nature and treatment of the haemoglobin in the substrate, e.g. the temperature at which it had been heated, and also by the amount of serum in the medium.



III. HEATED PLASMA IN SUBSTRATE.

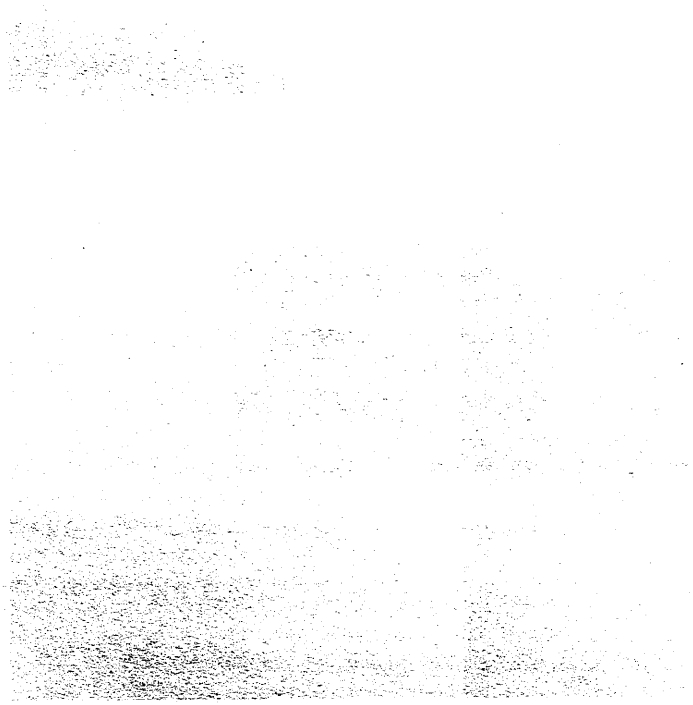
III. HEATED PLASMA IN SUBSTRATE.

Fibrinolysis in heated plasma-agar by staphylococci has been reported by a number of workers, e.g. Chapman (1942), Christie and Wilson (1941), Fisk and Mordvin (1943) and Lack (1957). Discrete plaques of clearing and irregular areas of lysis in association with the zones of fibrinolysis on heated plasma-agar plates was mentioned by Fisk and Mordvin (1943), although they did not say that this appearance was in fact Muller's phenomenon. The phenomenon, however, has been reported on similar media although less frequently than fibrinolysis. Thus, Christie, Graydon and Woods (1945) obtained Muller's phenomenon by growing staphylococci in appropriate conditions on a special heat precipitated fibrinogen. Moreover, attention should be drawn to the production of Muller's phenomenon on heat coagulated serum to which fresh serum was added, as reported by Packalén (1941).

In view of these observations it did seem desirable to examine the production of Muller's phenomenon in media containing heated blood proteins (other than haemoglobin) and to ascertain the conditions necessary for its demonstration.

Accordingly, on one experiment (App. 109-110) in which plates of heated plasma-agar were used, a demonstration of Muller's phenomenon was obtained with two of the three strains of staphylococci grown on them. Three plates of this medium were prepared by incorporating 25% human plasma, preheated at 65°C. for 30 minutes in the customary "Lab-Lemco" agar base and two of them received 0.25 ml. and 0.5 ml. of fresh serum respectively. To the third plate no serum was added. Three strains of coagulase-positive staphylococci were stab-inoculated in each of the three plates which were incubated at 37°C. for 24 hours, and then left at room temperature. Readings were recorded after 3 days at room temperature. All three strains produced slight indefinite lysis on the plate without added fresh serum and this lysis was reminiscent of the type seen around colonies of the majority of strains of staphylococci growing in milk-agar without added serum. On the plates with added fresh serum all three strains produced zones of complete lysis which in the case of two strains were associated with a rough edge and plaque-formation (i.e. Muller's phenomenon). The third strain, although not giving Muller's phenomenon (plaque-formation) had an extensive zone of clear-cut lysis around its colonies on the plates containing serum and this lysis differed markedly from the limited zone of indefinite lysis yielded on the

FIG. XXVIII - The production of fibrinolysis by three strains of coagulase-positive staphylococci on a heated-plasma-agar medium with 0.5 ml. of added serum. A rough-edge and plaques - "Muller's phenomenon" - are visible around the zone of clearing resulting from the fibrinolysis with two of the strains. (Incubated at 37°C. for 24 hours and then left at room temperature for 3 days.)



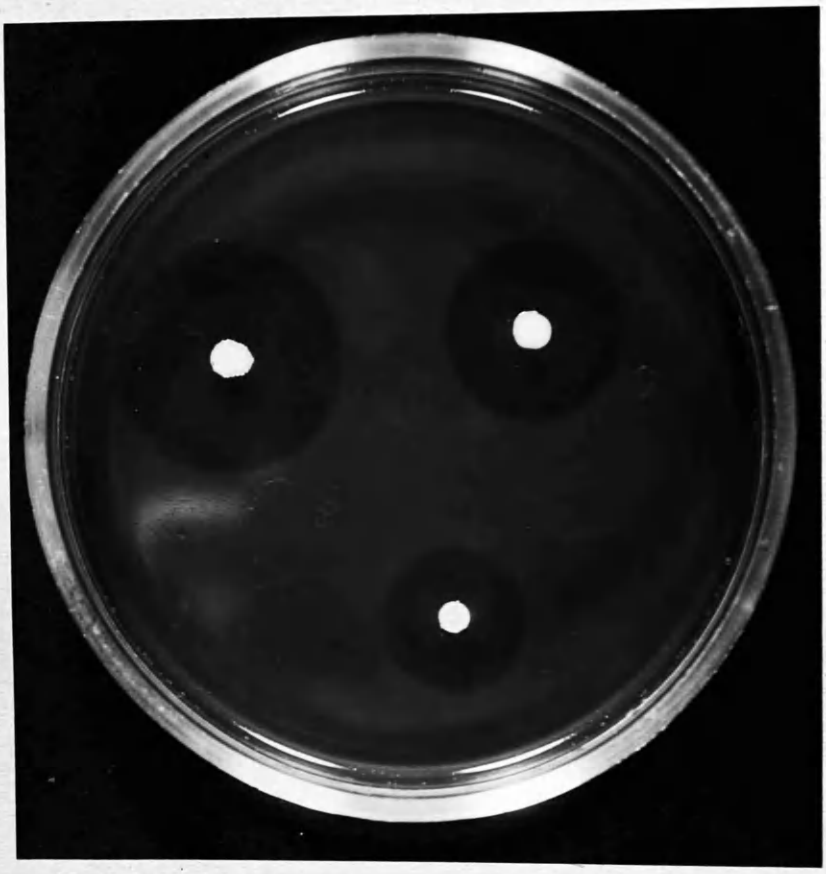


Fig. XXVIII

plate without serum. It was thus demonstrated that Muller's phenomenon can be produced by at least certain strains of staphylococci growing on heated plasma-agar. The plate with 0.5 ml. of added serum was photographed and is reproduced as Fig. XXVIII.

The irregular extensions and other variations of the lytic zones around colonies of staphylococci on heated plasma-agar described by Fisk and Mordvin (1943) are reminiscent of the irregularities and interference with plaque-production which can be observed on serum-milk-agar plates to which sodium chloride has been added, particularly in concentrations over 1.0%. Fisk and Mordvin had employed Chapman's medium (Chapman, 1942) which was based on that of Christie and Wilson (1941) and which contained beef extract, Bacto-Peptone and sodium chloride (final concentration 0.8%). To this basic medium citrated rabbit plasma (13.3%) was added and the mixture heated at 56°C. for 10-15 minutes before being poured into Petri plates.

In view of the established effect of sodium chloride in modifying Muller's phenomenon in serum-milk-agar it was decided to repeat the Fisk and Mordvin type of experiment using the modified Chapman's medium with and without sodium chloride. As Bacto-Peptone was not available at the time the basic medium was

prepared with "Lab-Lemco" and peptone (Oxoid) but without sodium chloride. The method of preparation is given in App. 480-481.

To tubes containing 13.0 ml. of this basic medium, melted and at 50°C., 2.0 ml. amounts of human plasma were added and also amounts of sterile 20% sodium chloride solution to give concentrations of from 0.2% to 1.6%. A control without added sodium chloride was included. These tubes of medium were heated at 56°C. for 10 minutes to precipitate the fibrinogen and were then poured as plates. Each plate was stab-inoculated with seven strains of coagulase-positive staphylococci and incubated at 37°C. for 48 hours. Readings were made after incubation at 37°C. for 24 and 48 hours (App. 111-112).

Muller's phenomenon was produced by all seven strains but not in the presence of all the concentrations of sodium chloride. The plaques produced in the control plate without added sodium chloride were small and were not far removed from the rim of the zone of lysis surrounding the colonies. Plaque-production was best developed in the plates with 0.2% and 0.4% sodium chloride. No plaques were present in the plates which contained sodium chloride in excess of 0.8% but around the growth of six of the seven strains of staphylococci on these plates there were to be seen the irregular extending areas of lysis described by Fisk and Mordvin.

Although added sodium chloride did not prove necessary for the production of Muller's phenomenon, concentrations of the salt in excess of 0.8%, interfered with plaque-production, giving rise to irregular areas of lysis extending from the main zone of lysis around the staphylococcal growth. The fact that Fisk and Mordvin had obtained this appearance on their medium, which contained only 0.8% of added sodium chloride may be accounted for by the fact that they used a different basic medium whose constituents, e.g. meat extract may have contained rather more sodium chloride or other salts than those used in the present experiment.

Moreover, it may be mentioned that on re-examination of the plates after three days at room temperature following on the 48 hours' incubation at 37°C., four of the strains each showed a large irregular "plaque" or extension of the zone of lysis surrounding its growth on the plate containing 0.6% sodium chloride. The breadth of the zones of lysis was greatest around colonies on the plate without added sodium chloride and decreased with increasing concentration of the salt. Thus the average diameter of the zones of lysis in the plate containing 1.6% sodium chloride was approximately half that in the plate without added sodium chloride.

Christie, Graydon and Woods (1945) with their heat-precipitated fibrinogen medium claimed that the time-temperature relationship used in heating to precipitate the fibrinogen from the plasma was critical as regards the subsequent development of Muller's phenomenon. They found that fibrinogen precipitated at 53°C. for 3 minutes was satisfactory but that fibrinogen precipitated at 56°C. for 30 minutes was unsatisfactory. The former yielded plaques and lysis: the latter only lysis, but its ability to yield plaques followed incorporation of fresh serum in the substrate.

One of a number of experiments carried out to confirm or refute these findings is shown in App. 113-115. To tubes containing 13.0 ml. of a modified Chapman's medium melted at 50°C., 2.0 ml. of preheated plasma were added and also 0.15 ml. of 20% sodium chloride. The pre-heating of the plasma was carried out by heating three tubes, each containing 2.0 ml. of plasma at 56°C. for 5, 10 and 15 minutes respectively and a further three tubes at 60°C. for the same time intervals. The tubes of medium containing the preheated plasma were poured as plates. A duplicate set of tubes containing the modified Chapman's medium with similarly preheated plasma samples was also prepared but to each tube in the set 0.5 ml. of fresh serum was added immediately before pouring.

Every plate was stab-inoculated with seven strains of coagulase-positive staphylococci, incubated at 37°C. for 24 hours and then left at room temperature for 48 hours. Readings were taken after 24 hours at 37°C. and after a further 48 hours at room temperature. All seven strains showed Muller's phenomenon (plaques and lysis) after 24 hours' incubation at 37°C. in the plates containing plasma heated at 56°C. for 5, 10 or 15 minutes, irrespective of their containing added fresh serum or not. The plates containing plasma heated at 60°C., but without added serum showed no plaque-formation but only lysis, slight lysis or no change, whereas the corresponding plates with added fresh serum showed typical Muller's phenomenon although not with every one of the staphylococcal strains.

After a further period of 48 hours at room temperature there were changes in the appearances of the plates but these changes in the appearances of the plates but these changes were essentially a progressive development of the zones of lysis, with or without plaque-formation around the staphylococcal growths. This was most marked in the plates containing added fresh serum, in which plaques had largely disappeared, as a result of their merging in the extending zones of lysis.

There is one point which merits mention in relation

to the differing character of the lysis which developed in plates containing plasma heated at 60°C. depending on whether or not fresh serum was present in the substrate. In the absence of serum, the lysis is of the indefinite type such as is produced by some strains of staphylococci growing on milk-agar (without added serum). In the presence of serum the lysis is of the clear-cut type associated with Muller's phenomenon.

Thus it was established that Muller's phenomenon can be demonstrated on a heated plasma-agar medium but that the temperature and length of time of the heating are of importance; that in the case of a heated plasma-agar the time-temperature relationship of whose heating has been excessive its activity with regard to the production of the phenomenon can be restored by the addition of fresh serum; and that sodium chloride, although not essential, improved plaque-production if present in small amounts but interfered with it in concentrations greater than 0.8%.

In an extended series of investigations presented in App. 116-143, the production of Muller's phenomenon in heated plasma-agar and in heat-precipitated fibrinogen-agar was examined more closely. These investigations were performed in three main groups:- Group I (Experiment 44) with heated plasma incorporated in agar containing 1.0% "Lab-Lemco"; Group II

(Experiment 45) with heated plasma incorporated in modified Chapman's medium; Group III (Experiment 46) with heat-precipitated fibrinogen incorporated in agar containing 1.0% "Lab-Lemco".

The heated plasma incorporated in Group I and in Group II media, was used in plates of these media in 1.0 ml., 2.0 ml. and 3.0 ml. amounts. The heat precipitated fibrinogen incorporated in Group III media was prepared by heating plasma, centrifuging it and resuspending the precipitated fibrinogen recovered, in distilled water of half the original volume of the plasma. The amounts of heat-precipitated fibrinogen used in the preparation of plates in media in Group III were 0.5 ml., 1.0 ml. and 1.5 ml., the equivalents of 1.0 ml., 2.0 ml. and 3.0 ml. of plasma respectively.

The plasma used in these three groups was heated at three different temperatures, and at each of these temperatures for four different periods of time. Thus the media prepared in each group contained plasma heated for twelve different temperature-time combinations. The three temperatures were 53°C., 56°C. and 60°C. and the four periods of time, 3 minutes, 10 minutes, 15 minutes and 30 minutes. Since these twelve differently heated plasma preparations were incorporated in the agar base in amounts of 1.0 ml., 2.0 ml. and 3.0 ml. per plate of medium, in all

thirty-six different media were available in each of the three groups.

These thirty-six media were modified in two different ways, firstly by the addition of sodium chloride and secondly by the addition of serum. The thirty-six media were prepared in triplicate - one set of media to receive sodium chloride final concentration 0.2%, a second set to receive sodium chloride 0.8%, and a third set a control set to which no sodium chloride was added. The introduction of this modification thus resulted in 108 different media being produced in each of the three groups. The second modification applied to these 108 media and it consisted in their preparation in duplicate. One set of media had serum added, 0.5 ml. of serum sufficient for each plate of medium; the other set had no serum added and acted as a control set. Thus for each of the three groups 216 different media were prepared, a total of 648 media for all the investigations carried out in these three groups.

One plate of each medium was prepared and stab-inoculated from 6 hour old "Lab-Lemco" broth cultures of six strains of coagulase-positive staphylococci. The plates were incubated at 37°C. for 24 hours and then left at room temperature. Readings were made after 24 hours' incubation at 37°C. and after a

further 24 hours at room temperature.

Muller's phenomenon was produced by all six strains of staphylococci growing on all three basic media, viz. heated plasma in "Lab-Lemco" agar, heated plasma in the modified Chapman's medium and the heat-precipitated fibrinogen in "Lab-Lemco" agar although plaque-formation did not occur in all the media prepared from them, e.g. by addition of serum. Usually the demonstration of the phenomenon was apparent after incubation of the plates for 24 hours at 37°C. but sometimes a further period of 24 hours at room temperature was necessary before the plaques were seen. It did happen, not infrequently, that strains of staphylococci which showed Muller's phenomenon after 24 hours' incubation no longer did so after a further 24 hours at room temperature, the plaques having been engulfed by the advancing lysis.

All the three groups of media showed Muller's phenomenon on one or more of the sets of plates made with heated plasma or heat-precipitated fibrinogen at each of the different temperature-time combinations. This demonstration of Muller's phenomenon in these three groups of media was dependent on the presence or absence of fresh serum, the concentration of sodium chloride and the amount of heated plasma or heat precipitated

fibrinogen contained. Moreover the nature of the basic medium was of some importance.

As examples of some of these points, the occurrence of Muller's phenomenon on the three groups of media with plasma heated at 53°C. for 15 minutes and 60°C. for 30 minutes will be discussed. With plasma heated at 53°C. for 15 minutes incorporated in "Lab-Lemco" agar, plaque-formation occurred only in the plates with 0.8% added sodium chloride, both with and without serum. With plasma heated at the same temperature for the same period of time and incorporated in the modified Chapman's medium, Muller's phenomenon was seen in every plate prepared in this series, with serum or without serum, with sodium chloride or without sodium chloride, except in those plates which contained 1.0 ml. of heated plasma and 0.5 ml. of serum without sodium chloride or containing only 0.2% of it; that is, in only two of the eighteen plates in this series, Muller's phenomenon did not develop. With heat-precipitated fibrinogen (prepared by heating plasma at 53°C./15 minutes Muller's phenomenon developed in plates of medium with added sodium chloride both with and without serum. The plates without added sodium chloride also showed the phenomenon when serum was present.

Thus it can be seen that serum, sodium chloride, the

nature of the agar base, the nature of the test protein all influence plaque-formation. In all these plates around every strain a zone of fibrinolysis was present whether or not plaques developed.

In the plates containing plasma heated at 60°C. for 30 minutes, incorporated in "Lab-Lemco" agar or modified Chapman's medium, plaque formation was not observed unless serum had been added. The observation also applied to the heat-precipitated fibrinogen agar prepared from plasma heated at 60°C. for 30 minutes. These demonstrations of Muller's phenomenon occurred principally in the plates with 0.8% added sodium chloride although they also appeared in the plates with 0.2% sodium chloride.

The fact that added sodium chloride is not necessary for the production of Muller's phenomenon in heated plasma in the modified Chapman's medium was shown by the demonstration given by all six strains of staphylococci grown on plates without added sodium chloride, without added serum with plasma heated at 53°C. for 15 minutes and with some strains with plasma heated at 60°C. for 3 minutes in the same medium. Muller's phenomenon, however, was not demonstrated in any of the plates made with either heated plasma in "Lab-Lemco" agar or the heat precipitated fibrinogen in "Lab-Lemco" agar without added serum unless 0.2% or 0.8% sodium

chloride was also included. The heat-precipitated fibrinogen in "Lab-Lemco" agar, however, did show Muller's phenomenon in some plates without added sodium chloride provided serum was present, e.g. those prepared by heating plasma at 53°C. for 15 minutes. It should be noted here that the "Lab-Lemco" agar was prepared using 15% "Lab-Lemco" which contained no added sodium chloride.

The importance of the addition of sodium chloride to heated plasma in "Lab-Lemco" agar for the production of the phenomenon is seen in the fact that it is in the plates of medium, irrespective of the time-temperature combination of the heating of plasma, which contain 0.8% sodium chloride in which it principally occurs. To a much lesser extent the phenomenon occurs in the plates with the added 0.2% sodium chloride.

A similar finding was made with heated plasma in the modified Chapman's medium although as has been pointed out the phenomenon occurs in plates of this medium without added sodium chloride. The appearance of the phenomenon is, however, more common in the plates of modified Chapman's medium with 0.2% sodium chloride, than it is in the corresponding plates in "Lab-Lemco" agar. The plates containing heat-precipitated fibrinogen, however, show the phenomenon more often in the plates with 0.8% added sodium

chloride than in the plates with 0.2% of this salt.

The influence of added fresh serum is apparent in the plates containing plasma heated at 60°C. for 10, 15 or 30 minutes. In its absence plaque-formation does not occur on such heated plasma-agar plates, although zones of fibrinolysis will still develop around the staphylococcal colonies.

IV. SUMMARY.

IV. SUMMARY.

In this investigation the optimal conditions for the production of Muller's phenomenon on serum-milk-agar by strains of staphylococci were ascertained. The effect of certain conditions on plaque-formation in serum-milk-agar, serum-haemoglobin-agar and heated-plasma-agar was also examined.

For the preparation of serum-milk-agar reconstituted skim milk powder was found to be as satisfactory as liquid milk, more convenient to store and more easily handled. The concentration of milk in serum-milk-agar necessary for a good demonstration of plaque-production was not critical, and in practice, 13.3%, the lowest concentration in the optimal range was used. Although Muller's phenomenon will develop in plates containing a concentration of serum up to 30% or thereabouts (depending on the particular serum employed), the optimal concentration was usually in the range 3.3% to 6.6%, i.e. 0.5 ml. to 1.0 ml. in a 15 ml. amount of medium. In the standard serum-milk-agar plates commonly used to ascertain the ability of strains of staphylococci to produce Muller's phenomenon, 0.5 ml. of serum was the amount

employed. The thermolability of the serum factor, first reported by Muller, was confirmed.

The optimum temperature for plaque-production in serum-milk-agar and in serum-haemoglobin-agar was 37°C. but plaques would develop in both these media from 22°C. to 42°C., although earlier workers report that Muller's phenomenon (plaque-formation) does not develop at temperatures other than 37°C. or thereabouts. Under anaerobic conditions plaques developed in both serum-milk-agar and in serum-haemoglobin-agar although Elek (1953) and others claim that plaque-formation does not occur with staphylococci growing in the absence of oxygen. The observations of Packalén and others that plaque-formation occurs in gels of varied strengths and of different compositions were confirmed but it was found that, when a greater concentration of agar was employed resulting in a stiffer gel, the size of plaques was much smaller than usual. This effect of the stiffer gel, however, was overcome by increasing the concentration of serum incorporated in the medium, normal-size plaques developing. The effect of the addition of different nutrients to serum-milk-agar medium was investigated and 1% "Lab-Lemco" solution was found to be a satisfactory nutrient for incorporation in such medium since it did not interfere with plaque-production. Peptone (Oxoid) in concentrations in excess of 0.5% did interfere with the development

of plaques when incorporated in serum-milk-agar. Sodium chloride was found to have a similar action when added in concentrations of 0.5% or more.

Good growth of staphylococcal colonies was obtained on a sheet of cellophane interposed between the staphylococcal inoculum and the surface of serum-milk-agar but plaques were not produced in the serum-milk-agar, on account of the factor being unable to pass through the cellophane. Plaque-formation and the definite lysis associated with it was inhibited by soya-bean-trypsin-inhibitor, although the indefinite lysis, seen in milk-agar medium without serum around colonies of certain strains of staphylococci and brought about by staphylococcal protease was found to develop in serum-milk-agar in the presence of this inhibitor.

Muller's finding that plaque-formation occurs in human blood-agar was confirmed but serum-haemoglobin-agar was found to be a better medium for this purpose. Serum-haemoglobin-agar, however, was not such a good medium as the principal test medium used so extensively throughout this work, viz. serum-milk-agar. A new medium, at least for the demonstration of Muller's phenomenon was devised by heating either filtered haemoglobin or washed red cells (horse or human) and adding to it fresh human

serum. Such a medium - a modified chocolate medium was superior to unheated haemoglobin-agar with fresh serum for the demonstration of Muller's phenomenon.

The use of heated plasma, with or without the addition of fresh serum, as a test protein for the demonstration of the phenomenon was also investigated. Confirmation of the development of plaques in these media was obtained, but, although zones of fibrinolysis were easily recognisable around staphylococcal colonies, the production of plaques was seldom good. The plaques which did develop were less numerous, less well-defined, more difficult to recognise and altogether less satisfactory than the plaques which developed with the same strains of staphylococci in serum-milk-agar or serum-haemoglobin-agar media. Plaque-production in heated plasma depended on the temperature-time combination employed in heating this test-protein, on the addition of serum, the sodium chloride concentration of the medium, the amount of the test-protein incorporated and the nature of the agar base employed.

PART V.

THE STAPHYLOCOCCAL FACTOR

IN

MULLER'S PHENOMENON.

PART V.

THE STAPHYLOCOCCAL FACTOR IN MULLER'S PHENOMENON.

A. PRELIMINARY EXPERIMENTS ON THE PRODUCTION OF THE STAPHYLOCOCCAL FACTOR.

B. DETAILED INVESTIGATIONS ON THE PRODUCTION OF THE STAPHYLOCOCCAL FACTOR.

C. THE CONCENTRATION AND PURIFICATION OF STAPHYLOCOCCAL FACTOR.

A. PRELIMINARY EXPERIMENTS ON THE PRODUCTION OF THE STAPHYLOCOCCAL FACTOR.

It is no new observation that when attempts are made to elucidate a particular bacterial action, there is much to be gained by the separation of the products of a micro-organism from its cells. When such a separation has been achieved the study of the action of these bacterial substances can then be undertaken without the complications which arise through the presence of living organisms and it permits the purification and concentration of these sterile bacterial products. Moreover it affords the opportunity for their chemical composition to be ascertained. This applies to many products of bacteria, be they toxins, enzymes or other substances, more or less complicated, which are to be found in the medium in which the cells have grown. Progress in our understanding of the nature and action of staphylocoagulase, that staphylococcal product which clots the plasma of man, rabbits and certain other animals was only made when it was separated from living staphylococcal cells. It was believed that this procedure of separating bacterial products from the cells might be applicable

also to the staphylococcal factor necessary for the demonstration of Muller's phenomenon, and if this factor could be obtained free from the living staphylococci that it would then be possible to dissociate its action from that of the multiplying organisms, thereby gaining a better understanding of the phenomenon. So long as living and multiplying staphylococci were used to show this effect, it was difficult, if not impossible, to assess whether any change in the experimental conditions affected directly the staphylococci, the production of the staphylococcal factor, its diffusion through the agar gel or the production of the phenomenon itself. The action of the staphylococcal factor could not be studied under conditions inimical to the growing staphylococci, a quantitative estimation of its activity could not be made, nor could its properties and nature be ascertained because it diffused into the agar gel on which the organisms were growing.

A. PRELIMINARY EXPERIMENTS ON THE PRODUCTION OF THE STAPHYLOCOCCAL FACTOR.

I. PREVIOUS ATTEMPTS TO PRODUCE CELL-FREE STAPHYLOCOCCAL FACTOR.

II. FIRST ATTEMPTS BY THE PRESENT WORKER TO PRODUCE CELL-FREE STAPHYLOCOCCAL FACTOR.

(a) BY ELEK'S METHOD.

(b) BY PACKALEN'S METHOD (MODIFIED).

III. INVESTIGATION OF POINTS CONSIDERED TO BE OF IMPORTANCE IN THE PRODUCTION OF THE STAPHYLOCOCCAL FACTOR AND IN THE DEMONSTRATION OF ITS ACTIVITY.

(a) DAY OF HARVESTING OF THE STAPHYLOCOCCAL CULTURE.

(b) INFLUENCE OF HUMAN SERUM ON THE PRODUCTION OF THE STAPHYLOCOCCAL FACTOR.

(c) AMOUNT OF HUMAN SERUM REQUIRED IN SERUM-MILK-AGAR PLATES FOR THE DEMONSTRATION OF ACTIVITY OF THE STAPHYLOCOCCAL FACTOR.

(d) THE USE OF STANDARD LABORATORY MEDIA.

(e) THE USE OF DIFFERENT INOCULA.

(f) SURFACE TO VOLUME RATIO IN STAPHYLOCOCCAL CULTURES.

(g) SEITZ FILTRATION OF STAPHYLOCOCCAL CULTURE.

(i) Effect of filtration on staphylococcal factor produced in fluid medium.

(ii) Effect of filtration on staphylococcal factor produced by Elek's method of preparation.

IV. DISCUSSION.

I. PREVIOUS ATTEMPTS TO PRODUCE CELL-FREE STAPHYLOCOCCAL FACTOR.

A number of workers have reported their attempts to produce the staphylococcal factor responsible for Muller's phenomenon, free from the staphylococcal cells. Some of these attempts were completely unsuccessful, while others would appear to have had some degree of success. Segre (1929) added to a human blood-agar plate the filtrate obtained by passing a four day old culture of Staphylococcus aureus through a Berkefeld filter, without any haemophagic spots developing. Cucco (1929) also added a staphylococcal filtrate to a blood-agar plate, presumably made with human blood, and because no spots of haemophagic clearing were produced, he concluded that staphylococci possess the property of producing the haemophagic phenomenon only when they are alive.

Rhodes (1938) tested out on human blood-agar plates the filtrates of staphylococci, which were known to produce the phenomenon when grown on the surface of human blood-agar plates. Cultures of these staphylococci which had been grown for periods of 20 hours, 5 days and 12 days in "plain beef extract broth" were filtered through a Berkefeld N filter. These filtrates incorporated in or placed on the surface of the blood-agar medium failed to

produce Muller's phenomenon.

Packalén (1941) reported that

> "after many failures a staphylococcal broth culture was obtained which remained active even after removal of the bacteria. The staphylococci were incubated for 7 days in an atmosphere containing 20 per cent. carbon dioxide ..... The staphylococcal broth was made sterile by passing it through a Seitz E.K. Filter. This filtrate produced typical hemolytic (sic) zones on the blood agar plate where placed, one or two drops at a time, for several successive days."

Packalén showed that the staphylococcal factor in the filtrate was not identical with either the α or β toxin of the staphylococcus and that it retained its haemophagic property after heating at 100°C. for 30-60 minutes, or after treatment with 1 or 2 per cent. phenol for 10 days.

Elek (1953) is the only other worker in this field who has described his attempts to produce this staphylococcal factor free from the organisms which produce it. His method is given here in full.

> "The preparation of active filtrates presented considerable difficulty. Filtrates of broth cultures prepared to yield

high titres of a haemolysin, lipase or fibrinolysin were inactive. In order to approximate the conditions under which staphylococci produce plaques, plates were prepared containing the basal medium (App. 463) and serum but no haemoglobin. Another set of plates contained the basal medium only. Both sets were inoculated in parallel streaks about 4 cms. apart with a strain of staphylococcus previously tested for activity. The plates were incubated in 30% CO_2 in air for four days, then frozen and allowed to thaw. The liquid which exuded was filtered through a Gradocal filter with an average pore diameter of 700 mμ. and tested in 1 ml. quantities. The addition of this amount of filtrate to 1 ml. of fresh serum, 0.5 ml. of haemoglobin and 10 ml. of basal medium led to the development of numerous plaques after about 2 days' incubation. Active filtrates were obtained whether serum was present or not in the plates used for making the extract thus demonstrating that staphylococcal factor does not require serum for its production, although its yield was greater in

the presence of serum. Similar experiments showed that meat extract was a desirable but not an essential constituent of the medium. Results were somewhat erratic and at times filtrates of low activity were obtained which produced plaques when added in 5 ml. volumes to the indicator system, but not in lesser amounts. At other times no activity of any sort was obtained."

Elek noted that the activity of the filtrate with regard to its ability to produce plaques of clearing in a serum-haemoglobin-agar plate was related to the age of the staphylococcal culture from which it was obtained. The filtrates from 3 day old cultures were the most active, those from 4 day old cultures were less active, while those from 5 and 6 day old cultures were completely inactive. An active filtrate required a period of from 24 to 48 hours to produce the haemophagic effect on the serum-haemoglobin-agar indicator plate.

In view of the success of both Packalén and Elek in producing cell-free staphylococcal factor the present worker decided to attempt to obtain this substance by similar means.

II. FIRST ATTEMPTS BY THE PRESENT WORKER TO PRODUCE CELL-FREE STAPHYLOCOCCAL FACTOR.

(a) BY ELEK'S METHOD.

A trial of Elek's method (Elek, 1953) was made first of all. Twelve Petri dishes of 14 cm. diameter, containing a mixture of 2.5 ml. fresh human serum and 25 ml. of Elek's Basal Medium (App. 463) in 1% New Zealand agar were inoculated from a 6 hour old "Lab-Lemco" broth culture of Staphylococcus aureus, Strain A which was known to be an active producer of plaques on serum-milk-agar plates. The inoculation of the plates was made with a platinum wire loop, in parallel streaks 4 cm. apart, as recommended by Elek. The Petri dishes, which were sealed with plasticine, were incubated at 37°C., 6 of them for 3 days and 6 for 4 days. On removal from the incubator after the specified time at 37°C., these plates were quickly frozen and then allowed to thaw. The fluid which exuded from the agar was pipetted off and sterilised by filtration through a Seitz filter fitted with an S.B. (sterilising) pad. The filtrate obtained was tested for its ability to produce plaques in standard serum-milk-agar plates (App. 477) to which it was added in amounts of 1 ml. to 4 ml.

No plaques developed in any of the serum-milk-agar plates with the amounts of filtrate tested, neither from a 3 nor from a 4 day old culture of Staphylococcus aureus, Strain A. After several unsuccessful attempts had been made to produce an active filtrate by this method, other ways of obtaining the cell-free staphylococcal factor were considered. It should be noted here that the failure to obtain an active filtrate by Elek's method was most likely one of technique in the filtering of the fluid and not because the staphylococcal fluid from the plates was necessarily inactive. This apparent failure to obtain active Staphylococcal factor by Elek's method will be discussed more appropriately at a later stage.

(b) BY PACKALÉN'S METHOD (MODIFIED).

The method advocated by Packalén (1941) or some modification of it appeared to be worthy of a trial. He had been able to obtain a staphylococcal broth culture which was active even after it had been sterilised by filtering it through a Seitz filter fitted with an E.K. (sterilising) pad. This sterile filtrate produced typical plaques of clearing where it was placed one or two drops at a time for several successive days on the surface of a human blood-agar plate. What was of especial interest was his finding that the staphylococcal factor was heat stable, it being able to withstand a temperature of 100°C., for periods of from 30 to 60 minutes. This suggested that it would be possible to sterilise a staphylococcal broth culture by heat and that preliminary filtration would be unnecessary. Packalén had incubated his broth culture for a period of 7 days in an atmosphere containing 20% carbon dioxide but in view of Elek's finding that the best time of harvesting the staphylococcal factor was from the second to the fourth day of incubation and that an atmosphere of carbon dioxide was unnecessary, the method adopted by the author in this attempt involved the incubation of the staphylococcal broth culture for 72 hours in air. Thus the first attempt to produce the staphylococcal factor responsible for plaque-production was made

in the following way. Two 250 ml. Pyrex conical glass flasks each containing 100 ml. of 1% "Lab-Lemco" broth (App. 468) were sterilised by autoclaving at 15 lbs./sq. inch for 15 minutes. When cold they were inoculated each with 2 ml. of a 6 hour old "Lab-Lemco" broth culture of Staphylococcus aureus, Strain A and incubated at 37°C. After 48 hours one flask was withdrawn from the incubator, the culture centrifuged and then placed in a number of thin walled glass test tubes 6 x $\frac{3}{4}$" in size. The upper one and a half inches of these test tubes were thoroughly heated in the bunsen flame before the cutton wool plugs were replaced, in order to sterilise this part of the tube because of the impossibility of ensuring that the upper ends of the test tubes would be sterilised when the tubes were placed in the water bath. When the test tubes had cooled sufficiently they were placed in a water-bath at 65°C. for 10 minutes in order to sterilise the culture. This time and temperature were known to kill this particular Staphylococcus aureus, Strain A. The other flask of culture, after 72 hours' incubation at 37°C. was similarly treated. These two sterile cultures were incorporated in serum-milk-agar plates in amounts from 1 ml. to 5 ml. to ascertain if they contained any of the staphylococcal factor necessary to demonstrate Muller's phenomenon. After 24 hours' incubation at 37°C. plaques of clearing indicative of this proteolytic

action were observed in the serum-milk-agar plates in which the 48 hour old culture filtrate had been incorporated in 4.0 and 5.0 ml. amounts and in those to which the 72 hour old culture filtrate had been added in amounts from 1.0 ml. to 5.0 ml. The plates which showed the plaques of clearing had them scattered throughout the whole of the medium, and in some plates there were many more plaques than in others. The plates which had been prepared with the 72 hour old culture in 3.0 ml. and 4.0 ml. amounts had the greatest number of plaques while the plate which had only 1.0 ml. of filtrate had only a few plaques. When these plates were examined after a further 24 hours, during which time they had been allowed to remain at room temperature, all of those which had previously shown plaques now showed an increased number of plaques (App. 144-145).

The results obtained in this preliminary experiment were considered sufficiently encouraging to persist in the use of a fluid medium as the means of producing the staphylococcal factor. It was apparent, however, that a number of points would have to be investigated if this method of preparing the staphylococcal factor was to be placed on a sound basis.

III. INVESTIGATION OF POINTS CONSIDERED TO BE OF IMPORTANCE IN THE PRODUCTION OF THE STAPHYLOCOCCAL FACTOR AND IN THE DEMONSTRATION OF ITS ACTIVITY.

(a) DAY OF HARVESTING OF THE STAPHYLOCOCCAL FACTOR.

Although Elek had found that the best day of harvesting the staphylococcal factor under the conditions of his experiments, was the third day of incubation and that a marked and rapid fall of activity occurred from the fourth day onwards, it was realised that this would not necessarily hold good when a different medium was employed in different conditions. It was considered to be of prime importance to ascertain the best day of harvesting the culture in order to obtain as large a yield of the staphylococcal factor as possible. Six 250 ml. flasks, each containing 100 ml. of 1% "Lab-Lemco" broth were each inoculated with 2.0 ml. of a 6 hour old "Lab-Lemco" broth culture of Staphylococcus aureus, strain A and incubated at 37°C. One flask of culture was removed each day from the incubator from the second to the seventh day after inoculation, centrifuged and then sterilised by heating at 65°C. for 10 minutes by the method previously described. The sterilised culture was tested in amounts from 1.0 ml. up to 7.0 ml. for its ability to produce plaques of clearing in serum-milk-agar plates (App. 146-147).

Plaques developed on some of the serum-milk-agar plates on each of the 6 days on which the tests were carried out. After 24 hours' incubation at 37°C. plaques of clearing were observed on three plates made with the 2, 6 and 7 day old cultures, on five plates with the 3 and 4 day old cultures and 4 plates with the 5 day old culture. After the plates had been left at room temperature for a further 24 hours, an increase in the amount of the proteolytic activity was observed in all plates. This was indicated by an increase in the number of plaques on the plates which had previously shown plaques after the period of incubation at 37°C. and by the presence of plaques for the first time in those plates where no plaques had been seen before. In the 2, 3 and 7 day old culture plates there were plaques in 6 out of the 7 plates in each set; where there had been plaques at the end of the previous incubation period of 24 hours, they were now greatly increased in numbers. In the 4, 5 and 6 day old culture plates there had been a marked extension of the destruction of the milk in these plates manifest in the following way. Whereas at the end of the first 24 hours' incubation the plates in these sets had still retained much of their whiteness, the plaques appearing as small circular punched-out areas of clearing against the white background, after the further 24 hours at room temperature, they

had now increased in numbers and size and had fused, destroying completely or almost completely the white opacity of the serum-milk-agar. Where this destruction was complete the agar was seen to be quite transparent over its entire area, no trace of the milk being seen, its whiteness and opacity having disappeared. This degree of complete destruction is recorded in experiments as lysis and is denoted by "L". When the destruction was not complete an assessment was made of the degree of destruction, e.g. 50% lysis indicates that half the area of whiteness of a serum-milk-agar plate had been destroyed. In the recording of the experiments, to indicate that plaques were present, "P" accompanies the percentage lysis figure given. Thus "P.80% L." conveys that plaques were present in a serum-milk-agar plate in which the area of destruction of the milk amounted to 80%, due to the presence of these punched-out areas of clearing, - plaques.

Control plates were set up in all these experiments, to show that in the absence of either serum or the sterilised staphylococcal culture plaque-formation did not occur. From this experiment, it appeared that the best time for harvesting the staphylococcal culture with regard to its content of the factor required for Muller's phenomenon was from the third to the fourth day, a finding confirmed by many other experiments.

(b) INFLUENCE OF HUMAN SERUM ON THE PRODUCTION OF THE STAPHYLOCOCCAL FACTOR.

Although many different nutrient media will support the growth of a particular micro-organism it is not unusual to find that only some of them will permit the production of a certain toxin, enzyme or other substance by that organism. Often the amount of such a substance produced in one nutrient medium may be very great while that produced in another is very small. If one is interested in obtaining the maximum possible yield of a bacterial product, it is essential that attention be paid to the nutrient used. Thus, it has been found possible to increase the yield of a particular bacterial product which appears in a standard laboratory medium by adding additional nutrients, such as proteins or carbohydrates, or mineral salts or growth substances. Blood, serum or ascitic fluid are commonly added to a medium to enhance the yield of organisms or bacterial toxin, enzyme or other product. Elek (1953) found that the addition of 10% heated human serum to his basal medium resulted in increased activity of the staphylococcal extract prepared in this enriched medium. Even greater activity was obtained if fresh instead of heated human serum was added. In view of Elek's finding

it was thought worthwhile to grow Staphylococcus aureus, Strain A in a "Lab-Lemco" broth containing 10% fresh human serum to ascertain if the staphylococcal factor produced had a greater activity than that obtained from a "Lab-Lemco" broth without added serum. Such an experiment was performed and is presented in App. 148-149.

There was a marked enhancement of the activity of a 2 day old culture in this 10% serum-"Lab-Lemco" broth compared with that of a similarly aged culture in "Lab-Lemco" broth in the previous experiment. The activity of the 3, 4 and 5 day old cultures in 10% serum-"Lab-Lemco" broth, however, was much less than that of the 2 day old culture in the same medium and also that of the 3, 4 and 5 day old cultures in the "Lab-Lemco" broth without serum used in the previous experiment.

In view of the fact that serum is necessary to demonstrate Muller's phenomenon it may be objected that its inclusion in the medium in which the staphylococcal factor is being produced may be the reason for any increased activity detected in the staphylococcal culture. It was shown, however, that the heat-killed staphylococcal culture itself when added to a milk-agar plate without serum was inactive, it being possible to produce plaques only when fresh serum was added to the milk-agar plate containing the staphylococcal culture. This would indicate that

the serum factor, which plays an essential part in plaque-production, was destroyed by staphylococci growing in "Lab-Lemco" broth containing serum.

It was known from experiments carried out with strains of staphylococci on serum-milk-agar plates that the addition in sufficiently high concentration of sodium chloride or peptone to a medium in which Muller's phenomenon could be regularly demonstrated inhibited plaque-formation. The 1% "Lab-Lemco" broth which had been used in the experiments up to date had contained 0.5% sodium chloride and 1% peptone (Oxoid). A batch of 1% "Lab-Lemco" medium was prepared without the usual 0.5% sodium chloride and 1% peptone (Oxoid) but with 10% fresh human serum and was inoculated with Staphylococcus aureus, Strain A (App. 150-151).

It was found, however, that in this medium the staphylococcal factor necessary to demonstrate Muller's phenomenon was not produced.

(c) AMOUNT OF HUMAN SERUM REQUIRED IN SERUM-MILK-AGAR PLATES FOR THE DEMONSTRATION OF ACTIVITY OF STAPHYLOCOCCAL FACTOR.

Up to this time the batches of staphylococcal factor had been tested in serum-milk-agar plates which contained 1.5 ml. of pooled human serum. This particular amount of serum had been arrived at during the course of the early experiments with strains of staphylococci as a quantity of serum which allowed a zone of plaques to develop around the colonies of the organisms. The use of this amount of serum appeared to have been satisfactory in that the proteoclast activity of a number of staphylococcal cultures had been demonstrated without any great difficulty. It was decided, however, that it would be better at this stage to confirm that this quantity of serum, i.e. 1.5 ml. in the 15 ml. serum-milk-agar mixture was indeed the best to use. A new batch of the staphylococcal factor was prepared in1% "Lab-Lemco" broth, and on this occasion its activity was tested out with quantities of serum from 0.5 ml. up to 2.0 ml. incorporated in the milk-agar plates. (App. 152-153.) Three day and four day old heat-killed cultures were tested out with these different amounts of serum and, although this was not a very active batch of staphylococcal factor it was apparent from the examination of the plates after 48 hours'

incubation, that those which contained 0.5 ml. of serum showed activity of the staphylococcal factor in the form of plaques of clearing while those plates which contained 1.0 ml., 1.5 ml. and 2.0 ml. serum did not. This observation was also made with the next batch of staphylococcal factor produced and as a result it was decided that in future the activity of staphylococcal factor with regard to its proteoclast effect would be estimated in a serum-milk-agar plate containing 0.5 ml. of serum and not 1.5 ml. as used hitherto. No explanation could be given of the fact that it was sometimes not possible to demonstrate Muller's phenomenon with a given staphylococcal culture on a serum-milk-agar plate containing a large quantity of serum although an equal amount of the same staphylococcal culture with a smaller quantity of the same serum would in fact show the phenomenon. It did suggest that the larger quantity of serum contained an inhibitor of some kind which prevented the development of plaques. This point, however, was not investigated at this time.

(d) THE USE OF STANDARD LABORATORY MEDIA.

Although the staphylococcal factor responsible for Muller's phenomenon had been produced in useful amounts in 1% "Lab-Lemco" broth it was considered advisable to obtain some information about its production in other media. Four media were chosen for the first experiment in this series. There were

(a) 1% "Lab-Lemco" broth,

(b) 1% "Lab-Lemco" broth containing 5% fresh human serum,

(c) meat broth, and

(d) meat broth containing 5% fresh human serum.

The results of this experiment are given in App. 154-155.

The activity of the staphylococcal factor prepared from the two media with the added serum was greater than that of the same two basic media without the added serum when the cultures were 1 and 2 day old. With 3 day old cultures, however, it was found that the activity of the meat broth was equal to that of the meat broth with serum and of the "Lab-Lemco"-serum broth, while the activity of the "Lab-Lemco" broth was not far below. The 4 and 5 day old cultures of the meat broth and the meat broth with serum were superior in activity, when used in 1.0 ml. amounts to the "Lab-Lemco" broth and "Lab-Lemco" broth with serum cultures used

in the same amounts.

The other interesting point which emerged from this experiment was that the activity in the form of plaques of clearing could be detected with the 3 and 4 day old cultures after only $2\frac{1}{4}$ hours' incubation at $37^{\circ}C$. This is in advance of the finding of Elek who, noting that the more active the staphylococcal culture was, the earlier plaques appeared, did not observe plaques in serum-haemoglobin-agar in less than 12 hours with his staphylococcal factor preparations. It should be noted, however, that different proteins are not all broken down at the same rate by the same enzyme. The effect of the staphylococcal factor on serum agar plates containing different test proteins milk, haemoglobin, heated haemoglobin and heated plasma is dealt with in Part VI.

On account of the fact that there was no difference in the amount of activity detected in the staphylococcal cultures in meat broth with serum and meat broth without serum, it was decided that when meat broth was used as a medium for the production of the staphylococcal factor no serum would be added. The finding made in the experiment just presented (App. 154-155) in which the activity of the staphylococcal factor produced in meat broth was greater, even if only slightly greater, than that

in "Lab-Lemco" broth, was confirmed in a number of other experiments.

A number of different standard laboratory media were used in a series of experiments designed to test the suitability of such media for the production of the staphylococcal factor. One such experiment in which the media tested were meat broth, casein digest broth, 1% peptone water (Oxoid), 1% "Lab-Lemco" broth and horse heart digest broth is given in App. 156-157. The activity of the staphylococcal factor was greater in the meat broth medium than in any of the other media, including the 1% "Lab-Lemco" broth.

In another experiment estimations of the activity of a meat broth culture and a "Lab-Lemco" broth culture of Staphylococcus aureus, Strain A made over a period of a week, indicated that the meat broth medium was better than the "Lab-Lemco" medium for the production of the staphylococcal factor (App. 158-159).

(e) THE USE OF DIFFERENT INOCULA.

All the flasks of media in which the staphylococcal factor had been produced, had received up to this time a standard inoculum, viz. 2.0 ml. of a 6 hour old "Lab-Lemco" broth culture of Staphylococcus aureus, Strain A. It did appear possible that the age and nature of the inoculum might exert some influence on the production of the staphylococcal factor and a number of experiments were designed to ascertain if this suggestion was correct or not.

In one of these experiments (App. 160-161) in which four flasks of meat broth medium each received a different type of inoculum the activity of the four batches of staphylococcal factor produced differed one from another when tested out in quantities of 1.0 ml. in serum-milk-agar. The differences were not great but were sufficient to warrant notice. Complete clearing (100% lysis) of a serum-milk-agar plate was brought about by incorporating 1.0 ml. of staphylococcal culture inoculated from the 6 weeks' old ice-chest culture while a corresponding serum-milk-agar plate containing 1.0 ml. of culture inoculated from a 7 day old room temperature culture showed only plaques.

In another experiment (App. 162-163) in which the

activity of staphylococcal factor prepared in meat broth inoculated from a 6 hour old meat broth culture of Staphylococcus aureus, Strain A was compared with the activity of staphylococcal factor prepared in the same meat broth medium but inoculated from a 4 day old meat broth culture no difference was detected. When, however, the activities of these preparations of staphylococcal factor were compared with three subsequent batches each of which had been inoculated from the 4 day old staphylococcal culture used to prepare the immediately previous batch of staphylococcal factor, marked differences were observed. The reason for these differences in the activity of the staphylococcal factor preparations was not apparent; the strain of organism was the same, the age of the inocula was the same and the medium used to cultivate the organisms came from the same batch.

The activities of ten subsequent batches of staphylococcal factor, which were prepared during the month of July, 1956 are given in App. 172-173. These batches fall into two groups, one of six lots, the other of four. The former group, which had as the source of its inoculum a culture of Staphylococcus aureus, Strain A which had been preserved in the ice-chest for nine months, had high activity which was consistent throughout the six batches in this group. The latter group, which consisted of four

batches had as the source of its inoculum a 12 month old waxed culture of Staphylococcus aureus, Strain A. The activity of the staphylococcal factor of these four batches was less than that of the six batches in the former group.

In seemed from these experiments that the nature, age and source of the inoculum used in the production of staphylococcal factor could play some part in its activity but the manner in which it exerted its influence was not clear at this time.

(f) SURFACE TO VOLUME RATIO IN STAPHYLOCOCCAL CULTURES.

Conical flasks of 250 ml. volume containing 100 ml. of the chosen medium had been used in almost all of the experiments on the production of the staphylococcal factor carried out up to this time. It seemed possible that the surface to volume ratio of the culture medium might play a significant part in the production of the staphylococcal factor.

In one experiment carried out to find if, indeed, the surface to volume ratio was of importance, Kolle flasks were employed. App. 164-165). One Kolle flask was incubated vertically and in this position held 200 ml. of meat broth with a very small surface area; another Kolle flask holding 60 ml. was incubated horizontally and gave a very large surface area. Although the activity of the staphylococcal factor produced from the 2 day old horizontal culture was greater than that of the vertical culture of the same age no great difference in the activity of the factor prepared from 4 day old cultures. was observed.

It was concluded from this experiment, in which the maximum difference in surface to volume ratio had been achieved, that this alteration in one of the important physical conditions,

had little or no effect on the production of the staphylococcal factor.

Five batches of staphylococcal factor were produced in meat broth in 250 ml. flasks. In place of the usual 100 ml. of medium only 60 ml. were used (App. 166-167). The activity of the batches of staphylococcal factor obtained was about the level usually reached in other experiments where 100 ml. of meat broth was employed in a 250 ml. flask.

(g) SEITZ FILTRATION OF STAPHYLOCOCCAL CULTURES.

As an alternative to heat-killing a staphylococcal culture at 65°C. for 10 minutes in order to sterilise it, a culture can be Seitz-filtered without loss of activity of the staphylococcal factor provided that either the sterilising filter pad is washed by passing distilled water or meat broth through it or that the first 10 ml. of filtrate is discarded.

(i) Effect of filtration on staphylococcal factor produced in fluid medium.

A heat-killed culture of Staphylococcus aureus, Strain "A", of known activity on serum-milk-agar was used in this experiment (App. 168-169). A 50 ml. sample of this sterilised culture was passed through a Seitz filter, fitted with a sterilising pad. The first 10 ml. of filtrate was collected and tested separately from the remainder of the filtrate. The first 10 ml. of filtrate was found to be completely inactive with regard to the production of plaques in serum-milk-agar when tested out under the same conditions as a sample of the original heat-killed culture, and the rest of the filtrate from the Seitz filtration. The rest of the filtrate

showed very slight loss of activity when compared with the original heat-killed staphylococcal factor.

When the staphylococcal factor was mixed with human serum before being Seitz-filtered its activity was reduced in the first 10 ml. of filtrate; the rest of the staphylococcal factor - serum mixture filtrate was only slightly less active than a similar mixture unfiltered.

Other experiments which will be reported later showed that if 20 ml. distilled water or meat broth was passed through a Seitz filter before such a staphylococcal culture was filtered it was unnecessary to discard the first 10 ml. of the filtrate.

Although the experiment reported here was carried out with an already heat-killed culture, in order to find out how great if any was the reduction in the activity of the staphylococcal factor, Seitz-filtration of a living staphylococcal culture is just as satisfactory. An experiment comparing the activity of staphylococcal factor in heat-killed and Seitz-filtered samples of the same staphylococcal culture is given in App. 208–209.

When it was realised that the first 10 ml. of filtrate of a staphylococcal culture was inactivated by passage through a Seitz filter, a possible explanation was found for the fact that

failure had attended the first attempts to produce the staphylococcal factor by Elek's method. In view of this a further attempt to obtain the staphylococcal factor by Elek's method was made.

(ii) Effect of filtration on staphylococcal factor produced by Elek's method of preparation.

Staphylococcal factor was prepared by Elek's method on solid medium (Elek, 1953). After freezing and thawing the agar plates, fluid was found to exude from the agar and in all 60 ml. of this fluid was collected (App. 170-171). It was filtered through a Seitz filter fitted with a sterilising pad which had been previously washed with 20 ml. of sterile distilled water. The filtrate obtained was found to possess staphylococcal factor of a high degree of activity when tested out in serum-milk-agar plates. The filtrate was also active when tested out in serum-haemoglobin-agar plates as recommended by Elek and to be as active as the preparations obtained by him.

It is not without interest to note that the degree of activity detected on these two test-proteins differed markedly. The filtrate incorporated in serum-milk-agar plates in amounts of 0.5 ml. to 4.0 ml. brought about complete clearing of the medium,

i.e. 100% lysis and this was the result of the fusion of the numerous plaques which had developed earlier. The filtrate incorporated in serum-haemoglobin-agar in amounts of 0.5 ml. to 4.0 ml., however, caused the appearance of plaques only, in numbers less numerous than in the corresponding serum-milk-agar plates. The reason for this may be that a 1.5 ml. amount of serum was used in each serum-haemoglobin-agar plate (the equivalent of the 1.0 ml. amount used in Elek's serum-haemoglobin-agar plates of 10 ml. volume) whereas a 0.5 ml. amount was used in the serum-milk-agar plate. This is not the complete explanation of the finding of greater activity in the milk-containing plates. It was found in a number of experiments that milk is much more easily broken down in such a system, than is haemoglobin, or heated haemoglobin, even when the same concentration of serum is used. One experiment in which this difference between milk and haemoglobin is shown is given in App. 276-277.

IV. DISCUSSION.

The first attempt to produce the staphylococcal factor responsible for Muller's phenomenon, free from staphylococcal cells by the method recommended by Elek, was a failure. The reason for this failure lay not in Elek's method as such, but in the filtration of the culture. The staphylococcal factor was absorbed or inactivated by the sterilising pad of the Seitz filter employed. It was found later that this difficulty could be overcome either by discarding the first 10 ml. of filtrate obtained or by washing the pad with distilled water or "Lab-Lemco" broth before filtering the culture. It was thus found possible to obtain by this method a filtrate with a high degree of activity with regard to Muller's phenomenon, but, by the time this had been achieved, another method of preparation had been successfully investigated.

Packalén (1941) prepared, after some considerable difficulty, a filtrate which would produce Muller's phenomenon when tested on human blood-agar and he reported that this filtrate could withstand heating at 100°C. for a period of from 30 to 60 minutes without loss of its ability to produce plaques. It seemed to the present worker, if this report of the thermostability of

staphylococcal factor was correct that it should be possible to sterilise a staphylococcal culture without loss of activity of any staphylococcal factor it might contain. This method of preparation of sterile staphylococcal factor was employed with success. A staphylococcal culture, heated at 65°C. for 10 minutes was found to be sterile and capable of producing Muller's phenomenon in serum-milk-agar.

In view of the success achieved by this simple method of preparation of staphylococcal factor, it was thought instructive to re-examine the accounts of previous workers who had attempted, without success, to produce the staphylococcal factor to ascertain, if possible, the reasons for their failures.

Rhodes (1938) had tried Berkfeld N filtrates of beef broth cultures, 20 hours, 5 days and 12 days old, in and on human blood-agar plates, without success. Segre (1929) also had used unsuccessfully a Berkfeld filtrate of a 4 day old culture of a staphylococcus as had Cucco (1929). Muller too recorded his failures to produce haemophagy with filtrates.

It is evident from the experiments carried out and reported above that one or more of a number of factors may have been the cause of their failure. The day of harvesting of the staphylococcal factor plays an important part in obtaining a

preparation of high activity. Although staphylococcal factor was present in cultures of Staphylococcus aureus, Strain A, 2 to 7 days' old, the activity of the factor was greatest in the 4 and 5 day old cultures. That these are necessarily the best days for harvesting with all strains of staphylococci, or in all media, or under all circumstances, is not claimed, but it is likely that Rhodes missed the peak of activity in the 20 hours' and 12 days' old culture filtrates which she examined, although some activity might have been expected with her 5 days' old culture filtrate.

The media used by some of the previous workers may well have played an important part in their lack of success in producing the staphylococcal factor. It has been shown that some media are much better than others in that they permit more of the staphylococcal factor to be produced. It has also been shown that in certain other media, in which the strain of staphylococcus used grew well staphylococcal factor was not produced.

Whether Muller's phenomenon will be manifested or not in a plate containing a test protein and staphylococcal factor will depend on the amount of serum present. If there is only a small amount of staphylococcal factor present it may not be detected unless the amount of serum is also small. If serum is in excess, Muller's phenomenon will not develop and it might be concluded that

there was no staphylococcal factor present. It is possible that some of the earlier workers may not have been able to demonstrate Muller's phenomenon on account of the fact that there was an excess of the serum factor in the human blood-agar plates which they employed compared with the small amount of staphylococcal factor present in their filtrates.

It is known from previous work on staphylococcal haemolysins that filtration of a staphylococcal culture through a Berkefeld filter gives a filtrate with a reduced amount of haemolysin. It may be that the staphylococcal cultures of Rhodes and Segre which were filtered through Berkefeld filters had the contained staphylococcal factor inactivated by the filtration.

There is evidence that milk may be broken down more easily than haemoglobin (App. 276-277). This would suggest that more staphylococcal factor would be required to break down a human blood-agar plate than a serum-milk-agar plate. Thus, a small amount of staphylococcal factor prepared by some of the earlier workers in their cultures may have escaped detection on account of the fact that human blood was used as the test protein.

When all the points mentioned above are taken into consideration, the failure of Rhodes and Segre, and the difficulty of Packalén to produce staphylococcal factor free from living

staphylococci can be readily understood.

It is clear from the experiments carried out and reported above that a number of factors can influence the production and activity of the staphylococcal substance which is necessary for the demonstration of Muller's phenomenon. The medium in which the staphylococcus is grown and the day on which the culture is harvested are both of importance. No less important is the method of preparation of the staphylococcal factor and the nature of the protein on which its activity is measured.

So far no attention has been given to the strain of staphylococcus and the way in which it is used, although these points also are of importance. Staphylococcus aureus, Strain A was the organism used in all these experiments and was chosen for this work, on account of the fact that it was a very active producer of Muller's phenomenon on serum-milk-agar medium.

The results given in App. 162-163 show that batches of staphylococcal factor prepared under the same conditions in the same medium but which had received inocula which varied in age, possessed different activities. It can be understood that the vigour of different-aged inocula would not be the same and that this might explain small differences in the activity of various batches of staphylococcal factor harvested. There were much

bigger differences, however, in the activity of the batches of staphylococcal factor prepared in the experiments reported in App. 162-163 and App. 172-173. It appeared from these experiments that on certain occasions a culture of Staphylococcus aureus, Strain A lost its ability to produce staphylococcal factor with the customary high degree of activity. The reason for this unpredictability of Strain A was unknown but it was considered essential that this staphylococcus should be "tamed" if highly active staphylococcal factor were to be produced when required. Until the action of Strain A in producing staphylococcal factor in a reliable medium could be predicted with certainty further investigation into the optimal conditions for the production of staphylococcal factor would be pointless.

Accordingly investigations were undertaken to ascertain why certain cultures of Staphylococcus aureus, Strain A produced batches of staphylococcal factor of low activity. Once this was achieved a more thorough examination of the conditions affecting the production of staphylococcal factor was made.

B. DETAILED INVESTIGATIONS ON THE PRODUCTION OF THE STAPHYLOCOCCAL FACTOR.

I. INVESTIGATIONS OF THE PURITY AND STABILITY OF STRAINS OF STAPHYLOCOCCUS AUREUS.

(a) INVESTIGATION OF STRAIN A.

(b) EXAMINATION OF STRAIN A, TYPES I AND II.

(c) INVESTIGATIONS OF STRAINS 72822 and 73016.

II. THE PRODUCTION OF STAPHYLOCOCCAL FACTOR BY STAPHYLOCOCCUS AUREUS, STRAIN AII.

(a) THE USE OF STANDARD LABORATORY MEDIA.

(b) COMPARISON OF HEAT-KILLING AND FILTRATION IN THE PREPARATION OF STERILE STAPHYLOCOCCAL FACTOR.

(c) THE EFFECT OF THE GASEOUS ENVIRONMENT ON THE PRODUCTION OF STAPHYLOCOCCAL FACTOR.

(d) THE EFFECT ON THE PRODUCTION OF STAPHYLOCOCCAL FACTOR OF THE ADDITION OF SERUM TO THE CULTURE MEDIUM.

(e) THE EFFECT ON THE PRODUCTION OF THE STAPHYLOCOCCAL FACTOR ON DIFFERENT INOCULA OF STRAIN AII.

(f) THE EFFECT OF THE pH REACTION ON THE ACTIVITY OF THE STAPHYLOCOCCAL FACTOR.

(g) THE TESTING OF STAPHYLOCOCCAL FACTOR WITH DIFFERENT SPECIMENS OF HUMAN SERUM.

(h) THE ACTIVITY OF THE STAPHYLOCOCCAL FACTOR PRODUCED IN MEDIUM D7 BY STRAINS OF STAPHYLOCOCCI OTHER THAN AII.

III. SUMMARY.

I. INVESTIGATIONS OF THE PURITY AND STABILITY OF STRAINS OF STAPHYLOCOCCUS AUREUS.

(a) INVESTIGATION OF STRAIN A.

Originally isolated from a staphylococcal lesion this coagulase-positive staphylococcus, strain A, had proved itself over many months to be one of the best producers of Muller's phenomenon when stab-inoculated in to serum-milk-agar plates. On account of its consistent ability to demonstrate Muller's phenomenon so well, it had been chosen for the experiments on the production of the staphylococcal factor in culture. Although it did not seem likely that it was primarily this "good-producer" of staphylococcal factor which was to blame for the occasional batches of factor of low activity it was considered necessary nonetheless to subject it to close examination.

The stock cultures of Strain A were kept on "Lab-Lemco" agar slopes at room temperature and subcultured every 7-10 days. Cultures suitable for the inoculation of serum-milk-agar plates or for the flasks of medium used in the production of the staphylococcal factor were made in 1% "Lab-Lemco" broth inoculated directly from these stock cultures. Examinations of the purity of

the stock cultures were made repeatedly over the months and always the growth was found to be pure consisting only of a coagulase-positive Staphylococcus aureus which was able to produce Muller's phenomenon when stab-inoculated into serum-milk-agar plates.

A tube of 1% "Lab-Lemco" broth was inoculated with Strain A directly from a stock culture and incubated at 37°C. for 21 days. Subcultures were made from it on to serum-milk-agar plates and 10% horse blood-agar plates after 6, 18 and 24 hours' and after 5, 7, 8, 12, 14, 19 and 21 days' incubation (App. 174-175). Colonies of staphylococci grew on all the serum-milk-agar plates and around them lysis and plaques developed. Two types of staphylococcal colony, however, were observed on the serum-milk-agar plates inoculated from the 12, 14, 19 and 21 days' old "Lab-Lemco" broth culture - Type I which had around it a narrow zone of indefinite lysis without plaques and Type II which was smaller and had around it a zone of lysis with plaques. This latter type of colony adhered to the medium and was sticky when touched with a platinum wire, properties not shared with the Type I colony. Previously, however, two types of colony, one a flat haemolytic colony (Type I) and a smaller raised non-haemolytic colony (Type II) had been observed on the horse blood-agar plates inoculated from the 24 hours', the 5, 7 and 8 days' old cultures.

The haemolytic and non-haemolytic colonies which had grown from the 5 days' old "Lab-Lemco" broth culture were further investigated on horse blood-agar plates and on serum-milk-agar plates (App. 176-177). "Lab-Lemco" broth cultures of these two types of colony gave rise on the horse blood-agar plates to pure cultures of the haemolytic and of the non-haemolytic types. The haemolytic type of colony gave rise to colonies surrounded by a narrow zone of indefinite lysis on the serum-milk-agar plates while the non-haemolytic colony give rise to colonies surrounded by lysis and plaques. The Type I colony distinguished on the blood-agar plates by being haemolytic and flat corresponds to the Type I colony observed on serum-milk-agar plates and distinguished by the indefinite lysis around it. The Type II colony which was non-haemolytic on blood-agar corresponds to the Type II colony seen on serum-milk-agar and distinguished by a zone of lysis and plaques around it. The Type II colony which was noted as non-haemolytic on horse blood-agar did in fact produce slight haemolysis but only after 48 hours' incubation at 37°C.

The Type I and Type II colonies which were grown on the blood-agar plates inoculated from the 7, 8, 14 and 19 days' old culture of Strain A gave similar results to those obtained with the colonies isolated from the 5 days' old culture, when subcultured

on serum-milk-agar and horse blood-agar plates. "Lab-Lemco" broth cultures of the two types found on all the plates, both blood-agar and serum-milk-agar, were coagulase-positive.

From one set of horse blood-agar plates and serum-milk-agar plates Type I and Type II colonies were picked up for further investigation. They were grown in 1% "Lab-Lemco" broth and in meat broth for 6 hours at 37°C., when these broth tubes were used to inoculate horse blood-agar and serum-milk-agar plates. These plates were incubated at 37°C. for 24 hours when readings were taken (App. 178-179). The 1% "Lab-Lemco" broth and meat broth cultures were also incubated at 37°C. for 24 hours.

(b) EXAMINATION OF STRAIN A, TYPES I AND II.

All the cultures of the Type I strain whether in 1% "Lab-Lemco" broth or in meat broth, inoculated either from the horse blood-agar plate or from the serum-milk-agar plate gave a similar appearance. In broth cultures of Type I uniform turbidity with sediment was present while in the serum-milk-agar plates there was indefinite lysis around the colonies and on the horse blood-agar plates all the colonies were surrounded with a zone of haemolysis. So also with the Type II strain all the cultures of it, whether in 1% "Lab-Lemco" broth or in meat broth, inoculated from the horse blood-agar plate or serum-milk-agar plate gave similar appearance. The growth in broth, whether 1% "Lab-Lemco" or meat, had a very slight turbidity accompanied with a marked sediment. In the serum-milk-agar plate there was a zone of lysis with plaques around each colony while in the horse blood-agar plates none of the colonies showed haemolysis. All the "Lab-Lemco" broth and meat broth cultures of Type I and Type II colonies when subjected to testing for coagulase, were found to be coagulase-positive by the tube method.

From these experiments it appeared that the cultures of Type I and Type II colonies were pure and in view of the fact

that further investigations of these two types would have to be carried out stock cultures of them were prepared, Twelve "Lab-Lemco" agar slope cultures of Strain A, Type I and Type II were prepared in addition to a similar number of cultures of the parent Strain A. After incubation at 37°C. for 24 hours these cultures were waxed and stored at room temperature. The activities of Strain A, Type I and Type II (referred to hereafter as AI and AII respectively) were checked on horse blood-agar and serum-milk-agar plates before the cotton wool plugs of the test-tubes were waxed and these stock cultures stored at room temperature.

The biochemical activities of Staphylococcus aureus, Strains AI and AII were compared by inoculating 20 hour old peptone water cultures of these organisms into 'sugar' sets made up of lactose, glucose, sucrose, mannitol and dulcite. Tubes of litmus milk and 15% nutrient gelatin were also inoculated in addition to plates of 10% horse blood-agar and serum-milk-agar. All the inoculated media were incubated at 37°C. except the nutrient gelatin which was left at room temperature. The plates were examined after 24 hours' incubation and the 'sugar' sets, litmus milk and gelatin tubes after 5 days' incubation (App. 180-181).

As was to be expected from previous examinations of Strains AI and AII on serum-milk-agar and horse blood-agar Muller's

phenomenon was demonstrated on the former plate around colonies of Strain AII only, a narrow zone of indefinite lysis being present around colonies of Strain AI; on the horse blood-agar plates Strain AI was haemolytic, and AII non-haemolytic. The activities of Strain AI and AII on the rest of the media used in this experiment were identical. These results have been summarised in Table XXIII (App. 448).

The 'sugar' sets, the litmus milk, the gelatin and the peptone water cultures of AI and AII from the previous experiment were used as sources of inocula for serum-milk-agar plates. These plates were stab-inoculated and then incubated at 37°C. for 24 hours. The colonies of Strain AI which grew from all the different media were surrounded by the usual zone of indefinite lysis. Strain AII produced Muller's phenomenon around its colonies grown from all the media.

Serum-milk-agar plates were inoculated from 6 hour old cultures of Strains AI and AII in nitrate broth, glucose broth, glucose phosphate broth and Robertson's meat broth. The plates were incubated at 37°C. for 24 hours when examination revealed that all colonies of Strain AII showed Muller's phenomenon while those of Strain AI did not.

These two strains of Staphylococcus aureus were

FIG. XXIX. - A standard serum-milk-agar plate showing colonies of Staphylococcus aureus, Strain AI and Strain AII. A narrow zone of indefinite lysis can be seen around the colonies of Strain AI, while around the colonies of Strain AII, Muller's phenomenon (plaque-formation) and zones of definite lysis can be observed. (Incubated at 37°C. for 24 hours.)

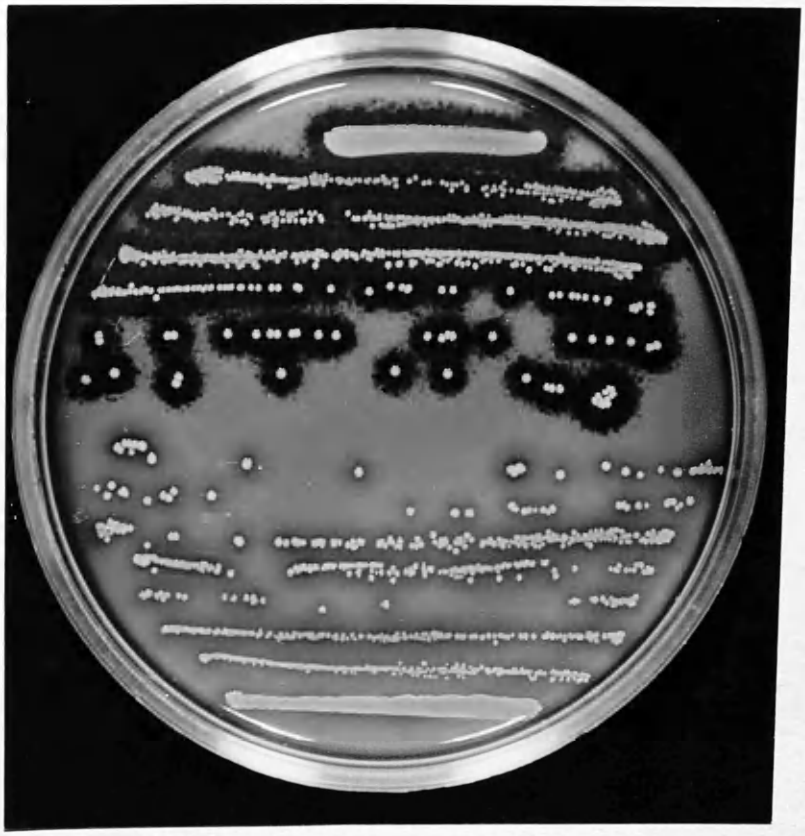


Fig. XXIX

cultivated on many occasions in the standard laboratory media and on each occasion when they were subcultured on to serum-milk-agar plates, AII always showed around its colonies a zone of lysis and plaques - Muller's phenomenon, while around colonies of AI only a narrow zone of indefinite lysis was to be seen. It appeared from these experiments that Strains AI and AII were stable, at least with regard to their inability and ability respectively to produce Muller's phenomenon on serum-milk-agar plates. One such serum-milk-agar plate showing colonies of Strain AI and AII was photographed and is reproduced as Fig. XXIX.

Coagulase tests were performed on many occasions with broth cultures of the two strains and always they were coagulase-positive. Overnight cultures of these two strains added to human plasma would clot it in about 30 minutes. The clot produced by Strain AII lysed within 18 hours but that produced by Strain AI did not lyse within 48 hours. The fibrinolytic action of these two strains AI and AII will be discussed later.

A comparison of the activities of Strains AI and AII on a number of different media is given in Table XXIII (App. 448).

Strains AI and AII were phage-typed by the Department of Bacteriology, Royal Victoria Infirmary and were found to belong

to the same phage types. Moreover, when these two strains were subjected to antibiotic-typing they gave similar patterns of sensitivity and resistance to penicillin, streptomycin, chloramphenicol, tetracycline and erythromycin.

The ability of Strain AII to produce the staphylococcal factor in meat broth was ascertained and at the same time the inability of Strain AI to produce it was also demonstrated. Seven flasks, each containing 50 ml. of meat broth were each inoculated with 1 ml. of a 6 hour old "Lab-Lemco" broth culture of Strain AII, prepared from a waxed stock culture. Another set of flasks was prepared in the same way and inoculated with 1 ml. of a 6 hour old "Lab-Lemco" broth culture of Strain AI prepared also from a waxed stock-culture. These two sets of flasks were incubated at 37°C. One flask from each set was withdrawn after 24 hours' incubation. Similar pairs of flasks were withdrawn after each subsequent interval of 24 hours until no further flasks remained in the incubator.

The cultures were heat-killed at 65°C. for 10 minutes and the activity of the staphylococcal factor was tested by incorporating in serum-milk-agar plates amounts from 0.25 up to 4.0 ml. (App. 182-183). Strain AII showed itself very active in producing the staphylococcal factor while Strain AI was quite

unable to do so. Subcultures of these different flasks of meat broth on to serum-milk-agar plates revealed that AI and AII were inactive and active respectively with regard to the production of Muller's phenomenon on this substrate.

Over a period of six months all the waxed cultures of Strain AII were used in a number of experiments and fresh batches of these waxed stock cultures were prepared and used. In every culture of AII grown from these stock cultures all the colonies of staphylococci which developed on serum-milk-agar plates showed plaque-formation around them and there were no colonies which did not show Muller's phenomenon. One such waxed stock culture of Strain AII, about 5 months' old, was used to inoculate 2 tubes each of 1% "Lab-Lemco" broth, meat broth and glucose broth of which one set was incubated at 37°C., the other at room temperature for a period of 30 days. After 6 and 24 hours, and after 4, 5, 6, 7 and 30 days subcultures were made from each tube on to serum-milk-agar plates (App. 184-185).

There was a zone of lysis and plaques around all the colonies of staphylococci grown from the meat broth, the 1% "Lab-Lemco" broth and the glucose broth cultures incubated at 37°C. and at room temperature up to the sixth day of incubation. The 7 day old "Lab-Lemco" broth culture, grown at 37°C., when plated out

on serum-milk-agar, gave rise to a few colonies of staphylococci which did not produce lysis and plaques. The 30 day old cultures - meat broth, 1% "Lab-Lemco" and glucose broth (this latter grown at room temperature only) all gave rise to a few colonies of staphylococci which did not produce lysis and plaques while all the other colonies were surrounded by zones of lysis and plaques. These two different types of colony were both coagulase-positive.

It appeared that, in the main, waxed stock cultures of Staphylococcus aureus, Strain AII were stable but if this strain were grown in broth for 7 days or longer a few organisms could develop which were unable to produce Muller's phenomenon. Thus if a fresh waxed stock culture of the strain was used for each experiment and if that stock culture had been prepared from a pure culture, all of the colonies of which produced Muller's phenomenon when plated out on serum-milk-agar it was unlikely that difficulty would arise in any experiment due to a variant developing in the culture. Such waxed stock cultures were used in the experiments undertaken to ascertain the suitability of different media for the production of the staphylococcal factor and were found to be satisfactory. The precaution was always taken, however, of plating out the culture to obtain single colonies on serum-milk-agar to check its purity.

A freeze-dried culture of Strain AII, reconstituted in meat broth and grown at 37°C. for up to 30 days when inoculated on to serum-milk-agar plates gave rise to staphylococcal colonies all of which showed zones of lysis and plaques around them. It would seem that freeze-dried cultures of AII may be more stable than waxed cultures of the same organism but this information was not obtained until after the experiments on the production of the staphylococcal factor had been completed, using the waxed stock cultures.

(c) INVESTIGATIONS OF STRAINS 72822 and 73016.

Investigations of cultures of Staphylococcus aureus, Strains 72822 and 73016 to ascertain if they were pure and stable were carried out at the same time as the investigation of Strain A just described. With these two cultures no colonies were found which did not produce Muller's phenomenon, as had been the case with Strain A, but differently pigmented colonies were detected (App. 186-187).

1% "Lab-Lemco" broth cultures of these two strains inoculated from their Dorset's egg medium stock cultures, were incubated at 37°C. for 21 days. Subcultures were made on serum-milk-agar plates and 10% horse blood-agar plates.

The 14, 19 and 21 days' old culture of Strain 72822 when plated out on serum-milk-agar plates gave rise to two types of colony both of which demonstrated Muller's phenomenon, one having an orange pigmented colony, the other being white. On the corresponding horse blood-agar plates, some colonies were haemolytic, others were non-haemolytic. These latter were small orange coloured, the former orange and white. The 21 days' old culture of Strain 73016 plated out on the horse-blood-agar and serum-milk-agar plates gave rise to white and to orange coloured colonies.

In view of the fact that all these variant colonies produced lysis and plaques they were not further investigated at this time.

At a later stage Strains 73016 and 72822 were again examined, 24 hour old "Lab-Lemco" broth cultures of these organisms being plated out on serum-milk-agar and chocolate-agar plates. Strain 73016 gave rise to two types of colony both of which produced lysis and plaques, one an orange colony, the other a white colony. Strain 72822 gave rise to three different types of colony, all of which produced lysis and plaques - a white colony, a small orange colony and a pale orange colony. These colonies were designated 73016 'W', 73016 'O', 72822 'W', 72822 'O' and 72822 'P'. Broth cultures of these five different types were coagulase-positive. Further investigations of these types on different media including serum-milk-agar showed that they were stable variants which produced Muller's phenomenon. Stock cultures of these five types were prepared on "Lab-Lemco" agar slopes, waxed and stored at room temperature. The ability of each of these types to produce the staphylococcal factor necessary for Muller's phenomenon was measured at a later stage (P. 301-302).

II. THE PRODUCTION OF STAPHYLOCOCCAL FACTOR BY STAPHYLOCOCCUS AUREUS, STRAIN AII.

(a) THE USE OF STANDARD LABORATORY MEDIA.

In the preliminary experiments already reported, (P. 248-250), both meat broth and 1% "Lab-Lemco" broth had been found to be suitable media for the production of the staphylococcal factor by Staphylococcus aureus, Strain A, but, now that the strain of staphylococcus being used was Strain AII, the purified form of A, it seemed desirable to examine more thoroughly the whole range of media readily available in the laboratory. It was hoped that it would be possible to find a medium easily prepared, and, more important, readily reproducible which would permit the production by Strain AII of staphylococcal factor with high activity.

Eighteen different media were used in the first experiment (App. 188-193). 60 ml. amounts of each medium employed were placed in each of two 250 ml. conical flasks, both of which received 2 ml. of a 6 hour old "Lab-Lemco" broth culture of Staphylococcus aureus, Strain AII, as inoculum. The flasks were incubated at 37°C. for 7 days and from a flask of each different medium 15 ml. amounts of culture were withdrawn on each of these

seven days (excluding Sunday). The culture was heat-killed at 65°C. for 10 minutes and incorporated in serum-milk-agar plates in amounts of 0.5 ml. to 4.0 ml. to ascertain the activity of the contained staphylococcal factor.

Only five of the eighteen media tested produced staphylococcal factor of high activity. These were meat broth and 1% "Lab-Lemco" broth, (both of which contained 1% "Oxoid" peptone), meat broth and 1% "Lab-Lemco" broth (both of which contained 1% Evan's peptone), and lastly double strength "Bacto" broth which had 5% human serum added to it. The continued use of this last medium was not considered desirable in view of the fact that it contained serum and that it was not possible to provide batches of serum which did not differ one from another. The addition of serum to a medium would result in the introduction of an ingredient of varied composition.

It was decided, however, to continue the use of a broth made with "Lab-Lemco" but to drop the use of meat broth, although the activity of the staphylococcal factor produced in the meat broth was slightly better than that in the "Lab-Lemco" broth. Whereas it was possible to prepare repeatedly batches of "Lab-Lemco" broth each one being the same as its predecessor, at least so long as the stock bottles of "Lab-Lemco" and peptone lasted, it was not

possible to prepare two batches of meat broth which were the same. Indeed, the results obtained with a single batch of meat broth medium may be different if the medium has been stored for a long time.

The result obtained in 1% "Lab-Lemco" broth with 1% Oxoid peptone - complete lysis of the serum-milk-agar plate with 0.5 ml. of the heat-killed culture was very slightly better than that obtained in 1% "Lab-Lemco" broth with 1% Evan's peptone which gave complete lysis with 1.0 ml. of the heat-killed culture and slight lysis of the serum-milk-agar with 0.5 ml. of culture. The production of staphylococcal factor both in 1% Oxoid peptone and 1% Evan's peptone was very poor, but it did appear that the Evan's peptone was slightly better than the Oxoid peptone.

On these findings it was decided to set aside for experimental use a bottle of "Lab-Lemco", and a bottle of Evan's peptone for the sole purpose of making different media for the production of staphylococcal factor. It seemed possible by using different concentrations of "Lab-Lemco", Evan's peptone and Sodium chloride to make one or more media, which, permitting the growth of Strain AII, would be suitable for the production of staphylococcal factor of high activity. If "Lab-Lemco" was used in concentrations of 0.5%, 1.0%, 1.5% and 2.0% Evan's peptone in 0.5%, 1.0%, 1.5% and 2.0% and sodium chloride in the same concentrations, it would be

possible to make sixty-four different media with these substances. It was found, however, in the course of this undertaking, that it was unnecessary to prepare all these different media since some of them were obviously going to be unsuitable. Forty of them were made and tested and a number of them were found to be satisfactory and at least as good as many batches of meat broth for the production of staphylococcal factor with high activity.

The forty different media which were prepared in 300 ml. amounts and distributed in 50 ml. amounts in each of six 150 ml. conical flasks, were inoculated with 1 ml. of a 6 hour old "Lab-Lemco" broth culture of Strain AII and incubated at 37°C. for 7 days (App. 194-205). One flask of each medium was withdrawn after 24 hours' incubation and one other flask after each subsequent 24 hours' interval up to 7 days after inoculation (including Sundays). The culture in each different medium was heat-killed at 65°C. for 10 minutes, and incorporated in serum-milk-agar plates in amounts from 0.5 ml. to 1.0 ml. These plates were incubated at 37°C. for 24 hours when the results were noted. Two points of importance must be stressed here - viz. the human serum used in the making of the serum-milk-agar plates in all the experiments carried out on the production of the staphylococcal factor in this section came from a single batch of 1 litre of pooled human serum set aside specially

for this purpose. It was kept frozen in the ice-chest in small bottles until required. The second point is that from each flask of medium in which Strain AII was grown, a loopful was plated out on to a standard serum-milk-agar plate before the culture was heat-killed. In this way it was possible to check if any variants which did not produce Muller's phenomenon arose in the course of the experiments. No such variant was discovered in these experiments with the forty different media used.

These forty media were examined in four main groups - Groups A, B, C and D, having 0.5%, 1.0%, 1.5% and 2.0% respectively of "Lab-Lemco". The media in Group D, i.e. with 2% "Lab-Lemco", as a whole produced staphylococcal factor of greater activity than those media in the other three groups. Of the individual media in Group D, Numbers 5, 6, 7 and 8 were slightly more active than Numbers 1, 2, 3 and 4. If any one medium was to be chosen as a routine medium in which staphylococcal factor was to be produced regularly preference would go to Medium D7. The activity of the staphylococcal factor produced in Medium D7 by Strain AII was such that 0.5 ml. of the heat-killed culture incorporated in a standard serum-milk-agar plate produced complete lysis (clearing) of the medium.

In one experiment (App. 206-207) in which four different media were retested, A9, B5, C5 and D7, this latter medium

produced staphylococcal factor of such an order of activity that not only did 0.5 ml. produce complete lysis of the medium but 0.25 ml. also brought about some lysis of the medium. The results obtained with Medium C5 were almost as good as those given by D7, except that the 0.25 ml. amounts of it were inactive in producing Muller's phenomenon. Medium B5, however, was just as poor a medium for the development of staphylococcal factor as it had been when previously tested. Complete clearing of a plate (lysis) was observed on one occasion only, when 4.0 ml. of heat-killed culture was tested on serum-milk-agar.

Thus, Medium D7 came to be used routinely for the production of staphylococcal factor and many litres of staphylococcal culture were produced over a period of twelve months, each batch containing staphylococcal factor of high activity. In addition, Medium D7 and also the "poor" medium, medium B5 were used in a number of experiments designed to test the effect on the production of staphylococcal factor, of altering the composition of the atmosphere in which the culture was grown and of altering its pH reaction.

(b) COMPARISON OF HEAT-KILLING AND FILTRATION IN THE PREPARATION OF STERILE STAPHYLOCOCCAL FACTOR.

Although it had been shown in the preliminary experiments that passage of a heat-killed staphylococcal culture through a Seitz filter, (the pad of which had been previously washed) had virtually no effect on the activity of the contained staphylococcal factor, it was thought necessary to compare the effect of heat-killing of a staphylococcal culture with filtering another portion of it. One such experiment is reported here in which medium D7 was employed for the production of the staphylococcal factor (App. 208-209).

Four flasks, each containing 60 ml. of Medium D7 were inoculated with 2 ml. of a 6 hour old "Lab-Lemco" broth culture of Staphylococcus aureus, Strain AII and incubated at 37°C. After 24 hours' incubation sufficient culture was withdrawn from the flasks and prepared for testing the activity of the contained staphylococcal factor. The heat-killed culture was prepared by heating it at 65°C. for 10 minutes while the filtered culture was centrifuged and filtered through a Seitz filter, the pad of which had been previously washed with 10 ml. distilled water and 10 ml. of uninoculated medium. The filtrate of the culture and the

heat-killed culture were incorporated in serum-milk-agar plates to ascertain the activity of the staphylococcal factor. These were prepared daily for 7 days (excluding Sunday).

There was no difference detected in the activity of the staphylococcal factor contained in the heat-killed culture and in the Seitz-filtered culture. The method employed to sterilise a staphylococcal culture without inactivating the staphylococcal factor is thus largely a matter of choice, these two methods being equally good.

(c) THE EFFECT OF THE GASEOUS ENVIRONMENT ON THE PRODUCTION OF STAPHYLOCOCCAL FACTOR.

Although it has been shown (P. 176) that staphylococci growing on the surface of a serum-milk-agar plate, under anaerobic conditions were able to demonstrate Muller's phenomenon it was not known what effect anaerobiosis would have on the production of the staphylococcal factor in a fluid medium.

Three flasks of Medium D7 inoculated with Strain AII were incubated at 37°C. each in a McIntosh and Fildes anaerobic jar. One flask was withdrawn after 2 days, one after 4 days and the remaining flask after 6 days under anaerobic conditions at 37°C. These 2, 4 and 6 day old cultures were heat-killed at 65°C. for 10 minutes and the activity of the staphylococcal factor contained tested by incorporation in serum-milk-agar plates in amounts of 0.5 ml. to 4.0 ml. (App. 210-211).

The staphylococcal factor in the 2 and the 4 day old anaerobic cultures had much less activity than that usually obtained under aerobic conditions. The 6 day old culture also had reduced activity when tested in 0.5 ml. and 1.0 ml. amounts, these quantities causing only plaques with 50% lysis of the plates.

It had been demonstrated that the staphylococcal

factor is produced under anaerobic conditions, although with reduced activity. The effect on the production of the staphylococcal factor by Strain AII of growing this organism in atmospheres to which different concentrations of carbon dioxide were added was investigated (App. 212-213).

Eight conical flasks, each containing 50 ml. of Medium B5, were each inoculated with 2 ml. of a 6 hour old "Lab-Lemco" broth culture of Strain AII. Two flasks were placed in a closed jar which had 5% carbon dioxide in its atmosphere, two flasks similarly in a jar with 10% carbon dioxide and two flasks in a jar with 15% carbon dioxide. The remaining two flasks were incubated under fully aerobic conditions. All the flasks were incubated at 37°C. Heat-killed cultures were prepared from each set of flasks after 2 days' and after 4 days' incubation and tested in serum-milk-agar plates. Plaques were produced in the serum-milk-agar plates containing 2 ml., 3 ml. and 4 ml. of heat-killed culture which had been grown under fully aerobic conditions. This poor activity of the staphylococcal factor produced in Medium B5 was in keeping with previous findings. It was found, however, that Strain AII growing for 2 days in this same Medium B5, in an atmosphere to which 5%, 10% or 15% carbon dioxide had been added was able to produce staphylococcal factor active in 0.5 ml. amounts causing

complete lysis of the serum-milk-agar plates. With the 4 day old staphylococcal culture grown in an atmosphere with 5%, 10% or 15% carbon dioxide, it was found that 0.25 ml. of it, after heat-killing, would completely lyse a serum-milk-agar plate.

When this experiment was repeated using Strain AII and Medium D7, it was found that the activity of the staphylococcal factor was increased also when the culture was grown in an atmosphere of 5%, 10% or 15% carbon dioxide. The activity of the staphylococcal factor grown in these conditions was such that 0.1 ml. of the heat-killed culture produced 90% lysis of a serum-milk-agar plate (App. 214-215).

(d) THE EFFECT ON THE PRODUCTION OF STAPHYLOCOCCAL FACTOR OF THE ADDITION OF SERUM TO THE CULTURE MEDIUM.

In view of the fact that double strength "Bacto" broth with 5% human serum added was a better medium for the production of the staphylococcal factor than the same medium without serum (P. 283) it seemed worthwhile to look into the effect of adding human serum to Medium D7 and to Medium B5.

The addition of 1%, 5% or 10% human serum to Medium D7 was found to have no effect in increasing the activity of the staphylococcal factor produced in this Medium by Strain AII (App. 216-217). Heating of the serum at 56°C. or 65°C. for 10 minutes before it was added to this medium also made no difference to the activity of the staphylococcal factor produced (App. 218-219). On the other hand, the addition of 1%, 5% or 10% human serum added to Medium B5, resulted in the production by Strain AII of staphylococcal factor of enhanced activity (App. 220-221). Thus a 6 day old staphylococcal culture in Medium B5 with added human serum to the extent of 10% when used in as small a quantity as 0.5 ml. was able to produce 100% lysis of a serum-milk-agar plate; of the corresponding medium without added serum 4.0 ml. of culture was required to bring about the same degree of lysis.

(e) THE EFFECT ON PRODUCTION OF STAPHYLOCOCCAL FACTOR OF DIFFERENT INOCULA OF STRAIN AII.

It had appeared from the preliminary experiments carried out with Staphylococcus aureus, Strain A that the age, the nature, and source of the inoculum might play some part in the production of staphylococcal factor in a fluid medium. The standard inoculum used in the experiments carried out in this section was a 6 hour old culture in 1% "Lab-Lemco" broth of Strain AII, used in amounts of 2 ml. for each 50 ml. of medium inoculated. The results obtained with this inoculum in Medium D7 and B5 were constant on the many occasions this was carried out and estimates of the activity of the staphylococcal factor made.

On a few occasions it was necessary to inoculate Medium D7 or B5 with a 24 hour old culture of AII in 1% "Lab-Lemco" broth but this departure from the use of a 6 hour old inoculum did not appear to have any marked difference in the activity of the staphylococcal factor produced.

The amount of culture used as inoculum had no effect on the activity of the staphylococcal factor produced in a medium. Inocula of 0.5 and 2.0 ml. of a 1% "Lab-Lemco" broth culture of AII used for 60 ml. amounts of Medium D7 resulted in cultures all of

which contained the staphylococcal factor with the same degree of activity. A similar result was obtained with a 1 ml. suspension in distilled water of the centrifuged deposit obtained from 10 ml. of a 6 hour old culture of AII (App. 222-223).

(f) THE EFFECT OF THE pH REACTION ON THE ACTIVITY OF THE STAPHYLOCOCCAL FACTOR.

A check on the pH reaction was kept on every flask of medium used in the course of the experiments described in this section. The initial reaction of the culture Medium D7 was adjusted to pH 7.4. After 24 hours' growth of Strain AII it fell to pH 6.7 rising to pH 7.0 at 48 hours. Thereafter it rose further to pH 7.3 to 7.6 a level around which it stayed for a period of 7 to 10 days.

It was thought worthwhile finding out the optimum pH reaction for demonstrating the activity of the staphylococcal factor.

A four day old staphylococcal culture in Medium D7 which had been heat-killed in the usual way at 65°C. for 10 minutes and which had a pH reaction 7.3 showed some activity in serum-milk-agar plates when tested in 0.25 ml. amount (App. 224-225). Portions of this culture had added to them different amounts of N/5 Sodium hydroxide or N/5 Hydrochloric acid to give a series of tubes of sterile culture with reactions within the pH range of pH 4 to pH 9.0. Although activity was detected in the culture adjusted to give a reaction pH 9.0 the culture at pH 4.0 was completely inactive. The cultures at pH 6.1 and pH 8.2 showed almost as great activity as the culture at pH 7.3.

In another experiment for which filtered staphylococcal culture was available, it was tested over the pH range from pH 5.5 to pH 7.9 with smaller steps between (App. 226-227). From this it was found that the same degree of activity was demonstrated within the pH range from pH 6.4 to pH 7.9. The activity obtained with the culture at pH 7.0 was slightly better than that obtained with the cultures adjusted to pH 6.4 and pH 7.9 but the difference was certainly not significant.

In all the experiments so far described the reaction of Medium D7 had been adjusted to pH 7.4 a suitable reaction at which to grow staphylococci.. In one experiment (App. 228-230), three batches of Medium D7 were adjusted to pH 6.0, pH 7.0 and pH 8.0 respectively before being inoculated with 2.0 ml. of a 6 hour old culture of Strain AII, to find out what effect, if any, these initial pH reactions had on the activity of the staphylococcal factor produced. Each of these media was tested up to 15 days after inoculation each being tested without adjustment of their pH reaction and also after adjustment to pH 7.0.

The reactions of all three lots of medium had adjusted themselves to pH 7.0 within 48 hours of inoculation after incubation at 37°C. No difference of any significance could be detected in the activity of the staphylococcal factor produced in the media at

the initial pH value, pH 6.0 and pH 7.0; the medium at pH 8.0 initially, was not quite so active with regard to its staphylococcal factor, e.g. at 8 days the media with initial pH 6.0 and with initial pH 7.0 produced 90% lysis of serum-milk-agar plates when used in 0.25 ml. amounts whereas a similar amount of culture derived from the medium with initial pH 8.0 was inactive. The difference of activity was, of course, trivial.

(g) THE TESTING OF STAPHYLOCOCCAL FACTOR WITH DIFFERENT SPECIMENS OF HUMAN SERUM.

A single large batch of pooled human serum was set aside for all the experiments described in this section on the production of staphylococcal factor. In this way it would be possible to compare the activities of different batches of staphylococcal factor directly one with another in the knowledge that the content of the serum factor would always be the same. It is known that different specimens of human serum differ in their content of the serum factor necessary to demonstrate Muller's phenomenon. This is shown in an experiment carried out with two specimens of serum and presented in App. 231-232.

The activity of the staphylococcal factor contained in a filtrate of a 4 day old culture of AII grown in Medium D7, was ascertained in the usual way by incorporating amounts of the filtrate in serum-milk-agar plates. This test was carried out in duplicate, one set of plates having serum "4", the other set serum "5". The difference between the two sera was shown by the fact that serum "5" brought about 95% lysis in a serum-milk-agar plate containing 0.25 ml. culture, while serum "4" brought about only "slight lysis" in a comparable plate. Moreover serum "5" showed complete lysis of the

medium with 3.0 ml. and 4.0 ml. of staphylococcal culture while serum "4" showed plaques with 95% lysis. The differences between these two sera are faily small; much greater differences between sera will be demonstrated in later experiments (e.g. App. 317-319).

(h) THE ACTIVITY OF THE STAPHYLOCOCCAL FACTOR PRODUCED IN MEDIUM D7 BY STRAINS OF STAPHYLOCOCCI OTHER THAN AII.

In view of the fact that Medium D7 was so satisfactory for the production of the staphylococcal factor by Strain AII and that the factor had consistently had a high degree of activity, it was decided to find out how good this medium was for the production of the factor by other strains of staphylococci.

In one experiment the activity of the staphylococcal factor produced by Strain AII was compared with that produced by Strains 73016 'W', 73016 'O', 72822 'W', 72822 'O' and 72822 'P' (App. 233-235). The activity of the factor produced by Strain 72016 'W' was not far short of that produced by AII; similar activity to that by AII was given by the factor produced by Strain 72822 'W'. The activities of the factor produced by the rest of the strains examined although not as high as those of the strains mentioned were nonetheless worthy of note.

In another experiment carried out with other strains of staphylococci, viz. 72493, 72871, 73008 and 72959, in Medium D7, the activity of the factor produced by them was very low indeed, coming nowhere near the level of activity of the factor produced by

Strain AII (App. 236-238).

Comparison of the activities of the staphylococcal factor produced by Strain AII and its parent Strain A revealed that in Medium D7 the former strain produced the factor with a higher degree of activity than the latter (App. 239-241). Moreover when a loopful from each culture was plated out on serum-milk-agar daily over a week all the colonies from culture of Strain AII were surrounded by lysis and plaques whereas from culture of Strain A two types of colonies were to be seen, the one showing Muller's phenomenon, the other only the indefinite lysis.

Included in this comparison was Strain AI which had also been derived from Strain A but which did not produce Muller's phenomenon. Examination of cultures of this organism failed to reveal any evidence that it had produced the staphylococcal factor and on plating out on serum-milk-agar from this culture, the colonies which grew failed to produce Muller's phenomenon.

III. SUMMARY.

The investigations carried out and reported in this section achieved two important objectives with regard to the production of the staphylococcal factor required for Muller's phenomenon. Firstly, a critical examination of Staphylococcus aureus, Strain A, the strain used in the preliminary experiments on the production of the staphylococcal factor, revealed that this strain consisted of two types of coagulase-positive staphylococci, identical with regard to all their biochemical characteristics but differing in one important respect, viz. in their abilities to produce Muller's phenomenon on serum-milk-agar plates and to produce in fluid medium the factor necessary to demonstrate the phenomenon.

Secondly, it was found possible to obtain a medium in which Strain AII, derived from the parent Strain A, produced the staphylococcal factor regularly and consistently with a high degree of activity. Thus it became possible to produce as required sufficient staphylococcal of high activity for investigations being carried out on other aspects of Muller's phenomenon.

The fact that Strain A was composed of two different

types of staphylococci, AI and AII provides a possible explanation of the results obtained in the preliminary experiments on the production of the staphylococcal factor. Under certain circumstances the number of AI cells in a culture may increase with a resultant drop in the amount of staphylococcal factor produced in that culture.

Variation in staphylococci generally and in particular in relation to the production of Muller's phenomenon and the staphylococcal factor necessary for its demonstration will be discussed in Part IX.

No major obstacle was encountered in the search for an easily made and readily reproducible medium for the production of the staphylococcal factor, once Strain AII was available. Thus it was possible to obtain staphylococcal factor which would bring about complete lysis of a serum-milk-agar plate when the culture in which it was produced was used in as small a quantity as 0.25 ml. The addition of 10% carbon dioxide to the atmosphere in which the organism was growing increased the activity of the staphylococcal factor so that 0.1 ml. would bring about the same change as the 0.25 ml. used previously. A very marked increase in the activity of the staphylococcal factor produced in a poor medium resulted either from the addition of carbon dioxide to the atmosphere in which the culture was grown or of human serum to the medium itself.

It was established that the optimum pH reaction for the demonstration of Muller's phenomenon, using staphylococcal factor in heat-killed culture was pH 7.0 but that there was no loss of activity of the staphylococcal factor when tested in the range pH 6.4 and pH 7.9. Moreover, Strain AII at least, will grow in culture medium with a pH reaction from pH 6.0 to pH 8.0 and produce active staphylococcal factor.

On one of the best media for the production of the staphylococcal factor by Strain AII, a number of other strains of coagulase-positive staphylococci were grown. All of them produced the factor, and although some gave it only with a very low activity, others were as active in this respect as Strain AII. Not all strains of staphylococci have the same ability to produce the factor necessary for Muller's phenomenon and, if it is desired to obtain staphylococcal factor with a high degree of activity it is essential not only to use a suitable medium but also to pick a strain of staphylococcus which has the capacity for doing so.

C. THE CONCENTRATION AND PURIFICATION OF STAPHYLOCOCCAL FACTOR.

I. EXPERIMENTS ON CONCENTRATION AND PURIFICATION OF STAPHYLOCOCCAL FACTOR.

(a) The first attempt to concentrate Staphylococcal Factor.

(b) Concentration and Purification.

(c) Further purification of staphylococcal factor.

II. SUMMARY.

I. EXPERIMENTS ON CONCENTRATION AND PURIFICATION OF STAPHYLOCOCCAL FACTOR.

As soon as it was established that cell-free staphylococcal factor could be prepared, attention was directed towards the consideration of methods which might be employed for its concentration and purification. It seemed that many advantages would accrue if this factor could be concentrated, purified and dried, such as a possible increase in its activity due to removal of inhibitory substances, the availability of a preparation which could be used in a wider range of concentrations, and greater ease in handling and storing the factor as a powder or concentrated solution. In addition it was felt that the activity of such a preparation might be more easily measured and that its interaction with the serum factor and the protein substrate become more apparent. Not all these objectives were attained in the present studies but a certain amount of success can be reported in those which were investigated. It was possible to concentrate the staphylococcal factor, to effect some purification of it and to dry it so that it could be stored for at least six months without loss of activity. At least 12 batches of staphylococcal factor were concentrated and purified successfully and in addition the factor was prepared as a dried powder on four occasions.

(a) THE FIRST ATTEMPT TO CONCENTRATE STAPHYLOCOCCAL FACTOR.

The first attempt to concentrate staphylococcal factor was made shortly after it had been shown that it was possible to prepare in meat broth satisfactory cell-free staphylococcal factor. 1,500 ml. of this staphylococcal factor preparation was collected and its activity was estimated in standard serum-milk-agar plates, containing 0.5 ml. serum and 2.0 ml. of milk (App. 242-243). The Department of Chemistry, King's College, Newcastle upon Tyne undertook the evaporating of this 1500 ml. (under pressure within the temperature range 25°C. - 30°C.) and achieved a concentration of 8.3 times, reducing the volume to 180 ml. The activity of this concentrated staphylococcal factor was tested in standard serum-milk-agar plates (using the same serum as before) and was found to be at the level expected if no loss occurred in the course of concentration, viz. the original staphylococcal factor was active in 1.0 ml. amounts, the concentrated factor was active in 0.1 ml. amounts when tested under the same conditions.

An attempt to freeze-dry this concentrated staphylococcal factor was unsuccessful but a further reduction in

volume was achieved, viz. from 180 ml. to 55 ml. This twice-concentrated staphylococcal factor produced a precipitate on standing in the ice-chest. The supernatant fluid was collected after centrifugation and it was tested in standard serum-milk-agar plates in which it was found to be active in a volume of 0.0312 ml.

Thus it had been found possible by removal of the water alone to concentrate a volume of 1500 ml. of staphylococcal factor (active in 1.0 ml. amounts) 27 times to a volume of 55 ml. (active in 0.0312 ml. amounts).

No further attempts at concentration of staphylococcal factor were made until the investigations reported in Part V, B were completed and it had been established that, using Staphylococcus aureus, Strain AII and a medium such as D7 it was possible to provide consistently staphylococcal factor of high activity.

(b) CONCENTRATION AND PURIFICATION.

The next attempt to concentrate staphylococcal factor was made with a 330 ml. volume (Batch 2) which had been prepared by growing Strain AII in Medium D7 (App. 244-247). The activity of this staphylococcal factor was ascertained by testing it in standard serum-milk-agar plates and it was compared with that of the staphylococcal factor after concentration to a volume of 28 ml. by evaporation under pressure and at 25°C. The increase in activity observed was in keeping with the result expected with a 12 fold-concentration of the original staphylococcal factor.

With this concentrated staphylococcal factor two experiments were performed in an attempt to purify it. In the first of these, 2.0 ml. of the concentrated factor had added to it an equal volume of saturated ammonium sulphate, the mixture being left at room temperature for 3½ hours before being centrifuged. The deposit obtained was taken up in 2.0 ml. of distilled water and dialysed overnight against running water. Dialysis was also carried out with the supernatant from the mixture. The redissolved deposit and the supernatant were tested for activity in standard serum-milk-agar plates, allowance being made for the slight increase

in volume which had taken place during dialysis. Whereas the supernatant was found to be as active as the concentrated factor before ammonium sulphate precipitation, the redissolved deposit was completely inactive.

The second experiment was concerned in finding out the effect of adding charcoal to staphylococcal factor. 0.1 gm. of animal charcoal was added to 1.0 ml. of concentrated staphylococcal factor, and, after having stood at room temperature overnight, the staphylococcal factor and charcoal suspension were centrifuged. The supernatant, and the charcoal, resuspended in 1.0 ml. of distilled water, were tested for activity in standard serum-milk-agar plates. The charcoal suspension was completely inactive but the supernatant was as active as the untreated concentrated staphylococcal factor.

Twenty ml. of the concentrated factor was then subjected to freeze-drying and the resultant brown crystalline material was found to become sticky and amorphous on exposure to air. This freeze-dried material was dissolved in half of its original volume of distilled water and tested out on standard serum-milk-agar plates. It had retained its activity apparently unimpaired.

All the standard serum-milk-agar plates included

in these and later experiments in this section had added to them sufficient "Merthiolate" to give a final concentration of 1/100,000, in order to prevent the growth of any organisms which might have contaminated the staphylococcal factor during the half-saturation with ammonium sulphate or during the subsequent dialysis.

In view of the fact that half-saturation with ammonium sulphate removed inactive material from the concentrated staphylococcal factor with no apparent loss of activity it appeared worthwhile to incorporate this procedure in the purification of succeeding batches of staphylococcal factor.

(c) FURTHER PURIFICATION OF STAPHYLOCOCCAL FACTOR.

Seven hundred and thirty-two ml. of staphylococcal factor (Batch 4) was concentrated 12.6 times by evaporation with the expected increase in activity (App. 248-251). This concentrated staphylococcal factor had added to it different amounts of three other similarly concentrated preparations of staphylococcal factor previously made and this resultant batch was mixed with an equal volume of saturated ammonium sulphate. After standing at room temperature for 1 hour this mixture was centrifuged, the supernatant collected and dialysed overnight against running water. This dialysed supernatant was found to be active in standard serum-milk-agar plates in 0.5 ml. amounts.

In all 550 ml. of this dialysed supernatant was available for further evaporation under pressure and subsequent freeze-drying. A brown powder was obtained of total weight 2.09 gm. This dried preparation of the staphylococcal factor was reconstituted in distilled water as a 2% solution and its activity tested in standard serum-milk-agar plates. 0.2 ml. completely cleared such a plate while 0.1 ml. produced 75% lysis of it.

Thus it was possible to prepare active staphylococcal factor as a powder, which when stored at room temperature retained

its activity for at least six months. This particular batch of staphylococcal factor was further purified by the use of acetone. 0.2 gm. of the powder was shaken up with 10 ml. of acetone and left in the ice-chest for 2 hours. After centrifugation, the supernatant consisting of the acetone and a whitish material suspended in it was discarded, and the brown deposit which remained was washed a further three times with 10 ml. of fresh acetone, each washing occupying 10 minutes. The brown deposit was thoroughly dried, dissolved in 5 ml. of distilled water and its activity tested in standard serum-milk-agar plates. This 2% solution of the staphylococcal factor was found to produce 75% lysis of a plate when as little as 0.05 ml. was used. There was required to effect the same amount of lysis 0.1 ml. of a 2% solution of the same powdered staphylococcal factor before the acetone treatment. In this way the staphylococcal factor could be further purified.

Two litres of staphylococcal factor, designated Batch 6, prepared in Medium D7 with Staphylococcus aureus, Strain AII were also subjected to concentration and purification successfully (App. 252-254). 0.25 ml. of this unconcentrated preparation effected 75% lysis in a standard serum-milk-agar plate while 0.025 ml. of the staphylococcal factor, concentrated about 12 times to 165 ml. by evaporation, caused a similar amount of lysis

in a comparable plate. One hundred and sixty-four ml. of this concentrated staphylococcal factor was mixed with an equal volume of saturated ammonium sulphate, and allowed to stand in the ice-chest for 2 hours, before being centrifuged. The supernatant fluid was collected and dialysed overnight against running water. During this procedure its volume increased to 975 ml., a dilution of 3.2 times having taken place. 0.1 ml. of this diluted supernatant was required to bring about a 75% lysis, of a standard serum-milk-agar plate.

The 975 ml. of staphylococcal factor was evaporated under pressure at a temperature of 25°C. and then subjected to freeze-drying. The brown residue obtained after rubbing with pestle in mortar gave a fine powder of 10.82 gm. A 1% preparation of this staphylococcal factor when it was used in 0.2 ml. amounts gave 75% lysis of a standard serum-milk-agar plate after 24 hours' incubation at 37°C. If the incubation of the plates was continued for a further 24 hours at 37°C. 0.05 ml. of the preparation produced plaques in a similar serum-milk-agar plate.

The object of these experiments was achieved in that it was found possible to prepare concentrated staphylococcal factor in a dried and stable form, which on being reconstituted was active on standard serum-milk-agar plates. Attention must be drawn to the

fact that plaque-production was not seen so regularly after incubation of plates for 24 hours at 37°C. - the activity of the staphylococcal factor being manifest by complete clearing, i.e. lysis of the serum-milk-agar medium. Moreover, on many occasions, as has been noted in the results, the change in the plate when not complete is given as e.g. 75% lysis; slight clearing. No plaques were visible in these plates at this time and the assessment of the degree of clearing was made by comparing the plates with a control plate.

Plaques could be observed in these plates, however, if they were examined after $2\frac{1}{2}$ - $3\frac{1}{2}$ hours' incubation at 37°C. These plaques usually faded very rapidly, resulting in a partial clearing of the plate which in some instances, depending on the amount of staphylococcal factor in the plate, might proceed to complete lysis. Thus, although the clearing or lysis was not complete and plaques were not visible in many plates when they were examined after 24 hours' incubation there was no reason to suspect that plaques had not been formed at some earlier stage of incubation and that this type of partial clearing was not due to the same staphylococcal factor which is responsible for the production of Muller's phenomenon. Control plates containing staphylococcal factor but without serum revealed no alteration in

the opacity of the medium nor any other change neither plaques nor lysis.

The 1% preparation of reconstituted staphylococcal factor was incorporated in different amounts in serum-milk-agar plates with different amounts of serum and the results obtained were similar to those observed in other experiments of the same kind performed with unconcentrated staphylococcal factor. After 24 hours' incubation some plates showed complete lysis and others showed only plaques (App. 255-256). There were, however, in addition others which showed only partial clearing, e.g. 75% lysis, but no plaques, as would have been expected, if unconcentrated staphylococcal factor had been used. Plaques, however, had been observed after $2\frac{1}{2}$ - $3\frac{1}{2}$ hours' incubation although they were no longer apparent when the plates were inspected after 24 hours' incubation.

Horse serum in place of human serum could be used with concentrated staphylococcal factor for the demonstration of Muller's phenomenon in serum-milk-agar plates (App. 257-258). In one experiment carried out three serum-milk-agar plates were made containing respectively 0.5, 1.0 and 2.0 ml. of horse serum. Two drops of a concentrated staphylococcal factor undiluted, and two drops of serial doubling dilutions of this factor, from 1/2 to

1/32 were placed respectively in six unglazed porcelain cups on the surface of each of these three serum-milk-agar plates. Complete lysis of the medium around many of the cups was seen after 24 and 48 hours' incubation at 37°C., while around others in addition to the lysis, plaques of clearing could be seen. The zones of lysis around the cups decreased, the greater the dilution of the staphylococcal factor contained in the cup.

In view of the fact that the staphylococcal factor prepared as a dried powder might be contaminated and that the plates prepared with it had included "Merthiolate" (1/100,000) to prevent possible growth it did seem worthwhile to examine the effect of Seitz-filtering a 1% preparation of reconstituted staphylococcal factor powder (App. 259-260). Fifty ml. of such a preparation was filtered using a small Seitz filter fitted with 3 cm. sterilising pad in the following way. Ten ml. of "Lab-Lemco" broth was first filtered through the pad to wash it and this was followed by 5 ml. of the 1% staphylococcal factor preparation. The filtrate obtained from these was discarded. Thirty five ml. of the 1% staphylococcal factor was then filtered, the first 6 ml. of filtrate also being discarded. The next 27 ml. of filtrate was collected in three lots, designated Filtrates I, II and III. A further 10 ml. of the 1% staphylococcal factor was then filtered. The remaining 13 ml. of

filtrate obtained was collected in two lots as Filtrates IV and V.

These Filtrates I to V were tested out in amounts of 0.1, 0.25, 0.5 and 1.0 ml. in standard serum-milk-agar plates and the results obtained compared with that given by the same amounts of unfiltered 1% staphylococcal factor tested also in standard serum-milk-agar plates which contained 1/100,000 "Merthiolate".

The Filtrates II to V gave a similar result to that given by the unfiltered material but Filtrate I had a slightly reduced activity.

Thus it was possible to filter reconstituted concentrated staphylococcal factor with only very slight loss of activity.

II. SUMMARY.

By evaporation under pressure at temperatures below 30°C. it is possible to concentrate staphylococcal factor preparations. Such concentrated staphylococcal factor can be purified by removal of inactive material by half saturation with ammonium sulphate. The dialysed supernatant fluid can be concentrated and freeze-dried to provide a fine brown powder which has the ability on reconstitution with water to produce Muller's phenomenon when appropriately tested. This dried brown powder is stable at room temperature for at least six months. The reconstituted staphylococcal factor can be filtered through a Seitz filter with little loss of activity.

PART VI.

INVESTIGATION OF THE CONDITIONS INFLUENCING THE PRODUCTION OF MULLER'S PHENOMENON BY CELL-FREE STAPHYLOCOCCAL FACTOR.

PART VI.

INVESTIGATION OF THE CONDITIONS INFLUENCING THE PRODUCTION OF MULLER'S PHENOMENON BY CELL-FREE STAPHYLOCOCCAL FACTOR.

I. MILK IN SUBSTRATE.

(a) AMOUNT OF MILK.

(b) THE EFFECTS OF VARYING THE TEMPERATURE OF INCUBATION, THE GASEOUS ENVIRONMENT AND OF ALTERING THE CHARACTERS OF THE GEL.

(c) THE EFFECT OF HEATING STAPHYLOCOCCAL FACTOR.

(d) CONSIDERATION OF THE SERUM FACTOR.

- (i) Varied amounts of serum.
- (ii) Effect of heating serum.
- (iii) The activity of serum and plasma compared.

(e) INTERFERENCE WITH PLAQUE-PRODUCTION.

- (i) By the addition of nutrients.
- (ii) By the addition of Sodium chloride.

(f) MISCELLANEOUS EXPERIMENTS.

I. MILK IN SUBSTRATE.

The suitability of milk as a test protein on which Muller's phenomenon could be demonstrated with cell-free staphylococcal factor need not be emphasised here in view of the fact that it was used so successfully as the indicator protein on which many preparations of staphylococcal factor were tested in experiments already described in Part V. A standard serum-milk-agar medium has already been referred to which was used extensively in experiments in which staphylococci were grown (App. 476) and it seemed worthwhile to devise a similar type of standard serum-milk-agar medium specifically for testing varied amounts of staphylococcal factor preparations (App. 477). Such a medium was employed from an early period in these studies and it differed from the standard serum-milk-agar medium already referred to only in the omission of the added nutrient, i.e. "Lab-Lemco". Reference has already been made to the observation which resulted in the use of 0.5 ml. of fresh serum in each 15.0 ml. volume of serum-milk-agar. The amount of milk contained in this volume of medium was 2.0 ml., and theexperiment on which the decision, that this was a suitable quantity of milk, was based, will now be described.

(a) AMOUNT OF MILK.

A series of plates of serum-milk-agar were prepared in which amounts of milk from 0.5 ml. to 6.0 ml. were incorporated in the customary agar base with 0.5 ml. of serum and 2.0 ml. of a staphylococcal factor preparation. These plates were incubated at 37°C. for 24 hours and then left at room temperature for a further 48 hours. Readings were taken after 6 hours and 24 hours at 37°C. and after a further 48 hours at room temperature (App. 261-262).

The staphylococcal factor employed in this experiment was not a very active one and that fact together with the amount of the factor used resulted in a marked difference in the activity detectable in the plates of medium with different amounts of milk. Thus, after 24 hours' incubation at 37°C. plaque-formation was entirely absent in the plate containing 0.5 ml. of milk due to the fact that plaques, previously detected after 6 hours' incubation had coalesced, resulting in a complete clearing or lysis of the medium. In the plate containing 6.0 ml. of milk many plaques could be seen after 24 hours' incubation of the plate at 37°C., although they were small and not so easily seen as those in some of the other plates in the series. In the plate containing 2.0 ml. of milk, the plaques were very numerous, well developed and easily seen.

It was estimated that the degree of lysis or clearing resulting from the numerous plaques present in this plate was about 75%.

The decision as to the optimal amount of milk to be incorporated in serum-milk-agar for the demonstration of Muller's phenomenon was influenced by two main considerations, viz. the less milk incorporated in the medium the more easily it would be broken down and the necessity of having sufficient milk in the medium to ensure that the plaques of clearing could be readily differentiated from the unaltered medium. Thus although the plates with 0.5 ml., 1.0 ml. and 1.5 ml. of milk showed a more rapid clearing of the medium by plaque-formation and fusion of the plaques than the plates with larger amounts of milk it was only in the plates with 2.0 ml. or more of milk that there was a sufficiently good contrast of altered with unaltered medium to afford a ready differentiation of plaques from the rest of the medium. Thus 2.0 ml. of milk was considered the optimal amount for incorporation in 15.0 ml. of serum-milk-agar, the volume of medium used for one plate, and this amount of milk was used routinely in the standard serum-milk-agar medium used with staphylococcal factor (App. 477).

(b) THE EFFECTS OF VARYING THE TEMPERATURE OF INCUBATION, THE GASEOUS ENVIRONMENT AND OF ALTERING THE CHARACTERS OF THE GEL.

The effect on the production of Muller's phenomenon of varying the temperature of incubation of serum-milk-agar plates containing staphylococcal factor was investigated in several experiments one of which is given in App. 263-264. Four serum-milk-agar plates each containing 2.0 ml. of staphylococcal factor and four containing 4.0 ml. of the factor were prepared. Four milk-agar plates without added serum but each with 4.0 ml. of staphylococcal factor were also prepared, as controls. A set of three plates, i.e. one serum-milk-agar plate with 2.0 ml., another with 4.0 ml. of staphylococcal factor and a milk-agar control plate without added serum was incubated at 37°C. for 48 hours. A similar set of plates was placed at 30°C., another at 22°C. and the fourth set at 4°C. Readings were made after 5 hours, 24 hours and 48 hours at these different temperatures.

Muller's phenomenon was not produced on any of the milk-agar control plates without added serum, irrespective of the temperature at which they had been held. Muller's phenomenon was observed on the serum-milk-agar plates with 2.0 ml. and 4.0 ml. of the staphylococcal factor, irrespective of the temperature at which

they had been held. Plaques were seen first in the plates incubated at 37°C. after 1¾ hours' incubation; at 30°C. after 2¾ hours; and at 22°C. after 3½ hours. Complete clearing (lysis) of the medium in the plates held at these temperatures was observed after 24 hours. The plates held at 4°C., showed plaques after 24 hours and complete clearing of the medium after 48 hours. Thus, it appeared that Muller's phenomenon develops at temperatures below 37°C. even at as low a temperature as 4°C., although at this latter temperature plaque-production is slower in making its appearance.

The fact that Muller's phenomenon can be demonstrated in serum-milk-agar plates containing staphylococcal factor incubated in anaerobic conditions was demonstrated in an experiment presented in App. 265-266. Four serum-milk-agar plates containing staphylococcal factor were prepared, one with 0.5 ml., a second with 1.0 ml., a third with 1.5 ml. and the fourth with 2.0 ml. of serum. These four plates were incubated immediately in a McIntosh and Fildes anaerobic jar at 37°C. A similar series of four plates were incubated aerobically (as control plates) at 37°C. The plates incubated aerobically all showed plaque-formation when examined after 4 hours at 37°C. but after 24 hours the medium in all these plates was completely cleared (lysis) due to increased plaque-

production and fusion of the plaques. The plaques incubated anaerobically all showed complete clearing of the medium when they also were examined after 24 hours' incubation. Since the anaerobic jar had not been opened until after 24 hours it was not possible to ascertain if plaque-formation had occurred in these plates held anaerobically as in the aerobic control plates after 4 hours' incubation. In view of the fact that the plaques had progressed to give complete clearing of the medium in the plates incubated aerobically it seemed likely that a similar development of plaques had preceded the clearing of the medium observed in the plates incubated anaerobically. The fact that plaques can develop in serum-milk-agar plates under anaerobic conditions was demonstrated in another experiment in which a smaller amount (1.0 ml.) of the same staphylococcal factor was used.

The fact that the agar concentration in serum-milk-agar plays a not unimportant part in the demonstration of Muller's phenomenon with staphylococcal factor was shown in a number of experiments. In one experiment (App. 267-268) a set of plates with agar concentrations of 0.6%, 1.2%, 1.8%, 2.4% and 3.0% respectively was prepared, each plate containing 0.5 ml. of serum. Similar sets of plates were also made but with 1.0 ml., 2.0 ml., 3.0 ml. or 4.0 ml. of serum in place of the 0.5 ml. of serum used in the first

set. These five sets of plates were incubated at 37°C. for 48 hours and readings were made after 24 and 48 hours' incubation at this temperature. Complete clearing (lysis) of all the plates with 0.5 ml. and 1.0 ml. of serum was seen after 24 hours' incubation irrespective of their agar concentrations. Neither plaque-formation nor clearing of the medium was detected in any of the plates with 3.0 ml. or 4.0 ml. of serum. In the series of plates with 2.0 ml. of serum, however, Muller's phenomenon (plaque-formation) was observed, but the pattern and number of plaques was dependent on the agar-concentration of the medium. Thus in the three plates with 0.6%, 1.2% and 1.8% agar concentration there were numerous plaques with a resultant clearing of about half of the medium (i.e. 50% lysis). In the plates with an agar concentration of 2.4% and 3.0% respectively Muller's phenomenon had been produced and was apparent as a network of numerous fine plaques, 0.7 - 0.8 mm. in diameter. After a further 24 hours' incubation of all the plates at 37°C. no change had occurred in the plates with 4.0 ml. of serum with any of the concentrations of agar used. The plates with 3.0 ml. of serum, however, now showed plaque-formation for the first time but only in the plates with 3.0%, 2.4% or 1.8% agar concentrations. No plaques nor any other change was detected in the plates in the same series with agar concentrations of 1.2% or 0.6%. Thus it was found that

the agar concentration of a serum-milk-agar medium could influence the development of Muller's phenomenon.

In another experiment reported in App. 269-270, in which agar concentrations from 1.5% to 6.0% were used in sets of plates with 0.5 ml., 1.0 ml., 2.0 ml. and 4.0 ml. of serum, further evidence of the effect of agar concentration on the development of Muller's phenomenon. In the series of plates with 0.5 ml. of serum Muller's phenomenon (plaque-formation) and clearing due to fusion of plaques was observed in the plate with an agar concentration of 1.5%. The plaques in this medium were of the usual size with a diameter of 1.0 - 1.2 mm. Plaques were also present in the plate with an agar concentration of 2.5% but they were fine plaques having an average diameter of 0.8 mm. No plaque-formation, however, occurred in the plates with an agar-concentration of 4.0% or 6.0%. In the series of plates with 1.0 ml. of serum, a picture similar to that described for the series with 0.5 ml. of serum was seen. No change in the medium was detected in any of the plates with 2.0 ml. and 4.0 ml. of serum, irrespective of agar concentration. It would appear that for the demonstration of Muller's phenomenon a suitable concentration of agar must be employed.

(c) THE EFFECT OF HEATING STAPHYLOCOCCAL FACTOR.

Packalén (1941) reported that his staphylococcal filtrate could be heated at 100°C. for 30 - 60 minutes without loss of activity and Elek (1953) reported that his staphylococcal extract could withstand boiling for at least 2 minutes without a noticeable decrease in its activity. In was these findings of Packalén and Elek which suggested the method of preparation of the staphylococcal factor reported in this thesis, viz. the heat-killing of a suitable staphylococcal culture using 65°C. for 10 minutes. This method, as has been indicated previously, was found reliable and serviceable over a period of months in the preparations of many batches of staphylococcal factor. It did seem worthwhile, however, to investigate further the effect of heat on the activity of the factor. To this end a number of experiments were performed in which staphylococcal factor was heated for varied temperature-time combinations. One such experiment is presented in App. 271-273.

Portions of staphylococcal factor were heated at 55°C. for periods of 10 minutes, 30 minutes and 60 minutes respectively. Other portions were heated for these same times, at 65°C., at 80°C., at 90°C. and at 100°C. In addition, further portions were heated at 100°C. for longer periods, viz. for 2 hours, 2½ hours, 3 hours,

$3\frac{1}{2}$ hours and 4 hours. Amounts of staphylococcal factor, from 1.0 ml. to 4.0 ml. heated at the different temperatures for the times specified were incorporated in standard serum-milk-agar plates. Unheated staphylococcal factor in amounts of 1.0 ml. to 4.0 ml. were incorporated in serum-milk-agar to give a series of control plates.

Heating of the staphylococcal factor did bring about a loss of activity which ranged from a very slight loss with a low temperature-short time combination to a complete loss of activity with a high temperature-long time combination, e.g. 2.0 ml. of unheated staphylococcal factor in a standard serum-milk-agar gave rise to plaques and 90% lysis of the medium; a similar amount of staphylococcal factor heated at 55°C. for 10 minutes tested in the same way gives plaques and 70% lysis of the medium; heated at 65°C. for 60 minutes plaques and 50% lysis; heated at 90°C. for 60 minutes - plaques only; and heated at 100°C. for 60 minutes - very few plaques.

(d) CONSIDERATION OF THE SERUM FACTOR.

Although the serum factor necessary for the production of Muller's phenomenon is dealt with more fully in Part VII three experiments involving serum are considered here.

(i) Varied amounts of serum.

The effect of varied amounts of serum on the production of Muller's phenomenon in serum-milk-agar plates by staphylococcal factor was investigated in a number of experiments, two of which are presented here. In one experiment (App. 274-275) a series of serum-milk-agar plates was prepared with amounts of serum from 0.1 ml. to 4.0 ml., and incubated at 37°C. for 24 hours. Plaque-production was observed in the plates with 0.1 ml., 0.25 ml., 0.5 ml and 3.0 ml. of serum. The plates with 1.0 ml. and 2.0 ml. of serum showed no plaques at this time but the medium was completely cleared (lysis), the result of fusion of the numerous plaques which had been visible at an earlier stage. The plate with 4.0 ml. of serum, however, showed neither plaques nor clearing and no change in the medium was detected.

Thus with increasing amounts of serum up to 2.0 ml., there were corresponding increases in the activity manifested by the appearance of more numerous plaques and by the degree of clearing (lysis) of the medium, resulting from fusion of the plaques. A further increase in the serum content of serum-milk-agar plates, however, resulted in inhibition of Muller's phenomenon, partial in the plate with 3.0 ml. of serum, complete in the plate with 4.0 ml.

In a second experiment of this nature, presented in App. 276-277, a series of serum-milk-agar plates were prepared with amounts of serum from 0.5 ml. to 4.0 ml. A duplicate series of plates with the same range of serum incorporated in haemoglobin-agar was also prepared. A similar result to that in the previous experiment was obtained with the largest amounts of serum used, viz. complete inhibition of Muller's phenomenon with 3.5 ml. and 4.0 ml. of serum in both the serum-milk-agar and the serum-haemoglobin-agar plates and partial inhibition of the phenomenon in the plates with 3.0 ml. of serum. This inhibition of Muller's phenomenon is of importance and will be discussed more appropriately later. There was slightly less activity manifested in the haemoglobin-containing plates than in the plates with milk as test-protein.

(ii) Effect of heating serum.

Muller (1927a) reported that the serum factor which plays so essential a part in plaque-production could be inactivated by heat. This was confirmed in experiments described in Part IV by growing strains of staphylococci in a series of serum-milk-agar plates containing serum which had been heated at different temperatures for different periods of time. It seemed likely that this thermolability of serum with regard to the demonstration of Muller's phenomenon could also be shown in serum-milk-agar plates with staphylococcal factor. This thermolability of serum was investigated and confirmed in a number of experiments, one of which is reported here (App. 278-279). Two portions of serum were heated at 55°C. one for 15 minutes, the other for 30 minutes; another two at 60°C. for 15 minutes and 30 minutes respectively and a third set of two portions at 65°C., one for 15 minutes, the other for 30 minutes. 0.5 ml. amounts of the portions of serum, subjected to heating for the different temperature-time combinations, were incorporated in standard serum-milk-agar plates and each of the six plates prepared contained 4.0 ml. of staphylococcal factor. A control serum-milk-agar plate with 0.5 ml. of unheated serum was also prepared. All the plates were incubated for 24 hours and

then placed at room temperature for 3 days.

The control plate with the unheated serum showed Muller's phenomenon (plaque-production) after 2 hours' incubation at 37°C. and the medium was completely cleared (lysis) after 18 hours at this temperature. The plate with serum heated at 55°C. for 15 minutes showed complete clearing (lysis) of the serum-milk-agar when examined after 24 hours' incubation, the plaques seen earlier having coalesced. The plate with serum heated at 55°C. for 30 minutes showed only plaque-formation when examined after 24 hours' incubation at 37°C. No extension of plaques nor subsequent clearing of the plate was observed even after 3 days at room temperature. This finding was consistent with partial destruction of the serum factor. The plates containing serum heated at 60°C. and 65°C. did not show Muller's phenomenon indicating that the serum factor had been destroyed.

(iii) <u>The activity of serum and plasma compared.</u>

In all the experiments carried out in these investigations on Muller's phenomenon in serum-milk-agar, serum from clotted blood had been used. It seemed worthwhile to compare the

ability of serum to produce Muller's phenomenon in serum-milk-agar plates with that of plasma tested under the same conditions. The serum and plasma were obtained from the same individual. Blood was collected in a test-tube, allowed to clot naturally, and the serum was removed on contraction of the clot. Another sample of blood was collected in citrate solution. After standing, the plasma was separated and divided into two portions. One portion was used to prepare plasma-milk-agar plates, the other portion was clotted by the addition of calcium chloride and the serum was collected. This serum was used to prepare another series of serum-milk-agar plates (App. 280-281).

The three sets of plates - one set of plasma-milk-agar, one set of serum-milk-agar prepared with serum obtained from naturally clotted blood, and the remaining set with serum from recalcified plasma were incubated at 37°C. for 24 hours and then left at room temperature for a further 24 hours.

All the plates in these three sets showed Muller's phenomenon and there was no significant difference in activity demonstrated by the two serum preparations and the plasma, all obtained from the blood of one individual.

(e) INTERFERENCE WITH PLAQUE-PRODUCTION.

(i) By the addition of nutrients.

Interference with the production of Muller's phenomenon resulting in either partial or complete inhibition, was noted on a number of occasions when large amounts of staphylococcal factor had been incorporated in serum-milk-agar plates. For example, in one experiment in which staphylococcal in amounts of 1.0 ml. to 7.0 ml. had been incorporated in a series of serum-milk-agar plates, plaque-production was observed with all the amounts of staphylococcal factor up to 5.0 ml. Subsequent complete clearing of the medium occurred in all these plates except that with 1.0 ml. of the factor. The plates with 6.0 ml. and 7.0 ml. respectively showed only a few plaques which were abnormally large, up to 5 mm. in diameter. This observation was further investigated in a number of experiments some of which are presented here.

It seemed possible that the inhibition observed might have resulted from the meat broth in which the staphylococcal factor had been produced. In one experiment carried out (App. 282-284) a series of standard serum-milk-agar plates were prepared with amounts of a staphylococcal factor preparation from 1.0 ml. to

7.0 ml., sterile distilled water being used as the diluent, as usual, to bring the total volume of the plate to 15.0 ml. A similar series of plates was prepared differing from the previous set only in the fact that the diluent used was meat broth instead of distilled water. In addition, a serum-milk-agar plate with 8.0 ml. of staphylococcal factor but without added diluent was prepared.

All the plates were incubated at 37°C. for 24 hours and then left at room temperature for a further 24 hours. The series of plates with distilled water asdiluent gave complete clearing of the medium in the plates with amounts of staphylococcal factor from 2.0 ml. to 6.0 ml., while in the plates with 1.0 ml. and 7.0 ml. plaques of clearing were present. The plaques in the plate with 1.0 ml. of the factor were all of normal size (1.0 - 1.2 mm. in diameter) but those in the plate with 7.0 ml. were abnormally large being in the range of 1.0 to 5.0 mm. in diameter.

The set of plates in which meat broth had been used as diluent showed a marked inhibition of plaque-production. This inhibition was greatest in the plates with the largest amounts of staphylococcal factor, i.e. with 6.0 ml. and 7.0 ml. to which meat broth had been added as diluent in 2.0 ml. and 1.0 ml. amounts respectively. The size of the plaques observed in this set of

plates increased from 1.0 - 2.0 mm. in the plate with 2.0 ml. of staphylococcal factor and 6.0 ml. of meat broth as diluent, to 7.0 mm. in diameter in the plate with 7.0 ml. of staphylococcal factor and 1.0 ml. of meat broth. The plate with 8.0 ml. of staphylococcal factor, but no diluent, showed only 7 plaques, all of which were about 7 mm. in diameter.

Re-examination of the plates after 24 hours at room temperature revealed a further development of plaques and a resultant increased clearing of the medium but only in the plate with 3.0 ml. of staphylococcal factor and 5.0 ml. of meat broth as diluent was the medium completely cleared (i.e. lysis).

Although the addition of meat broth in place of distilled water as diluent in these plates resulted in inhibition, it was in the plates with the largest amount of staphylococcal factor (7.0 ml.) irrespective of the nature of the diluent and in the plate with 8.0 ml. of staphylococcal factor with no added diluent that the inhibition of Muller's phenomenon was most marked. This finding suggested that there was present in the staphylococcal factor preparation in particular and to a lesser extent in meat broth an inhibitor for Muller's phenomenon.

The plates with 2.0 ml., 4.0 ml. and 7.0 ml. of staphylococcal factor with the added meat broth or distilled water

FIG. XXX - The inhibition of complete lysis of a serum-milk-agar plate containing 0.5 ml. of serum and 4.0 ml. of staphylococcal factor by the addition of 4.0 ml. of meat broth. Note the production of large plaques. (Incubated at 37°C. for 24 hours, then left at room temperature for a further 24 hours.)

FIG. XXXI - Complete lysis of a serum-milk-agar plate containing 0.5 ml. of serum and 4.0 ml. of staphylococcal factor. 4.0 ml. of distilled water was employed as diluent in place of the same volume of meat broth as used above. (Incubated at 37°C. for 24 hours and then left at room temperature for a further 24 hours.)

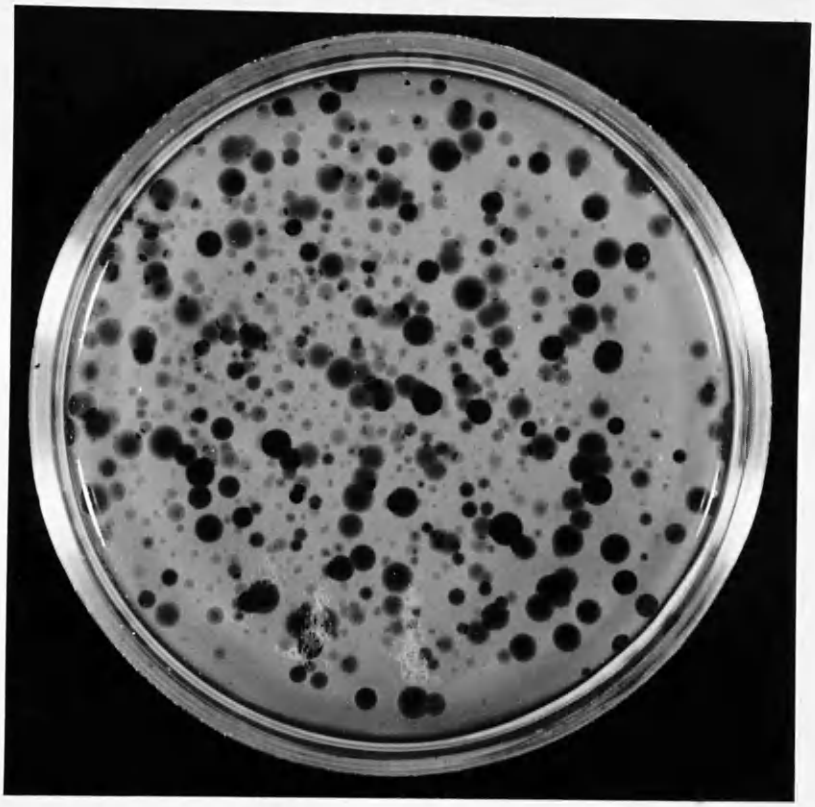


Fig. XXX

Fig. XXXI

FIG. XXXII - The inhibition of complete lysis of a serum-milk-agar plate containing 0.5 ml. of serum and 2.0 ml. of staphylococcal factor by the addition of 6.0 ml. of meat broth. (Incubated at 37°C. for 24 hours, then left at room temperature for a further 24 hours.)

FIG. XXXIII - Complete lysis of a serum-milk-agar plate containing 0.5 ml. of serum and 2.0 ml. of staphylococcal factor. 6.0 ml. of distilled water was employed as diluent in place of the same volume of meat broth as used above. (Incubated at 37°C. for 24 hours, then left at room temperature for a further 24 hours.)

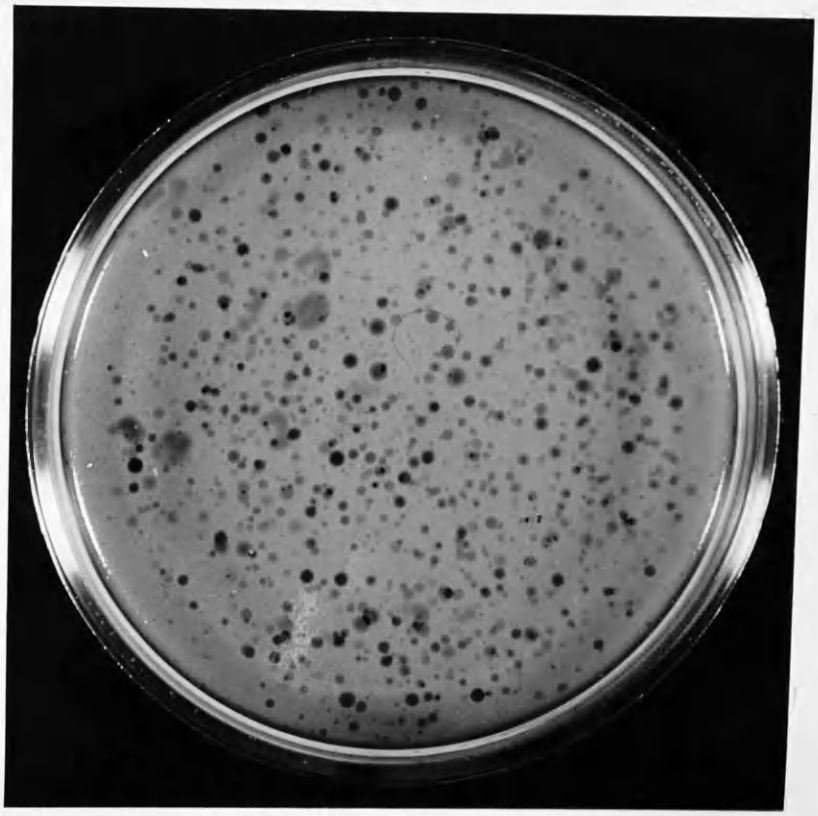


Fig. XXXII

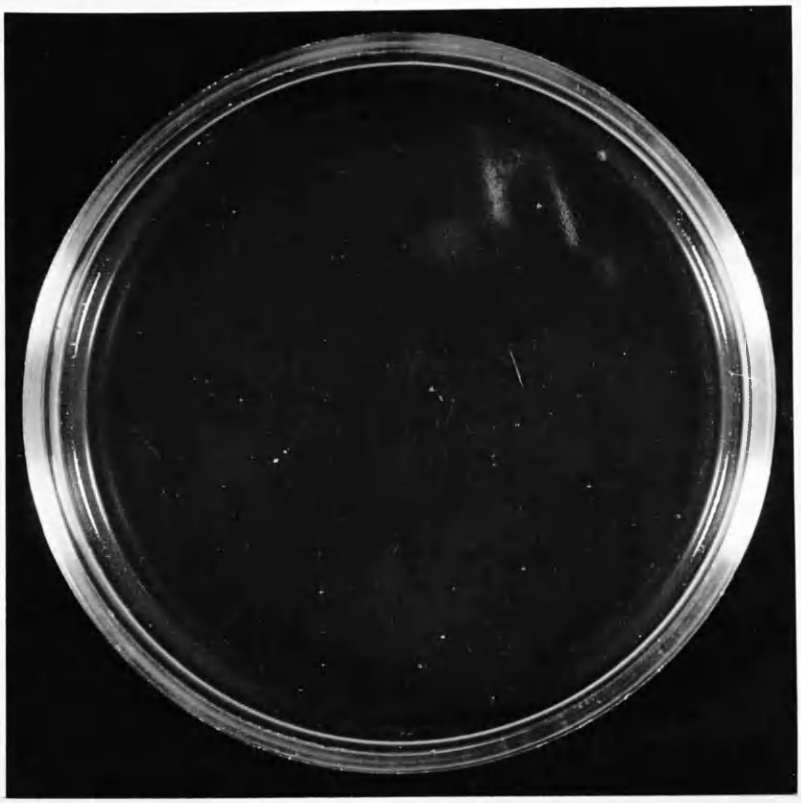


Fig. XXXIII

FIG. XXXIV - The inhibition of complete lysis in a serum-milk-agar plate containing 0.5 ml. of serum, 7.0 ml. of staphylococcal factor and 1.0 ml. of meat broth. Only a few large plaques indicate activity. (Incubated at 37°C. for 24 hours, and then left at room temperature for a further 24 hours.)

FIG. XXXV - The production of many plaques in a serum-milk-agar plate containing 0.5 ml. of serum, 7.0 ml. of staphylococcal factor, and 1.0 ml. of distilled water. The distilled water was employed as a diluent in place of the 1.0 ml. of meat broth as used above. (Incubated at 37°C. for 24 hours, and then left at room temperature for a further 24 hours.)

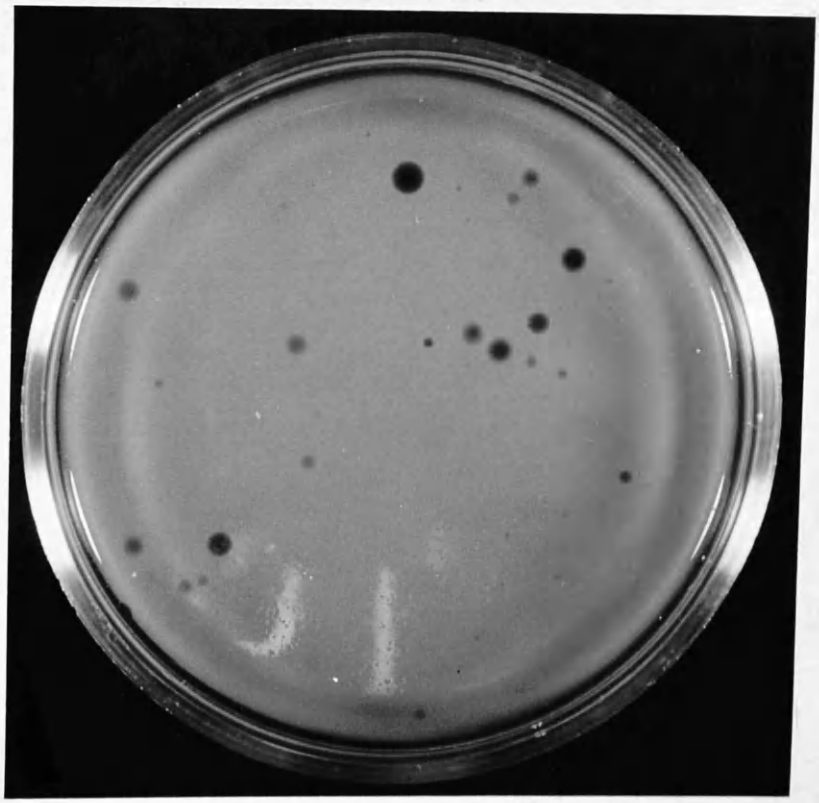


Fig. XXXIV

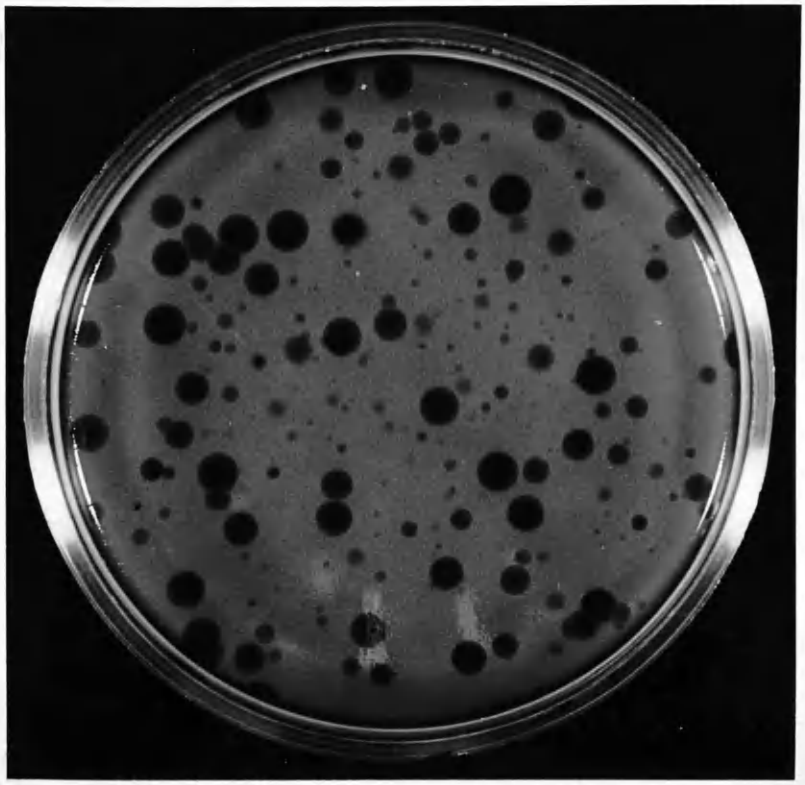


Fig. XXXV

FIG. XXXVI - Partial inhibition of Muller's phenomenon in a serum-milk-agar plate containing 0.5 ml. serum and 1.0 ml. of staphylococcal factor by the addition of 0.25% peptone (Oxoid). (Incubated at 37°C. for 24 hours.)

Fig. XXXVI

were photographed and are reproduced as Figs. XXX - XXXV.

In view of Burnet's finding (Burnet, 1928) that peptone was definitely inhibitory towards the manifestation of Muller's phenomenon it was decided to ascertain if peptone in any way interfered with the plaque-production by staphylococcal factor. This point was investigated in an experiment presented in App. 284-285. To a series of four serum-milk-agar plates amounts of a 15% solution of peptone (Oxoid) were added to give final concentrations of 0.25%, 0.5%, 0.75% and 1.0% of peptone. A control serum-milk-agar plate without added peptone was also prepared. These plates were incubated at 37°C. for 24 hours when readings were made. The control plate without added peptone showed plaques and almost complete clearing of the medium due to fusion of many plaques. The plates with 0.25% and 0.5% peptone added, showed fewer plaques than the control plate while the plates with 0.75% and 1.0% peptone showed no plaques whatsoever, nor any other change in the medium. Thus Muller's phenomenon is inhibited by peptone (Oxoid) either completely or partially, depending on the concentration of it present. The serum-milk-agar plate with 0.25% peptone (Oxoid) added was photographed and is reproduced as Fig. XXXVI.

In another experiment a comparison of the inhibitory

effect of the addition to serum-milk-agar plates of varied amounts of 1.0% "Lab-Lemco" broth and of 1.0% "Lab-Lemco" solution in distilled water (App. 286-287). The addition of the "Lab-Lemco" solution to a series of serum-milk-agar plates, in amounts from 1.0 ml. to 6.0 ml. in no way interfered with the development of plaques but the addition of 3.0 to 6.0 ml. amounts of 1.0% "Lab-Lemco" broth did interfere markedly with the number and size of the plaques which were produced. This finding can be accounted for by the fact that the 1.0% "Lab-Lemco" broth contained 1.0% peptone and 0.5% sodium chloride.

(ii) By the addition of sodium chloride.

The effect of sodium chloride in inhibiting the development of Muller's phenomenon by growing staphylococci either partially or completely has already been referred to in Part IV (P. 182-184). It did not seem likely that this inhibition was the result of interference with the growth of staphylococci but rather that it was due either to a failure of the staphylococci to produce the necessary factor in the presence of excess sodium chloride or a direct inhibition of the action of the staphylococcal factor

already produced. It was also considered that the sodium chloride might affect the serum factor.

The effect of sodium chloride on the development of plaque-production in serum-milk-agar was examined in a number of experiments, one of which is presented in App. 288-289. A series of five serum-milk-agar plates were prepared with 0.25%, 0.5%, 0.75%, 1.0% and 2.0% of sodium chloride respectively. In addition a serum-milk-agar plate without added serum was also prepared. All the plates were incubated at 37°C. for 24 hours.

The control serum-milk-agar plate without added sodium chloride was completely cleared (lysis) after 24 hours' incubation, fusion of the numerous plaques seen previously having occurred. The plates containing added sodium chloride, however, all showed inhibition of plaque-production, either partial as in the plates with 0.25% and 0.5% or complete as in the plates with 0.75%, 1.0% and 2.0%. Such a finding indicated that sodium chloride could inhibit the action of preformed staphylococcal factor.

(f) MISCELLANEOUS EXPERIMENTS.

In the course of certain investigations, e.g. fractionation of serum, it was necessary to incorporate in milk-agar plates certain fractions of serum whose sterility could no longer be relied upon. Since the temperature necessary to sterilise these serum fractions would also inactivate them and since the available amounts of these serum fractions were so small that they could not be easily filtered, it seemed worthwhile to consider the employment of chemical sterilising agents. It was found possible to obtain such agents which could be incorporated in standard serum-milk-agar plates containing staphylococcal factor or in other milk-agar test plates with serum fractions without interfering with the development of Muller's phenomenon and which would ensure the sterility of the media.

One of a number of experiments carried out with different antiseptics is given in App. 290-291. In this experiment in which acriflavine and "Merthiolate" were employed, certain concentrations of these substances were found not to interfere with the development of Muller's phenomenon. Two sets of standard serum-milk-agar plates containing 2.0 ml. of staphylococcal factor were prepared. To the three plates in one set, just before they

were poured amounts of acriflavine solution were added to give final concentrations of 1/10,000, 1/20,000 and 1/50,000 respectively. To the three plates in the other set amounts of "Merthiolate" solution were added to give final concentrations of 1/10,000, 1/20,000 and 1/50,000 respectively. A control serum-milk-agar plate without added antiseptic solution was also prepared. All the plates were incubated at 37°C. for 24 hours and then placed at room temperature. Readings were taken after 24 hours' incubation at 37°C. and after a further 48 hours at room temperature.

The demonstration of Muller's phenomenon in the three plates containing "Merthiolate" and in the plate with 1/50,000 acriflavine was in no way different from that observed in the control plate after 24 hours' and 48 hours' incubation at 37°C. The plates with 1/10,000 and 1/20,000 acriflavine showed however after 24 hours' incubation a complete and a partial inhibition of Muller's phenomenon but after a further 48 hours' incubation the plate with 1/10,000 acriflavine showed only a slight inhibition of the phenomenon compared with the control plate, which was then showing complete clearing of the medium (lysis). The plate with 1/20,000 acriflavine showed complete clearing of the medium also, after the further 48 hours at room temperature.

The inhibitory action of soya-bean on the production

FIG. XXXVII - Production of Muller's phenomenon in a serum-haemoglobin-agar plate containing 4.0 ml. staphylococcal factor and 2.0 ml. serum. (Incubated at 37°C. for 24 hours.)

FIG. XXXVIII - Production of Muller's phenomenon on a serum-heated-haemoglobin-agar plate containing 4.0 ml. staphylococcal factor and 2.0 ml. serum. (Incubated at 37°C. for 24 hours.)

Fig. XXXVII

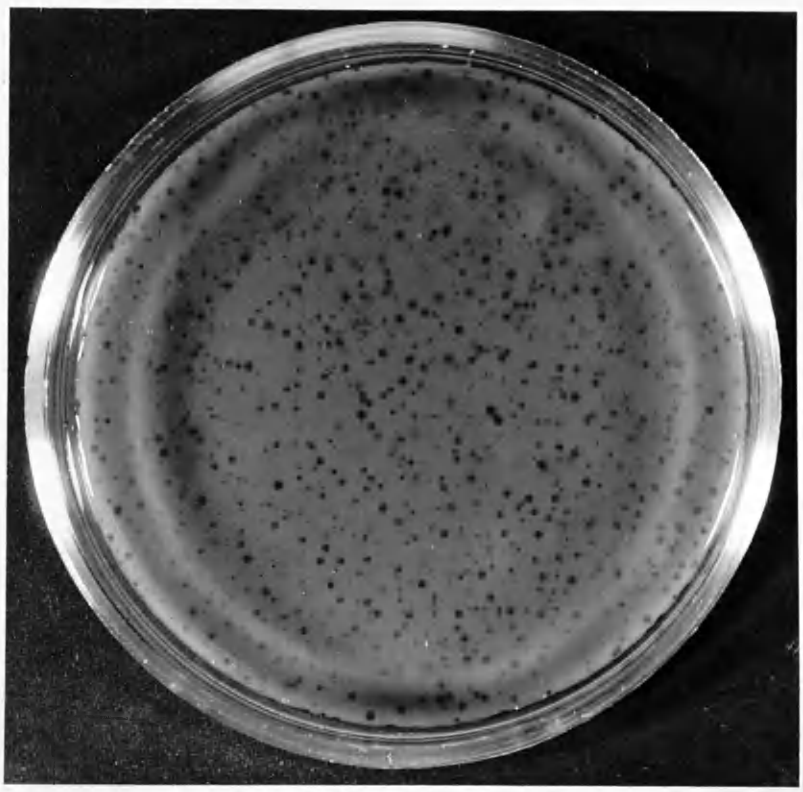


Fig. XXXVIII

FIG. XXXIX - Inhibition of Muller's phenomenon in a serum-milk-agar plate containing 0.5 ml. serum and 2.0 ml. staphylococcal factor by soya-bean-trypsin-inhibitor contained in two porous cups in the medium. The concentrations of the inhibitor in two of the cups were 5 mg./ml. and 2.5 mg./ml. respectively. There is no inhibition of the phenomenon around the third cup which contains distilled water only. (Incubated at 37°C. for 24 hours.)

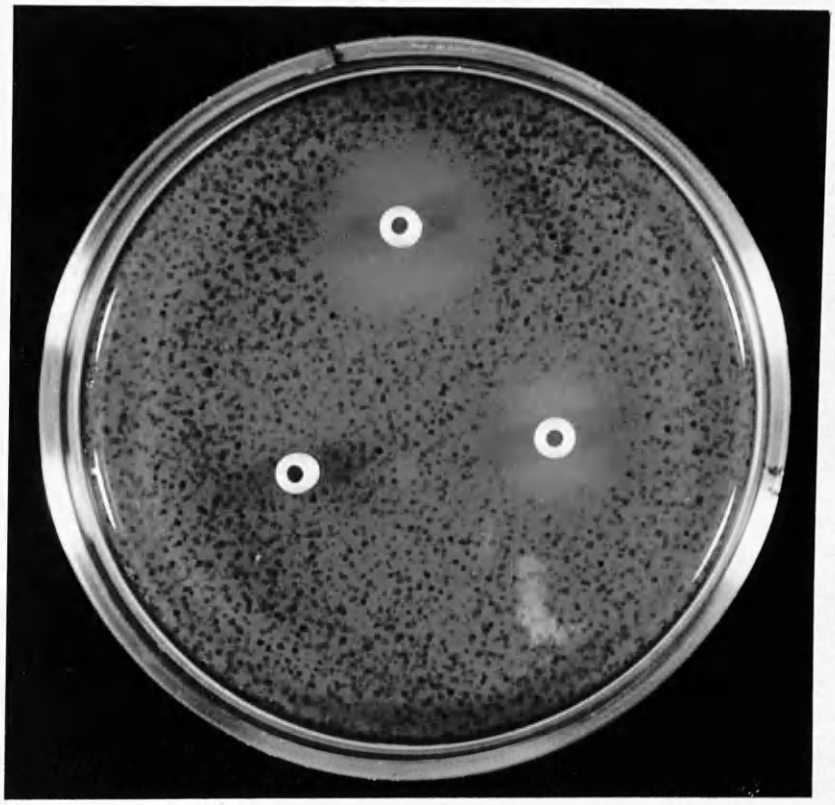


Fig. XXXIX

of Muller's phenomenon by growing staphylococci has already been dealt with. The fact that plaque-formation in serum-milk-agar containing staphylococcal factor can also be inhibited by a preparation of soya-bean was shown in an experiment presented in App. 292-293. Three sterile porous test cups were placed on the surface of a standard serum-milk-agar plate containing 2.0 ml. of a staphylococcal factor preparation and 1/30,000 merthiolate. To one cup was added 2 drops of a soya-bean-trypsin inhibitor preparation (S.B.I.) in a sterile distilled water (5 mgm./ml.); to a second cup, 2 drops of S.B.I. (2.5 mgm./ml.); and to the third cup, which acted as a control, 2 drops of sterile distilled water. The plate was incubated at 37°C. for 24 hours when readings were made. Plaque-formation was observed throughout the plate except in the vicinity of the two cups which contained the soya-bean-trypsin inhibitor (S.B.I.). Around each of these two cups there was a zone of unaltered medium. There was no such zone around the third (control) cup which contained only distilled water. This plate was photographed and is reproduced as Fig. XXXIX.

In many of the experiments performed with staphylococcal factor in serum-milk-agar, plaque-formation was observed after only 2 hours' incubation of the plates at 37°C. In the preparation of a serum-milk-agar plate the constituents were added

to a test-tube containing the melted agar held at 50°C. and, after the serum - the last ingredient to be incorporated - had been thoroughly mixed with the rest, the mixture was poured into a Petri dish. It seemed worthwhile to ascertain if, by holding the serum and staphylococcal factor together before adding them to the rest of the medium, the time which elapsed before plaques appeared could be shortened. An experiment of this nature was carried out and is given in App. 294-295. In this experiment similar serum-staphylococcal factor mixtures were held at 37°C. for varied periods of time before being added to the usual milk-agar base and poured as plates. In addition similar serum-staphylococcal factor-milk mixtures were held at 37°C. for varied periods before being added to the agar base. Control plates with the same amounts of serum, milk and staphylococcal factor were also prepared, but their constituents were poured immediately after mixing without any preliminary holding at 37°C.

All the plates were incubated at 37°C. and examined every 15 minutes until plaque-formation was apparent. Plaques were first visible in all plates, including the control plates, after $1\frac{3}{4}$ hours' incubation at 37°C. Thus preliminary holding of the active constituents at 37°C. in no way enhances the development of Muller's phenomenon in serum-milk-agar.

II. HAEMOGLOBIN IN SUBSTRATE.

(a) AMOUNT OF HAEMOGLOBIN.

(b) EFFECT OF VARIED AMOUNTS OF STAPHYLOCOCCAL FACTOR.

(c) EFFECTS OF ALTERING THE TEMPERATURE OF INCUBATION AND THE GASEOUS ENVIRONMENT.

(d) VARIED AMOUNTS OF SERUM.

(e) THE USE OF HEATED HAEMOGLOBIN.

II. HAEMOGLOBIN IN SUBSTRATE.

Although both the crude and the filtered haemoglobin solutions detailed by Elek (1953) were found to be suitable indicator protein preparations for the demonstration of Muller's phenomenon with staphylococcal factor under the appropriate conditions, the filtered haemoglobin solution was preferred to the crude haemoglobin and was used in the majority of the experiments carried out with this blood protein. In all but one of the experiments reported in this section, filtered haemoglobin was employed; this one exception was an experiment performed with heated haemoglobin, in which washed red cells were used as the source of haemoglobin.

(a) AMOUNT OF HAEMOGLOBIN.

A number of experiments were performed in which varied amounts of filtered haemoglobin solution were incorporated in serum-agar mixtures with staphylococcal factor to ascertain their effect on the production of Muller's phenomenon. The optimal amount of haemoglobin to be incorporated in serum-haemoglobin-agar was determined in these experiments, one of which is given in App. 296-297. A series of serum-haemoglobin-agar plates with staphylococcal factor was prepared containing amounts of filtered haemoglobin solution from 0.5 ml. to 5.0 ml. These plates were incubated at 37°C. for 24 hours and then left at room temperature. Readings were made after 24 hours at 37°C. and after a further 24 hours at room temperature. After 24 hours at 37°C. and a further 24 hours at room temperature the medium in the plates with amounts of haemoglobin from 0.5 ml. to 2.0 ml. was completely cleared, the many plaques which had been observed earlier having fused; the medium in the plates with amounts of haemoglobin from 3.0 ml. to 5.0 ml. was not completely cleared and showed still a few plaques which had not coalesced. The optimal amount of haemoglobin solution for such a serum-haemoglobin-agar plate was fixed at 1.5 ml. having been decided on not only by the speed of development of Muller's phenomenon with subsequent fusion of the plaques but also by the ease with which plaques of clearing could be distinguished from the unaltered medium.

(b) EFFECT OF VARIED AMOUNTS OF STAPHYLOCOCCAL FACTOR.

The effect of adding varied amounts of staphylococcal factor to a series of serum-haemoglobin-agar plates was ascertained with a number of different staphylococcal factor preparations. The results obtained with one of these preparations is given in App. 298-299. Amounts of staphylococcal factor from 1.0 ml. to 6.0 ml. were added to a series of six tubes of serum-haemoglobin-agar, mixed and then poured as plates. The plates were incubated at 37°C. and then left at room temperature for 24 hours. During the period of incubation and subsequent leaving at room temperature, the plates were examined frequently and readings were taken after 24 hours at 37°C. and after 24 hours at room temperature. The plates containing 3.0 ml. and 4.0 ml. of staphylococcal factor were completely cleared (lysis) when examined after 24 hours at 37°C., all the plaques which had been seen earlier having fused; the plates with 2.0 ml., 5.0 ml. and 6.0 ml. respectively, still showed a few plaques, the majority of those seen earlier having fused to bring about at least 90% lysis of the medium. The plate with 2.0 ml. of factor was completely cleared when re-examined after 24 hours at room temperature, while a reduction was noted in the number of plaques in the plates with 5.0 ml. and 6.0 ml. of staphylococcal factor with a corresponding

extension of the degree of lysis. No change - neither plaques nor clearing - was noted in the plate with 1.0 ml. of staphylococcal factor during the 24 hours it was incubated at 37°C., but during the subsequent 24 hours at room temperature plaques developed and coalesced resulting in complete clearing of the medium in this plate. Thus, with varied amounts of a staphylococcal factor, haemoglobin in the substrate behaves in a similar fashion to milk incorporated in such a substrate. Nowhere in his report on the use of his staphylococcal factor incorporated in serum-haemoglobin-agar, does Elek (1953) mention fusion of the plaques taking place with a resultant complete clearing of the medium.

(c) THE EFFECTS OF ALTERING THE TEMPERATURE OF INCUBATION AND THE GASEOUS ENVIRONMENT.

The effect of varied temperatures on the development of Muller's phenomenon in serum-haemoglobin-agar containing staphylococcal factor was ascertained in an experiment given in App. 300-301. Four serum-haemoglobin-agar plates and four haemoglobin-agar control plates (without added serum) were prepared. One serum-haemoglobin-agar plate and a control plate were placed at each of the following temperatures: (1) 4°C., (2) 22°C., (3) 30°C., and (4) 37°C. and left for 7 days at these temperatures. As was to be expected none of the haemoglobin-agar (control) plates, irrespective of the temperature, showed Muller's phenomenon. After 18 hours at 22°C., 30°C. and 37°C. the three serum-haemoglobin-agar plates placed at these respective temperatures all showed Muller's phenomenon (plaque-formation). The plate kept at 4°C., however, even after 7 days showed no change in its medium, neither plaques nor lysis.

This inability to demonstrate Muller's phenomenon at 4°C. in serum-haemoglobin-agar with staphylococcal factor is surprising on account of the fact that at the same temperature plaques can be readily demonstrated in serum-milk-agar. This is

possibly another example of the fact, previously noted, that haemoglobin is less readily broken down than is milk by staphylococcal factor. In this case the low adverse temperature has magnified the usually slight difference between these two proteins to attack by this proteolytic system. It was possible, however, to demonstrate in serum-haemoglobin-agar that plaque-formation occurs under anaerobic conditions. One experiment in which this was carried out is presented in App. 302-303. Two sets of serum-haemoglobin-agar plates were prepared with amounts of serum from 1.5 ml. to 5.0 ml. One set was incubated in a McIntosh and Fildes anaerobic jar at 37°C.; the other set which acted as a control was incubated aerobically at 37°C. Examination of the plates after 24 hours' incubation at 37°C. revealed that Muller's phenomenon (plaque-formation) was present in all the plates incubated anaerobically as well as all those incubated aerobically.

(d) VARIED AMOUNTS OF SERUM.

The effect of varied amounts of serum on the development of Muller's phenomenon in haemoglobin-agar plates with staphylococcal factor was investigated in a number of experiments, one of which is detailed in App. 304-305. Amounts of serum from 0.5 ml. to 5.0 ml. were added to a series of ten tubes of melted haemoglobin-agar containing 4.0 ml. of staphylococcal factor held in a water-bath at 50°C. The serum added to each tube was thoroughly mixed with the contents and poured as a plate. A control plate of haemoglobin-agar without added serum was also prepared. All the plates were incubated at 37°C. for 48 hours and readings were made after 24 and 48 hours' incubation. Muller's phenomenon (plaque-formation) did not occur, of course, in the control plate without serum. At 24 hours all the test plates with the exception of those which contained 3.5 ml. and 4.0 ml. of serum respectively showed plaque-formation. There was an increasing number of plaques in the plates with 0.5 ml. up to that with 3.0 ml. and in addition, clearing of the medium was marked in the plates with 1.5 ml. to 3.0 ml. of serum. The plate with 4.5 ml. of serum, with regard to its number of plaques and degree of lysis gave a picture similar to that with 2.0 ml. of serum while the

plate with 5.0 ml. was comparable with that containing 1.0 ml. of serum. The plates with 3.5 ml. and 4.0 ml. of serum were completely cleared (lysis) when examined after 24 hours' incubation, due to fusion of the numerous plaques seen at an earlier stage of incubation. After a further 24 hours' incubation at 37°C., except in those plates with 3.5 and 4.0 ml. of serum there was an increase in the number of plaques present in all plates with some increase in the amount of lysis due to fusion of plaques. The plate with 2.0 ml. of serum was photographed and is reproduced as Fig. XXXVII.

This result is similar to those previously obtained in many experiments carried out with serum-milk-agar and in the experiment reported in App. 276-277 in which varied amounts of serum were used in milk-agar and in haemoglobin-agar plates. In that previous experiment it was observed that with the largest amounts of serum used (3.5 ml. and 4.0 ml.) complete inhibition of Muller's phenomenon occurred. In the experiment reported here only partial inhibition of the phenomenon occurred with the largest amounts of serum (4.5 ml. and 5.0 ml.). Such a finding, however, is not unexpected in view of the fact that different sera were used in these two experiments and it is known that different samples of serum differ in their activity when used in small amounts and in their inhibitory action when used in large amounts.

(e) THE USE OF HEATED HAEMOGLOBIN.

In view of the fact that heated-haemoglobin had been found to be a good indicator protein for the demonstration of Muller's phenomenon with living staphylococci in appropriate conditions, indeed a better indicator than unheated haemoglobin, it seemed desirable to test it also with staphylococcal factor.

In one experiment (App. 306-307) washed human red cells were used as the haemoglobin source. A series of 4 tubes each containing agar, distilled water and washed human red cells were heated at 75°C. for 10 minutes. The tubes were cooled to 50°C. and to each 1.0 ml. of staphylococcal factor was added. Amounts of serum - 0.5 ml., 1.0 ml. and 4.0 ml. were added respectively to three tubes while the fourth tube received no serum. Similar sets of plates were made, one set with 2.0 ml., one with 3.0 ml. and the third with 4.0 ml. of staphylococcal factor. All plates were incubated at 37°C. for 24 hours when readings were taken.

None of the plates without serum, as was to be expected, showed Muller's phenomenon; all the plates with serum showed plaque-formation with the exception of that plate which contained 4.0 ml. of serum and 1.0 ml. of staphylococcal factor. In this latter plate the absence of plaque-formation was due apparently to inhibition by the large amount of serum in the plate.

Such inhibition, however, did not occur with this same quantity of serum in the other plates which contained larger amounts of the staphylococcal factor. Indeed, the plaques in the plates with 4.0 ml. of serum and 3.0 ml. of staphylococcal factor and that with 4.0 ml. of staphylococcal factor were so numerous that coalescence had occurred resulting in clearing of the medium to the extent of causing "80% Lysis" and "95% Lysis" respectively.

In another experiment (App. 308-309) filtered haemoglobin was used in place of the washed human red cells employed in the previous experiment. Three tubes containing agar, distilled water and filtered haemoglobin were heated at 75°C. for 10 minutes. The tubes were cooled to 50°C. and to each the same amount of staphylococcal factor was added. To one tube 0.5 ml. of fresh serum was then added, to another 1.0 ml. and to the third 2.0 ml. The contents of each tube were mixed thoroughly and the mixtures poured as plates. The plates were incubated at 37°C. for 24 hours when readings were made.

Plaque-formation was observed in all three plates. The plate containing 2.0 ml. of serum was photographed and is reproduced as Fig XXXVIII.

III. HEATED PLASMA IN SUBSTRATE.

(a) TEMPERATURE OF HEATING PLASMA.

(b) AMOUNTS OF HEATED PLASMA AND FRESH SERUM.

(c) ADDITION OF SODIUM CHLORIDE.

III. HEATED PLASMA IN SUBSTRATE.

The production of Muller's phenomenon with staphylococcal factor in heated plasma-agar was studied in a small number of experiments. Although it was possible to produce plaques of clearing in such a test protein in appropriate conditions, heated plasma was considered a poor indicator protein for this purpose and more extensive investigations with it were not carried out.

(a) TEMPERATURE OF HEATING PLASMA.

In one experiment in which plasma heated at 56°C. for 10 minutes was used with varied amounts of staphylococcal factor, no plaque-production was observed although the plates in which the largest amounts of the staphylococcal factor were incorporated (2.0 ml. to 4.0 ml.) were completely cleared (lysis). In a similar experiment with plasma heated at 65°C. for 30 minutes, to which a small amount of fresh serum was added, in addition to the staphylococcal factor, plaque-production was observed in one plate of medium before it was completely cleared after 48 hours' incubation at 37°C. The plaque-production, however, was poor, the plaques being very faint and ill-defined, compared with those usually seen in demonstrations of Muller's phenomenon with milk or haemoglobin as the test protein.

(b) AMOUNTS OF HEATED PLASMA AND FRESH SERUM.

The amount of heated-plasma (preheated at 65°C. for 30 minutes) and the amount of fresh serum added to the agar base was of importance, not only with regard to the clearing of the plate by the staphylococcal factor but also for the demonstration of plaque-production. In one experiment in which 1.0 ml. of heated plasma was used as the test protein in a series of plates with varied amounts of fresh serum from 0.5 ml. to 4.0 ml., clearing of the medium in the plate with 0.5 ml. of fresh serum was noted after 24 hours' incubation at 37°C. and plaque-production was observed in the plate with 1.0 ml. of fresh serum. The rest of the plates in the series, however, showed no change in the medium, neither clearing (lysis) nor plaques. A second series of plates containing 5.0 ml. of plasma, preheated at 65°C. for 30 minutes and with varied amounts of fresh serum, from 0.5 ml. to 4.0 ml., showed neither plaque-production nor clearing of the medium although the same amount of staphylococcal factor was present in these plates as had been included in the previous series of plates.

(c) ADDITION OF SODIUM CHLORIDE.

The effect of the addition of varied amounts of sodium chloride solution to heated plasma-agar on the production of Muller's phenomenon, was ascertained in another experiment. A series of heated plasma-agar plates (heated at 65°C. for 30 minutes), each containing 0.5 ml. of added fresh serum and a constant amount of a staphylococcal factor preparation were prepared and varied amounts of a sodium chloride solution were added to these plates to give a range of sodium chloride concentrations from 0.2% to 0.8%. One plate in the series received no sodium chloride and acted as a control. After incubation at 37°C. for 2½ hours, all the plates were completely cleared (lysis) except the plates with 0.6% and 0.8% added sodium chloride, both of which showed many plaques with up to 70% lysis of the medium. Examination of these two plates after 24 hours' incubation at room temperature revealed that no trace of the plaques previously noted, was to be seen and that the medium had completely cleared.

Muller's phenomenon can be demonstrated therefore in a heated plasma-agar medium in appropriate conditions but there is a marked tendency for any change brought about by the staphylococcal factor to be in the nature of a complete clearing of the medium, without visible plaque-formation. In view of this finding the use of heated plasma as a test protein in further studies on Muller's phenomenon was not continued.

IV. SUMMARY.

This investigation of the conditions influencing the production of Muller's phenomenon by staphylococcal factor was carried out on three main indicator proteins, viz. milk, haemoglobin and heated plasma. Heated haemoglobin was also employed successfully for this purpose. Although the phenomenon could be demonstrated with a wide range of concentrations of milk and of haemoglobin there was a much narrower range of optimal concentrations whose use resulted in quicker production and easier recognition of plaques. With milk as the test protein plaque-production was demonstrated at a temperature as low as 4°C. although this was not so with haemoglobin. With staphylococcal factor Muller's phenomenon was demonstrated in milk and in haemoglobin test-plates, incubated under anaerobic conditions.

Plaques of clearing were produced in serum-milk-agar plates with agar concentrations from 0.6% to 3.0%, but not with concentrations of 4.0% or 6.0%. The plaques developing in agar concentrations of 2.5% and 3.0% were half the average size of those usually found in the plates with the customary 1.3% concentration of agar. The thermostability of staphylococcal

factor and the thermolability of the serum factor were confirmed. The effect of varied concentrations of serum incorporated in milk-agar and haemoglobin-agar was investigated. There was an optimal concentration of serum for incorporation in a 15 ml. volume of medium and addition of serum in excess of that concentration resulted in the complete absence of plaques. There was also an optimal concentration of staphylococcal factor and staphylococcal factor in excess of this concentration resulted in inhibition of plaque-formation. This was most likely due to the presence of an inhibitor in the meat broth used to prepare the staphylococcal factor. "Lab-Lemco" broth, peptone (Oxoid) and sodium chloride were all found to have an inhibitory action on plaque-production, when incorporated in serum-milk-agar plates in final concentrations of 0.5%, 0.25% and 0.25% respectively. The fact that a "Lab-Lemco" solution did not inhibit plaque-formation when used in 0.6% concentration, suggested that it was the content of peptone and sodium chloride which imparted the inhibitory power to the 1% "Lab-Lemco" broth. Acriflavine and "Merthiolate" solutions in sterilising concentrations did not interfere with plaque-production when incorporated in serum-milk-agar plates. Soya-bean-trypsin-inhibitor inhibited Muller's phenomenon.

Heated plasma, with and without the addition of

fresh serum incorporated in the customary agar base could be used for the demonstration of Muller's phenomenon but it was a poor indicator protein for this purpose compared with milk, haemoglobin or heated haemoglobin. The temperature and time of heating of plasma, the amount of plasma used, the amount of fresh serum added and the addition of sodium chloride were all factors of importance in the production of plaques on this indicator protein. It was much easier to demonstrate complete clearing (lysis) of this medium than it was to demonstrate plaque-formation.

Heated-haemoglobin was used in a few experiments with staphylococcal factor and when tested in the appropriate conditions was found to be a satisfactory indicator-protein for the demonstration of Muller's phenomenon.

P A R T VII.

INVESTIGATION OF THE ROLE OF SERUM IN MULLER'S PHENOMENON.

P A R T VII.

INVESTIGATION OF THE ROLE OF SERUM IN MULLER'S PHENOMENON.

The essential role of serum in the production of Muller's phenomenon has been recognised since the original description of "l'hémophagie staphylococcique" by Muller and all subsequent workers have agreed that serum is necessary for plaque-production. There is no general agreement, however, as to the nature of the contribution which serum makes to the production of the phenomenon and little or nothing is known of the serum factor itself. The essential role of serum has been confirmed in the course of many experiments thus far carried out in this thesis. Control plates of milk-agar or haemoglobin-agar without added serum always failed to show plaque-formation while corresponding plates of the same medium with the addition of serum showed Muller's phenomenon.

It was found possible to carry out only a limited investigation of the serum factor necessary for Muller's phenomenon. A few experiments dealing with the part played by serum have already been presented, for convenience in Parts IV and VI but the main section of this restricted investigation on the role of serum, dealing with methods of titration of serum, the thermolability of serum, the suitability of sera from different animals and the attempts to isolate the active factor from serum, is now presented.

PART VII.

INVESTIGATION OF THE ROLE OF SERUM IN MULLER'S PHENOMENON.

I. TITRATIONS OF HUMAN SERUM.

II. THE EFFECT OF HEATING SERUM.

III. THE SUITABILITY OF SERUM FROM OTHER ANIMALS.

IV. THE ACTIVE FACTOR IN SERUM.

V. SUMMARY.

P A R T VII.

I. TITRATIONS OF HUMAN SERUM.

(a) STANDARD METHODS OF TITRATION.

(i) With growing staphylococci.

(ii) With staphylococcal factor.

(iii) Comparison of the activity of three pooled sera.

(iv) Checkerboard Titrations of eight sera.

(v) Titrations of sera from cases of disease.

(vi) Titration of plasma.

(vii) Titrations of placental serum.

(b) OTHER METHODS OF TITRATION.

(i) The effect of spreading serum on milk-agar containing staphylococcal factor; the effect of spreading staphylococcal factor on serum-milk-agar.

(ii) The use of the "disc method" of titration.

(iii) The applicability of the "disc method" for staphylococcal factor titrations.

(iv) Titration of serum by the test-tube method.

I. TITRATIONS OF HUMAN SERUM.

(a) STANDARD METHODS OF TITRATION.

Reference has already been made in this thesis to the importance of the concentration of serum in a serum-milk-agar plate for the demonstration of Muller's phenomenon. It was recognised initially that plaque-production could be demonstrated in a serum-milk-agar plate if a concentration of less than 50% of human serum was employed (as had been used by Fisk and Mordvin, (1943)). The highest concentration of serum which permits the development of plaques in serum-milk-agar varies from one serum to another but is usually a concentration of about 20% to 30%. The procedure by which the range over which a given serum can bring about plaque-production is referred to here as the "titration" of that serum. The titrating of sera, with regard to their ability to produce Muller's phenomenon, was carried out in a number of different ways, which will now be described.

(i) With growing staphylococci.

This type of experiment has already been referred to on a number of occasions. A series of serum-milk-agar plates with amounts of serum from 0.1 ml. to 7.0 ml. were prepared and each plate stab-inoculated with seven strains of coagulase-positive staphylococci from 6 hour old "Lab-Lemco" broth cultures. These plates were incubated at 37°C. for 24 hours and then left at room temperature for a further 24 hours. Readings were made after 24 hours at 37°C. and after 24 hours at room temperature (App. 310-311). A control milk-agar plate without added serum was also prepared, and Muller's phenomenon did not develop, of course, in this plate. Plaque-formation occurred with all strains of staphylococci in the plates with 0.1 ml. to 3.0 ml. of serum, within 24 hours at 37°C. The remainder of the plates, i.e. those with 4.0 ml. to 7.0 ml. of serum showed no plaques but only zones of clear cut definite lysis around the colonies. After the plates had been held at room temperature for a further 24 hours, the only change which had occurred was in the plate with 3.0 ml. of serum. Around the colonies in this plate, plaques were no longer to be seen, only zones of lysis with a cleanly cut regular edge to them. If the result of this

titration is taken after 24 hours' incubation at 37°C., then 3.0 ml. of this serum is the greatest amount which will permit plaque-production under the conditions of this experiment.

Different strains of staphylococci are known to produce different amounts of staphylococcal factor and from colonies of such staphylococci growing on the surface of serum-milk-agar plates varied amounts of the factor are likely to diffuse into the surrounding medium. One result of this would be that not all strains growing on the same set of serum-milk-agar plates would give the same end point for the serum. This has been observed with some titrations of serum in which a number of strains of staphylococci were used although it has not occurred in the titration presented here. It is apparent that no great importance can be attached to the titration of a serum with growing staphylococci as the source of staphylococcal factor, in view of the conditions which may interfere either with the growth of these organisms or their production of staphylococcal factor.

(ii) <u>With staphylococcal factor.</u>

Much greater reliance can be placed on the titration of sera with a standard preparation of staphylococcal factor. One

experiment in which a serum was titrated with a given amount of a staphylococcal factor preparation is presented in App. 312-313. Amounts of this serum from 0.1 ml. to 5.0 ml. were incorporated in milk-agar plates, each of which contained 1.0 ml. of a staphylococcal factor preparation. These plates were incubated at 37°C. when readings were taken. Only plaques were observed in the plates with 0.1 ml. and 0.25 ml. of serum, while in the plate with 0.5 ml. a few plaques were seen but about 90% of the medium was lysed due to fusion of the numerous plaques which had been present earlier, i.e. after a shorter period of incubation. The plate with 3.0 ml. of serum also showed plaques and lysis of the medium due to fusion of plaques but in this case the lysis or clearing amount to about 20%. The plates with 1.0 ml. and 2.0 ml. of serum were completely lysed due to the fusion of plaques which had been present earlier but the plates with 4.0 ml. and 5.0 ml. of serum showed neither plaques nor lysis. What is the greatest amount of this serum which will permit the demonstration of Muller's phenomenon? Is 3.0 ml. the end-point of this titration? The fact that complete clearing of the serum-milk-agar plates with 1.0 ml. and 2.0 ml. of serum had occurred in a shorter space of time than 24 hours and that that clearing had resulted from the fusion of plaques present in these plates would suggest that the optimal amount of serum for the

FIG. XL - Production of Muller's phenomenon in a serum-milk-agar plate containing 0.1 ml. human serum, and 1.0 ml. of staphylococcal factor. (Incubated at 37°C. for 24 hours.)

FIG. XLI - Production of Muller's phenomenon in a serum-milk-agar plate containing 3.0 ml. human serum and 1.0 ml. of staphylococcal factor. (Incubated at 37°C. for 24 hours.)

Fig. XL

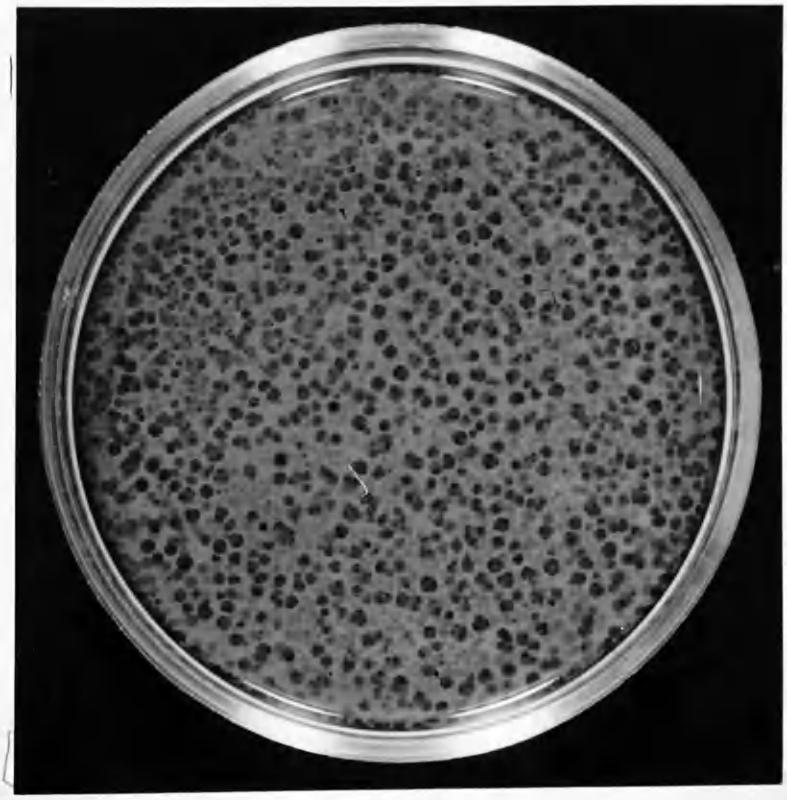


Fig. XLI

demonstration of Muller's phenomenon lay between 1.0 ml. and 2.0 ml. of serum. In the plate with 3.0 ml. of serum, although plaques are present there are fewer plaques and a smaller amount of clearing of the medium than there was with the 2.0 ml. of serum. This finding suggests that an inhibitor of some kind may be exerting its effect. So also with the plates containing 4.0 ml. and 5.0 ml. of serum in which no activity of any kind was observed, it would appear that the absence of plaque-formation is associated with these greater amounts of serum and might well be due to the presence in them of an inhibitor. Two of the plates in this series, the one with 0.1 ml., the other with 3.0 ml. of serum were photographed and are reproduced as Fig. XL and Fig. XLI.

(iii) Comparison of the activity of three pooled sera.

In another experiment presented in App. 314-316 three different pooled sera, "PI", "P2" and "P3" were titrated with the same sample of a staphylococcal factor preparation. This titration was carried out as a "checkerboard" titration so that amounts of 0.5 ml., 1.0 ml., 2.0 ml., 3.0 ml. and 4.0 ml. of each serum were tested with 1.0 ml., 2.0 ml., 3.0 ml. and 4.0 ml. of the staphylococcal

factor respectively. Different results were obtained with these three different pooled sera. From these results it was seen that as a general rule plaque-formation or complete lysis of the medium would occur with larger amounts of serum if a larger amount of staphylococcal factor was used. Thus with Serum P2, after incubation of the plates at 37°C. for 24 hours, 1.0 ml. of staphylococcal factor with 1.0 ml. of the serum resulted in plaques with 90% lysis of the medium, and with 3.0 ml. of serum neither plaques nor lysis. With 4.0 ml. of the staphylococcal factor, however, the plate with 3.0 ml. of serum showed complete lysis of the medium. Not all sera, however, will be activated by a larger amount of staphylococcal factor. In the plate containing 4.0 ml. of Serum P3, the presence of even 4.0 ml. of staphylococcal factor was unable to bring about either plaque-formation or lysis in the medium.

(iv) Checkerboard titrations of eight sera.

A similar checkerboard titration was carried out with eight different specimens of serum and the results are presented in App. 317-319. Amounts of these eight sera, 0.5 ml., 1.0 ml., 2.0 ml.,

3.0 ml. and 4.0 ml. were incorporated in milk-agar plates with 1.0 ml., 2.0 ml., 3.0 ml. and 4.0 ml. respectively of staphylococcal factor. The plates were incubated at 37°C. for 24 hours when readings were taken. Marked differences in the overall titration results were obtained with these different sera, e.g. 4.0 ml. of serum "B335" brought about complete clearing of a plate containing 2.0 ml. of the staphylococcal factor, whereas 4.0 ml. of serum "A333" was completely inactive with all amounts of staphylococcal factor.

(v) Titrations of sera from cases of disease.

Specimens of serum were obtained from eleven hospital patients with different diseases and they were titrated with 1.5 ml. amounts of a staphylococcal factor preparation in milk-agar plates. The results of these titrations are given in App. 320-321. Again differences were found in the ability of different sera to produce Muller's phenomenon and under the conditions of the test they gave different end-points. Thus serum from one case of thrombophlebitis produced plaques with 0.5 ml. but not with 1.0 ml.; serum from a patient who had a ureteric transplantation performed

produced complete lysis of the plate containing 3.0 ml. of serum. No more serum was available from this last patient so that an extension of the titration of this serum was not possible. With a method of titration such as has been described in which the only variable is the serum, in different amounts, information is obtained about the ability of the serum to produce Muller's phenomenon or its extension, viz. complete lysis of the medium. It would appear that there is an upper limit, (which varies from one serum to another) to the amount of serum which can be used to produce this phenomenon and this suggests that an inhibitor of some kind is present in the larger amounts of serum. In the results obtained from titrations of the few sera it has been possible to test it can be seen that these differences between one serum and another may be marked. At the present time, in the absence of knowledge of the normal range of this activity of serum it is not possible to say if these differences in the titrations of serum have any significance, e.g. clinically.

One disadvantage of this method of titration of sera lay in the large amounts of it required. Some other methods of titration will be described later in which smaller amounts of serum were used. (Part VII, I (b)).

(vi) Titration of plasma.

In a previous experiment reported in this thesis plasma from one individual was found to be as satisfactory for the demonstration of Muller's phenomenon as serum derived from clotted blood of the same individual. In an experiment presented in App. 322-323 a titration of plasma was made in milk-agar plates using growing staphylococci. Two sets of milk-agar plates with amounts of plasma from 0.5 ml. to 6.0 ml. were prepared. One set of plates was stab-inoculated from 6 hour old "Lab-Lemco" broth cultures of five strains of coagulase-positive staphylococci and incubated at 37°C. for 24 hours before being left at room temperature for a further 24 hours. All five strains of staphylococci developed plaques around their colonies in all the plates with plasma from 0.5 ml. to 4.0 ml. (i.e. 27%). Two strains also produced plaques in the plate with 5.0 ml. of plasma and two strains in the plate with 6.0 ml. of plasma. Thus it is possible to titrate a specimen of plasma. This titration, however, differs from that of the serum reported in App. 310-311, in that all the strains of staphylococci do not give the same end-point.

The second set of plasma-milk-agar plates prepared in this experiment and left uninoculated were incubated with the

test set of plates. The reason for this provision was the necessity of having a control with which to compare the inoculated set. In the preparation of these plasma-milk-agar plates it was noted that in the plates with the larger amounts of plasma, the milk in the plate lost much of its opacity, and it appeared that the medium had been partially cleared.

(vii) Titrations of placental serum.

A titration of two specimens of placental serum which became available was carried out with staphylococcal factor and the results obtained are presented in App. 324-325. Amounts of serum from 0.5 ml. to 3.0 ml. were incorporated in milk-agar plates, each of which contained 2.0 ml. of staphylococcal factor and the plates were incubated at 37°C. for 24 hours. Complete clearing of the media in all the plates prepared with these two sera was observed after 24 hours' incubation at 37°C. Plaques and 40% lysis of the plates with 1.0 ml. and 2.0 ml. of both the specimens of serum was observed after only 5 hours' incubation.

The end-point of the titration of these sera was not reached due to the fact that sufficient serum was not available.

This method of titration is extravagant of serum and other methods of titration were investigated which would permit a fuller titration using less serum. These other methods of titration are now presented.

(b) OTHER METHODS OF TITRATION.

(i) The effect of spreading serum on milk-agar containing staphylococcal factor; the effect of spreading staphylococcal factor on serum-milk-agar.

The effect of spreading a known amount of serum over the surface of a milk-agar plate containing a known amount of a staphylococcal factor preparation was investigated and the result obtained in one experiment is given in App. 326-327. The opportunity was taken also to observe the effect of spreading staphylococcal factor over the surface of a serum-milk-agar plate.

Two milk-agar plates each containing 2.0 ml. of staphylococcal factor and two serum-milk-agar plates each containing 0.5 ml. of serum were prepared. One plate of milk-agar had 0.5 ml. of serum spread over its surface by means of a sterile glass-spreader; the other plate of milk-agar had 0.2 ml. of serum spread over its surface in a similar manner. Over the surface of one serum-milk-agar plate was spread 1.0 ml. of staphylococcal factor and over the surface of the other was spread 0.2 ml. of staphylococcal factor. The plates were incubated at 37°C. for 24 hours when readings were made. Over the whole of the two milk-agar plates containing

staphylococcal factor many faint plaques were seen; over the whole of one of the serum-milk-agar plates, viz. the one over which 1.0 ml. of staphylococcal factor was spread, many faint plaques were seen. No change, however, was observed in the other serum-milk-agar plate, over which 0.2 ml. of staphylococcal factor was spread.

A more elaborate experiment on the same lines was carried with dilutions of serum and of staphylococcal factor. Drops of serum were placed on the surface of a milk-agar plate (containing staphylococcal factor) and drops of staphylococcal factor on a serum-milk-agar plate. One of a number of experiments carried out in this fashion is presented in App. 328-329. Two milk-agar plates were prepared, one containing 2.0 ml., the other 4.0 ml. of staphylococcal factor. Two serum-milk-agar plates were also prepared, one with 0.5 ml. of serum, the other with 1.0 ml. of serum. On the surface of each of the two milk-agar plates one drop of serum (undiluted) was placed. Drops of serum diluted 1/2, 1/4, 1/8, 1/16 and 1/32 were placed on the surface of these two milk-agar plates, i.e. in all, 6 drops of serum and its different dilutions on each milk-agar plate. Drops of staphylococcal factor - undiluted, and diluted 1/2, 1/4, 1/8, 1/16 and 1/32 were placed on

the surface of both serum-milk-agar plates. A dropping-pipette was used to deliver the drops, and it was calibrated to give 30 drops per ml. The plates were incubated at 37°C. for 24 hours and then left at room temperature for a further 48 hours. Readings were taken after 24 hours at 37°C. and after a further 24 and 48 hours at room temperature.

Plaques were produced in both the milk-agar plates with staphylococcal factor over the drops of serum undiluted, and diluted 1/2 and 1/4 after 24 hours at 37°C.; after a further 24 hours at room temperature there were in addition plaques over the 1/4 dilutions of serum while after 48 hours at room temperature, plaques and an area of lysis developed over all the drops of serum spotted on these two plates, i.e. over the undiluted serum and the dilutions from 1/2 to 1/32. Neither plaques nor lysis developed over the drops of staphylococcal factor spotted on the serum-milk-agar plates.

It did seem possible therefore from the results of these experiments to devise a method of titrating sera by spotting drops of dilution on milk-agar plates which contained staphylococcal factor. One difficulty, however, had to be overcome, viz. the fact that the drops tended to run over the surface of the milk-agar and unless care was taken these drops would coalesce. Moreover, all these drops did not cover the same area, but this difficulty

was overcome, partially at least, by the introduction of discs of milk-agar on which the drops of serum were placed.

(ii) The use of the "disc method" of titration.

A number of experiments were carried out using discs of milk-agar containing staphylococcal factor on which dilutions of serum were placed as drops. One such experiment is presented in App. 330-331, and in this experiment two sets of milk-agar plates were prepared, one with staphylococcal factor (a), the other with staphylococcal factor (b). Each set consisted of four plates with 0.5 ml., 1.0 ml., 2.0 ml. and 4.0 ml. of the staphylococcal factor. A milk-agar control plate without staphylococcal factor was also prepared. After drying these plates had agar discs 16 mm. in diameter cut out. Dilutions of serum made in 0.1 ml. volumes were prepared and 0.1 ml. of each dilution of serum was placed on the milk-agar discs. This amount of serum was imbibed by the agar disc. The Petri dishes containing the discs were incubated at 37°C. for 24 hours when the discs were transferred to the surface of a sheet of black plastic to facilitate the reading of results which was carried out immediately.

The results of this titration of serum differed in the two sets of milk-agar discs prepared with the different staphylococcal factor preparations. They were not quite in keeping, however, with results given by full-plate titrations of the serum carried out with staphylococcal factor preparations. This method did have the advantage that only a small amount of serum was required for such a titration, but it had the disadvantage of being time-consuming and difficult to carry out and moreover unless the surface of the milk-agar was horizontal the serum dilution tended to run to one side of the disc and exert its influence unevenly. It did not appear to be a method worth pursuing.

(iii) The applicability of the "disc method" for staphylococcal factor titrations.

The disc method of titration was used in several experiments to ascertain the activity of staphylococcal factor preparations and one of these experiments is presented in App. 332-333. In this experiment a comparison of the disc method with a full plate titration was made. Staphylococcal factor in amounts from 0.1 to 3.5 ml. were incorporated in a series of serum-milk-agar plates each of which contained 0.44 ml. of serum. Two other sets

of plates, one containing 0.875 ml. of serum per plate, the other set 3.5 ml. per plate were prepared with the same range of staphylococcal factor. These plates represented full plate titrations of the staphylococcal factor with three different amounts of serum. A series of serum-milk-agar plates containing serum in amounts 0.11, 0.22, 0.44, 0.875, 1.75 and 3.5 ml. were prepared without any staphylococcal factor. These plates were dried at 37°C. for 30 minutes; 16 mm. discs were cut out of these plates with a sterile brass cutter. Dilutions of staphylococcal factor in 1 ml. amounts from 0.1 ml. to 0.0015 ml. were placed on the discs of serum-milk-agar containing the different amounts of serum. These discs were incubated in Petri dishes at 37°C. for 24 hours when readings were taken. It was found that the titration of Staphylococcal factor obtained by the plate method did not entirely agree with the titration results obtained by the disc method.

It was considered that the disc method of titration was not altogether satisfactory for estimating the activity of staphylococcal factor and its use in this connection was also discontinued.

(iv) Titration of serum by the test-tube method.

Another method of titrating serum which could be carried out with small volumes of serum was instituted. This method involved the preparation of doubling dilutions of serum in 0.4 ml. amounts from 1/2 to 1/256 in 2 x 3/8" tubes. These serum dilutions were added to a series of test-tubes, which were held in a water-bath at 50°C. and which contained 1.0 ml. amounts of a melted milk-agar-staphylococcal factor mixture. The contents of each tube were thoroughly mixed, the agar was allowed to set (hastened by placing the rack of tubes in the ice-chest for a short time), and the tubes incubated at 37°C. for 48 hours. A control tube containing 0.4 ml. of distilled water (in place of a serum dilution) was mixed with 1.0 ml. of the milk-agar and incubated at 37°C. with the rest of the tubes. All the tubes were closed with rubber bungs to prevent evaporation and drying of the medium. The readings were taken after 24 hours at 37°C. In one experiment, 14 different sera were titrated and the results are presented in App. 334-335. Muller's phenomenon was seen usually in the end tubes of the titrations, i.e. with the greatest and the smallest amounts of serum. Between these amounts there was complete clearing of the milk-agar medium with the majority of the sera titrated. As had been found with titrations of other

sera by other methods there were marked differences in the activity of the sera to produce Muller's phenomenon (plaque-formation) or its extension complete clearing of the medium. This appeared to be the most satisfactory method of titrating specimens of serum evolved so far.

II. THE EFFECT OF HEATING SERUM.

(a) MILK IN SUBSTRATE.

(i) Human Serum.

(ii) Horse Serum.

(iii) Protective Effect of "Lab-Lemco" and of Staphylococcal Factor during heating.

(iv) Effect of Merthiolate.

(b) HEATED HAEMOGLOBIN IN SUBSTRATE.

II. THE EFFECT OF HEATING SERUM.

(a) MILK IN SUBSTRATE.

(i) Human Serum.

The thermolability of the serum factor necessary for the production of Muller's phenomenon by staphylococci has already been demonstrated in experiments detailed in App. 43-47 and App. 278-279. A further experiment in which the thermolability of the serum factor was confirmed is presented in App. 336-337.

Aliquots of human serum were heated at 55°C., at 60°C. and at 65°C. Half the sample heated at 55°C. was subjected to this temperature for 15 minutes, the other half for 30 minutes. Similar periods of heating were employed for the samples heated at 60°C. and 65°C. The serum pre-heated in this way was incorporated in milk-agar plates in amounts from 0.5 ml. to 6.0 ml. Each serum-milk-agar plate thus prepared contained 2.0 ml. of a staphylococcal factor preparation. A control set of serum-milk-agar plates, each containing 2.0 ml. of the same staphylococcal factor as the test-plates, was prepared with amounts of unheated serum from 0.5 ml. to 6.0 ml. All

the plates were incubated at 37°C. for 48 hours after which readings were made.

The unheated serum-milk-agar plates showed activity only in the plates with 0.5 ml. and 1.0 ml. of serum. The plate with 0.5 ml. was completely cleared, while that with 1.0 ml. showed numerous plaques. No change was detected in any of the plates with amounts of unheated serum from 2.0 ml. to 6.0 ml. This was an "inhibitory" serum. Although Muller's phenomenon was produced in the plates with 0.5 ml. and 1.0 ml. of serum heated at 55°C. for 15 minutes there was a reduction in the number of plaques produced when compared with the corresponding control plates. A similar finding was made in the plates with 0.5 ml. and 1.0 ml. of serum heated at 55°C. for 30 minutes. No change was observed in the plates containing amounts of serum from 2.0 ml. to 6.0 ml., heated at 55°C. for either 15 or for 30 minutes. The samples of serum heated at 60°C. and 65°C., irrespective of the time of heating and of the amounts subsequently used, were completely inactive, no demonstration of Muller's phenomenon being obtained in any of the serum-milk-agar plates prepared with them.

Thus it was confirmed that the serum factor necessary for the production of Muller's phenomenon is thermolabile, being partially inactivated by heating at 55°C. for 15-30 minutes and completely inactivated at 60°C. for 15 minutes.

(ii) Horse Serum.

In another experiment presented in App. 338-339 the thermolability of the serum factor in horse serum necessary for the phenomenon was investigated. A set of serum-milk-agar plates was prepared with amounts of horse serum from 0.1 ml. to 4.0 ml., each plate containing 1.0 ml. of staphylococcal factor. Similar sets of serum-milk-agar plates were prepared with the same horse-serum, portions of which had been preheated at the following temperatures and times (1) 56°C. for 5 minutes, (2) 56°C. for 15 minutes, (3) 56°C. for 30 minutes, (4) 65°C. for 5 minutes, (5) 65°C. for 15 minutes and (6) 65°C. for 30 minutes. Milk-agar control plates without added horse serum were also prepared. All the plates were incubated at 37°C. for 24 hours when readings were made.

Muller's phenomenon did not develop, of course, in the milk-agar control plates without added serum. There was complete clearing (lysis) of the medium in the plates with 0.1 ml., 0.25 ml., 0.5 ml. and 1.0 ml. of unheated horse serum. Plaques were present in the plates with 2.0 ml. and 3.0 ml. of unheated serum although the plate with 4.0 ml. showed no change in the medium. In the set of plates containing serum preheated at 56°C. for five

minutes there was complete clearing of the medium in the plates with 0.5 ml. and 1.0 ml. amounts of serum, partial clearing with 0.25 ml. but no alteration, neither plaques nor clearing, was seen in the rest of the plates in this set, i.e. those with 0.1 ml., 3.0 ml. or 4.0 ml. of serum. The remaining five sets of plates prepared with horse serum heated at 56°C. for 15 minutes, at 56°C. for 30 minutes, at 65°C. for 5 minutes, at 65°C. for 15 minutes and 65°C. for 30 minutes respectively showed no change in the medium. Thus it would appear that the serum factor necessary for Muller's phenomenon in horse serum is thermolabile and, at least so far as the specimen used in this experiment is concerned, more easily inactivated by heat than the usual specimens of human serum tested.

(iii) Protective effect of "Lab-Lemco" and of staphylococcal factor during heating.

The fact that the serum factor could be protected from inactivation by heat through the presence of "Lab-Lemco" at the time of heating was shown in an experiment presented in App. 340-341. Aliquots of human serum were heated at 65°C. for the following periods of time:- (a) 5 minutes, (b) 10 minutes, (c) 20 minutes, and (d) 30 minutes. These samples of heated serum were incorporated in

amounts of 0.5 ml. and 1.0 ml. in milk-agar plates each of which contained 1.0 ml. of 15% "Lab-Lemco". These plates acted as the heated-serum control plates, the serum having been preheated by itself. Two serum-milk-agar plates containing respectively 0.5 ml. and 1.0 ml. of unheated serum, acted as the serum(unheated)-milk-agar control plates. A milk-agar control plate, without added serum, was also prepared. The series of temperatures in the presence of "Lab-Lemco" were prepared as follows:- A set of four test-tubes were prepared each containing 0.5 ml. of serum, 1.0 ml. of 15% "Lab-Lemco" and 7.0 ml. of distilled water. A second set of four test-tubes was also prepared with 1.0 ml. of serum, 1.0 ml. of 15% "Lab-Lemco" and 6.5 ml. of distilled water. A pair of test-tubes, one from each set, were heated at 65°C. for 5 minutes, a second pair at 65°C. for 10 minutes, a third pair at 65°C. for 15 minutes and the fourth pair at 65°C. for 30 minutes. The contents of each tube, i.e. the heated "Lab-Lemco"-serum mixture, were added to tubes of melted agar and milk (4.5 ml. of 4.5% agar and 2.0 ml. of milk), each sufficient to make one plate. All the plates were stab-inoculated from 6 hour old "Lab-Lemco" broth cultures of five strains of coagulase-positive staphylococci and incubated at 37°C. for 24 hours, after which they were left at room temperature for a further 24 hours. Readings were taken after 24 hours at 37°C. and after 24

hours at room temperature.

The milk-agar control plate without serum, as was to be expected, did not show Muller's phenomenon. Around four of the five strains of staphylococci growing on this plate a zone of the indefinite lysis, customarily found around certain strains of coagulase-positive staphylococci growing on milk-agar was present. The serum-milk-agar control plates which contained 0.5 ml. and 1.0 ml. of unheated serum, both gave a good demonstration of Muller's phenomenon around all five strains of staphylococci. The plates with the two amounts of serum, i.e. 0.5 ml. and 1.0 ml., heated at 65°C. for 5, 10, 20 or 30 minutes did not show Muller's phenomenon around the colonies of staphylococci. Around the colonies of four of the five strains, however, on all these eight plates, a zone of indefinite lysis was present; no change was detected around the colonies of the fifth strain. The serum-milk-agar plates prepared with the samples of serum heated at 65°C. for the varied periods of time with the "Lab-Lemco" solution showed Muller's phenomenon with all five strains of staphylococci. There was also a zone of definite lysis with a cleanly cut border around the colonies of the five strains of staphylococci. Thus it was shown that "Lab-Lemco" protects the serum factor necessary for Muller's phenomenon from the destructive effect of heating at 65°C. for up to 30 minutes, although possibly the protection was not complete.

In another experiment presented in App. 342-343, it was found that serum heated either with staphylococcal factor (prepared in a "Lab-Lemco"-peptone-sodium chloride medium or with meat broth) retained its ability to produce Muller's phenomenon when incorporated in a milk-agar plate or in a milk-agar plate with staphylococcal factor, respectively.

Eight tubes, each containing 1.0 ml. of serum and 4.0 ml. of staphylococcal factor were prepared. Four of the tubes were heated at 56°C. - one for 5 minutes, one for 10 minutes, one for 20 minutes and one for 30 minutes; the other four tubes were heated at 65°C., one tube for each of these same periods of time. These heated serum-staphylococcal factor mixtures were mixed with melted milk-agar and poured as plates. As controls tubes of 1.0 ml. of serum and 4.0 ml. of staphylococcal factor were heated separately at 56°C. and at 65°C. for the same periods of time as used above. The tubes of milk were then mixed with the corresponding tubes of staphylococcal factor, added to tubes of melted milk-agar and then poured as plates. In addition sets of serum-milk-agar plates with staphylococcal factor were prepared with serum heated (for the same temperatures and for the same periods of time as used above) in the presence of 1.0 ml., 2.0 ml., 3.0 ml. and 4.0 ml. of meat broth. All the plates prepared were incubated at 37°C. for 24 hours, when readings were made.

In all the plates prepared with serum heated at 56°C. with staphylococcal factor or with the different amounts of meat broth, complete clearing of the medium was observed, fusion of plaques seen at an earlier period having occurred. The control plates prepared from the serum and staphylococcal factor heated separately at 56°C., also showed complete clearing of the medium. The control plates prepared with the serum and staphylococcal factor heated separately at 65°C., however, showed no change. The test plates prepared with the serum and staphylococcal factor heated together at 65°C. did show activity. When the heating was only 5 minutes, complete clearing of the medium resulted but when the period of heating was 10, 20 or 30 minutes, numerous plaques only were observed in the medium. In the plates prepared with the serum heated with 3.0 ml. or 4.0 ml. of meat broth at 65°C., complete clearing of the medium occurred. The plates prepared with serum heated with 1.0 ml. or 2.0 ml. of meat broth showed only a few plaques when the period of heating had been 5 minutes or 5 minutes and 10 minutes respectively, longer periods of heating having completely inactivated the specimens of serum.

Thus it was ascertained that the serum factor necessary for the demonstration of Muller's phenomenon could be protected from inactivation by heat with staphylococcal factor or

with meat broth. With regard to meat broth this protective action is dependent on the amount of it present and may well be linked with the content of protein.

(iv) Effect of Merthiolate.

In view of the action of "Merthiolate", on preserving the serum factor necessary for the clotting of fibrinogen by staphylocoagulase, from destruction by heat (Menzies, 1952), it was thought worthwhile to ascertain if "Merthiolate" had a similar action on the serum factor necessary for Muller's phenomenon. An experiment in which this was investigated is presented in App. 344-345. It was found that 0.1% "Merthiolate" was unable to prevent the inactivation of the serum factor necessary for Muller's phenomenon in 0.5 ml. or 1.0 ml. amounts of serum heated at 56°C., at 60°C. or at 70°C. for 15 minutes. It may be that higher or lower concentrations might be effective but this aspect was not investigated further.

(b) HEATED HAEMOGLOBIN IN SUBSTRATE.

The thermolability of the serum factor necessary for Muller's phenomenon was also demonstrated in heated haemoglobin-agar on which strains of coagulase-positive staphylococci were grown. One such experiment is presented in App. 346-347.

In this experiment washed human red cells were used in place of filtered haemoglobin solution. Three tubes each containing mixtures of washed human red cells, "Lab-Lemco" solution, agar and distilled water were heated at 75°C. for 10 minutes and placed in a water-bath at 50°C. To the first tube 0.5 ml. of fresh serum was added, to the second 1.0 ml. and to the third 2.0 ml. The contents of each tube were thoroughly mixed and poured as plates. Five similar sets of plates were prepared but they differed only in the fact that heated serum replaced fresh serum. For these five sets of plates, aliquots of serum were heated at 56°C., 60°C., 65°C., 70°C. and 75°C. for 10 minutes. A heated haemoglobin-agar control plate without serum was also prepared. All the plates were stab-inoculated from 6 hour old "Lab-Lemco" broth cultures of 6 strains of coagulase-positive staphylococci and incubated at 37°C. for 24 hours. Readings were made after 24 hours at 37°C.

The heated haemoglobin-agar plate without serum did not, of course, show Muller's phenomenon although around five of the six strains of staphylococci there was a zone of lysis, of the indefinite type lacking a cleanly cut edge. Muller's phenomenon (plaque-formation) was observed around the growths of all six strains of staphylococci on the three serum-heated haemoglobin-agar plates with 0.5 ml., 1.0 ml. and 2.0 ml. of unheated serum respectively. There were no zones of lysis around the staphylococcal colonies on these plates containing unheated serum. Muller's phenomenon (plaque-formation) was seen also in the three plates containing serum heated at 56°C. for 10 minutes but the zones of lysis observed in the heated haemoglobin plate without serum were also absent from these plates. With serum heated at 60°C. for 10 minutes incorporated in heated haemoglobin-agar, it was found that whereas only one of the six strains showed plaque-formation in the plate containing 0.5 ml. of serum, three of the six strains showed it in the plate with 1.0 ml. and five of the six strains in the plate with 2.0 ml. In the absence of plaques around these strains no other change was observed in the surrounding medium. In the three plates with 0.5 ml., 1.0 and 2.0 ml. of serum heated at 65°C. for 10 minutes, no change in the medium around the staphylococcal growths was seen. Although plaque-formation was again absent from the plates with the different

amounts of serum heated at 70°C. for 10 minutes, or at 75°C. for 10 minutes, zones of lysis were observed around the colonies of five of the six staphylococcal strains. The type of lysis and the strains around which it was present were the same as in the heated haemoglobin-agar plate without serum. It would appear that this type of lysis which is not accompanied by plaque-production is inhibited by fresh serum and by serum heated up to a temperature of 65°C. for 10 minutes. Serum heated at 70°C. or 75°C. for a period of 10 minutes, no longer inhibits it. The plaque-formation which was seen in certain of the plates in this experiment, was unaccompanied by lysis after 24 hours' incubation. Subsequent lysis did develop in some of these plates after a further period of incubation and it was clearly the result of fusion of the plaques.

III. THE SUITABILITY OF SERUM FROM OTHER ANIMALS.

(i) SHEEP SERUM.

(ii) GUINEA-PIG SERUM.

(iii) DOG PLASMA.

(iv) RABBIT SERUM.

(v) HORSE SERUM.

III. THE SUITABILITY OF SERUM FROM OTHER ANIMALS.

The use of whole blood or serum from a variety of animal species in investigations on Muller's phenomenon has been reported by a number of workers. In addition to human blood, rabbit is the only one which has been found suitable for the demonstration of the phenomenon. Attempts to demonstrate plaque-formation on blood-agar prepared with the blood of horse, ox, sheep, hen, goose or guinea-pig have proved unsuccessful although a medium containing washed human red cells with the serum of sheep, guinea-pig or rabbit has been found suitable. The production of Muller's phenomenon in serum-milk-agar media prepared with sera from a number of different animals has been investigated in several experiments, a few of which are presented here.

(i) SHEEP SERUM.

In an experiment performed in the initial stages of this investigation on Muller's phenomenon and presented in App. 10-12 sheep serum was shown to be a satisfactory substitute for human serum. Twelve strains of coagulase-positive staphylococci all produced the phenomenon in serum-milk-agar prepared with sheep serum. It was also found possible to demonstrate plaque-production in sheep serum-milk-agar in which was incorporated staphylococcal factor. One such experiment is presented in App. 348-349.

A set of five serum-milk-agar plates with amounts of sheep serum from 0.5 ml. to 4.0 ml. were prepared, each plate containing 1.0 ml. of staphylococcal factor. A milk-agar control plate without added serum but containing 1.0 ml. of staphylococcal factor was also prepared. All the plates were incubated at 37°C. for 24 hours, when readings were made. The milk-agar control plate showed no change in the medium, neither plaque-formation nor any clearing. The plates with 3.0 ml. and 4.0 ml. of sheep serum gave a good demonstration of Muller's phenomenon while those with 0.5 ml., 1.0 ml. and 2.0 ml. showed complete clearing of the medium due to fusion of the plaques of clearing which had been present at an earlier stage. In another experiment with sheep serum-milk-agar,

FIG. XLII - Production of Muller's phenomenon in a serum-milk-agar plate containing 3.0 ml. guinea-pig serum and 1.0 ml. of staphylococcal factor. (Incubated at 37°C. for 4 hours.)

FIG. XLIII - Production of Muller's phenomenon in a serum-milk-agar plate containing 1.0 ml. horse serum and 1.0 ml. staphylococcal factor. (Incubated at 37°C. for 24 hours.)

Fig. XLII

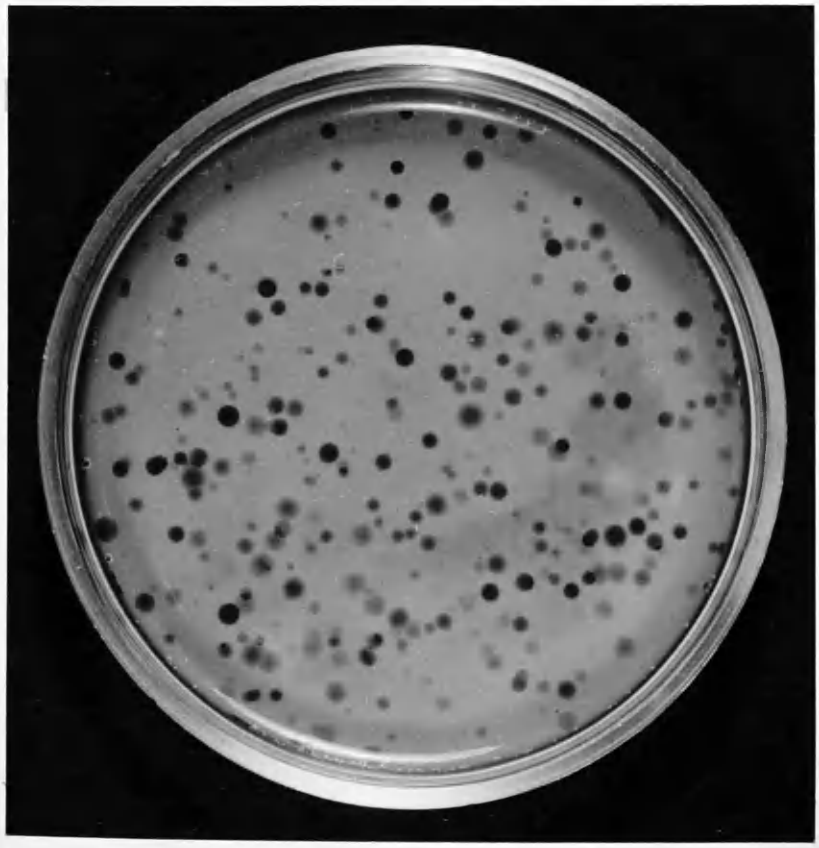


Fig. XLIII

FIG. XLIV - Production of Muller's phenomenon in a serum-milk-agar plate containing 3.0 ml. of sheep serum and 1.0 ml. of staphylococcal factor. (Incubated at 37°C. for 18 hours.)

FIG. XLV - Production of Muller's phenomenon in a serum-milk-agar plate containing 0.5 ml. of dog plasma and 0.25 ml. of staphylococcal factor. (Incubated at 37°C. for 24 hours.)

Fig. XLIV

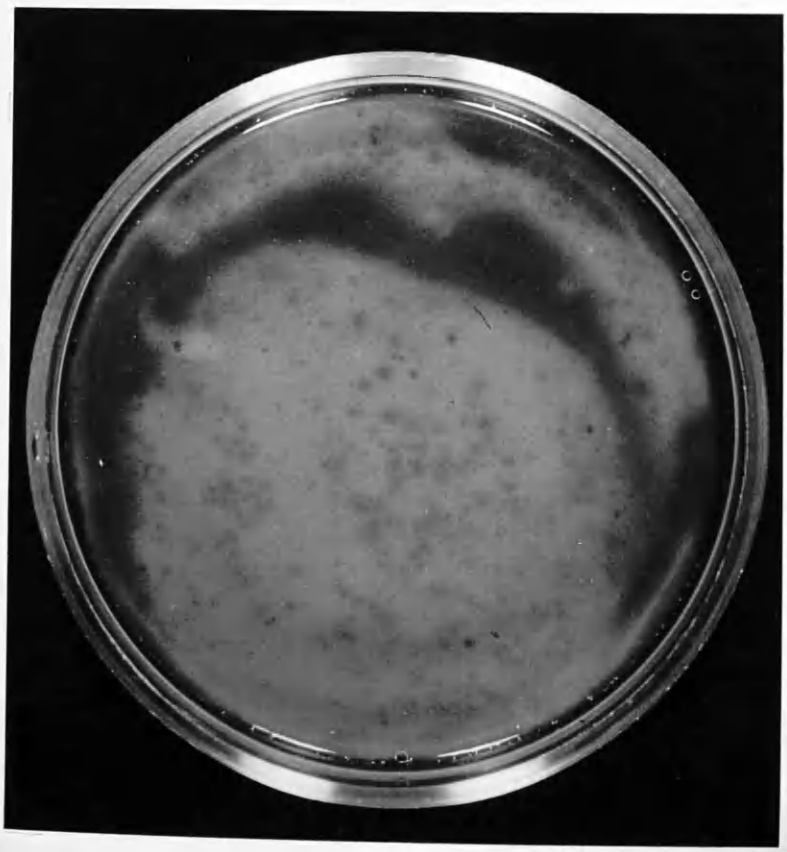


Fig. XLV

plaque-production was observed in the plate with 1.0 ml. of serum after 18 hours' incubation at 37°C. This plate was photographed and is reproduced as Fig. XLIV.

(ii) GUINEA-PIG SERUM.

Although guinea-pig blood-agar was found unsuitable for the production of Muller's phenomenon (Muller, 1929a, Packalén, 1938) a number of workers showed that guinea-pig serum added to washed human red cells in an agar medium permitted plaque-production by strains of staphylococci. (Burnet, 1928, Packalén, 1938, Rhodes, 1938). A satisfactory demonstration of Muller's phenomenon in serum-haemoglobin-agar containing guinea-pig serum was reported by Elek (1953). A serum-milk-agar medium containing guinea-pig serum was used in a number of experiments with different strains of staphylococci or with cell-free staphylococcal factor. One of these experiments in which staphylococcal factor was incorporated in guinea-pig serum-milk-agar is presented in App. 350-351.

Four serum-milk-agar plates with 0.5 ml., 1.0 ml., 2.0 ml. and 3.0 ml. of guinea-pig serum respectively were prepared, each plate containing 1.0 ml. of staphylococcal factor. A milk-agar control plate without added serum was also prepared with 1.0

ml. of staphylococcal factor. All the plates were incubated at 37°C. for 24 hours after which readings were made. No plaque-formation was seen in the milk-agar control plate without serum. All four plates with guinea-pig serum were completely cleared when examined after 24 hours' incubation, the clearing having resulted from fusion of the numerous plaques which were present at an earlier stage. The plate containing 3.0 ml. of serum, for example, showed numerous plaques after 4 hours' incubation. This plate was photographed at this time and is reproduced as Fig. XLII.

(iii) DOG PLASMA.

No report has been found of the use of dog blood in the preparation of blood-agar for the demonstration of Muller's phenomenon. On two occasions it was possible to obtain specimens of heparinized dog blood, and the separated plasma was used to prepare dog-plasma-milk-agar to which was added staphylococcal factor. One experiment with this medium is given in App. 352-353.

A set of seven plasma-milk-agar plates were prepared with amounts of dog-plasma from 0.1 ml. to 2.0 ml., each plate containing 1.0 ml. of staphylococcal factor. A milk-agar control plate without plasma was also prepared. All the plates were

incubated at 37°C. for 24 hours, when readings were made. No plaque-formation nor any other change was observed in the milk-agar control plate. Complete clearing of all the test plates was found after the period of 24 hours' incubation. Examination of these plates on several occasions during this time had failed to reveal any plaques of clearing but a gradual clearing of the medium was noted, a clearing which progressed to completion by the end of 24 hours' incubation period.

Further plates of plasma-milk-agar were prepared with amounts of the same sample of dog plasma from 0.1 ml. to 0.5 ml. but to each plate only 0.25 ml. of staphylococcal factor was added in place of the 1.0 ml. amounts used in the previous set of plates. The plates were incubated at 37°C. for 24 hours when readings were made. The plates with 0.1 ml. and 0.2 ml. of dog-plasma showed some clearing of the medium but no plaque-formation. The plates with 0.3 ml., 0.4 ml. and 0.5 ml., however, not only showed some clearing of the medium but also plaques. These plaques, which were indistinct and lacked the cleanly-cut regular edge normally found in plaques developing in serum-milk-agar prepared with human serum, tended to coalesce and bring about the clearing of the medium. The plate containing 0.5 ml. of plasma with 0.25 ml. of staphylococcal factor was photographed and is reproduced as Fig. XLV.

(iv) RABBIT SERUM.

Serum-milk-agar containing rabbit serum was used in a number of experiments and was found to be a satisfactory medium for the demonstration of Muller's phenomenon with strains of staphylococci or with staphylococcal factor. One experiment, in which a comparison of the abilities of strains of staphylococci to produce Muller's phenomenon in two sets of serum-milk-agar plates, one prepared with rabbit serum, the other with human serum, is presented in App. 354-356.

A set of serum-milk-agar plates with amounts of rabbit serum from 0.5 ml. to 3.0 ml. and a second set with amounts of human serum from 0.5 ml. to 4.0 ml. were prepared. A milk-agar control plate without added serum was also prepared. All the plates were stab-inoculated from 6 hour old "Lab-Lemco" broth cultures of five strains of coagulase-positive staphylococci and incubated at 37°C. for 24 hours before being placed at room temperature. Readings were made after 24 hours at 37°C. and after a further 24 hours at room temperature.

Muller's phenomenon did not develop, of course, in the milk-agar control plate without serum. All five serum-milk-agar plates prepared with human serum showed plaque-formation around all

five strains of staphylococci, after 24 hours' incubation at 37°C. Examination of these plates after a further 24 hours at room temperature, however, revealed only zones of lysis around the staphylococcal growths in the plates with 3.0 ml. and 4.0 ml. of serum, although plaques were still present in the other plates in this set. Plaque-formation was observed in all the plates prepared with rabbit serum when examined after 24 hours at 37°C. and also after a further 24 hours at room temperature. One strain of staphylococcus, however, failed to produce plaques on all the plates containing rabbit serum, although it did so in the plates with human serum.

(v) HORSE SERUM.

Serum-milk-agar prepared with horse serum was found suitable for the demonstration of Muller's phenomenon with staphylococci or cell-free staphylococcal factor in a number of experiments. On one experiment presented in App. 357-358 a set of serum-milk-agar plates with amounts of horse serum from 0.1 ml. to 4.0 ml. were prepared, each plate containing 0.5 ml. of staphylococcal factor. A milk-agar control plate without serum

was also prepared. All the plates were incubated at 37°C. for 24 hours and then left at room temperature for a further 24 hours. Readings were made after 24 hours at 37°C. and again after a further 24 hours at room temperature.

As was to be expected plaques did not develop in the milk-agar control plate without added serum. Muller's phenomenon (plaque-formation) was present in the plates containing 0.1 ml., 3.0 ml. and 4.0 ml. of horse-serum, after 24 hours' incubation at 37°C. The plates with 0.25 ml., 0.5 ml., 1.0 ml. and 2.0 ml., however, were completely cleared and showed no trace of plaques. The plaques which had been present in the plate with 0.1 ml. of horse serum were no longer visible after the plate had been left at room temperature for a further 24 hours, the medium having been completely cleared. A plate with 1.0 ml. of horse serum - a different specimen from that used in the above mentioned experiment was photographed after 24 hours at 37°C. and is reproduced as Fig. XLIII.

The production of Muller's phenomenon by staphylococci and by cell-free staphylococcal factor in serum-haemoglobin-agar and serum-heated haemoglobin-agar containing horse-serum was investigated in another experiment which is presented in App. 359-362.

A set of serum-haemoglobin-agar plates were prepared

with amounts of horse-serum from 0.1 ml. to 4.0 ml., each plate containing 1.0 ml. of cell-free staphylococcal factor. A second set of plates similar with regard to the agar, horse-serum, staphylococcal factor and distilled water content, but differing from the first set in that the haemoglobin had been preheated at 75°C. for 10 minutes was also prepared. In addition a haemoglobin-agar plate and a heated-haemoglobin-agar plate, both without serum, i.e. control plates, were made. All the plates were incubated at 37°C. for 48 hours and readings were made after 24 and 48 hours' incubation at this temperature. After 24 hours' incubation at 37°C. plaque-formation was observed in the serum-haemoglobin-agar plates containing 2.0 ml., 3.0 ml. and 4.0 ml. of horse-serum, partial clearing of the medium in the plate with 0.5 ml., complete clearing in the plate with 1.0 ml., and no alteration with 0.1 ml. and 0.25 ml. In the plates with serum-heated haemoglobin-agar plaques were seen in the plates with 0.5 ml., 1.0 ml., 2.0 ml., 3.0 ml. and 4.0 ml. of horse-serum but not in the plates with 0.1 ml. and 0.25 ml., in which the medium was unchanged. After a further 24 hours' incubation at 37°C. there was a slight increase in the number of plaques observed in these two sets of plates. The two control plates, without added serum, did not show plaque-formation.

In this same experiment, moreover, the production of Muller's phenomenon by fiwe strains of coagulase-positive staphylococci on haemoglobin and heated haemoglobin medium containing horse serum was also investigated. The horse serum-haemoglobin-agar and the horse serum heated-haemoglobin-agar plates on which these staphylococci were grown, were prepared in the same way as the corresponding plates of these two media used with staphylococcal factor although their constituents varied in that each plate contained 1.0 ml. of 15% "Lab-Lemco" in place of the 1.0 ml. of staphylococcal factor. Two control plates without added serum, i.e. a haemoglobin-agar and a heated-haemoglobin-agar plate were also prepared. All the plates were stab-inoculated from 6 hour old "Lab-Lemco" broth cultures of five strains of coagulase-positive staphylococci and incubated at 37°C. for 48 hours. Readings were made after 48 hours' incubation at 37°C.

Around all five strains of staphylococci on all the serum-haemoglobin-agar plates a zone of definite lysis was seen but only around one staphylococcal colony in the plate with 4.0 ml. of horse serum were plaques seen in addition to the lysis. The haemoglobin-agar control plate, without added serum, showed a zone of lysis around the growth of all five strains, but it

appeared to be of a different type from that seen in the plates with horse-serum being less definite and with edges less well defined. In the plates with serum-heated haemoglobin-agar, however, all five strains of staphylococci showed Muller's phenomenon. Plaque-formation was observed with all five strains in the plates with 1.0 ml., 2.0 ml., 3.0 ml. and 4.0 ml. of horse serum, with three strains in the plate with 0.5 ml. and with one strain in the plate with 0.25 ml. The plate which contained 0.1 ml. of horse serum showed a zone of lysis around the staphylococcal growths but no plaque-formation. In the heated-haemoglobin-agar control plate without added serum some lysis of the medium was observed around four of the strains of staphylococci. It was of the indefinite type however, and different from that observed in the plates with horse-serum.

IV. THE ACTIVE FACTOR IN SERUM.

PRECIPITATION OF THE ACTIVE FACTOR.

(i) By Ammonium sulphate.

(ii) By Sodium sulphate.

(iii) By Sodium chloride.

IV. THE ACTIVE FACTOR IN SERUM.

PRECIPITATION OF THE ACTIVE FACTOR.

Little or nothing is known of the active factor in serum which is essential for the production of Muller's phenomenon. The limited information of this necessary constituent for plaque-formation given by earlier workers has already been reviewed in Part II of this thesis. Of particular interest is the work reported by Muller (1927b) who extended the work of his colleague Dormal on the precipitation of the active principle from human serum. This Muller did by the use of ammonium or magnesium sulphate and he obtained the active factor in the redissolved precipitate. It did seem worthwhile to repeat this work on the precipitation of the active factor and this was carried out on a number of occasions with ammonium sulphate and on a few occasions with sodium sulphate. One experiment in which ammonium sulphate was used in presented in App. 364-366 and another experiment in which sodium sulphate was used is presented in App. 367-368.

(i) BY AMMONIUM SULPHATE.

To 20 ml. amounts of a specimen of serum in large test-tubes amounts of ammonium sulphate (previously weighed) were added to give final concentrations (w/v) of 10% to 30%. These mixtures were left at room temperature for 2 hours. Precipitates developed in all the test-tubes of serum except that containing 10% of ammonium sulphate. These precipitates were resuspended in 20 ml. distilled water after centrifugation of the serum. These resuspended deposits, the supernatant fluids from these deposits and the 10% ammonium sulphate-serum mixture were placed in 'Visking' dialysis tubing and dialysed against running water until free of ammonium sulphate. These supernatants and resuspended deposits were incorporated in milk-agar plates containing staphylococcal factor, in amounts from 0.1 ml. up to 5.0 ml., allowance being made for any increase in volume which had occurred during dialysis. In addition, a control plate containing fresh serum in amounts of 0.1 ml. to 5.0 ml. were incorporated in milk-agar plates containing staphylococcal factor. Acriflavine (1/5000) was added in 1.0 ml. amounts to each plate to ensure that no growth of organisms took place during the period of 24 hours' incubation of the plates.

Readings were taken after 24 hours' incubation at 37°C. and after a further 48 hours at room temperature. The plates with 0.25 ml. and 2.0 ml. of fresh serum produced plaques, and those with 1.0 and 2.0 ml. of fresh serum complete clearing of the medium. None of the deposits nor supernatants showed activity in less than 0.25 ml. amounts but it was found that the deposits from the 15%, the 17%, and the 19% ammonium sulphate-serum mixtures were active in 3.0 ml. amounts, and indeed that the deposit from the 17% ammonium sulphate-serum mixture was also active in a 4.0 ml. amount. This finding suggested that inhibitor had been removed in the process of precipitation of the active factor.

(ii) BY SODIUM SULPHATE.

In an experiment presented in App. 367-368 an attempt was made to precipitate the active factor from serum by sodium sulphate. 2.7 gm. of sodium sulphate were mixed with 20 ml. of serum, allowed to stand in the incubator for 3 hours and the precipitate which formed was collected after centrifugation. This precipitate was resuspended in 20 ml. of distilled water. The resuspended precipitate and the supernatant from the precipitate

were dialysed in 'Visking' tubing against distilled water overnight and tested in amounts of 0.1 to 5.0 ml. in milk-agar plates containing staphylococcal factor. A set of plates containing similar amounts of fresh serum were also prepared. These plates were incubated at 37°C. for 24 hours and then placed at room temperature for a further 48 hours. The fresh serum brought about complete lysis of the medium when used in amounts of from 0.1 to 1.0 ml. Plaques with 70% lysis and plaques with 20% lysis were produced by the 2.0 and 3.0 ml. amounts of this serum. The 13.5% sodium sulphate did not precipitate all the active factor from the serum; this is apparent since plaques of clearing appeared with amounts of the supernatant from 0.25 ml. to 2.0 ml. The precipitate (i.e. deposit) caused complete clearing of the medium when used in amounts from 0.25 ml. to 4.0 ml. Thus, it would appear that once again an inhibitor had been lost in the course of precipitation of the active factor. The fact that the precipitate is not active in 0.1 ml. amounts suggests that some of the active factor was also lost in the course of the precipitation.

(iii) BY SODIUM CHLORIDE.

Reference has been made on several occasions in this thesis to the inhibitory effect of sodium chloride on the production of Muller's phenomenon. It has been shown that this effect is not on the growing staphylococci nor on the production of the staphylococcal factor as it can occur in a serum-milk-agar plate containing preformed factor. It did seem possible, however, that this action of sodium chloride might be on the serum factor. In an experiment presented in App. 369-370 amounts of 15% sodium chloride were added to aliquots of serum to give a series of concentrations of sodium chloride in the sodium chloride-serum mixtures from 5% to 12%. Amounts of these different sodium chloride-serum mixtures were added to a set of milk-agar plates containing staphylococcal factor so that the final concentration of sodium chloride in this set of plates ranged from 0.25% to 2.0%, each plate containing 0.5 ml. of serum. A control plate containing 0.5 ml. of fresh serum was also prepared. A second set of sodium chloride-serum mixtures were prepared and dialysed in 'Visking' tubing against running water overnight to remove the sodium chloride. A control plate containing 0.5 ml. of fresh serum which had been dialysed was also prepared. These dialysed sodium chloride-serum mixtures were then incorporated in milk-agar plates in

amounts comparable with those used in undialysed mixtures referred to above. All the plates were incubated at 37°C. The control plates containing fresh serum dialysed, or undialysed, showed complete clearing of the medium. The sodium chloride serum-mixtures which were undialysed showed plaques with 80% lysis of medium with the mixture containing 0.25%, plaques with the mixture containing 0.5% sodium chloride, and again plaques which were faint, in the mixture containing 0.75% sodium chloride. The mixtures which contained 1% and 2% sodium chloride were without effect, i.e. neither plaques nor lysis developed in the plates in which they were incorporated. On the other hand the dialysed serum-sodium chloride mixtures produced complete lysis of the medium in the plates to which they were added. Thus it would appear that the action of the sodium chloride in preventing Muller's phenomenon is not the result of permanent or irreversible damage to the serum factor.

In another experiment presented in App. 371-372 amounts of sodium chloride 1.0 gm., 1.5 gm., 2.0 gm. and 2.5 gm. of sodium chloride were added to 10 ml. volumes of human serum to give concentrations of sodium chloride in serum of 10%, 15%, 20% and 25% respectively. These serum-sodium chloride mixtures were left at room temperature for one hour during which time precipitates appeared

in the 20% and 25% sodium chloride containing mixtures. These two latter mixtures were centrifuged, the precipitates collected and resuspended in 10 ml. amounts of distilled water. The serum-sodium chloride mixtures containing 10% and 15% of sodium chloride, the supernatants and resuspended precipitates from the 20% and 25% sodium chloride mixtures were dialysed overnight in running water to remove sodium chlcride. The activity of the sodium chloride treated samples of serum was tested by incorporating them in volumes of 0.1 to 3.0 ml. (allowance being made for any increase in volume which may have occurred during dialysis) in milk-agar plates containing staphylococcal factor. Fresh serum, i.e. serum untreated with sodium chloride was also incorporated in amounts of 0.1 ml. to 3.0 ml. in a series of milk-agar plates containing staphylococcal factor. "Merthiolate" was incorporated in all these plates in a final concentration of 1-25,000. Plates were incubated at 37°C. for 24 hours after which readings were made. The plates containing fresh serum produced Muller's phenomenon in amounts from 0.1 ml. to 1.0 ml., the plate containing 1 ml. showing in addition to plaques, 50% clearing of the medium. No change was detected in the plates with 2.0 ml. and 3.0 ml. of this serum. The samples of serum treated with 10% and 15% sodium chloride produced Muller's phenomenon in the plates with 0.1 ml. to 2.0 ml. amounts

of them, and the supernatant obtained from the 20% sodium chloride-serum mixture showed similar activity when used in the same amounts. The deposit from the 20% sodium chloride-serum mixture although active in amounts up to 3.0 ml. produced no change when used in 0.1 ml. and 0.25 ml. amounts. The deposit from the 25% sodium chloride-serum mixture, showed activity in all the plates prepared with amounts of it from 0.1 ml. to 3.0 ml. The active factor, however, had not been completely precipitated by the 25% sodium chloride since the supernatant from the mixture still showed some activity when tested in amounts of 0.25 ml., 0.5 ml. and 1.0 ml. Thus it would appear that an active factor can be precipitated from serum and that an inhibitor can be lost in the process of precipitation. This is evidenced by the fact that 3.0 ml. of the resuspended deposit obtained by treating serum with 25% sodium chloride produced Muller's phenomenon with complete clearing of a milk-agar plate containing staphylococcal factor whereas 1.0 ml. of the untreated serum produced only plaques with 50% clearing, and no change when incorporated in 2.0 ml. or 3.0 ml. amounts in similar milk-agar plates.

V. SUMMARY.

A limited investigation of the role of serum in the production of Muller's phenomenon was carried out.

A suitable method of titrating serum was devised using cell-free staphylococcal factor and a small volume of serum in tubes of milk-agar. Sera differ with regard to their ability to produce Muller's phenomenon.

The thermolability of serum was confirmed. If "Lab-Lemco" or staphylococcal factor was heated with serum, the active factor in serum was not destroyed. "Merthiolate", at least in the concentrations used, had no such protective action on serum.

Specimens of serum from sheep, guinea-pig, rabbit and horse have been found suitable for the demonstration of Muller's phenomenon. Two specimens of dog plasma were also active in this respect.

The active factor can be precipitated from serum by ammonium sulphate, sodium sulphate or sodium chloride.

P A R T VIII.

FURTHER INVESTIGATION OF CONDITIONS AFFECTING THE PRODUCTION OF PLAQUES.

PART VIII.

FURTHER INVESTIGATION OF CONDITIONS AFFECTING THE PRODUCTION OF PLAQUES.

I. EXPERIMENTS ON PLAQUE-PRODUCTION.

(a) Plaque-production with different sera.

(b) Plaque-production with different strains of staphylococci.

(c) Effect of charcoal on plaque-production.

(d) Plaque-production with varied amounts of serum.

II. SUMMARY.

I. EXPERIMENTS ON PLAQUE-PRODUCTION.

The size and morphology of the plaques of clearing which characterise Muller's phenomenon have been the subject of comment by a number of workers. Muller (1928) found that most blood-agar plates showed a mixture of large and small plaques, the latter usually the more distinct and clearly-cut. Specimens of blood from different individuals were associated with variations in the overall picture of plaques obtained. Packalén (1938) found that there were slight differences in the size, number and clarity of the plaques developing in blood-agar in addition to the rapidity of their appearance, with different strains of Staphylococcus aureus. He observed, as had Burnet (1928), that solid particles were often to be seen in the centre of plaques and suggested that such particles might act as foci for the adsorption of the haemophagic agent, although he was unable to confirm this by experiment. Elek (1953) also made observations on the size and morphology of plaques which developed in serum-haemoglobin-agar. Such a medium prepared with adult human serum gave a crop of medium-sized plaques, that prepared with human cord serum numerous small plaques while that with rabbit serum gave mixtures of large and

small plaques. He observed four main morphological plaque-patterns: giant plaques, very small plaques, a mixture of very small and medium-sized plaques and plaques surrounded by a halo. Giant plaques, he considered to be due to an aggregation of the proteoclast while the other three plaque-types appeared to be associated with the sample of serum employed as though in some way the morphology was characteristic of the serum.

In the course of the various studies which make up this thesis a number of observations on the size and morphology of plaques have been made and these have been included in the relevant parts. Thus, for example, the amount of sodium chloride, the agar concentration and the nature of the protein in the test medium were all found to influence the size and morphology of plaques. There are now presented four additional experiments which indicate other factors of importance with regard to the size and morphology of plaques.

(a) PLAQUE-PRODUCTION WITH DIFFERENT SERA.

A comparison of the size and morphology of plaques produced in serum-milk-agar prepared with different sera was made in a number of experiments, one of which is presented in App. 373-374. Two series of serum-milk-agar plates were prepared, one with amounts of 0.15 ml. to 1.0 ml. of serum "13", the other with similar amounts of serum "17". Two milk-agar control plates without added serum were also prepared. All the plates were stab-inoculated from 6 hour old "Lab-Lemco" broth cultures of seven strains of coagulase-positive staphylococci and incubated at 37°C. for 24 hours, when readings were made. No plaques were observed around the colonies of staphylococci in the control plates without serum but plaques sometimes associated with lysis were seen around all seven strains on all the plates with the different amounts of the two sera "13" and "17". The plaques which developed in the plates with 0.15 ml. and 0.25 ml. of serum "13" were very fine, with an average diameter of 0.5 mm. In the rest of the plates containing serum "13", small, medium and large plaques developed with diameters in the range 1.0 mm. to 2.5 mm. Both large and small plaques developed in all the plates containing serum "17". Although the overwhelming majority of the plaques in the plates

with 0.15 ml. and 0.25 ml. of serum "17" were from 0.5 mm. to 1.0 mm. in diameter, a few plaques with a diameter greater than 1.0 mm. did develop. Plaques, mainly in the range 1.0 mm. to 2.0 mm. were produced in the remaining plates prepared with serum "17" although even in the plate with 1.0 ml. of this serum, a few plaques less than 1.0 mm. in diameter appeared. Thus, with these two different sera there were differences in the plaque-patterns produced. It should be noted also that in one series of serum-milk-agar plates prepared with varied amounts of the same serum, there were marked differences in the size of plaques produced. Within limits it appeared that with increasing amounts of serum there was an increase in the size of plaques which developed although the plates with the larger plaques nearly always showed a number of smaller plaques as well.

(b) PLAQUE-PRODUCTION WITH DIFFERENT STRAINS OF STAPHYLOCOCCI.

In many experiments in which stab-inoculations of different strains of coagulase-positive staphylococci were made on plates of serum-milk-agar, it was noted that the resultant plaque-patterns around the growth of staphylococci were dissimilar. They differed in the number and size of the plaques and in the breadth of the plaque zone. Moreover, the area of lysis associated with Muller's phenomenon and, believed to result from fusion of plaques, also differed in size around the growth of different strains.

The plaque-pattern produced by different strains of coagulase-positive staphylococci was the object of special study in a number of experiments, one of which is presented in App. 375-376. Two serum-milk-agar plates, one containing 0.15 ml., the other 1.0 ml. of serum were prepared in duplicate and stab-inoculated four times with each strain from 6 hour old "Lab-Lemco" broth cultures of them, and incubated at 37°C. for 24 hours before being placed at room temperature for 3 days.

After 24 hours' incubation at 37°C. the four stab-inoculations of one strain on one plate gave plaque-patterns which

showed only minimal differences although showing marked differences from the patterns produced around the four stab-inoculations of the other strain in the same plate. There were also only minimal differences in plaque-patterns occurring in one plate and in its duplicate. There were differences, however, in the plaque-patterns produced by the two strains in the plates with the different amounts of serum.

After 3 days at room temperature, around each stab-inoculation of one strain on the plates with 0.15 ml. of serum there was a marked zone of lysis with few medium-sized plaques, while around each stab-inoculation of the other strain, although there was only a small zone of lysis there was a broad zone of plaques, both large and small. The plates containing 1.0 ml. of serum, however, showed similar plaque-patterns around the stab-inoculations of both strains, viz. a zone of lysis with a rough edge and a few plaques of medium-size lying in the otherwise unaltered medium beyond the zone of lysis.

(c) EFFECT OF CHARCOAL ON PLAQUE-PRODUCTION.

Packalén (1938) was unable to confirm by experiment, his own suggestion that minute solid particles, e.g. of fibrin or charred cotton, often seen in the centre of plaques might act as foci for the adsorption of the haemophagic agent. He found that the addition of minute sterile particles of glass or coal added to blood-agar medium just before it was poured, in no way influenced the production of plaques. It thus appeared unlikely that plaque-production depended on such foci for their development. A number of experiments, however, were carried out with staphylococcal cultures and with cell-free staphylococcal factor with a view to confirming or otherwise Packalén's finding. One of these experiments in which sterile animal charcoal was added to serum-milk-agar is given in App. 377-378.

A series of three serum-milk-agar plates containing 0.25 ml., 0.5 ml. and 1.0 ml. of serum respectively was prepared. A duplicate set was also prepared but to each of the three tubes of serum-milk-agar in this set 0.5 ml. of a 0.1% suspension of sterile animal charcoal was added just before they were poured as plates. Both sets of plates were stab-inoculated from 6 hour old

"Lab-Lemco" broth cultures of seven strains of coagulase-positive staphylococci and incubated at 37°C. for 24 hours when readings were made. There was no essential difference found in the production of Muller's phenomenon on these two sets of serum-milk-agar plates and it was concluded that animal charcoal in no way influenced plaque-production. The same conclusion was reached as a result of a number of other experiments carried out with cell-free staphylococcal factor.

(d) PLAQUE-PRODUCTION WITH VARIED AMOUNTS OF SERUM.

The importance of the concentration of serum on the demonstration of plaque-formation has already been stressed in the preliminary experiments reported earlier in this thesis. It was the use of serum in concentrations below 50% the concentration used by Fisk and Mordvin (1943) which first revealed that Muller's phenomenon (plaque-formation) could be produced in serum-milk-agar. A concentration of 40% or more of serum in such a medium permitted a clearly-cut zone of caseinolysis around growths of coagulase-positive staphylococci to develop but Muller's phenomenon was never observed. Since these serum-milk-agar plates were incubated at 37°C. overnight for a period of 18-24 hours before examination, it seemed possible that plaque-formation might have occurred in the plates with serum in concentrations of 40% or more at an earlier period of incubation and been swallowed up by the development of the zone of lysis. It was known that lesser concentrations of serum which did permit plaque-formation often showed fusion of plaques after 2-3 days' incubation resulting in a zone of lysis, sometimes indented with plaques (a "rough edge") but on other occasions showing a zone of lysis with a regular clearly cut edge without plaques and which differed in no way from the picture obtained in

serum-milk-agar with 40% or more of serum. It did appear worthwhile to make hour-to-hour observations on the development of the zones of lysis and the plaque-formation which occurred in a series of serum-milk-agar plates containing serum in varied concentrations up to almost 50%. One such experiment is presented in App. 379-381.

A set of serum-milk-agar plates with amounts of serum from 0.5 ml. to 7.0 ml. (i.e. 3.3% to 47%) was prepared and each plate stab-inoculated from a 16 hour old "Lab-Lemco" broth culture of Staphylococcus aureus, Strain A. Five similar sets of plates were prepared and inoculated with the same strain of staphylococcus, the inoculations into the plates of these sets being made 2 hours, 4 hours, 6 hours, 7 hours and 11 hours respectively after the inoculation of the first set. All the plates were incubated at 37°C. In this way it was possible by examining these six sets of plates to have (almost) hourly observations on the development of plaques and/or lysis around the staphylococcal growths, continuously over a period of 24 hours or longer. In practice the readings at 8, 10, 14 and 15 hours were omitted during the first 24 hours' incubation.

It was possible to identify plaques in the serum-milk-agar plate containing 1.0 ml. (6.6%) serum after 5 hours'

incubation at 37°C. and, after 9 hours' incubation, all the plates showed plaques with the exception of those containing 6.0 ml. and 7.0 ml. (i.e. 40% and 46.6%) of serum respectively. At least one hour before plaques were observed around the staphylococcal growths in the different plates some lysis was apparent. The plates with 6.0 and 7.0 ml. of serum, however, also showed some lysis as early as the fourth hour of incubation but instead of the development of plaques around the staphylococcal growths these areas of lysis increased in size until, on examination after 18-24 hours' incubation typical zones of lysis, definite and with a regular edge were to be seen. Thus it was found that plaques did not develop at any stage around staphylococci growing in serum-milk-agar plates containing a concentration of 40% or more of serum.

It was of interest also to note that in the serum-milk-agar plates with 5.0 ml. of serum (33.3%) isolated plaques were not seen during any period of the observations. The plaques which did appear were all associated with the advancing edge of the zone of lysis. Indeed, after 27 hours' incubation no further plaques were seen, the edge of the zone of lysis having become regular and was no longer indented with plaques, i.e. a "rough edge".

II. SUMMARY.

Plaque-patterns, (resulting from the number, size and morphology of plaques) which develop on serum-milk-agar plates around growing staphylococci were found to be influenced by the particular serum used, the amount of serum in the plate and by the strain of staphylococcus used as the test organism. The addition of sterile animal charcoal to a plate of serum-milk-agar was not found to influence the plaque-pattern obtained.

PART IX.

DISCUSSION.

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The origin of this investigation of "one of the minor mysteries of bacteriology" was the chance discovery that a reduction in the serum concentration in the serum-milk-agar devised by Fisk and Mordvin (1943) from 50% to 30% or less, resulted in the development of plaques of clearing around colonies of staphylococci, reminiscent of Muller's phenomenon. No claim is made that the results of this investigation of the phenomenon, on serum-milk-agar - a new substrate - have solved this mystery, but it is believed that certain aspects of it, formerly obscure, have been rendered more clear. In the discussion which follows - the results of this investigation of Muller's phenomenon are related to previous work on this subject and to other known proteolytic activities of staphylococci.

PART IX.

DISCUSSION.

I. A COMMON CHARACTER OF COAGULASE-POSITIVE STAPHYLOCOCCI.

II. LYSIS IN SERUM-MILK-AGAR AND MILK-AGAR MEDIA.

III. VARIATION IN STAPHYLOCOCCAL CULTURES.

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VI. OPTIMAL CONDITIONS FOR PLAQUE-PRODUCTION BY STAPHYLOCOCCAL FACTOR.

VII. THE EFFECT OF SODIUM CHLORIDE IN MULLER'S PHENOMENON.

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IX. THE SERUM FACTOR IN MULLER'S PHENOMENON.

X. FUTURE RESEARCH IN MULLER'S PHENOMENON AND FIBRINOLYSIS.

I. A COMMON CHARACTER OF COAGULASE-POSITIVE STAPHYLOCOCCI.

The view of Burnet (1928), Packalén (1938) and Elek (1953) that '*aureus*' strains or coagulase-positive strains of staphylococci were commonly able to produce Muller's phenomenon was based on the examination of relatively few strains of staphylococci. The author has confirmed their view by examining 515 strains of coagulase-positive staphylococci, and 41 strains of coagulase-negative staphylococci. 96% of the coagulase-positive strains produced Muller's phenomenon while none of the coagulase-negative strains did so. It was considered unnecessary to conduct a special investigation of organisms other than staphylococci on account of the fact that 130 strains of micrococci, diphtheroids, coliforms, yeasts, spore-bearing bacilli and others grew from the bacteriological specimens inoculated on serum-milk-agar plates used in the investigation reported above and that not one of them was found to produce Muller's phenomenon. Thus for the first time it is possible to state clearly and unequivocably based on the examination of a sufficiently large number of strains of organisms that Muller's phenomenon is produced by coagulase-positive staphylococci and by

these organisms only.

This close correlation of Muller-positivity with coagulase-positivity is of interest and importance in view of the acceptance by almost all bacteriologists, of the coagulase-test for the recognition of pathogenic staphylococci. Thus it would follow that if an organism is capable of producing Muller's phenomenon, it can be regarded straightaway as a coagulase-positive strain and as a pathogen.

Many investigations were carried out on the 21 undoubted coagulase-positive strains of staphylococci which failed to produce the phenomenon on serum-milk-agar and other suitable media for the purpose. As far as could be ascertained these strains lacked the ability to produce plaques. It is pointless to speculate as to why a percentage of strains of any organism lack one or other character. It may be that these 21 strains lost their ability to produce Muller's phenomenon by a mutation which affected their capacity to make available the factor essential for plaque-production. Such a mutation need only affect a proportion of the cells in a culture; the effects of environment may bring about a selective action which would result in the survival of the mutant cells at the expense of the normal cells which produced the factor.

II. LYSIS IN SERUM-MILK-AGAR AND MILK-AGAR MEDIA.

The term "lysis" has been frequently used in this thesis and care has been taken to distinguish between the definite lysis with a cleanly cut regular edge associated with plaques and the indefinite lysis with an ill-defined edge which merges almost imperceptibly into the surrounding unaltered medium. The former type of lysis occurs in serum-milk-agar plates around colonies of staphylococci which produce Muller's phenomenon, the latter type of lysis around colonies of certain coagulase-positive staphylococci on milk-agar plates without serum.

This indefinite lysis in the milk-agar medium without serum with some staphylococci is sometimes very slight being restricted to a lessening of the opacity of the milk around the staphylococcal colony. With other strains of staphylococci this partial lysis with ill-defined edge constitutes a slightly broader zone but its breadth does not exceed 0.5 cm. All the strains of staphylococci used in the investigations were always tested on milk-agar as well as serum-milk-agar. The results of these examinations are given in App. 384-395 and App. 397-401. This

action of staphylococci on milk-agar is believed to be due to the action of a staphylococcal protease. It is not produced by all strains of coagulase-positive staphylococci and it is not related to the ability of staphylococci to liquefy gelatin. It does not appear to be related to the lysis which occurs around certain strains on haemoglobin-agar media prepared without added serum.

The definite lysis which is observed in serum-milk-agar around colonies of coagulase-positive staphylococci is entirely or almost entirely the result of the breakdown of milk caused by a proteolytic enzyme of serum, activated by the staphylococcal factor. The breadth of the zone of definite lysis with a cleanly cut edge which occurs around staphylococcal colonies in a serum-milk-agar plate depends on the amount of the serum present in the plate. In a plate with a large amount of serum, e.g. 7.0 ml., a zone of this definite lysis develops around the growing colony after a few hours and it increases in size with the passage of time but with such an amount of serum no plaques will ever be seen; with a slightly smaller amount of serum, e.g. 5.0 ml., a zone of lysis with a "rough edge" develops, this "rough edge" being due to the development of plaques at the regular edge of the zone of advancing lysis. Such a "rough edge" may be swallowed up in the advancing lysis, the edge

of which will again become regular. Smaller amounts of serum in serum-milk-agar plates will show plaques in zones of different breadth around the staphylococcal colonies but almost always there is a zone of lysis separating the zone of plaques from the colony and this lysis is of the definite type (e.g. App. 379-381).

What part does the indefinite lysis found in milk-agar plates play in the development of lysis of the definite type in serum-milk-agar plates? It is the author's belief very little. It would appear that the amount of indefinite lysis of the protease type which can develop on a serum-milk-agar plate is no greater than that which can be produced by a strain of staphylococcus in a milk-agar plate without serum. This was seen in the experiment described in App. 86-87 in which the addition of a preparation of soya-bean-trypsin-inhibitor to a milk-agar plate and to two serum-milk-agar plates containing respectively 0.5 ml. and 1.0 ml. of serum permitted the development of the indefinite ('protease') lysis around four strains of staphylococci on all three plates. Around these four strains there was neither the development of plaques nor of the definite type of lysis which occurred in the control serum-milk-agar plates. The fifth strain of staphylococcus which failed to give the indefinite (protease) lysis on the control milk-agar

plate without the soya-bean-trypsin-inhibitor gave a good demonstration of Müller's phenomenon, i.e. plaques, and with it a zone of definite lysis in the two serum-milk-agar plates without soya-bean-trypsin-inhibitor. No change in the medium was to be seen in any of the three plates with soya-bean-trypsin-inhibitor around the colony of this strain. Moreover, it was observed on a number of occasions with strains of staphylococci which did not produce the indefinite ('protease') lysis on milk-agar that around their colonies on serum-milk-agar plates containing only small amounts of serum, e.g. 0.15 ml. to 0.2 ml., they gave rise to plaques of clearing only. These same strains produced lysis of the definite type on serum-milk-agar plates with greater amounts of serum, e.g. 1.0 ml. to 2.0 ml. Thus it would appear that the staphylococcal protease which produces this indefinite lysis in milk-agar plates plays little part in the broader zones of definite lysis which is associated with plaque-production in serum-milk-agar plates.

III. VARIATION IN STAPHYLOCOCCAL CULTURES.

In view of the large number of examples of variation in strains of staphylococci reported by different workers over many years (most recently reviewed by Elek (1959)) and from the present worker's own experience with fast-clotting variants of coagulase-positive staphylococci isolated from synthetic media, the finding of variants in the investigations on coagulase-positive staphylococci reported in this thesis came as no surprise. With regard to the variants of staphylococci encountered it was, of course, those which failed to produce Muller's phenomenon, although coming from a culture which also included cells which did produce the phenomenon, that attracted particular attention. Since these 'Muller-negative' variants appeared to breed true, an explanation was of course afforded of the 21 strains of coagulase-positive staphylococci which failed to produce the phenomenon, viz. that these strains may well have been mutants which occurred in cultures of 'Muller-positive' cells and owing to some environmental change these 'Muller-negative' cells survived at the expense of the normal 'Muller-positive' cells. There is reason to believe that many if not all staphylococcal strains

are indeed mixed populations and that under particular circumstances certain cells thrive and outgrow others. Staphylococcus aureus, Strain A is a good example of a strain containing 'Muller-positive' and 'Muller-negative' cells, the latter only making their appearance after fairly long cultivation in the one medium.

Other examples of variation which have occurred are perhaps more complex, involving sometimes poor producers of Muller's phenomenon which did not breed true, giving rise to some colonies which did not produce the phenomenon at all, and to other colonies which produced it only poorly.

IV. THE OPTIMAL CONDITIONS FOR PLAQUE-PRODUCTION BY COAGULASE-POSITIVE STAPHYLOCOCCI.

The circumstances in which strains of staphylococci will produce Muller's phenomenon were investigated using several different indicator-protein containing media. It was found possible to ascertain the optimal conditions for the production of plaques on serum-milk-agar, serum-haemoglobin-agar and serum-heated-haemoglobin-agar. The same conditions were required for these three media but the conditions required for the production of the phenomenon in heated plasma-agar were somewhat different and they will be discussed later.

The essentials for plaque-production would appear to be quite clear, viz. a strain of staphylococcus which can produce the staphylococcal factor, an adequate source of nutriment for the organism, a suitable indicator protein on which to demonstrate plaques, fresh serum as a source of the serum factor and a gel of suitable strength. There are a number of other conditions, which although of less importance, can influence plaque-formation.

(a) Strain of Staphylococcus:- The investigations of the 515

strains of coagulase-positive staphylococci have already been dealt with in detail and little further comment is required here. It has been noted that some strains are much better producers of Muller's phenomenon than others and that different strains give different plaque-patterns although growing under the same conditions (App. 375-376). The most likely explanation of these observations is that varied amounts of the staphylococcal factor are elaborated by colonies of the different strains of staphylococci.

(b) Nutrition:- Experiments with different nutrients added to serum-milk-agar plates for the purpose of providing sufficient nutriment for adequate growth of staphylococci, so that they would be independent of the serum content of the medium, revealed that 1% "Lab-Lemco" (final concentration) was eminently satisfactory, although smaller amounts of meat broth could also be used. Peptone (Oxoid) was found to interfere with plaque-production as did sodium chloride, when added in concentrations of 0.5% or more. Plaques could be demonstrated, however, in the absence of the entity present in meat extract referred to by Burnet (1928) who stated that it was necessary for plaque-production.

(c) Indicator Protein:- The part played by the indicator or test protein in the demonstration of plaque-production is an important

one. The use of milk (either liquid or reconstituted skim milk powder) was found to be eminently satisfactory in these investigations and superior to human blood and to haemoglobin, used either as a crude or filtered solution. Two points would appear to seal the superiority of milk over blood cells or haemoglobin, viz. the greater ease with which milk appeared to be broken down and the more easily observed contrast of the lysed to the unaltered protein of milk. The usefulness of heated haemoglobin-agar with added fresh serum, i.e. a modified chocolate medium has been stressed on account of the fact that this is an excellent indicator protein for the demonstration of plaques, which can be easily seen against the unaltered brown colour of the medium. This protein preparation is not quite so easily broken down as milk but there is less tendency for the plaques to coalesce, at least when the smaller amounts of serum are incorporated in the medium, e.g. 0.5 ml. and 1.0 ml. amounts of serum.

One point of interest was observed with plates of heated haemoglobin which should be mentioned here. Plaques only without lysis were found around all five strains of staphylococci growing on two plates of serum-heated haemoglobin-agar containing 0.5 ml. and 1.0 ml. of serum respectively. Around each of four of the five strains of staphylococci growing on the control plate

without serum, an area of lysis was seen, presumably the action of a staphylococcal protease (App. 107-108). The action of this protease, however, is inhibited by the presence of fresh serum, since no such lysis was observed around the staphylococcal colonies in the plates with serum (0.5 ml. and 1.0 ml. amounts). Other experiments, not reported in this thesis, but carried out by the author have shown that serum heated at 70°C. for 10 minutes no longer inhibited this protease type of lysis of heated haemoglobin by staphylococci. With serum heated at this temperature, Muller's phenomenon of course did not develop, the serum factor necessary for its production having been destroyed at this temperature.

(d) Serum Factor:- A more detailed consideration of the serum factor in the production of Muller's phenomenon will be considered later. Fresh serum is essential and all of the many samples of human serum tested, pooled samples, and individual samples from those in health and disease, were able to produce Muller's phenomenon. There were differences, however, in the suitability of these varied samples of sera - differences which were apparent when cell-free staphylococcal factor was employed but not noticeable when growing staphylococci were used. Different samples of serum incorporated in serum-milk-agar plates gave rise to different

plaque-patterns with the same organisms growing on them (App. 373-374).

(e) The Gel:- Earlier workers have reported that the nature and strength of the gel on which Muller's phenomenon is to be demonstrated are of no importance. The present worker, however, although able to demonstrate the production of plates in different gels of varied strengths has shown that in a series of plates of different agar concentrations plaques of different sizes develop (App. 58-61). It is believed that the effect of increased agar concentration in a plate is to slow the diffusion of the staphylococcal factor from the colonies and the spread of the activated proteolytic enzyme through the substrate.

(f) Other Conditions:- The optimum temperature was found to be around 37°C., although plaque-production will occur over a wide range (20C.°) with growing staphylococci. With staphylococcal factor in serum-milk-agar plates plaques will develop at 4°C. The optimum pH reaction is around pH 7.0 to pH 7.2 but with serum-milk-agar at least the pH reaction is not critical for the development of plaques. Plaques will develop in serum-milk-agar and serum-

haemoglobin-agar with staphylococci growing under anaerobic conditions, but it would seem that aerobic conditions are optimal.

V. PRODUCTION OF THE STAPHYLOCOCCAL FACTOR.

It has been shown that the staphylococcal factor essential for the production of Muller's phenomenon can be obtained free from living staphylococcal cells. It has been concentrated, partially purified and stored without loss of activity, as a dry powder for at least six months. This finding is believed by the author to represent an important step forward to the final elucidation of the mystery of Muller's phenomenon. It is clear, however, that much remains to be done to produce a more highly purified preparation of the staphylococcal factor.

Most of the work presented in this thesis with the cell-free staphylococcal factor was carried out with the unconcentrated batches of it. Both the concentrated and the unconcentrated preparations were without apparent activity when tested on a protein-containing substrate, which did not contain serum. It would appear that these preparations did not contain a staphylococcal protease capable of breaking down milk or haemoglobin in the absence of serum. Tests carried out with many of the staphylococcal factor preparations revealed the presence in them of

staphylocoagulase.

Do the staphylococcal factor preparations contain fibrinolysin? The answer is that it does, if the presence of fibrinolysin is indicated by lysis of a heated-plasma agar medium containing plasma heated at 56°C. for 20 minutes to which the staphylococcal factor has been added. In one experiment, 1.0 ml. of a staphylococcal factor was placed within a polythene ring sitting on a heated plasma-agar plate prepared with plasma heated at 56°C. for 20 minutes. Complete clearing of the heated plasma-agar occurred within the ring, while the rest of the medium was unchanged. In a similar experiment in which plasma was heated at 65°C. for 30 minutes no such fibrinolytic zone developed, but when 0.5 ml. of fresh serum was added a zone of fibrinolysis developed.

With a staphylococcal factor preparation it was possible to show both Muller's phenomenon, i.e. plaque-formation, and fibrinolysis in heated-plasma-agar. It was not difficult to demonstrate the fibrinolysis in such media but it was only by paying attention to the amount of plasma in the plate, the temperature and time of heating, the amount of fresh serum added, that it was possible to demonstrate plaque-formation. The addition of sodium chloride was found to be useful in helping the formation of plaques. Such experiments have been described in

pages 361-363.

Thus it would appear that staphylococcal factor contains two factors, one that is necessary for the production of Muller's phenomenon and a second factor necessary for the production of fibrinolysis, i.e. staphylokinase. There is, of course, another possibility, viz. that the staphylococcal factor preparation contains only one factor which is responsible for both Muller's phenomenon and for fibrinolysis. The action of this staphylococcal factor is to activate the necessary serum factor and the resulting proteolytic enzyme is then able to attack a number of different proteins causing them to break down. Plaque-production, i.e. Muller's phenomenon, might be considered a special manifestation of this proteolytic action. This point can be discussed more profitably later.

Although it has been possible to provide staphylococcal factor preparations with a high degree of activity it should be possible to provide even more active preparations in the future. Importance is attached to the provision of a highly nutritious medium for the organism and to the selection of a highly active strain of staphylococcus.

One line of approach lies in the use of a synthetic medium to enable the investigator to ascertain the requirements of

of the staphylococcus for the production of the staphylococcal factor. Such an approach was successfully made to the production of staphylocoagulase by Lominski et al (1950).

VI. OPTIMAL CONDITIONS FOR PLAQUE-PRODUCTION BY STAPHYLOCOCCAL FACTOR.

Although plaque-production could be demonstrated with staphylococcal factor over a wide range of conditions, the optimal conditions lay in a fairly narrow range.

The importance of the test-protein employed has already been emphasised - milk and heated haemoglobin being superior to haemoglobin, which in turn was better than heated plasma. Not only is the nature of the test protein important but also the concentration of it incorporated in the medium. There was a wide range of concentrations of the test-protein in which the phenomenon could be demonstrated but a much narrower range in which there was an optimal demonstration of plaques.

Comment has already been passed on the difference in the breakdown of milk and haemoglobin. It is the author's experience that a serum-milk-agar medium is more easily lysed by the fusion of plaques than is a serum-haemoglobin-agar medium. It is of course difficult to equate amounts of these different proteins and it may be that much more is being asked of a given amount of staphylococcal factor to break down 1.5 ml. of haemoglobin

solution than 2.0 ml. of milk. The difference in the speed of breakdown of these two proteins by staphylococcal factor is magnified when the tests are carried out at 4°C., at which temperature plaque-formation can still be demonstrated with milk as the indicator protein but not with haemoglobin.

Comment has also been made on the range of agar concentrations which can be employed for the demonstration of the phenomenon and although previous workers have stated that the gel strength was unimportant it has been shown that it influences the appearance and the size of the plaques. The customary agar concentration of 1.3% was found to be eminently satisfactory for plaque-production and was considered the optimal concentration. The effect of the higher concentrations of agar in giving a network of fine plaques, is overcome by increasing the serum concentration in the medium. This action of serum may be due to its facilitating the diffusion of the enzyme through the medium or to greater activity of the proteolytic enzyme in the medium due to the greater amount of serum present.

The question of an optimal concentration of serum for the production of Muller's phenomenon has already been raised and discussed briefly on Page 373. It is a question which is difficult to answer in view of the fact that the plates which might

contain an optimal amount of serum show within a relatively short space of time clearing of the medium, whereas another plate, with less serum or more serum than the "optimal", although slower very often in showing plaques, manifests a good crop of plaques long after the medium containing the "optimal" amount has been lysed completely. Such a statement of an "optimal" amount of serum for the demonstration of plaques is applicable only to a given amount of staphylococcal factor. Use a greater or a lesser amount of serum or use a different amount of serum for the demonstration of plaque-production is altered as has been shown in checkerboard titrations of serum described previously (App. 317-319).

Since Muller's phenomenon (plaque-production) is apparently a manifestation of proteolysis and since that proteolysis will go on to completion, i.e. complete lysis of the medium, provided there is sufficient serum factor and staphylococcal factor in the medium, it would seem better to ascertain the optimal amount of serum for complete lysis of the medium than for a good demonstration of plaques. The former can be accurately measured, but not the latter. (It will be appreciated that the measurements of plaque-formation, e.g. "P. 50% L." given in the results of experiments as an indication of the activity in a particular plate are only crude measurements made by the eye.)

Thus it may well be that if measurements of this proteolytic activity are to be made they will have to be based on the complete breakdown of the test-protein, i.e. lysis, and not on a stage of proteolysis, i.e. plaques, which cannot be measured accurately.

VII. THE EFFECT OF SODIUM CHLORIDE IN MULLER'S PHENOMENON.

The effect of sodium chloride on the production of Muller's phenomenon in serum-milk-agar plates by developing staphylococci or with cell-free staphylococcal factor is of great interest. Plaque-formation was partially inhibited around colonies of staphylococci when sodium chloride was added to give a final concentration of 0.25% or 0.5%, the partial inhibition being manifest by a reduction in the number of plaques, those which did develop being abnormally large (App. 80-81). Complete inhibition of plaque-formation and of the definite type of lysis was observed with the addition of sodium chloride in final concentrations greater than 0.5%. The indefinite lysis associated with the staphylococcal protease, however, was not inhibited and developed on the serum-milk-agar plates. The indefinite lysis which appeared on these plates was identical with the indefinite lysis observed in milk-agar plates without serum. Thus it was observed that inhibition of plaque-formation and definite lysis in serum-milk-agar plates by varied concentrations of sodium chloride,

occurred while the staphylococcal protease induced lysis persisted - a picture similar to that produced by the addition of soya-bean-trypsin-inhibitor being provided.

Concentrations of 0.25% and 0.5% of sodium chloride, added to serum-milk-agar plates containing staphylococcal factor, caused a partial inhibition of the development of Muller's phenomenon or rather the extension of Muller's phenomenon, viz. the complete clearing of the serum-milk-agar, which a control plate of serum-milk-agar without added sodium chloride manifested. Serum-milk-agar plates with amounts of sodium chloride in excess of 0.5% concentration showed no sign of activity, i.e. complete inhibition of plaque-formation and lysis (App. 208-209). With the passage of time, however, plaques did develop. This inhibition was only temporary. It is believed that the action of sodium chloride is one of precipitating the serum factor and thus rendering it non-available for activation by the staphylococcal factor. Possibly an aggregation of the serum factor occurs. The fact that in time plaques can develop in a plate with an inhibitory concentration of sodium chloride suggests that the salt may be released from the aggregation of serum factor and that a new equilibrium of salt dispersion occurs. The serum factor is then able to play its normal part as a component of a proteolytic

serum enzyme.

This view is substantiated by the evidence available from the experiment reported (App. 371-372) in which sodium chloride was used to precipitate the active factor from serum and in which the recovery of the serum factor was effected unimpaired and indeed enhanced, after dialysis of the precipitate.

It must not be forgotten that a standard serum-milk-agar plate will contain a certain amount of sodium chloride and other salts in its constituent milk, serum and "Lab-Lemco" solution (although the latter, of course, contains no added sodium chloride). The addition of sodium chloride in a concentration of 0.25% to a plate of medium may raise the content of sodium chloride and other salts to such a level that precipitation of the serum factor occurs.

This action of sodium chloride will be mentioned later when its effect on the production of plaques in heated-plasma agar-media has been dealt with.

VIII. HEATED PLASMA-AGAR AS A TEST-PROTEIN FOR MULLER'S PHENOMENON AND FOR THE DEMONSTRATION OF FIBRINOLYSIS.

The suggestion has already been made on Page 455 that the staphylococcal factor necessary for the production of Muller's phenomenon may be the same as that required for the production of fibrinolysis. If this suggestion were correct it would seem that the staphylococcal factor referred to throughout this thesis as being necessary for plaque-production was in fact staphylokinase the name given to the staphylococcal contribution to the production of staphylococcal fibrinolysis. Such a suggestion will bear closer inspection and accordingly the available evidence will now be presented.

The 197 coagulase-positive staphylococci which produced Muller's phenomenon on serum-milk-agar plates were also found to be able to produce fibrinolysis when grown in heated plasma-agar plates (App. 435). This would indicate a close correlation of the ability to produce Muller's phenomenon with the ability to produce fibrinolysis. When the 5 strains of coagulase-positive staphylococci in this group which did not produce Muller's

phenomenon on serum-milk-agar, were tested on heated plasma-agar they were found to be unable to produce fibrinolysis. Perhaps of even greater interest were the three strains of coagulase-positive staphylococci which were Muller-positive and fibrinolysis-positive. Each of these three strains produced a variant which was recognised on account of the fact that it was unable to produce Muller's phenomenon. When these three Muller-negative variants were grown on heated plasma-agar they were found to be unable to produce fibrinolysis around their colonies (App. 423). There is no denying that there is a close correlation between the ability of a strain to produce Muller's phenomenon with its ability to produce fibrinolysis on heated plasma-agar.

Christie, Graydon and Woods (1945) reported that 37 strains of fibrinolytic staphylococci produced Muller's phenomenon on lysed human blood-agar while 42 non-fibrinolytic strains failed to do so. They were of the opinion that staphylococcal fibrinolysin was the agent responsible for Muller's phenomenon. Elek (1953) objected to the correlation of fibrinolysis-negativity of these coagulase-positive strains with their Muller negativity. "This correlation cannot, however, be taken as proof that fibrinolysin is the actual factor concerned in plaque-formation for fibrinolysin dissolves heat precipitated fibrinogen in the absence of fresh serum

while the Muller phenomenon requires the latter." Elek says in this same paper that staphylococcal filtrates rich in alpha haemolysin, lipase and fibrinolysin were inactive in producing Muller's phenomenon, although he does not mention the test protein used or the conditions in which the filtrates were tested.

Lack (1948) and Lack and Wailling (1954) have shown that on heated plasma-agar plates (prepared by heating at 56°C. for 20 minutes) the action of the staphylococcal fibrinolysin is inhibited by soya-bean-trypsin-inhibitor but that the action of the staphylococcal protease is not. Reference has already been made to the action of soya-bean-trypsin-inhibitor in inhibiting the production of plaques and of the definite lysis in serum-milk-agar while permitting the development of the indefinite lysis caused by the staphylococcal protease (App. 86-87) and it is considered that this is a significant observation showing another point of similarity of the proteolytic enzyme required for Muller's phenomenon with that of the staphylococcal fibrinolysin.

What is the record of development of Muller's phenomenon on heated plasma-agar? If Muller's phenomenon is brought about by the action of staphylococcal fibrinolysin surely heated-plasma-agar would be the best medium for the demonstration of Muller's phenomenon. Although Muller's phenomenon can be demonstrated on

heated plasma-agar it is more difficult to produce plaques on this test-protein than on serum-milk-agar for example.

Reference has already been made to the 197 coagulase-positive Muller-positive staphylococci which produced fibrinolysis on heated plasma-agar prepared by heating at 56°C. for 20 minutes. These plates contained 15% of plasma. In heated plasma-agar plates prepared by heating plasma at 65°C. for 30 minutes and to which 1.0 ml. of fresh serum was added before pouring 199 coagulase-positive, Muller-positive strains produced zones of fibrinolysis on these plates whose plasma content was 25%. Moreover 75 of them, i.e. 37.5% also produced plaques of clearing - Muller's phenomenon. These plaques of clearing were small and poor compared with those seen in other test-protein containing media. Why should only a little more than a third of the strains of staphylococci which produce a good zone of fibrinolysis in these heated-plasma plates give rise to Muller's phenomenon? It could be said that the conditions were not optimal for the majority of these strains to give rise to plaques. Such a reply is unhelpful unless some attempt is made to define the conditions necessary for plaque-production in heated plasma-agar. It should possibly be stated, first of all, that the author believes that the production of plaques in a heated-plasma medium around colonies of staphylococci

occurs only when conditions are not optimal for the usual development of fibrinolysis, but are not sufficiently adverse to bring about complete inhibition of fibrinolysis. A similar state of affairs in a heated plasma-agar plate containing staphylococcal factor will result in plaque-formation in place of the usual complete clearing of the medium. The usual zones of fibrinolysis around staphylococcal colonies in heated plasma-agar are comparable with the zones of lysis which occur around staphylococcal colonies in a serum-milk-agar plate containing more than 40% of human serum. In this latter type of plate by a reduction in the concentration of serum plaques will develop, i.e. conditions are no longer optimal for the full activity of the proteolytic enzyme. With test proteins other than heated plasma it is possible to produce fairly easily the conditions which are not the optimal and this range of sub-optimal conditions in which plaques will develop is fairly wide. Not so, however, is the case of heated plasma. There is only a narrow range of sub-optimal conditions in which it is possible to have plaque-formation.

The conditions which are necessary for plaque-production in heated plasma-agar with growing staphylococci are to be found in the results of the experiments given in App. 109-143k and discussed in Pages 206-220. The amount of plasma, the

temperature and time of heating, the addition of serum, the addition of sodium chloride and the nature of the basic agar were all found to be important for plaque-production in heated plasma media. These conditions fall into two groups (a) the amount of plasma, the temperature and time of heating, and the addition of serum, (b) the addition of sodium chloride and the nature of the basic agar medium. Group (a) conditions are concerned with the amount of test protein and more important the supply of the "plasma factor" which is activated by the staphylococcal factor. Group (b) conditions are concerned with the concentration of sodium chloride and other salts contained in the basic medium and which have been shown to exert some influence on plaque-production.

Elek (1953) was quoted above as saying that fibrinolysin dissolves heat-precipitated fibrinogen in the absence of fresh serum while the Muller phenomenon requires fresh serum. It is true that plaque-production occurs best with fresh serum but the thermolability of this serum factor is such that a temperature greater than 56°C. and a time longer than 30 minutes is required (usually) to destroy the factor completely. When, however, the heating of plasma is considered cognizance must be taken of the fact that the heating brings about precipitation of fibrinogen and that the precipitated fibrinogen may well protect the serum or

plasma factor necessary for fibrinolysis. It was certainly shown that the serum factor could be protected from heat-destruction by the presence of "Lab-Lemco" or staphylococcal factor during heating. If as Lack (1948) and Lack and Wailling (1954) suggest staphylokinase activates plasminogen then the protection of the plasma factor will be the protection of plasminogen. Lassen (1952) has indicated that to ensure the destruction of the plasminogen in a bovine fibrinogen plate a temperature of 80°C. for 30 minutes was required. Such a finding is not necessarily applicable to human plasma-agar plates.

It has been shown with regard to the serum factor necessary for Muller's phenomenon that an increase in the sodium chloride concentration precipitated it. It would appear that the sodium chloride concentration and concentration of other contained salts present for example in modified Chapman's medium, might well play a part in plaque-production in heated plasma-agar.

IX. THE SERUM FACTOR IN MULLER'S PHENOMENON.

Only a limited investigation was carried out on the serum-factor necessary for the demonstration of Muller's phenomenon and for the definite lysis which results from the fusion of plaques in milk or haemoglobin containing media. The results obtained from this study have been reported in Part VII.

Some additional information has been obtained from experiments (not reported in this thesis) by paper-strip electrophoresis of serum and by-preparative (column) electrophoresis of serum-staphylococcal-factor mixtures. By the paper strip method it was observed that the activity of serum for Muller's phenomenon was situated near the gamma globulins. In the experiment carried out by preparative (column) electrophoresis in which a mixture of staphylococcal factor and serum was fractionated, a number of active fractions were recovered, grouped closely together and associated with the gamma globulins. These active fractions produced definite lysis and some plaques in milk-agar test medium.

X. FUTURE RESEARCH IN MULLER'S PHENOMENON AND FIBRINOLYSIS.

It is abundantly clear that much more work is required on the serum factor necessary for Muller's phenomenon. In the limited study undertaken as part of these investigations it has not been possible to prove the identity of this serum factor with plasminogen, if that is in fact the serum (plasma) factor required for fibrinolysis brought about by staphylococci. It is clear, however, the direction in which future work on this problem should take. Investigations of plaque-formation with more highly purified materials is necessary.

Much work has already been done by the American workers, Gerheim et al (1948); Gerheim and Ferguson (1949), and Lewis and Ferguson (1949) on the different aspects of staphylococcal fibrinolysis. These workers have contributed much to our understanding of this proteolytic activity of staphylococci but it may be that some of their work will require revision in view of the recent contributions of Astrup and others to the whole field of fibrinolysis. The American workers regarded staphylokinase as

activating plasminogen (prolysin) directly to plasmin (lysin), in a fashion similar to the then accepted direct activiation of plasminogen by streptokinase. Astrup (1956) has brought forward fresh evidence of the manner in which plasminogen is activated to give plasmin. A group of lysokinases (which includes streptokinase) are thought to activate a pro-activator present in blood and other body fluids and which is then capable of activating plasminogen to plasmin. In view of this our concepts of the mechanism of fibrinolysis of heated plasma may well need to be revised.

There is a great need for correlating the information available on the different aspects of staphylococcal fibrinolysin which have been studied by the different workers and for examining it against the background of the fibrinolytic activities of the blood and body fluids generally. The presence of inhibitors and antibodies to the various constituents of the fibrinolytic system have been recognised. The use of commercial staphylococcal antitoxin in a small number of experiments carried out by the author (but not reported in this thesis) revealed inhibition of Muller's phenomenon and the definite lysis associated with it. It may be that antibody to the staphylococcal factor was involved in this inhibition. The presence of an inhibitor to Muller's phenomenon was revealed in the experiments on fractionation of serum.

It may be held that the formation of plaques depends on the presence of a particulate serum factor which has not hitherto been recognised apart from its action in demonstrations of Muller's phenomenon. It may be that ultra-centrifugation studies will reveal such a particulate serum factor and in this way differentiate it from the factor necessary for fibrinolysis, if indeed it is different. The present worker, however, is of the opinion that this serum factor is not necessarily particulate. Reference has already been made to the fact that no plaques were visible with growing staphylococci in a serum-milk-agar plate containing 50% serum. When conditions are optimal plaques may not be seen. Also, when staphylococcal factor of high activity is used with the optimal amount of serum, plaques may not be seen in serum-milk-agar - a fading or clearing of the medium only being apparent. Or again it sometimes happens that plaques are only seen at an early stage of incubation/such plates and when observed are very small, appearing as a fine network and fading before they reach 1.0 mm. in diameter. The evidence available at the moment would suggest that the serum factor is highly susceptible to the salt concentration in the medium, being readily aggregated and that the effect of this aggregation is readily seen and recognised as Muller's phenomenon when only sub-optimal amounts of the serum

factor and/or the staphylococcal factor or present.

It has seemed unnecessary to the author to discuss the theory that Muller's phenomenon is due to a virus in the serum in view of the evidence presented in these investigations, which makes such a theory untenable. Elek (1953) discussing the possibility that a virus in serum might be the active factor mentioned that "the initial lag period and the appearance of pin-point areas of clearing growing rapidly to a certain size and then no further are all points which have their counterpart in the cultivation of microbes. The striking lack of confluence and the smallness of the plaques in places of crowding are also more difficult to explain on a chemical basis, but would fit the concept of biological competition." In this work it has been shown that the initial lag period is often less than 2 hours, a good demonstration of plaques and partial lysis of the medium being often seen by the fourth hour of incubation. Confluence of plaques, has of course been a feature of the demonstration of Muller's phenomenon with the staphylococcal factor used in these investigations. Elek never found plaques in serum-haemoglobin-agar plates in less than 12 hours and he does not appear to have observed complete lysis of clearing of his medium due to fusion of plaques.

Rubenstein (1958) has indicated that coagulase clots differ from normal clots produced by recalcification of plasma, in the structure of their fibrin. It is not known, but it may well be that there is a difference in the susceptibility of these two types of fibrin to breakdown by staphylococcal fibrinolysin. Since staphylococcal factor often contains staphylocoagulase investigations on fibrinogen or plasma-containing media with the staphylococcal factor may require special care, in view of the possible action by the staphylocoagulase.

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"STUDIES IN MULLER'S PHENOMENON WITH SPECIAL REFERENCE TO THE USE OF A MILK-AGAR SUBSTRATE."

T H E S I S

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V O L U M E II.

A P P E N D I C E S.

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APPENDICES.

ABBREVIATIONS.

L.	=	Lysis.
P.	=	Plaques.
Ppt.	=	Precipitation.
S.	=	Slight.
R.E.	=	Rough Edge.
-	=	No change.
...	=	No growth.
(...)	=	No result.
B.A.	=	Blood-Agar.
S.M.A.	=	Serum-Milk-Agar.
S.Hb.A.	=	Serum-Haemoglobin-Agar.
Hb.A.	=	Haemoglobin-Agar.

EXPERIMENTS.

EXPERIMENT 1 .

To repeat the experiment of Fisk and Mordvin (1943) in which they demonstrated increased caseinolysis by staphylococci on milk agar plates enriched by addition of human serum.

MEDIA: (a) Serum-milk-agar plates prepared by method of Fisk and Mordvin (App. 473).

(b) Milk agar plates without serum (App. 473).

INOCULUM: A loopful of 18 hour old 1% "Lab-Lemco" broth cultures of five strains of coagulase positive Staphylococcus aureus and one of Bacillus subtilis is plated out on media (a) and (b), one plate of each medium for each strain.

INCUBATION: The plates were incubated at 37°C. for 48 hours.

READINGS: Taken at 24 hours and 48 hours.

EXPERIMENT 1.

RESULTS: At 24 and 48 hours.

ORGANISMS	TIME OF READING (Hrs.).	MILK-AGAR WITH 50% HUMAN SERUM	MILK-AGAR WITH "LAB-LEMCO" BROTH
Strains of Staph. aureus			
Strain I.	24	L.	S.Ppt.
	48	"	"
Strain II.	24	S.L.	S.Ppt.
	48	"	"
Strain RVI.	24	S.L.	-
	48	L.	-
Strain IV.	24	L.	Ppt.
	48	"	"
Staph. citreus.	24	S.L.	-
	48	L.	-
Bacillus subtilis.	24	-	L.
	48	-	"

EXPERIMENT 2.

To ascertain the caseinolytic abilities of strains of coagulase positive Staphylococcus aureus, Bacillus subtilis and other organisms on modified Fisk and Mordvin serum-milk-agar plates.

MEDIA:

(a) Serum-milk-agar plates prepared by method of Fisk and Mordvin (App. 473).

(b) Similar plates modified so that they contained 10% and 30% serum, (App. 473).

(c) Milk agar plates without serum (App. 473).

INOCULUM: A loopful of 18 hour old 1% "Lab-Lemco" broth cultures of four strains of coagulase positive staphylococci and seven strains of other caseinolytic organisms was plated out on (a), (b) and (c), a half plate of each medium for each strain used.

INCUBATION: The plates were incubated at 37°C. for 72 hours.

READINGS: Taken at 24 hours and 72 hours.

EXPERIMENT 2 .

RESULTS: At 24 hours and 72 hours.

ORGANISMS	TIME OF READING (Hrs.).	MILK-AGAR PLATES.	10% SERUM IN MILK-AGAR.	30% SERUM IN MILK-AGAR.	50% SERUM IN MILK-AGAR.
Strains of Staph. aureus					
Oxford	24	S.L.Ppt.	L.P.	L. R.E. P.	L.
	72	"	"	L.P.	"
Strain I	24	-	S.L.	L. R.E.	L.
	72	L.	L.P.	L.	"
Strain II	24	-	S.L.	L. R.E.	L.
	72	Ppt.	"	L.P.	"
Staph. citreus	24	S.L.Ppt.	L.P.	L. R.E. P.	L.
	72	"	"	L.P.	"
Pr. vulgaris	24	L.	-	-	-
	72	"	L.	L.	L.
S. marcescens	24	L.	L.	L.	L.
	72	"	"	"	"
Ps. pyocyanea	24	L.	S.L.	S.L.	S.L.
	72	"	L.	L.	L.
B. subtilis	24	L.	-	-	-
	72	"	L.	L.	L.
B. anthracoides	24	-	-	-	-
	72	S.L.	S.L.	-	-
B. megatherium	24	L.	-	-	-
	72	"	L.	L.	L.
B. mycoides	24	-	-	-	-
	72	S.L.	S.L.	S.L.	S.L.

EXPERIMENT 3 .

To ascertain if alteration in milk concentration of and the addition of nutrient to serum-milk-agar media interferes with caseinolysis by Staphylococcus aureus, Bacillus subtilis and Bacillus anthracoides.

MEDIA: As detailed below.

Milk - Cow's milk (App. 472).

Serum - Pooled Human Serum from blood bank (App. 484).

Nutrient - 1% "Lab-Lemco" broth (App. 468).

Agar - Oxoid Agar 4% in distilled water pH 7.4 (App.452).

Using the above substances the following plates were prepared in triplicate.

Plate Number	1	2	3	4	5	6	7	8
Milk	3.0 ml.	3.0	3.0	1.0	1.0	1.0	1.0	1.0
Human Serum	0.0	1.0	2.0	0.0	1.0	2.0	3.0	4.0
1% "L-L" Broth.	2.0	1.0	0.0	4.0	3.0	2.0	1.0	0.0
4% Agar	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Percentage of serum in plate	0.0	10.0	20.0	0.0	10.0	20.0	30.0	40.0

INOCULUM: A loopful of 18 hour old 1% "Lab-Lemco" broth cultures of Staphylococcus aureus, Strain 4, Bacillus subtilis and Bacillus anthracoides were inoculated on to the surface of each of the eight types of medium above-mentioned, one plate of each medium for each organism.

INCUBATION: The plates were incubated at 37°C. for 48 hours.

READINGS: Readings were taken at 24 hours and 48 hours.

EXPERIMENT 3.

RESULTS: At 24 hours and 48 hours.

NUMBER OF PLATE	TIME OF READING (Hrs.).	ORGANISMS.		
		Staph. aureus, Strain 4.	B. subtilis	B. anthracoides
1.	24	Ppt.	L.	L.
	48	"	"	"
2.	24	L.P.	-	-
	48	L.P.Ppt.	L.	-
3.	24	L.	-	-
	48	L.Ppt.	S.L.	-
4.	24	S.L.	L.	S.L.
	48	"	"	"
5.	24	L. R.E. P.	L.	-
	48	" "	"	-
6.	24	L.	S.L.	-
	48	"	"	-
7.	24	L.	-	S.L.
	48	"	S.L.	-
8.	24	L.	-	S.L.
	48	"	-	-

EXPERIMENT 4.

To ascertain the caseinolytic ability of a number of organisms on serum-milk-agar and milk-agar plates.

MEDIA: (a) Serum-milk-agar plates containing 10%, 20% and 40% pooled human serum were made as detailed below. They contained no added nutrient.

(b) Milk-agar plates with no serum but with 4.5 ml. of 1% "Lab-Lemco" broth added as nutrient.

Ten plates of each of the following types of media were made:-

Milk	2.5 ml.	2.5	2.5	2.5
Human Serum	0.0	1.0	2.0	4.0
1% "Lab-Lemco	4.5	0.0	0.0	0.0
4.5% Agar.	3.0	3.0	3.0	3.0
Distilled Water	0.0	3.5	2.5	0.5
Percentage of serum in plate	0.0	10.0	20.0	40.0

INOCULUM: A loopful of an 18 hour old 1% "Lab-Lemco" broth culture of each of the nineteen organisms to be tested was plated out on half-plates of each of the four different media used.

INCUBATION: The plates were incubated at 37°C. for 48 hours.

READINGS: Readings were taken at 24 hours and 48 hours.

EXPERIMENT 4.

RESULTS: At 24 hours and 48 hours.

ORGANISM	TIME OF READING (Hrs.).	MILK-AGAR PLATE	10% SERUM IN MILK-AGAR.	20% SERUM IN MILK-AGAR.	40% SERUM IN MILK-AGAR.
STAPH. AUREUS "A"	24 48	S.L.Ppt. S.L.	L. R.E. L.P.	L. R.E. L.	L. "
"B"	24 48	- -	L. R.E. L.P.	L. R.E. L.	L. "
"C"	24 48	- -	L. L.P.	L. L.P.	L. "
"D"	24 48	- -	L. L.P.	L. L.P.	L. "
"E"	24 48	- -	L. L.P.	L. L.P.	S.L. L.
"F"	24 48	- -	L. L.P.	S.L. L.P.Ppt.	L. L.Ppt.
"G"	24 48	Ppt. "	L. L.P.Ppt.	L. S.L.Ppt.	L. Ppt.
"H"	24 48	Ppt. "	- L.P.	S.L. L.P.Ppt.	S.L. L.Ppt.
"I"	24 48	S.Ppt. "	- S.L.P.	- S.L.P.	- L.
"J"	24 48	- Ppt.	- -	- -	- S.L.
"K"	24 48	- -	L. P.	L. L.P.	L. "
"L"	24 48	- -	L. R.E. L.P.Ppt.	L. L.Ppt.	L. L.

/see over.

EXPERIMENT 4 (Continued).

ORGANISM	TIME OF READING (Hrs.).	MILK-AGAR PLATE.	10% SERUM IN MILK-AGAR.	20% SERUM IN MILK-AGAR.	40% SERUM IN MILK-AGAR.
B. mycoides.	24 48	- -	- -	- -	- -
S. marcescens.	24 48	S.L. S.L.Ppt.	- S.L.	- S.L.	- S.L.
B. anthracoides.	24 48	- -	- S.L.	- S.L.	- S.L.
B. megatherium.	24 48	L. "	- L.	- S.L.	- S.L.
B. subtilis.	24 48	L. "	- -	- -	- -
Pr. vulgaris.	24 48	- -	- -	- -	- -
Ps. pyocyanea.	24 48	L. "	S.L.Ppt. "	Ppt.L. "	S.L. Ppt.L.

EXPERIMENT 5 .

To repeat Experiment 4 using sheep serum in place of pooled serum, i.e. to ascertain the caseinolytic ability of a number of strains of organisms on serum-milk-agar plates, using sheep serum in place of pooled human serum.

MEDIA: (a) Serum-milk-agar plates, containing 10%, 20% and 40% sheep serum as detailed below. They contained no added nutrient.

(b) Milk-agar plates with no serum but with 4.5 ml. of 1% "Lab-Lemco" broth added as nutrient.

Ten plates of each of the following types of media were made:-

Milk	2.5	2.5	2.5	2.5
Sheep serum	0.0	1.0	2.0	4.0
1% "Lab-Lemco" Broth	4.5	0.0	0.0	0.0
4.5% Agar	3.0	3.0	3.0	3.0
Distilled Water	0.0	3.5	2.5	0.5
Percentage of Sheep serum in plate.	0.0	10.0	20.0	40.0

INOCULUM: A loopful of an 18 hour old 1% "Lab-Lemco" broth culture of each of the nineteen organisms to be tested was plated out on half-plates, of each of the four different media used.

INCUBATION: The plates were incubated at 37°C. for 48 hours.

READINGS: Readings were taken at 24 hours and 48 hours.

EXPERIMENT 5 .

RESULTS: At 24 hours and 48 hours.

ORGANISM	TIME OF READING (Hrs.).	MILK-AGAR PLATE	10% SHEEP SERUM IN MILK.	20% SHEEP SERUM IN MILK.	40% SHEEP SERUM IN MILK.
Staph. aureus "A"	24 48	S.L.Ppt. S.L.	L. L.Ppt.	L. L.Ppt.	L. L.P.
"B"	24 48	- -	L. L.P.	L. L.P.	S.L. S.L.P.
"C"	24 48	- -	S.L. L. R.E.	L. L.P.	L. L.P.
"D"	24 48	- -	L. L.P.	S.L. L.P.	S.L. S.L.P.
"E"	24 48	- -	S.L. L.P.	L. L.P.	- S.L.P.
"F"	24 48	- -	L. L.P.	S.L. L.P.	S.L. S.L.P.
"G"	24 48	Ppt. "	- P.	- P.Ppt.	S.L. Ppt.
"H"	24 48	Ppt. "	L. L.P.	S.L. L.P.	S.L. L.P.
"I"	24 48	S.Ppt. "	S.L. L.	S.L. L.	S.L. L.P.
"J"	24 48	- Ppt.	S.L. S.L.P.	S.L. S.L.P.	S.L. "
"K"	24 48	- -	S.L. P.	S.L. L.P.	S.L. S.L.P.
"L"	24 48	- -	S.L. L.P.	S.L. L.P.	S.L. "

/see over.

EXPERIMENT 5 (Continued).

ORGANISM	TIME OF READING (Hrs.).	MILK-AGAR PLATE	10% SHEEP SERUM IN MILK.	20% SHEEP SERUM IN MILK.	40% SHEEP SERUM IN MILK.
B. mycoides.	24 48	- -	- -	- -	- -
S. marcescens.	24 48	S.L. S.L.Ppt.	L. "	S.L. S.L.	S.L. S.L.
B. anthracoides.	24 48	- -	- -	- -	- -
B. megatherium.	24 48	L. "	- -	- -	- -
B. subtilis.	24 48	L. "	- S.L.	- -	- -
Pr. vulgaris.	24 48	- -	- -	- -	- -
Ps. pyocyanea.	24 48	L. "	S.L.Ppt. "	S.L.Ppt. "	S.L.Ppt. "

EXPERIMENT 6 .

To ascertain the caseinolytic ability of organisms on a further modification of Fisk and Mordvin serum-milk-agar plates.

MEDIA: As detailed below.

Milk - Cow's milk (App. 472).

Serum - Pooled Human Serum (App. 484).

Nutrient - 15% "Lab-Lemco" in distilled water (App.469).

Agar - Oxoid agar 4.5% in distilled water pH 7.4 (App. 452).

The following plates of media were prepared using the above ingredients.

	Without added nutrient			With added nutrient		
Milk	3.75 ml.	3.75	3.75	3.75	3.75	3.75
15% "Lab-Lemco" Broth	0.0	0.0	0.0	1.0	1.0	1.0
Human Serum	0.0	1.5	6.0	0.0	1.5	6.0
4.5% Agar	4.5	4.5	4.5	4.5	4.5	4.5
Distilled Water	6.75	5.25	0.75	5.75	4.25	0.0
Percentage of Serum	0.0	10.0	40.0	0.0	10.0	40.0

INOCULUM: The plates were inoculated by single strokes of "Lab-Lemco" agar slope cultures (24 hours old) of the four organisms used, viz. Pseudomonas pyocyanea, Bacillus anthracoides, Staphylococcus aureus, strain A and Serratia marcescens.

INCUBATION: The plates were incubated at 37°C. for 24 hours.

READINGS: The results were recorded at 24 hours.

EXPERIMENT 6.

RESULTS: At 24 hours.

MEDIUM	ORGANISMS			
Without "Lab-Lemco" Broth.	Ps. pyocyanea	B. anthracoides	Staph. aureus Strain A.	S. marcescens
Serum 0.0%	-	L.	-	L.
" 10.0%	-	-	L.P.	L.
" 40.0%	-	-	L. R.E. P.	S.L.
With "Lab-Lemco" Broth.				
Serum 0.0%	L.	L.	S.L.	L.
" 10.0%	S.L.	-	L.P.	L.
" 40.0%	"	-	L. R.E.	L.

EXPERIMENT 7 .

To ascertain the effect of human serum on the liquefaction of gelatin by Staphylococcus aureus, strain A and B. anthracoides.

MEDIA: As detailed below.

Gelatin - 20% gelatin (Gold Leaf) in "Lab-Lemco" broth (App. 459).

Human Serum - Pooled human serum (App. 484),

1% "Lab-Lemco" broth - standard laboratory broth (App. 468).

METHOD: Test tubes containing 5.0 ml. 20% Gelatin in "Lab-Lemco" broth were placed at 37°C. until the gelatin had melted. Different amounts of human serum and "Lab-Lemco" broth were added to the different tubes, (as given in the table below) and thoroughly mixed. The tubes were placed in the ice-chest until the gelatin mixture had solidified. The final concentration of gelatin was 10%.

20% Gelatin	5.0	5.0	5.0	5.0
Human Serum	0.0	1.0	2.0	4.0
"Lab-Lemco" Broth	5.0	4.0	3.0	1.0
Percentage of Serum	0.0	10.0	20.0	40.0

INOCULUM: Sets of serum-gelatin mixtures were inoculated with a straight platinum wire from 24 hour old "Lab-Lemco" broth cultures of Staphylococcus aureus, strain A and B. anthracoides.

INCUBATION: Tubes of the inoculated serum-gelatin mixture were placed at 20°C.

READINGS: Examination of the tubes was made every 24 hours until liquefaction of the gelatin was observed to have taken place.

EXPERIMENT 7.

RESULTS:

ORGANISM	AMOUNT OF SERUM IN TUBE (ml.).			
	0.0	1.0	2.0	4.0
Staph. aureus A.	Li 48 hrs	Li 72 hrs	Li 72 hrs	Li 72 hrs
B. anthracoides.	"	Li 48 hrs	"	"

Li = Liquefaction.

EXPERIMENT 8 .

To produce filtrates of Staphylococcus aureus, strain A, B. anthracoides and B. subtilis capable of liquefying gelatin.

MEDIA: 1% gelatin/"Lab-lemco" broth (App. 460).

METHOD: 100 ml. quantities of 1% gelatin (Gold Leaf) in 1% "Lab-Lemco" broth (pH 7.5) were placed in each of three 500 ml. flasks and inoculated respectively with 18 hour old "Lab-Lemco" broth cultures of Staphylococcus aureus, Strain A, B. anthracoides and B. subtilis. These flasks were incubated at 37°C. for three days when they were withdrawn from the incubator, the broth cultures centrifuged and supnatant broths filtered. The Seitz filters which were employed in these filtrations had had their filter pads previously washed by allowing 10 ml. of "Lab-Lemco" broth to pass through. The first 5 ml. of filtrate was discarded. The filtrates obtained were tested for gelatinase activity in the following way using 20% gelatin in distilled water as the substrate, with all aseptic precautions. (App. 460)

				CONTROL
20% Gelatin	1.0	1.0	1.0	1.0
Filtrate	1.0	1.5	2.0	0.0
Distilled Water	2.0	1.5	1.0	3.0

For each filtrate four tubes of 1 ml. 20% gelatin was used, one tube acting as control. The remaining three tubes contained 1.0 ml., 1.5 ml. and 2.0 ml. of the filtrate under test. The volume in each of the four tubes was made up to 4.0 ml. with sterile distilled water. Thorough mixing of all the materials was ensured and the tubes were then placed in the ice-chest until the gelatin mixture had set.

INCUBATION: Tubes were incubated at 20°C.

READINGS: These were made after incubation of tubes for 24 and 48 hours at 20°C.

EXPERIMENT 8.

RESULTS: After 24 and 48 hours' incubation at 20°C.

PERIOD OF INCUBATION, (Hrs.).	FILTRATE	AMOUNT (ml.)			
		0.0	1.0	1.5	2.0
24	Staph. aureus, Strain A.	-	-	-	-
24	B. anthracoides.	-	Li	Li	Li
24	B. subtilis.	-	"	"	"
48	Staph. aureus, Strain A.	-	Li	Li	Li
48	B. anthracoides.	-	"	"	"
48	B. subtilis.	-	"	"	"

Li = Liquefaction,

EXPERIMENT 9.

To ascertain the effect of fresh and heated human serum on liquefaction of gelatin by filtrates of Staphylococcus aureus A, B. anthracoides and B. subtilis.

MEDIA: As detailed below:-

Gelatin: Gold leaf gelatin 20% was prepared in distilled water and sterilised by autoclaving at 10 lbs. pressure 15 minutes. Dispensed in 1.0 ml. amounts in test-tubes.

Filtrates: As prepared for Experiment 8.

Serum: (a) Fresh human serum.

(b) Human serum heated at 65°C. for 30 minutes.

Two sets of tests were put up for each of the filtrates used, one set prepared with fresh serum, the other set with the serum heated at 65°C. for 30 minutes, in the following way:-

20% Gelatin	1.0	1.0	1.0	1.0	1.0
Filtrate	1.0	1.0	1.0	1.0	1.0
Serum	0.0	0.25	0.5	1.0	2.0
Distilled Water	2.0	1.75	1.5	1.0	0.0

A Control Plate containing 1 ml. of 20% gelatin and 3 ml. of distilled water was put up.

The tubes of gelatin were melted at 37°C., mixed with the filtrate, serum and distilled water and then placed in the ice-chest till the gelatin mixtures were firm.

INCUBATION: The sets of tubes were incubated at 20°C.

READINGS: Taken every 24 hours for 7 days. Those for 24 hours, 2 days and 7 days were recorded.

EXPERIMENT 9 .

RESULTS: After incubation at 20°C. for 24 hours, 2 days and 7 days.

At 24 hours:

FILTRATE	AMOUNT OF SERUM (ml.)					AMOUNT OF HEATED SERUM				
	0.0	0.25	0.5	1.0	2.0	0.0	0.25	0.5	1.0	2.0
Staph. aureus A.	-	-	-	-	-	-	-	-	-	-
B. anthracoides.	Li	Li	Li	Li	Li	Li	Li	Li	Li	Li
B. subtilis.	Li	-	-	-	-	Li	-	-	-	-
At 2 days:										
Staph. aureus A.	Li	-	-	-	-	Li	-	-	-	-
B. anthracoides.	Li	Li	Li	Li	Li	Li	Li	Li	Li	Li
B. subtilis.	Li	-	-	-	-	Li	-	-	-	-
At 7 days:										
Staph. aureus A.	Li	-	-	-	-	Li	-	-	-	-
B. anthracoides.	Li	Li	Li	Li	Li	Li	Li	Li	Li	Li
B. subtilis.	Li	-	-	-	-	Li	-	-	-	-

The control Plate without filtrate and serum showed no change.

Li = Liquefaction.

EXPERIMENT 10 .

To investigate 12 strains of coagulase positive staphylococci which had previously not shown Muller's phenomenon on serum-milk-agar plates.

MEDIA: Serum-milk-agar plates containing different amounts of human serum as follows:-

Milk	2.0	2.0	2.0	2.0	2.0
Serum	0.1	0.25	0.5	1.0	1.5
"Lab-Lemco" Broth (15%)	1.0	1.0	1.0	1.0	1.0
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5
Distilled Water	7.4	7.25	7.0	6.5	6.0

INOCULA: 18 hour old "Lab-Lemco" broth cultures of strains 71060, 71090, 71273, 71706, 71709, 71710, 72041, 72298, 72333, 72907, 72932, 73105, which had never previously shown Muller's phenomenon.

INCUBATION: The plates were incubated at 37°C. for 24 hours. Thereafter left at room temperature.

READINGS: Readings were recorded after 24 hours at 37°C.

EXPERIMENT 10.

RESULTS: After 24 hours at 37°C.

STRAIN	AMOUNT OF SERUM IN PLATE (ml.).				
	0.1	0.25	0.5	1.0	1.5
71060	L.P.Ppt.	L.P.Ppt.	L.Ppt.	L.Ppt.	L.Ppt.
71090	Ppt.	Ppt.	Ppt.	Ppt.	Ppt.
71273	L.Ppt.	L.Ppt.	L.Ppt.	L.Ppt.	L.Ppt.
71706	"	"	"	"	"
71709	"	"	"	"	"
71710	"	"	"	"	"
72041	-	"	"	"	"
72298	L.P.Ppt.	L.P.Ppt.	L.Ppt.	"	"
72333	L.Ppt.	L.Ppt.	" "	"	"
72907	-	-	-	-	-
72932	L.Ppt.	L.Ppt.	L.Ppt.	L.Ppt.	L.Ppt.
73105	L.	"	"	"	"

The lysis recorded in this experiment around the growth of all strains except that of 71060 and 72298 is of the "indefinite type" which lacks the clear cut edge associated with the lysis and plaques found in Muller's phenomenon.

EXPERIMENT 11.

The liquefaction of gelatin by 12 strains of staphylococci.

MEDIA: 15% Gold-Leaf Gelatin was made up in 1% "Lab-Lemco" broth (pH 7.4) and distributed in 3 ml. amounts in 5" x ½" test-tubes and sterilised by autoclaving at 10 lbs. for 15 minutes. These were the tubes of gelatin used to test the ability of strains of staphylococci with regard to liquefaction. Control tubes were prepared from the same batch of gelatin at the same time. These were the tubes of gelatin 15%, 14%, 12%, 10%, 8%, 6%, 4% and 2% concentrations made up in 1% "Lab-Lemco" broth.

INOCULUM: Each of the twelve strains of staphylococci was inoculated into tubes of 15% gelatin with a straight platinum wire directly from the Dorset's Egg stock cultures of these organisms. The control tubes were uninoculated.

INCUBATION: The tubes of 15% gelatin and the control tubes were incubated at 37°C. for 14 days in all. After 2, 4, 7, 10, 12 and 14 days' incubation the tubes were transferred to the ice-chest for a period of 3 hours and then returned once more to the 37°C. incubator.

READINGS: These were taken every ½ hour during the 3 hours the tubes were in the ice-chest. A record was taken of whether the gelatin had remained fluid, become semi-fluid or had solidified, these states being noted by the marks "-", "±" and "+" respectively.

EXPERIMENT 11.

RESULTS: After 2 days' incubation at 37°C.

STRAINS	TIME IN ICE-CHEST					
	30 mins.	1 hr.	1½ hr.	2 hr.	2½ hr.	3 hr.
89412	±	±	+			
89495	±	±	+			
89508	-	±	±	±	±	±
89578	+					
89627	+					
90410	+					
90478	+					
90742	±	±	±	±	±	±
90770	±	±	±	±	+	
90800	+					
90838	±	±	±	±	±	±
90839	±	±	+			
CONTROLS						
15%	+					
14%	+					
12%	+					
10%	-	+				
8%	-	+				
6%	-	-	±	+		
4%	-	-	-	-	-	±
2%	-	-	-	-	-	-

EXPERIMENT 11 (Continued).

RESULTS: After 4 days' incubation at 37°C.

STRAINS	TIME IN ICE-CHEST					
	30 mins.	1 hr.	1½ hr.	2 hr.	2½ hr.	3 hr.
89412	-	±	±	±	±	±
89495	-	±	+			
89508	-	-	-	-	-	-
89578	+					
89627	+					
90410	+					
90478	+					
90742	-	-	-	±	±	±
90770	-	±	±	±	±	±
90800	+					
90838	-	±	±	±	±	±
90839	-	±	+			
CONTROLS						
15%	+					
14%	+					
12%	+					
10%	±	+				
8%	-	+				
6%	-	-	±	+		
4%	-	-	-	±	±	±
2%	-	-	-	-	-	-

EXPERIMENT 11 (Continued).

RESULTS: After 7 days' incubation at 37°C.

STRAINS	TIME IN ICE-CHEST					
	30 mins.	1 hr.	1½ hr.	2 hr.	2½ hr.	3 hr.
89412	-	-	-	-	-	-
89495	-	-	±	+		
89508	-	-	-	-	-	-
89578	-	+				
89627	+					
90410	+					
90478	±	+				
90742	-	-	-	-	-	-
90770	-	-	±	±	±	±
90800	-	+				
90838	-	±	±	±	±	±
90839	-	±	+			
CONTROLS						
15%	+					
14%	+					
12%	+					
10%	±	+				
8%	-	+				
6%	-	±	±	+		
4%	-	-	-	±	±	±
2%	-	-	-	-	-	-

EXPERIMENT 11 (Continued).

RESULTS: After 10 days' incubation at 37°C.

STRAINS	TIME IN ICE-CHEST					
	30 mins.	1 hr.	1½ hr.	2 hr.	2½ hr.	3 hr.
89412	-	-	-	-	-	-
89495	-	-	-	-	±	±
89508	-	-	-	-	-	-
89578	-	+				
89627	-	+				
90410	-	+				
90478	-	+				
90742	-	-	-	-	-	-
90770	-	-	-	-	±	±
90800	-	±	+			
90838	-	-	-	±	±	±
90839	-	-	-	±	+	
CONTROLS						
15%	+					
14%	+					
12%	+					
10%	±	+				
8%	-	±	+			
6%	-	-	+			
4%	-	-	-	-	±	±
2%	-	-	-	-	-	-

EXPERIMENT 11 (Continued).

RESULTS: After 12 days' incubation at 37°C.

STRAINS	TIME IN ICE-CHEST					
	30 mins.	1 hr.	1½ hr.	2 hr.	2½ hr.	3 hr.
89412	-	-	-	-	-	-
89495	-	-	-	-	-	±
89508	-	-	-	-	-	-
89578	-	+				
89627	-	+				
90410	-	+				
90478	-	+				
90742	-	-	-	-	-	-
90770	-	-	-	-	-	-
90800	-	±	+			
90838	-	-	-	±	±	±
90839	-	-	-	-	±	±
CONTROLS						
15%	+					
14%	+					
12%	+					
10%	±	+				
8%	-	±	+			
6%	-	-	+			
4%	-	-	-	-	±	±
2%	-	-	-	-	-	-

EXPERIMENT 11 (Continued).

RESULTS: After 14 days' incubation at 37°C.

STRAINS	TIME IN ICE-CHEST					
	30 mins.	1 hr.	1½ hr.	2 hr.	2½ hr.	3 hr.
89412	-	-	-	-	-	-
89495	-	-	-	-	-	±
89508	-	-	-	-	-	-
89578	-	+				
89627	-	+				
90410	-	+				
90478	-	+				
90742	-	-	-	-	-	-
90770	-	-	-	-	-	-
90800	-	±	+			
90838	-	-	-	±	±	+
90839	-	-	-	-	±	±
CONTROLS						
15%	+					
14%	+					
12%	+					
10%	±	+				
8%	-	±	+			
6%	-	-	+			
4%	-	-	-	-	±	±
2%	-	-	-	-	-	-

EXPERIMENT 12.

To ascertain the effect on the production of Muller's phenomenon of increasing amounts of milk in serum-milk-agar plates.

MEDIA: A series of serum-milk-agar plates were made up with different amounts of milk as follows:-

Milk	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	5.0	6.0	Control 3.0
Serum	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.0
"Lab-Lemco" Broth (15%)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5
Distilled Water	8.0	7.5	7.0	6.5	6.0	5.5	5.0	4.5	3.5	2.5	6.5

INOCULUM: Stab inoculations of all these plates were carried out from 6 hour old "Lab-Lemco" broth cultures of strains of Staphylococcus aureus A, 401, 406, 061, 779 and 935.

INCUBATION: Plates incubated at 37°C. for 24 hours.

READINGS: These were noted after 24 hours at 37°C. and after a further 48 hours at room temperature.

EXPERIMENT 12.

RESULTS: After 24 hours' incubation at 37°C.

Milk (ml.)	A.	401.	406.	061.	779.	935.
0.5	L. R.E.	L. R.E.	L. R.E.	L. R.E.	L. R.E.	L. R.E.
1.0	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.
1.5	"	"	"	"	"	"
2.0	"	"	"	"	"	"
2.5	"	"	"	"	"	"
3.0	"	"	"	"	"	"
3.5	"	"	"	"	"	"
4.0	"	"	"	"	"	"
5.0	"	"	"	"	"	"
6.0	"	"	"	"	"	"

RESULTS: After 48 hours' at Room Temperature.

0.5	L. R.E.	L. R.E.	L. R.E.	L. R.E.	L. R.E.	L. R.E.
1.0	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.
1.5	"	"	"	"	"	"
2.0	"	"	"	"	"	"
2.5	L.P.Ppt.	"	"	"	"	"
3.0	"	"	"	"	"	"
3.5	"	L.P.Ppt.	"	"	L.P.Ppt.	"
4.0	"	L.P.	L.P.Ppt.	L.P.Ppt.	"	L.P.Ppt.
5.0	L.P.	"	L.P.	L.P.	L.P.	L.P.
6.0	L.P.Ppt.	"	L.P.Ppt.	L.P.Ppt.	L.P.Ppt.	L.P.

The control plates showed around the growth of each organism a narrow area of indefinite lysis and a broader zone of precipitation.

EXPERIMENT 13.

To compare the production of Muller's phenomenon by strains of staphylococci in serum-milk-agar plates made with differently prepared milk.

MEDIA: Serum-milk-agar plates were made as detailed below. Three different preparations of milk were used. (1) 'Oxoid' dried skim milk reconstituted 100 gms. milk powder to 1000 ml. distilled water. Sterilised by autoclaving 15 lbs./15 minutes. (2) As in (1), but sterilised by steaming at 100°C. for 20 minutes on three successive days. (3) Cow's milk (fresh) - skimmed. Sterilised by steaming at 100°C. for 20 minutes on three successive days. Plates were prepared containing 0.5, 1.0 and 2.0 ml. of serum. All these plates were made in triplicate.

Milk	3.0	3.0	3.0
Human Serum	0.5	1.0	2.0
"Lab-Lemco" (15%)	1.0	1.0	1.0
Agar (4.5%)	4.5	4.5	4.5
Distilled Water	6.0	5.5	4.5

INOCULUM: All plates were stab inoculated from 6 hour old "Lab-Lemco" broth cultures of strains A, 401, 406, 039, 061, 779 and 935.

INCUBATION: Plates were incubated at 37°C. for 24 hours.

READINGS: These were noted after 24 hours' incubation at 37°C.

EXPERIMENT 13.

RESULTS: After incubation at 37°C. for 24 hours.

Dried Milk (Steamed) - 1st. Set.

Serum (ml.)	"A"	401	406	039	061	779	935
0.5	L.P.	P.	P.	P.	P.	L.P.	P.
1.0	"	"	"	"	"	P.	L.P.
2.0	"	L.P.	"	L.P.	L.P.	L.P.	"

2nd. Set.

0.5	L.P.	P.	P.	P.	P.	P.	P.
1.0	"	"	"	"	"	"	"
2.0	"	L.P.	"	L.P.	L.P.	L.P.	L.P.

3rd. Set.

0.5	L.P.	P.	P.	P.	P.	L.P.	P.
1.0	"	"	"	"	"	P.	"
2.0	"	L.P.	"	L.P.	L.P.	L.P.	L.P.

Dried Milk (Autoclaved) - 1st. Set.

0.5	L.P.	P.	P.	P.	P.	P.	P.
1.0	"	"	"	"	"	"	L.P.
2.0	"	L.P.	"	L.P.	L.P.	L.P.	"

2nd. Set.

0.5	L.P.	P.	P.	P.	P.	P.	P.
1.0	"	"	...	"	"	"	L.P.
2.0	"	L.P.	P.	L.P.	"	"	"

EXPERIMENT 13 (Continued).

3rd. Set.

Serum (ml.)	"A"	401	406	039	061	779	935
0.5	L.P.	P.	P.	P.	P.	P.	P.
1.0	"	"	"	"	"	"	"
2.0	"	L.P.	"	L.P.	L.P.	L.P.	L.P.

Fresh Skimmed Milk - 1st. Set.

0.5	L.P.	P.	P.	P.	P.	P.	L.P.
1.0	"	"	"	"	"	"	P.
2.0	"	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.

2nd. Set.

0.5	L.P.	P.	P.	P.	P.	P.	P.
1.0	"	"	"	"	"	"	"
2.0	"	L.P.	"	L.P.	L.P.	L.P.	L.P.

3rd. Set.

0.5	L.P.	P.	P.	P.	P.	P.	P.
1.0	"	"	"	"	"	"	"
2.0	"	L.P.	"	L.P.	L.P.	L.P.	L.P.

EXPERIMENT 14.

To compare the production of Muller's phenomenon on serum-milk-agar plates using different methods of inoculation.

MEDIA: Serum-milk-agar plates were made up as follows:-

			Control
Milk	3.0	3.0	3.0
Serum	0.5	1.0	0.0
"Lab-Lemco" (15%)	1.0	1.0	1.0
Agar (4.5%)	4.5	4.5	4.5
Distilled Water	6.0	5.5	6.5

Five sets of plates were prepared.

INOCULUM: The different methods of inoculation were carried out using 6 hour old "Lab-Lemco" broth cultures of strains A, 401, 039, 061, 779 and 935. The methods of inoculation were (1) Stab, (2) Loopful (Rideal-Walker loop used), (3) Single stroke inoculum. Strain A only was used in the two remaining methods, viz. (4) Successive strokes, (5) Use of glass spreader.

INCUBATION: All plates incubated at 37°C. for 24 hours and then left at room temperature.

READINGS: These were taken after 24 hours' at 37°C. and after 24 hours' incubation at room temperature.

EXPERIMENT 14.

RESULTS: After 24 hours' incubation at 37°C. (1) Stab Inoculum.

Serum (ml.)	"A"	401	039	061	779	935
0.0	L.Ppt.	-	L.Ppt.	-	L.Ppt.	-
0.5	L.P.	P.	P.	P.	L.P.	L.P.
1.0	"	"	"	"	P.	P.

(2) Loop Inoculum.

0.0	L.Ppt.	-	-	-	L.Ppt.	-
0.5	P.	P.	P.	P.	P.	P.
1.0	"	"	"	"	"	"

(3) Single Stroke Inoculum.

0.0	L.	-	L.	-	L.	-
0.5	P.	P.	P.	P.	P.	P.
1.0	"	"	"	"	"	"

(4) Successive Strokes. "A" only.

0.0 Lysis (indefinite) around each stroke.

0.5 Lysis and Plaques " " "

1.0 " " " " " "

(5) Glass Spreader. "A" only.

0.0 Lysis (indefinite) and precipitation around growth.

0.5 Plaques beneath growth - Lysis and plaques around growth.

1.0 " " " " " " " "

Lysis in control plates without serum was of the indefinite type.

EXPERIMENT 14 (Continued).

RESULTS: After a further 24 hours' incubation at room temperature.

(1) Stab Inoculum.

Serum (ml.)	"A"	401	039	061	779	935
0.0	L.	-	L.	-	L.	-
0.5	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.
1.0	"	"	"	"	"	"

(2) Loop Inoculum.

0.0	L.Ppt.	-	Ppt.	-	L.Ppt.	-
0.5	P.	-	P.	P.	P.	P.
1.0	"	-	"	"	"	"

(3) Single Stroke Inoculum.

0.0	L.	-	L.	-	L.	-
0.5	P.	P.	P.	P.	P.	P.
1.0	"	"	"	"	"	"

(4) Successive Strokes. "A" only.

0.0 Lysis (indefinite) around each stroke.

0.5 Lysis and Plaques around each stroke.

1.0 " " " " " "

(5) Glass Spreader. "A" only.

0.0 Lysis (indefinite) around growth.

0.5 Plaques beneath the growth and some around edges of growth.

1.0 " " " " " " " " " "

Lysis in control plates without serum was of the indefinite type.

EXPERIMENT 15.

To ascertain the effect on the production of Muller's phenomenon of different amounts of human serum incorporated in serum-milk-agar plates.

MEDIA: Serum-milk-agar plates were made with different amounts of human serum as follows:-

Milk	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5
"Lab-Lemco" (15%)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Human serum	0.0	0.25	0.5	1.0	2.0	3.0	4.0	5.0
Distilled Water	6.5	6.25	6.0	5.5	4.5	3.5	2.5	1.5

INOCULUM: These plates were stab inoculated from 6 hour old "Lab-Lemco" broth cultures of strains of Staphylococcus aureus, A, 401, 406, 039, 061, 779 and 935.

INCUBATION: The plates were incubated at 37°C. for 24 hours.

READINGS: These were recorded after incubation of plates at 37°C. for 24 hours.

EXPERIMENT 15.

RESULTS: After incubation of plates at 37°C. for 24 hours.

Serum (ml.)	"A"	401	406	039	061	779	935
0.0	Ppt.S.L.	S.L.	S.Ppt.	Ppt.	Ppt.	Ppt.S.L.	Ppt.
0.25	L.P.Ppt.	P.	P.	P.	-	P.	L.P.Ppt.
0.5	L.P.	"	"	"	P.	S.L. P.	L.P.
1.0	"	L.P.	L.P.	L.P.	L.P.	L.P.	"
2.0	L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.
3.0	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.
4.0	"	"	"	"	L.	"	"
5.0	"	"	"	"	"	"	"

EXPERIMENT 16.

To ascertain the effect on the production of Muller's phenomenon in serum-milk-agar by different strains of staphylococci of different amounts of human serum.

MEDIA: Serum-milk-agar plates were made with different amounts of human serum in duplicate, one set having no added nutrient, the other set having 1 ml. of 15% "Lab-Lemco" added to each plate, as follows:-

Milk	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75
Serum	0.2	0.5	1.0	1.5	2.0	3.0	4.0	4.5	5.0	5.5	6.0
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5
Distilled Water	6.55	6.25	5.75	5.25	4.75	3.75	2.75	2.25	1.75	1.25	0.75

Another series of plates similar to the above except that each contained 1 ml. of 15% "Lab-Lemco" broth and 1 ml. less of Distilled Water.

INOCULATION: Each plate was stab inoculated from 6 hour old "Lab-Lemco" broth cultures of strains of A, 401, 406, 039, 061 and 779.

INCUBATION: Plates were incubated at 37°C. for 24 hours and then at room temperature for a further 24 hours.

READINGS: These were taken after 24 hours' and 48 hours' incubation.

EXPERIMENT 16.

RESULTS: At 24 hours - Plates without nutrient.

Serum (ml.)	"A"	401	406	039	061	779
0.2	S.L.Ppt.	-	-	-	-	-
0.5	L.P.	-	-	S.L.	-	-
1.0	"	L.P.	L.P.	L.P.	S.L.P.	S.L.P.
1.5	"	"	"	"	L.P.	L.
2.0	"	"	"	"	"	L.P.
3.0	"	"	"	"	"	"
4.0	"	L. R.E.	L.	L. R.E.	L. R.E.	L.
4.5	"	"	L. R.E.	"	"	"
5.0	L. R.E.	L.	L.	"	L.	"
5.5	L.	"	"	L.	"	L.
6.0	"	"	"	"	"	"

At 24 hours - Plates with nutrient.

0.2	Ppt.L.P.	P.	P.	P.	S.L.	-
0.5	P.	"	"	"	P.	P.
1.0	L.P.	"	"	"	"	"
1.5	"	"	"	"	"	"
2.0	"	L.P.	L.P.	L.P.	L.P.	L.P.
3.0	"	"	"	"	"	"
4.0	"	"	"	"	"	"
4.5	"	L. R.E.	L. R.E.	L. R.E.	L. R.E.	L. R.E.
5.0	L. R.E.	"	"	"	"	"
5.5	"	"	"	"	"	"
6.0	"	L.	L.	L.	"	L.

EXPERIMENT 16 (Continued).

RESULTS: At 48 hours - Plates without nutrient.

Serum (ml.)	"A"	401	406	039	061	779
0.2	Ppt.L.	-	-	-	-	-
0.5	L.P.	S.L.	S.L.	L.P.	L.	S.L.
1.0	"	L.P.	L.P.	"	L.P.	L.P.
1.5	"	"	"	"	"	"
2.0	L. R.E.	L. R.E.	L. R.E.	L. R.E.	L. R.E.	L. R.E.
3.0	"	"	"	"	"	"
3.5	"	"	"	"	"	L.
4.5	L.	L.	L.	L.	L.	"
5.0	"	"	"	"	"	"
5.5	"	"	"	"	"	"
6.0	"	"	"	"	"	"

At 48 hours - Plates with nutrient.

0.2	L.Ppt.	L.P.	L.P.	L.P.	L.P.	L.P.
0.5	L.P.	"	"	"	"	"
1.0	"	"	"	"	"	"
1.5	"	"	"	"	"	"
2.0	"	"	"	"	"	"
3.0	L. R.E.	L. R.E.	L. R.E.	L. R.E.	L. R.E.	L. R.E.
4.0	"	"	"	"	"	"
4.5	"	"	"	"	"	"
5.0	"	L.	L.	L.	L.	L.
5.5	L.	"	"	"	"	"
6.0	"	"	"	"	"	"

EXPERIMENT 17.

To ascertain the effect on the production of Muller's phenomenon in serum-milk-agar plates by staphylococci of using human serum pre-heated at different temperatures.

MEDIA: Batches of serum-milk-agar plates were made up as follows:-

Milk	3.75	3.75	3.75	3.75	3.75	3.75
Serum	1.0	2.0	3.0	4.0	5.0	6.0
"Lab-Lemco" (15%)	1.0	1.0	1.0	1.0	1.0	1.0
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5	4.5
Distilled Water	4.75	3.75	2.75	1.75	0.75	0.0

The serum used was divided into lots - one lot was left unheated, other lots were heated at 55°C. for 15 and 30 minutes, at 60°C. for 15 and 30 minutes and at 65°C. for 15 and 30 minutes. The unheated and the various lots of heated serum were used to make up the serum-milk-agar plates as detailed above.

INOCULATION: These batches of plates were stab inoculated from 6 hour old "Lab-Lemco" broth cultures of strains of staphylococci, A, 401, 406, 061, 779 and 935.

INCUBATION: The plates were incubated at 37°C. for 48 hours.

READINGS: These were taken after 24 and 48 hours' incubation.

EXPERIMENT 17.

RESULTS: After 24 hours' incubation.

Unheated Serum:

Serum (ml.)	"A"	401	406	061	779	935
1.0	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.
2.0	"	"	"	"	"	"
3.0	"	"	"	"	"	"
4.0	L. R.E.	L.	L.	L.	L. R.E.	L.
5.0	"	"	"	"	L.	"
6.0	L.	"	"	"	"	"

Serum heated at 55°C. for 15 minutes.

1.0	L.P.	Ppt.	Ppt.	Ppt.	Ppt.	L.P.
2.0	"	L.P.	L.P.	L.P.	L.P.	"
3.0	"	"	"	"	"	"
4.0	"	"	"	"	"	"
5.0	L.	L.	L.	L. R.E.	L.	L.
6.0	"	"	"	L.	"	"

Serum heated at 55°C. for 30 minutes.

1.0	L.P.	-	-	-	-	L.P.
2.0	"	S.L.P.	S.L.	S.L.	L.P.	"
3.0	"	L.P.	L.P.	"	"	"
4.0	"	"	"	L.P.	"	"
5.0	L.	L.	L.	L.	L.	L.
6.0	"	"	"	"	"	"

EXPERIMENT 17 (Continued).

Serum heated at 60°C. for 15 minutes.

Serum (ml.)	"A"	401	406	061	779	935
1.0	-	-	-	-	-	-
2.0	-	-	-	-	-	-
3.0	-	-	-	-	-	-
4.0	S.L.	S.L.	S.L.	S.L.	S.L.	S.L.
5.0	"	"	"	"	"	"
6.0	"	"	"	"	"	"

Serum heated at 60°C. for 30 minutes.

1.0	-	-	-	-	-	-
2.0	-	-	-	-	-	-
3.0	S.L.	S.L.	S.L.	S.L.	S.L.	S.L.
4.0	"	"	"	"	"	"
5.0	"	"	"	"	"	"
6.0	"	"	"	"	"	"

Plates with serum heated at 65°C. for 15 and 30 minutes showed no change in any plate with any quantity of serum.

RESULTS: At 37°C. after 48 hours. Unheated serum.

1.0	L.P.	L.P.	Ppt.	Ppt.	L.P.	L.P.
2.0	"	"	L.P.	L.P.	"	"
3.0	"	"	"	"	"	"
4.0	L.	L.	L.	L.	L.	L.
5.0	"	"	"	"	"	"
6.0	"	"	"	"	"	"

EXPERIMENT 17 (Continued).

Serum heated at 55°C. for 15 minutes.

Serum (ml.)	"A"	401	406	061	779	935
1.0	L.P.	Ppt.	Ppt.	Ppt.	Ppt.	L.P.
2.0	"	L.P.	L.P.	L.P.	L.P.	"
3.0	"	"	S.L.P.	S.L.	"	"
4.0	"	"	L.P.	L.P.	"	"
5.0	L.	L.	L.	L. R.E.	L.	L.
6.0	"	"	"	L.	"	"

Serum heated at 55°C. for 30 minutes.

1.0	L.P.	-	-	-	-	L.P.
2.0	"	S.L.	S.L.	S.L.	L.P.	"
3.0	"	L.P.	Ppt.	Ppt.	"	"
4.0	L.	L.	L.	L.	L.	L.
5.0	"	"	"	"	"	"
6.0	"	"	"	"	"	"

Serum heated at 60°C. for 15 minutes.

1.0	Ppt.S.L.	Ppt.	Ppt.	Ppt.	Ppt.	Ppt.
2.0	"	S.L.	"	"	"	"
3.0	Ppt.	"	"	S.L.	S.L.	S.L.
4.0	Ppt.S.L.	"	S.L.	"	"	"
5.0	"	"	"	"	"	"
6.0	"	"	"	"	"	"

EXPERIMENT 17 (Continued).

Serum heated at 60°C. for 30 minutes.

Serum (ml.)	"A"	401	406	061	779	935
1.0	Ppt.	-	-	Ppt.	Ppt.	-
2.0	Ppt.S.L.	S.L.	S.L.	S.L.	S.L.	S.L.
3.0	Ppt. L.	"	"	"	"	"
4.0	"	"	"	"	"	"
5.0	L.	"	"	"	"	"
6.0	"	"	"	"	"	"

Plates containing serum heated at 65°C. for 15 and 30 minutes show precipitation and slight lysis with all strains in all quantities of serum.

EXPERIMENT 18.

To ascertain the effect of different temperatures of incubation on the production of Müller's phenomenon.

MEDIA: Serum-milk-agar plates were made up as follows:-

			Control
Milk	2.0	2.0	2.0
Human Serum	1.0	1.5	0.0
"Lab-Lemco" (15%)	1.0	1.0	1.0
Agar (4.5%)	4.5	4.5	4.5
Distilled Water	6.5	6.0	7.5

Four sets of these plates were prepared.

INOCULUM: These plates were stab inoculated from 6 hour old "Lab-Lemco" broth cultures of Staphylococcus aureus, Strains A, 401, 406, 039, 061, 779 and 935.

INCUBATION: A set of plates was incubated at each of the following temperatures, 22°C., 26°C., 30°C., and 37°C.

READINGS: These were taken at 20 hours, 3 days, 4 days, 5 days and 7 days.

EXPERIMENT 18.

RESULTS: After 20 hours at the temperatures specified.

37°C. Plates.

Serum (ml.)	"A"	401	406	039	061	779	935
0.0	S.L.	-	-	S.L.	-	S.L.	-
1.0	L.P.	L.P.	P.	L.P.	P.	L.P.	P.
1.5	"	"	"	"	"	"	"

30°C. Plates.

0.0	S.L.	-	-	-	-	-	-
1.0	L.P.R.E.	L.P.R.E.	L.P.R.E.	L.P.R.E.	L.P.R.E.	L.P.R.E.	L.P.R.E.
1.5	"	"	"	"	"	"	"

(Plaques are not so numerous as in 37°C. Plate).

26°C. Plates.

0.0	-	-	-	-	-	-	-
1.0	L.P.R.E.	L.P.R.E.	L.P.R.E.	L.P.R.E.	L.P.R.E.	L.P.R.E.	L.P.R.E.
1.5	"	"	"	"	"	"	"

(Growth is slight, lysis small).

22°C. Plates.

0.0 1.0 1.5	Growing but no change observed.

EXPERIMENT 18 (Continued).

All plates were re-incubated at specified temperatures for 48 hours.

RESULTS: At 3 days - 37°C. Plates.

Serum (ml.)	"A"	401	406	039	061	779	935
0.0	S.L.	S.L.	S.L.	S.L.	S.L.	S.L.	S.L.
1.0	L.P.	L.P.	P.	L.P.	"	L.P.	L.P.
1.5	"	"	"	"	P	"	"

30°C. Plates.

0.0	S.L.	S.L.	S.L.	S.L.	S.L.	S.L.	S.L.
1.0	L.P.	L.P.	L.P.	L.P.	P.	L.P.	L.P.
1.5	"	"	"	"	S.L.P.	"	"

26°C. Plates.

0.0	S.L.	-	-	-	-	S.L.	-
1.0	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.
1.5	"	"	"	"	"	"	"

22°C. Plates.

0.0	S.L.	-	-	-	-	-	-
1.0	L.P.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.P.	L.R.E.
1.5	"	"	"	"	"	L.R.E.	"

EXPERIMENT 18 (Continued).

RESULTS: After 4 days' incubation.

37°C. Plates.

Serum (ml.)	"A"	401	406	039	061	779	935
0.0	L.	S.L.	S.L.	S.L.	S.L.	S.L.	S.L.
1.0	L.P.Ppt.	L.P.	P.	L.P.	"	L.P.	L.P.
1.5	L.P.	"	"	"	P.	"	"

30°C. Plates.

0.0	S.L.	S.L.	S.L.	S.L.	S.L.	S.L.	S.L.
1.0	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.
1.5	"	"	"	"	"	"	"

26°C. Plates.

0.0	S.L.	S.L.	S.L.	S.L.	S.L.	S.L.	S.L.
1.0	L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.
1.5	"	"	"	"	"	"	"

22°C. Plates.

0.0	S.L.	-	-	-	-	S.L.	-
1.0	L.R.E.P.	L.R.E.P.	L.R.E.	L.R.E.P.	L.R.E.P.	L.R.E.	L.R.E.
1.5	"	"	"	L.R.E.	L.R.E.	"	"

EXPERIMENT 18 (Continued).

RESULTS: At 5 days.

26°C. Plates.

Serum (ml.)	"A"	401	406	039	061	779	935
0.0	L.	S.L.	S.L.	S.L.	S.L.	S.L.	S.L.
1.0	L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.
1.5	"	"	"	"	"	"	"

22°C. Plates.

0.0	L.	-	-	S.L.	-	S.L.	-
1.0	L.R.E.P.	L.R.E.P.	L.R.E.	L.R.E.P.	L.R.E.P.	L.R.E.	L.
1.5	"	L.R.E.	"	"	L.R.E.	"	"

RESULTS: After 7 days.

26°C. Plates - In these plates with 1.0 and 1.5 serum - there are large plaques well outside ring of lysis.

0.0	L.	S.L.	S.L.	L.	S.L.	S.L.	S.L.
1.0	L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.
1.5	"	"	"	"	"	"	"

22°C. Plates.

0.0	L.	-	-	S.L.	-	S.L.	-
1.0	L.R.E.P.	L.R.E.P.	L.R.E.	L.R.E.	L.R.E.	L.R.E.P.	L.
1.5	"	L.R.E.	L.	L.R.E.P.	"	L.R.E.	"

EXPERIMENT 19.

To ascertain the effect of different temperatures of incubation on the production of Muller's phenomenon in serum-milk-agar and serum-haemoglobin-agar plates.

MEDIA: Serum-milk-agar and serum-haemoglobin-agar plates were made as follows:-

Milk	2.0	Haemoglobin	1.5
Serum	0.5	Serum	1.5
"Lab-Lemco" (15%)	1.0	"Lab-Lemco" (15%)	1.0
Agar (4.5%)	4.5	Agar (4.5%)	4.5
Distilled Water	7.0	Distilled Water	6.5

Four sets of these plates were made.

INOCULUM: Stab inocula were made on all plates from 6 hour old "Lab-Lemco" broth cultures of strains of Staphylococcus aureus, A, 401, 406, 039, 061, 779 and 935.

INCUBATION: One set of plates was incubated at each of the following temperatures - 4°C., 22°C., 37°C. and 42°C.

READINGS: The results were recorded after 24 hours', 2 days' and 3 days' incubation.

EXPERIMENT 19.

RESULTS: At 24 hours.

Serum-milk plates.

TEMP.	"A"	401	406	039	935	779	061
4°C.	-	-	-	-	-	-	...
22°C.	L.P.	-	-	-	-	L.P.	L.P.
37°C.	"	L.P.	L.P.	L.P.	L.P.	"	"
42°C.	"	"	"	"	"	"	"

Serum-haemoglobin plates.

4°C.	-	-	-	-	-	-	...
22°C.	-	-	-	-	-	-	-
37°C.	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.
42°C.	"	...	...	"	...	...	"

RESULTS: At 2 days.

Serum-milk plates.

4°C.	...	...	...	...	...	...	...
22°C.	L.P.	L.	L.	L.P.	L.	L.P.	L.P.
37°C.	"	L.P.	L.P.	"	L.P.	"	"
42°C.	"	"	"	"	"	"	L.P.

Serum-haemoglobin plates.

4°C.	...	...	...	...	...	...	...
22°C.	L.	L.	L.	-	L.	-	L.
37°C.	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.
42°C.	"	"	"	"	"	"	P.

EXPERIMENT 19 (Continued).

RESULTS: At 3 days.

Serum-milk plates.

TEMP.	"A"	401	406	039	935	779	061
4°C.	...	...	...	...	...	...	...
22°C.	L.P.	L.P.	L.P.	L.P.	L.	L.P.	L.P.
37°C.	"	"	"	"	L.P.	"	"
42°C.	"	"	"	"	"	"	"

Serum-haemoglobin plates.

4°C.	...	...	...	...	...	...	...
22°C.*	L.	L.	L.	L.	L.	L.	L.
37°C.	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.
42°C.	"	"	"	"	"	"	"

* All show lysis and plaques at 96 hours.

EXPERIMENT 20.

To ascertain if Muller's phenomenon is produced in serum-milk-agar and serum-haemoglobin agar plates under anaerobic conditions.

MEDIA: Plates of serum-milk-agar and serum-haemoglobin-agar (in duplicate) were prepared as follows:-

Serum-haemoglobin-agar (a)

Haemoglobin	1.5	1.5	1.5	1.5	1.5
Serum	1.5	2.0	3.0	4.0	5.0
"Lab-Lemco" (15%)	1.0	1.0	1.0	1.0	1.0
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5
Distilled Water	6.5	6.0	5.0	4.0	3.0

(b)

Haemoglobin	1.5	1.5
Serum	1.5	3.0
"Lab-Lemco" (15%)	1.0	1.0
Agar (4.5%)	4.5	4.5
Distilled Water	6.5	5.0

Serum-milk-agar (a)

Milk	2.0	2.0	2.0	2.0	2.0
Serum	0.5	1.0	2.0	3.0	4.0
"Lab-Lemco" (15%)	1.0	1.0	1.0	1.0	1.0
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5
Distilled Water	7.0	6.5	5.5	4.5	3.5

(b)

Milk	2.0	2.0
Serum	0.5	1.0
"Lab-Lemco" (15%)	1.0	1.0
Agar (4.5%)	4.5	4.5
Distilled Water	7.0	6.5

INOCULUM: All plates were stab inoculated from 6 hour old cultures of strains of Staphylococcus aureus, A, 401, 406, 039, 061, 779 and 935.

INCUBATION: The serum-haemoglobin-agar and serum-milk-agar plates (a) series were incubated at 37°C. in a McIntosh and Fildes jar. The (b) series of plates were set up as Fortner plates, a strain of Pseudomonas pyocyanea being used to inoculate the nutrient agar in the lid of the plate. These Fortner plates were incubated at 37°C. for 48 hours. The duplicate set were incubated aerobically at 37°C. for 48 hours.

READINGS: These were made after 48 hours' incubation.

EXPERIMENT 20.

RESULTS: After 48 hours' incubation at 37°C.

(a) series - McIntosh & Fildes Jar.

Serum-haemoglobin plates.

Serum (ml.)	"A"	401	406	039	061	779	935
1.5	L.P.	L.P.	L.	L.P.	L.	L.	L.P.
2.0	"	"	"	"	L.P.	L.P.	"
3.0	"	"	L.P.	"	"	"	"
4.0	"	"	"	"	"	"	"
5.0	"	"	"	"	"	"	"

Serum-milk plates.

0.5	L.P.	L.P.	L.	L.P.	L.P.	L.P.	L.P.
1.0	"	"	L.P.	"	"	"	"
2.0	"	"	"	"	"	"	"
3.0	L.R.E.	"	"	"	"	"	"
4.0	L.	"	"	L.	"	"	L.

(b) series - Fortner plates.

Serum-haemoglobin plates.

1.5	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.
3.0	"	"	"	"	"	"	"

Serum-milk plates.

0.5	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.
1.0	"	"	"	"	"	"	"

CONTROL PLATES (aerobic) - ALL SHOWED L.P.

EXPERIMENT 21.

To ascertain the effect on the production of Muller's phenomenon by strains of staphylococci of different concentrations of agar.

MEDIA: Serum-milk-agar plates were made up with agar concentrations from 1% to 5% and with amounts of human serum from 1 ml. - 4 ml., as follows:-

Milk	2.0	2.0	2.0	2.0	2.0
Serum	1.0	1.0	1.0	1.0	1.0
"Lab-Lemco" (15%)	1.0	1.0	1.0	1.0	1.0
Agar (10%)	1.5	3.0	4.5	6.0	7.5
Distilled Water	9.5	8.0	6.5	5.0	3.5
% Agar	1.0	2.0	3.0	4.0	5.0

Similar sets of plates made up with 2.0, 3.0 and 4.0 ml. of serum.

INOCULUM: These sets of plates were stab inoculated from 6 hour old cultures of strains of Staphylococcus aureus, A, 935, 039 and 401.

INCUBATION: These plates were incubated at 37°C. for 20 hours, thereafter at room temperature for 48 hours.

READINGS: These were recorded after 20 hours' incubation of the plates and after 48 hours' at room temperature.

EXPERIMENT 21.

RESULTS: After 20 hours' incubation at 37°C.

Serum 1 ml.	"A"	935	039	401	Remarks	
Agar 5%	L.P.	L.P.	L.P.	L.P.	Plaques very fine	All plates show rough edge around all strains.
4%	"	"	"	"	Plaques fine	
3%	"	"	"	"		
2%	"	"	"	"		
1%	"	"	"	"	Plaques fairly big.	
Serum 2 ml.						
Agar 5%	L.P.	L.P.	L.P.	L.P.	Plaques very fine	"
4%	"	"	"	"	Plaques fine	
3%	"	"	"	"		
2%	"	"	"	"	Average size.	
1%	"	"	"	"	Average size +.	
Serum 3 ml.						
Agar 5%	L.P.	L.P.	L.P.	L.P.	Plaques small	"
4%	"	"	"	"		
3%	"	"	"	"	Plaques average size	
2%	"	"	"	"	" " "	
1%	...	...	...	...		
Serum 4 ml.						
Agar 5%	L.R.E.	L.R.E.	L.R.E.	L.R.E.		
4%	"	"	"	"		
3%	"	"	"	"		
2%	"	"	"	"		
1%	"	"	"	"		

EXPERIMENT 21 (Continued).

RESULTS: After incubation at room temperature for 48 hours.

Serum 1 ml.	"A"	935	039	401	Remarks	
Agar 5%	L.P.	L.P.	L.P.	L.P.	Plaques are very fine.	All plates show rough edge around all strains
4%	"	"	"	"	Plaques are fine	
3%	"	"	"	"	Plaques big and small	
2%	"	"	"	"	Plaques mainly average, few small	
1%	"	"	"	"	Plaques are of average size, i.e. big compared with those in 5% and 4%.	
Serum 2 ml.						
Agar 5%	L.P.	L.P.	L.P.	L.P.	Plaques very fine	"
4%	"	"	"	"	Plaques mainly fine	
3%	"	"	"	"	Plaques few small average size.	
2%	"	"	"	"	Plaques average size	
1%	"	"	"	"	" " "	
Serum 3 ml.						
Agar 5%	L.P.	L.P.	L.P.	L.P.	Plaques are all about the same size. Separate plaques are few in number, rough edge predominating.	
4%	"	"	"	"		
3%	"	"	"	"		
2%	"	"	"	"		
1%	"	"	"	"		

EXPERIMENT 21 (Continued).

Serum 4 ml.	"A"	935	039	401	Remarks
Agar 5%	L.	L.	L.	L.	The rough edge noted here is very slight indeed. Areas of lysis are about same size.
4%	L.	L.R.E.	L.R.E.	L.R.E.	
3%	L.	L.	L.	"	
2%	L.	L.	L.	L.	
1%	L.	L.	L.	L.R.E.	

EXPERIMENT 22.

To ascertain if Muller's phenomenon can be demonstrated by strains of staphylococci in a gelatin-agar gel.

MEDIA: Gelatin powder (Gurr, G.T.) and agar powder (Oxoid) were melted in distilled water so that they constituted 20% and 1% respectively. The reaction of the gelatin-agar mixture was adjusted to pH 7.4. The gelatin-agar mixture was tubed in 7.5 ml. amounts and sterilised at 10 lbs./sq. in. for 15 minutes. Plates of medium were prepared as follows:-

Gelatin-agar	7.5	7.5	7.5	7.5
Milk	2.0	2.0	2.0	2.0
"Lab-Lemco" (15%)	1.0	1.0	1.0	1.0
Human Serum	0.0	0.5	1.0	2.0
Distilled Water	4.5	4.0	3.5	2.0

INOCULUM: All plates were stab inoculated from 6 hour old "Lab-Lemco" broth cultures of strains A, 401, 406, 039, 061, 779 and 935.

INCUBATION: The plates were incubated at room temperature.

READINGS: These were made after 48 hours' and 72 hours' incubation at room temperature.

EXPERIMENT 22.

RESULTS: After 48 hours' incubation at room temperature.

Serum (ml.)	"A"	401	406	039	061	779	935
0.0	S.L.	-	-	S.L.	-	-	-
0.5	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.
1.0	L.R.E.	"	"	L.R.E.	"	"	"
2.0	"	"	"	"	"	"	L.R.E.

Slight liquefaction around colonies of all strains.

RESULTS: After 72 hours' incubation at room temperature.

No further change in the production of plaques and area of lysis in the milk. The area of liquefaction had increased.

EXPERIMENT 23.

To ascertain the effect on the production of Muller's phenomenon of the addition of different amounts of different nutrients to serum-milk-agar plates.

MEDIA: Batches of serum-milk-agar plates were made up containing different amounts of different media as follows:-

Milk	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75
Serum	0.0	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Nutrient	0.5	0.5	1.0	1.5	2.0	3.0	4.0	5.0
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5
Distilled Water	6.25	4.75	4.25	3.75	3.25	2.25	1.25	0.25

The nutrients used were (1) 1% "Lab-Lemco" broth, (2) Meat Broth, (3) 1% Peptone/Water (Oxoid), (4) Whey Broth, (5) Casein digest broth. Each set contains a control plate with 0.5 ml. nutrient and no serum. A control plate with 1.5 ml. of serum but without added nutrient was also included.

INOCULUM: These plates were stab inoculated from 6 hour old "Lab-Lemco" broth cultures of strains of Staphylococci A, C, D, E, and 1339.

INCUBATION: All plates were incubated at 37°C. for 48 hours.

READINGS: These were taken after 24 and 48 hours' incubation at 37°C.

EXPERIMENT 23.

RESULTS: After incubation at 37°C.

Control Plates - Without added Nutrient.

Result	A	C	D	E	1339
24 hrs	L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.
48 "	"	"	"	"	"

1% "Lab-Lemco" Broth - 24 hour reading.

"Lab-Lemco"	A	C	D	E	1339
Control no serum 0.5	Ppt. L.	Ppt.	Ppt.	Ppt.	Ppt.
0.5	L.P.R.E.	P.	P.	L.P.	Ppt.L.P.
1.0	"	"	"	P.	"
1.5	"	"	"	"	"
2.0	"	"	"	"	"
3.0	Ppt.L.P. R.E.	P.	S.L.P.	"	"
4.0	"	S.L.P.	"	S.L.P.	"
5.0	"	L.	S.L.	S.L.	Ppt. P.

1% "Lab-Lemco" broth - 48 hour reading.

Control no serum 0.5	Ppt. L.	Ppt.	Ppt.	Ppt.	Ppt. L.
0.5	L.P.R.E.	L.P.R.E.	P.	L.P.	L.P.Ppt.
1.0	"	P.	Ppt.L.P.	P.	"
1.5	"	"	P.S.L.	"	"
2.0	"	"	"	"	"
3.0	"	"	L.P.	"	"
4.0	"	"	"	"	"
5.0	...	...	...	...	...

EXPERIMENT 23 (Continued).

Meat Broth Plates - 24 hour reading.

Meat Broth.	A	C	D	E	1339
Control no serum 0.5	Ppt. L.	Ppt.	-	Ppt.	Ppt. L.
0.5	L.P.R.E.	P.	P.	P.	Ppt. P.
1.0	"	"	"	"	"
1.5	"	"	"	"	L.P.Ppt.
2.0	"	"	"	"	"
3.0	"	"	L.	"	"
4.0	L.P.R.E. Ppt.	"	P.	"	"
5.0	"	"	L.	L.P.	"

Meat Broth Plates - 48 hour reading.

Control no serum 0.5	L.Ppt.	Ppt. L.	Ppt.	Ppt.	Ppt. L.
0.5	L.P.R.E. Ppt.	P.	P.Ppt.	P.	Ppt. P.
1.0	"	"	"	"	"
1.5	...	...	...	...	...
2.0	L.P.R.E. Ppt.	P.	Ppt. P.	P.	Ppt. P.
3.0	"	P.Ppt.	Ppt.	"	"
4.0	"	P.	"	Ppt. P.	"
5.0	"	"	"	Ppt.	Ppt.

EXPERIMENT 23 (Continued).

1% Peptone Water Plates - 24 hour reading.

Peptone Water	A	C	D	E	1339
Control no serum 0.5	S.L.Ppt.	Ppt.	Ppt.	Ppt.	Ppt.S.L.
0.5	L.P.R.E.	P.	P.	P.	L.P.
1.0	"	L.P.	"	L.P.	L.P.R.E.
1.5	"	"	L.P.	"	L.P.
2.0	"	L.P.R.E.	"	L.R.E.P.	"
3.0	"	"	L.R.E.	"	L.P.R.E.
4.0	"	L.R.E.	L.P.R.E.	"	"
5.0	"	L.	-	L.P.	"

1% Peptone Water Plates - 48 hour reading.

Control no serum 0.5	S.L.Ppt.	Ppt.	Ppt.	Ppt.	Ppt.S.L.
0.5	L.P.R.E.	P.	P.	P.	P. Ppt.
1.0	"	L.P.	"	L.P.	L.P.Ppt.
1.5	...	...	...	...	...
2.0	L.P.	P.	L.P.	L.P.	L.P.
3.0	L.P.R.E.	L.P.R.E.	L.P.R.E.	L.P.R.E.	L.P.R.E. Ppt.
4.0	"	L.P.	L.P.	"	"
5.0	"	P..	...	...	L. Ppt.

EXPERIMENT 23 (Continued).

Whey Broth Plates - 24 hour reading.

Whey Broth.	A	C	D	E	1339
Control no serum 0.5	Ppt.	-	-	-	Ppt.S.L.
0.5	L.P.	L.	L.P.	L.	L.P.Ppt.
1.0	"	L.P.	"	L.P.	"
1.5	"	"	"	"	"
2.0	"	L.	"	S.L.	"
3.0	"	S.L.	"	-	"
4.0	"	-	S.L.	-	"
5.0	L.	-	"	-	L. Ppt.

Whey Broth Plates - 48 hour reading.

Control no serum 0.5	Ppt.	-	-	-	Ppt. L.
0.5	L.P.	L. R.E.	L.P.	L.	L.P.Ppt.
1.0	"	L.P.	"	L.P.	"
1.5	"	"	"	"	"
2.0	"	S.L.R.E.	"	S.L.	L.R.E. Ppt.
3.0	"	-	S.L.P.	-	"
4.0	"	-	S.L.	-	L.P.Ppt.
5.0	L.	-	"	-	"

EXPERIMENT 23 (Continued).

Casein Digest Plates - 24 hour reading.

Casein Digest	A	C	D	E	1339
Control no serum 0.5	Ppt.	-	-	-	Ppt.S.L.
0.5	L.P.	S.L.	-	S.L.	L.P.
1.0	P.L.	"	-	"	L.P.Ppt.
1.5	-	-	-	-	P. Ppt.
2.0	-	-	-	-	Ppt.S.L.
3.0	-	-	-	-	"
4.0	Ppt.	-	-	-	"
5.0	-	-	-	-	"

Casein Digest Plates - 48 hour reading.

Control no serum 0.5	Ppt. L.	Ppt.	Ppt.	S.L.	Ppt. L.
0.5	L.P.Ppt.	S.L.	-	"	L.P.Ppt.
1.0	...	...	...	...	...
1.5	Ppt. L.	Ppt. L.	S.L.	S.L.	Ppt. L.
2.0	"	"	"	"	"
3.0	"	"	Ppt. L.	"	"
4.0	"	"	"	"	"
5.0	"	"	"	"	"

EXPERIMENT 24.

To ascertain the effect of different amounts of "Lab-Lemco" added to serum-milk-agar plates on the production of Muller's phenomenon by staphylococci.

MEDIA: Serum-milk-agar plates were prepared with different amounts of "Lab-Lemco" as follows:-

Milk	3.75	3.75	3.75	3.75	3.75	3.75	3.75
Distilled Water	5.75	5.25	4.75	3.75	2.75	1.75	0.75
"Lab-Lemco" (15%)	0.0	0.5	1.0	2.0	3.0	4.0	5.0
Serum	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5	4.5	4.5
% "Lab-Lemco"	0.0	0.5	1.0	2.0	3.0	4.0	5.0

INOCULATION: These plates were stab inoculated from 6 hour old "Lab-Lemco" broth cultures of strains of staphylococci, A, B, C, D, E, K, and 1339.

INCUBATION: The plates were incubated at 37°C. for 48 hours.

READINGS: These were made after 24 hours' and 48 hours' incubation at 37°C.

EXPERIMENT 24.

RESULTS: After 24 hours' incubation at 37°C.

% "Lab-Lemco	A	B	C	D	E	K	1339
0.0	L.P.	L.P.	L.P.	L.	L.P.	L.P.	L.P.Ppt.
0.5	"	"	"	L.P.	"	"	"
1.0	"	"	"	"	"	"	"
2.0	"	"	"	"	-	"	"
3.0	L. Ppt.	-	"	-	-	"	L. Ppt.
4.0	"	-	L.	-	-	L.	"
5.0	"	-	-	-	-	-	"

After 48 hours incubation at 37°C.

0.0	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.Ppt.
0.5	"	"	"	"	"	"	"
1.0	L.P. Ppt.	"	"	"	"	"	"
2.0	"	"	"	P.	S.L.	"	"
3.0	L.Ppt.	-	L.R.E. Ppt.	-	-	L.Ppt.	L.Ppt.
4.0	"	-	L.Ppt.	-	-	"	"
5.0	"	-	"	-	-	"	"

EXPERIMENT 25.

To ascertain the effect on the production of Muller's phenomenon on serum-milk-agar plates by staphylococci of the addition of different amounts of peptone.

MEDIA: Serum-milk-agar plates were made containing different amounts of peptone as follows:-

Milk	3.75	3.75	3.75	3.75	3.75	3.75	3.75
Distilled Water	5.75	5.25	4.75	3.75	2.75	1.75	0.75
Peptone (Oxoid) 15%	-	0.5	1.0	2.0	3.0	4.0	5.0
Serum	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5	4.5	4.5
% Peptone.	0.0	0.5	1.0	2.0	3.0	4.0	5.0

INOCULATION: These plates were stab-inoculated from 6 hour old "Lab-Lemco" broth cultures of strains of staphylococci, A, B, C, D, E, K and 1339.

INCUBATION: The plates were incubated at 37°C. for 48 hours.

READINGS: These were taken after 24 hours' and 48 hours' incubation at 37°C.

EXPERIMENT 25.

RESULTS: After incubation at 37°C. for 24 hours.

% Peptone	A	B	C	D	E	K	1339
0.0	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.Ppt.
0.5	L.P. Ppt.	S.L.	P.	-	S.L.	P.	Ppt.
1.0	"	"	Ppt.	-	"	Ppt.	"
2.0	L.Ppt.	"	"	-	"	"	L.Ppt.
3.0	Ppt.	"	"	-	L.	"	"
4.0	"	-	-	-	-	"	Ppt.
5.0	"	-	Ppt.	-	-	"	"

After incubation at 37°C. for 48 hours.

0.0	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.Ppt.
0.5	L.P. Ppt.	Ppt.	P.Ppt.	Ppt.	Ppt.	P.Ppt.	L.Ppt.
1.0	"	"	Ppt.	S.L.	"	Ppt.	"
2.0	L.Ppt.	L.	"	L.	L.	"	"
3.0	"	"	"	"	"	"	"
4.0	"	L.Ppt.	"	"	"	"	"
5.0	S.Ppt.	S.Ppt.	Ppt.	S.Ppt.	S.Ppt.	Ppt.	S.Ppt.

EXPERIMENT 26.

To ascertain the effect of the addition of Sodium chloride to serum-milk-agar plates on the production of Muller's phenomenon by staphylococci.

MEDIA: Serum-milk-agar plates were made up with different amounts of added Sodium Chloride as follows:-

							Control
Milk	2.0	2.0	2.0	2.0	2.0	2.0	2.0
"Lab-Lemco" (15%)	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Serum	0.5	0.5	0.5	0.5	0.5	0.5	0.0
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5	4.5	4.5
Distilled Water	7.0	6.75	6.5	6.25	6.0	5.0	6.5
NaCl (15%)	0.0	0.25	0.5	0.75	1.0	2.0	0.0
Final % NaCl.	0.0	0.25	0.5	0.75	1.0	2.0	0.0

INOCULUM: Stab inoculated from 6 hour old "Lab-Lemco" broth cultures of Strains A, 401, 406, 039, 061, 779 and 935.

INCUBATION: Incubated at 37°C. for 24 hours, thereafter at room temperature.

READINGS: These were noted after 24 hours' incubation at 37°C. and 72 hours' at room temperature.

EXPERIMENT 26.

RESULTS: After 24 hours at 37°C.

NaCl. %	"A"	401	406	039	061	779	935
0.0	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.
0.25	"	"	"	"	"	"	"
0.5	S.L.	L.*	S.L.	"+	S.L.	S.L.*	S.L.*
0.75	L.Ppt.	S.L.	"	S.L.	"	S.L.Ppt.	S.L.
1.0	"	"	"	S.L.Ppt	"	"	S.L.Ppt.
2.0	S.L.Ppt	-	-	-	-	-	-

* had 2 large plaques

\+ the plaques here were large.

After 72 hours at room temperature.

0.0	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.
0.25	"+	"+	"+	"+	"+	"+	"+
0.5	"+	"+	"+	"+	"+	"+	"+
0.75	L.	S.L.	L.	L.	L.	L.	L.
1.0	"	L.	"	"	"	"	"
2.0	"	"	"	"	"	"	"

\+ = plaques here were large.

Control S.L. L. L. S.L. L. L. L.

The lysis in the control plate without serum is of the indefinite type. So also is the lysis in the plates containing 2.0% Sodium chloride.

EXPERIMENT 27.

To ascertain the effect on the production of Muller's phenomenon of the addition of Sodium chloride to a series of serum-milk-agar plates containing different amounts of human serum.

MEDIA: Serum-milk-agar plates were made with different additions of Sodium Chloride as follows:-

Milk	3.75	3.75	3.75	3.75	3.75
Serum	0.5	0.5	0.5	0.5	0.5
NaCl (15%)	0.5	1.0	1.5	2.0	3.0
Distilled Water	5.75	5.25	4.75	4.25	3.25
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5
Final % NaCl.	0.5	1.0	1.5	2.0	3.0

Similar series of plates were made with 1.0 and 1.5 ml. serum. Three control plates with 0.5, 1.0 and 1.5 ml. serum respectively, but containing no added NaCl. were prepared.

INOCULUM: These plates were stab inoculated from 6 hour old "Lab-Lemco" broth cultures of strains of Staphylococcus aureus, A, B, C, E, K and 1339.

INCUBATION: Plates were incubated at 37°C. for 24 hours and then at room temperature for 24 hours.

READINGS: These were recorded after 24 hours' and 48 hours' incubation.

EXPERIMENT 27.

RESULTS: After incubation at 37°C. for 24 hours.

Serum 0.5 ml.

NaCl. %	A	B	C	E	K	1339
0.5	L.P.	L.P.	L.P.	L.P.	L.P.	L.Ppt.
1.0	L.	S.L.	-	-	-	"
1.5	L.Ppt.	-	-	-	-	"
2.0	"	-	-	-	-	L.
3.0	-	-	-	-	-	-

Serum 1.0 ml.

0.5	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.
1.0	"	L.	L.	L.	L.	L.
1.5	L.	-	-	-	"	"
2.0	"	-	-	-	-	"
3.0	-	-	-	-	-	S.L.

Serum 1.5 ml.

0.5	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.
1.0	"	"	L.	"	L.	"
1.5	L.	L.	"	-	-	L.
2.0	"	-	-	-	-	"
3.0	"	-	-	-	-	"

EXPERIMENT 27 (Continued).

RESULTS: After incubation at 37°C. for 48 hours.

Serum 0.5 ml.

NaCl. %	A	B	C	E	K	1339
0.5	L.P.	L.P.	L.P.	L.P.	L.P.	L.Ppt.
1.0	L.	L.	L.	L.	L.	"
1.5	"	-	-	-	-	L.
2.0	"	-	-	-	-	"
3.0	"	-	-	-	-	"

Serum 1.0 ml.

0.5	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.Ppt.
1.0	"	L.	L.	L.	"	L.Ppt.
1.5	L.	"	S.L.	"	L.	"
2.0	"	-	-	-	-	L.
3.0	-	-	-	-	-	"

Serum 1.5 ml.

0.5	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.
1.0	"	"	L.	"	"	L.Ppt.
1.5	L.	L.	"	L.	L.	"
2.0	"	-	-	-	-	L.
3.0	"	-	-	-	-	"

EXPERIMENT 27 (Continued).

CONTROL PLATES: Readings after 24 hours' incubation at 37°C. and 24 hours at room temperature.

Serum	A	B	C	E	K	1339
0.5 ml.	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.
1.0 "	"	"	"	"	"	"
1.5 "	"	"	"	"	"	"

EXPERIMENT 28.

To ascertain the effect of adding different amounts of Sodium chloride to serum-milk-agar and milk-agar plates.

MEDIA: Serum-milk-agar plates were prepared as follows:-

Milk	2.0	2.0	2.0	2.0	2.0	2.0
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5	4.5
"Lab-Lemco" (15%)	1.0	1.0	1.0	1.0	1.0	1.0
Serum	0.5	0.5	0.5	0.5	0.5	0.5
Distilled Water	7.0	6.75	6.5	6.25	6.0	5.0
NaCl. (15%)	0.0	0.25	0.5	0.75	1.0	2.0
Final % NaCl.	0.0	0.25	0.5	0.75	1.0	2.0

A similar set of plates but without serum were also prepared.

INOCULUM: Plates were stab inoculated from 6 hour old "Lab-Lemco" broth cultures of strains of Staphylococcus aureus, A, 401, 406, 039 and 779.

INCUBATION: At 37°C. for 24 hours and then at room temperature for 24 hours.

READINGS: Made after 48 hours' incubation.

EXPERIMENT 28.

RESULTS: After 24 hours at 37°C. and 24 hours at room temperature.

With serum NaCl. %	A	401	406	039	779
0.0	L.P.	L.P.	L.P.	L.P.	L.P.
0.25	L.R.E.P.*	L.P.*	L.R.E.P.*	L.R.E.P.*	L.R.E.P.*
0.5	L.R.E.	L.P.	S.L.	L.	L.
0.75	L.	S.L.	"	"	"
1.0	"	"	-	"	S.L.
2.0	S.L.	"	-	S.L.	"
Without serum NaCl. %					
0.0	S.L.	S.L.	-	L.	L.
0.25	"	"	-	"	"
0.5	"	"	-	"	S.L.
0.75	"	"	-	"	"
1.0	"	"	-	S.L.	"
2.0	"	"	-	"	"

* All plaques in these plates were large.

The nature of the Lysis observed in the plates with serum with 0.0% NaCl, 0.2% NaCl. and 0.5% NaCl. was different from that observed in the rest of the plates with the higher concentrations of NaCl. and in all the plates without serum. This latter type of lysis had an indefinite edge to its border.

EXPERIMENT 29.

To ascertain the effect on the production of Muller's phenomenon of killing strains of staphylococci on serum-milk-agar plates after different periods of growth.

MEDIA: The serum-milk-agar plates were made in triplicate as follows.

Milk	2.0	2.0	2.0
Serum	1.0	4.0	0.0
"Lab-Lemco" (15%)	1.0	1.0	1.0
Agar (4.5%)	4.5	4.5	4.5
Distilled Water	6.5	3.5	7.5

INOCULUM: Stab inoculations of seven strains of staphylococci were made from 6 hour old "Lab-Lemco" broth cultures.

INCUBATION: All three sets of plates were incubated at 37°C. One set of plates was withdrawn after 4½ hours' and another after 9 hours' incubation and the organisms killed by exposure to chloroform vapour for 15 minutes. They were then re-incubated at 37°C. for the remainder of the 24 hour period. The remaining set was kept as control - the organisms not being exposed to chloroform.

READINGS: These were taken after 24 hours' incubation of the plates at 37°C. and after 48 hours' incubation at room temperature.

EXPERIMENT 29.

RESULTS: After 24 hours' incubation at 37°C. Unkilled.

Control Serum	A	401	406	039	061	779	935
0.0	S.L.	-	-	S.L.	-	S.L.	-
1.0	L.P.	L.P.	P.	L.P.	P.	L.P.	P.
4.0	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.

Killed at 4½ hours.

0.0	-	-	-	-	-	-	-
1.0	S.L.P. R.E.	-	-	S.L. R.E.	-	S.L.P. R.E.	S.L.P. R.E.
4.0	L.	L.	L.	L.	L.	L.	L.

(Areas of Lysis very small).

Killed at 9 hours.

0.0	S.L.	-	-	S.L.	-	S.L.	-
1.0	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.
4.0	L.	L.	L.	L.	L.	L.	L.

(Areas of Lysis almost as big as control).

RESULTS: After 48 hours' incubation at room temperature. Unkilled.

0.0	S.L.	S.L.	S.L.	S.L.	S.L.	S.L.	S.L.
1.0	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.
4.0	L.	L.	L.	L.	L.	L.	L.

EXPERIMENT 29 (Continued).

Killed at 4½ hours.

Control Serum	A	401	406	039	061	779	935
0.0	-*	-*	-*	-*	-*	-*	-*
1.0	S.L.P.+	-	-	S.L.P.+	-	S.L.P.+	S.L.P.+
4.0	L.	L.	L.	L.	L.	L.	L.

* = Growth is minimal - just visible.

\+ = The plaques are small in number - one or two around each colony.

Killed at 9 hours.

0.0	S.L.	-	-	-	-	S.L.	-
1.0	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.
4.0	L.	L.	L.	L.	L.	L.	L.

The areas of lysis are much smaller than control plates now but they are larger (slightly) than 48 hours ago.

EXPERIMENT 30 (Continued).

INCUBATION: The plates were incubated at 37°C. for 24 hours.

READINGS: These were made after 24 hours at 37°C.

EXPERIMENT 30.

To ascertain the effect of soya-bean-trypsin-inhibitor on the production of Muller's phenomenon by staphylococci on serum-milk-agar plates.

MEDIA: A preparation of soya-bean-trypsin-inhibitor (S.B.I.) (5 mg./ml.) was made and three sets each of three plates with the following composition were prepared.

	Plate 1 Milk-Agar	Plate 2 Serum-Milk-Agar	Plate 3 Serum-Milk-Agar
Serum	0.0 ml.	0.5 ml.	1.0 ml.
1st Set S.B.I.	0.0	0.0	0.0
2nd " "	0.5	0.5	0.5
3rd " "	1.0	1.0	1.0

In each instance the medium contained the usual concentrations of milk "Lab-Lemco" and agar. In addition a 4th. Set duplicating the 1st Set was prepared and on each of its three plates three small porous cups were placed. These porous cups each received two drops of the soya-bean-trypsin-inhibitor preparation.

INOCULUM: Each plate was stab inoculated from 6 hour old "Lab-Lemco" broth cultures of Staphylococcus aureus Strains A, 406, 039, 935 and 779. The stab inoculations on the three plates with the porous cups were made 1.5 cm. from the centre of the cups.

EXPERIMENT 30.

RESULTS: After 24 hours at 37°C.

1st Set - (Control Plates; No S.B.I.)

Serum (ml.)	A	406	039	935	779
0.0	S.L.	S.L.	S.L.	-	S.L.
0.5	L.P.	P.	L.P.	P.	P.
1.0	"	"	"	"	L.P.

2nd Set - (with 0.5 ml. S.B.I.)

0.0	S.L.	S.L.	S.L.	-	S.L.
0.5	"	"	"	-	"
1.0	"	"	"	-	"

3rd Set - (with 1.0 ml. of S.B.I.)

0.0	S.L.	S.L.	S.L.	-	S.L.
0.5	"	"	"	-	"
1.0	"	"	"	-	"

4th Set - (with cups of S.B.I.)

0.0	S.L.	S.L.	S.L.	-	S.L.
0.5	Inhibition of lysis and plaques on that side of the staphylococcal colonies nearest the cups; lysis and plaques still visible around colonies on side away from the cups.				
1.0					

EXPERIMENT 31.

The demonstration of Muller's phenomenon in blood-agar plates.

MEDIUM: Human blood-agar plates were made as follows:-

Agar (4.5%)	4.5	4.5
"Lab-Lemco" (15%)	1.0	1.0
Distilled Water	8.75	8.0
Blood (Human Citrated).	0.75	1.5

The plates were prepared in duplicate.

INOCULA: The plates in one set were each inoculated with a loopful of a 6 hour old "Lab-Lemco" broth culture of Staphylococcus aureus, Strain A, in such a way as to give single colonies of the organism. The plates in the other set were inoculated with a loopful of the same culture spread as two streaks at right angles one to another, to give lines of heavy growth.

INCUBATION: The plates were incubated at 37°C. for 48 hours and at room temperature for 48 hours.

READINGS: These were taken after 96 hours' incubation.

EXPERIMENT 31.

RESULTS: After 96 hours' incubation.

Inoculum	Amount of Blood	
	0.75 ml.	1.5 ml.
Isolated colonies.	L.P.	L.P.
Streaks	L.P.	L.P.

EXPERIMENT 32.

To ascertain the effect on the demonstration of Muller's phenomenon in serum-haemoglobin-agar plates of different amounts of haemoglobin.

MEDIA: Serum-haemoglobin-agar plates were made up as follows:-

Haemoglobin (filtered)	0.5	1.0	1.5	2.0	3.0	4.0	5.0
Serum	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5	4.5	4.5
"Lab-Lemco" (15%)	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Distilled Water	7.5	7.0	6.5	6.0	5.0	4.0	3.0

INOCULA: Stab inoculations were made from 6 hour old "Lab-Lemco" broth cultures of A, 401, 406, 039, 061, 779 and 935.

INCUBATION: Plates were incubated at 37°C. for 48 hours.

READINGS: These were recorded after 24 and 48 hours' incubation at 37°C.

EXPERIMENT 32.

RESULTS: After 24 hours' incubation at 37°C.

Serum-haemoglobin plates.

Haemoglobin ml.	A	401	406	039	061	779	935
0.5	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.
1.0	"	"	"	"	"	"	"
1.5	"	"	L.	"	L.	L.	"
2.0	"	-	-	"	-	-	"
3.0	"	-	-	"	-	-	"
4.0	"	-	-	-	-	-	"
5.0	"	-	-	-	-	-	-

RESULTS: After 48 hours' incubation at 37°C.

Serum-haemoglobin plates.

0.5	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.
1.0	"	"	"	"	"	"	"
1.5	"	"	"	"	"	"	"
2.0	"	"	"	"	"	"	"
3.0	"	"	"	"	"	"	"
4.0	"	"	-	"	"	"	"
5.0	"	"	-	"	"	"	"

EXPERIMENT 33.

To ascertain the effect of different temperatures on the production of Muller's phenomenon by live staphylococci in serum-haemoglobin-agar plates.

MEDIUM: Five plates of serum-haemoglobin-agar were made as follows:-

Haemoglobin	1.5 ml.
Serum	1.5
"Lab-Lemco" (15%)	1.0
Agar (4.5%)	4.5
Distilled Water	6.5

INOCULA: Each plate was stab inoculated from 6 hour old "Lab-Lemco" broth cultures of A, 401, 406, 039, 061, 779 and 935.

INCUBATION: One plate was incubated at each of the following temperatures, 4°C., 22°C., 37°C., 42°C. and 46°C.

READINGS: These were taken after 24, 48, 72 and 96 hours' incubation.

EXPERIMENT 33.

RESULTS: After 24 hours' incubation.

Temperature	A	401	406	039	061	779	935
4°C.	...	...	...	...	...	...	...
22°C.	-	-	-	-	-	-	-
37°C.	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.
42°C.	"	S.L.	S.L.	"	"	"	S.L.
46°C.	...	...	...	...	...	...	...

After 48 hours' incubation.

4°C.	...	...	...	...	...	...	...
22°C.	L.	L.	L.	-	L.	-	L.
37°C.	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.
42°C.	P.	P.	P.	P.	P.	P.	P.
46°C.	...	...	...	...	...	...	...

After 72 hours' incubation.

4°C.	...	...	...	...	...	...	...
22°C.	L.	L.	L.	L.	L.	L.	L.
37°C.	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.
42°C.	"	"	"	"	"	"	"
46°C.	...	...	...	...	...	...	...

After 96 hours' incubation.

4°C.	...	...	...	...	...	...	...
22°C.	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.
37°C.	"	"	"	"	"	"	"
42°C.	"	"	"	"	"	"	"
46°C.	...	...	...	...	...	...	...

EXPERIMENT 34.

To ascertain the effect of anaerobic incubation on the production of Muller's phenomenon in serum-haemoglobin-agar plates.

MEDIA: The following serum-haemoglobin-agar plates were made:-

Haemoglobin	1.5	1.5	1.5	1.5	1.5
Serum	1.5	2.0	3.0	4.0	5.0
"Lab-Lemco" (15%)	1.0	1.0	1.0	1.0	1.0
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5
Distilled Water	6.5	6.0	5.0	4.0	3.0

The plates with 1.5 and 3.0 ml. of serum were made in duplicate.

INOCULA: Stab inoculations of all plates were made from 6 hour old "Lab-Lemco" broth cultures of A, 401, 406, 039, 061, 779 and 935.

INCUBATION: The complete set of plates were incubated at 37°C. for 48 hours in a McIntosh and Fildes jar. The two duplicate plates were incubated as Fortner plates (pseudomonas pyocyanea being the accompanying aerobic organism) at 37°C. also for 48 hours.

READINGS: These were made after 48 hours' incubation at 37°C.

EXPERIMENT 34.

RESULTS: After 48 hours' incubation at 37°C.

McIntosh and Fildes Jar.

Amount of Serum	A	401	406	039	061	779	935
1.5	L.P.	L.P.	L.	L.P.	L.	L.	L.P.
2.0	"	"	P.	"	L.P.	L.P.	L.P.
3.0	"	"	L.P.	"	"	"	"
4.0	"	"	"	"	"	"	"
5.0	"	"	"	"	"	"	"

Fortner Plates.

1.5	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.
3.0	"	"	"	"	"	"	"

EXPERIMENT 35.

To ascertain if Muller's phenomenon can be demonstrated in a modified "chocolate" agar medium to which fresh serum had been added.

MEDIA: Horse red cells were thrice-washed with sterile saline and made up to the original volume of the blood with saline. 0.75 ml. of red cells was heated at 75°C. for 10 minutes along with agar, "Lab-Lemco" and distilled Water. It was then poured into a Petri dish. A similar mixture was cooled from 75°C. to 50°C. and then 0.5 ml. human fresh serum was added before pouring into Petri dish. The plates were made as follows:-

Washed Horse red cells	0.75 ml.	0.75
Agar (4.5%)	4.5	4.5
"Lab-Lemco" (15%)	1.0	1.0
Distilled Water	8.75	8.25
Serum	0.0	0.5

Six plates of each type of medium were prepared. Three of each type were covered with cellophane before inoculation.

INOCULA: One loopful from 6 hour old "Lab-Lemco" broth cultures of 3 strains of coagulase positive staphylococci was used to inoculate the two different types of media.

INCUBATION: Plates were incubated at 37°C. for 24 hours.

READINGS: These were made after 24 hours' incubation.

EXPERIMENT 35.

RESULTS: After 24 hours' incubation at 37°C.

Medium	A	039	935
Chocolate Agar	S.L.	L.	L.
Chocolate Agar with Serum.	S.L.P.	S.L.P.	P.

Duplicate plates on which the organisms were inoculated over a layer of sterile cellophane which was lying on top of the medium, failed to show any change in the medium even after 48 hours' incubation although good growth of the organisms was obtained.

EXPERIMENT 36.

To ascertain the effect of adding different amounts of fresh human serum to a modified "chocolate" agar medium.

MEDIA: Human red cells, washed with sterile saline three times and made up to original volume with saline were heated at 75°C. for 10 minutes with agar, "Lab-Lemco" and distilled water. The mixture was cooled to 50°C., human serum was added in appropriate amounts, mixed thoroughly and poured into a Petri dish. These chocolate agar plates with fresh serum were made up as follows:-

Red Cells	0.75	0.75	0.75	0.75	0.75	0.75
"Lab-Lemco" (15%)	1.0	1.0	1.0	1.0	1.0	1.0
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5	4.5
Distilled Water	8.75	8.65	8.5	8.25	7.75	6.75
Serum	0.0	0.1	0.25	0.5	1.0	2.0

A serum-milk-agar plate containing 0.5 ml. serum and a milk-agar plate without serum were also prepared.

INOCULA: The serum-chocolate-agar plates and the milk-agar and serum-milk-agar plates were stab inoculated from 6 hour old "Lab-Lemco" broth cultures of 9 strains of coagulase positive staphylococci.

INCUBATION: All plates were incubated at 37°C. for 24 hours.

READINGS: These were taken after 24 hours' incubation at 37°C.

EXPERIMENT 36.

RESULTS: After incubation of plates at 37°C. for 24 hours.

Chocolate plates with serum.

Serum (ml.)	3	4	6	7	10	11	12	S2	S4
0.0	L.	L.	L.	L.	-	S.L.	-	-	L.
0.1	P.	P.	P.	P.	P.	P.	P.	P.	P.
0.25	"	"	"	"	"	"	"	"	"
0.5	"	"	"	"	"	"	"	"	"
1.0	"	"	"	"	"	"	"	"	"
2.0	"	"	"	"	"	"	"	"	"

Serum-milk plates.

0.0	S.L.	S.L.	S.L.	S.L.	S.L.	S.L.	S.L.	S.L.	S.L.
0.5	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.

In the chocolate-agar plates with serum after a further 24 hours' incubation at 37°C. the only change detected was an increase in the breadth of the zones of plaques but further incubation brought about the appearance of lysis around the colonies especially in the plates with the larger amounts of serum.

EXPERIMENT 37.

To demonstrate Muller's phenomenon on a "chocolate" agar medium containing added fresh serum.

MEDIA: The red cells from a sample of citrated human blood were washed three times with sterile saline made up to the original volume of the blood with saline and used in 0.25 ml., 0.5 ml. or 1.0 ml. amounts in plates as follows:-

Red cells	0.25	0.25	0.25	0.25	0.25	0.25	0.25
"Lab-Lemco" (15%)	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5	4.5	4.5
Distilled Water	9.25	8.75	8.25	7.25	6.25	5.25	4.25
Serum	0.0	0.5	1.0	2.0	3.0	4.0	5.0

The mixture of red cells, agar, "Lab-Lemco" and Distilled Water was heated at 75°C. for 10 minutes, cooled to 50°C. and to it was added the appropriate amount of serum. The contents of the tube were thoroughly mixed and poured into a Petri dish.

INOCULA: The plates were stab inoculated with five strains of coagulase positive staphylococci from 6 hour old cultures.

INCUBATION: The plates were incubated at 37°C. for 18 hours and then incubated at room temperature for 48 hours.

READINGS: These were taken after 18 hours' at 37°C. and after 48 hours' incubation at room temperature.

EXPERIMENT 37.

RESULTS: After 18 hours' incubation at 37°C.

Plates with 0.25 ml. red cells heated.

Serum	3	4	6	7	10
0.0	L.	S.L.	L.	L.	S.L.
0.5	P.	P.	P.	P.	P.
1.0	"	"	"	"	"
2.0	L.P.	L.P.	L.P.	L.P.	L.P.
3.0	"	"	"	"	"
4.0	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.
5.0	"	"	"	"	"

Plates with 0.5 ml. red cells heated.

0.0	L.	L.	L.	L.	-
0.5	P.	P.	P.	-	-
1.0	"	"	"	P.	P.
2.0	L.P.	L.P.	L.P.	L.P.	L.P.
3.0	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.
4.0	"	"	"	"	"
5.0	"	"	"	"	"

Plates with 1.0 ml. red cells heated.

0.0	L.	L.	L.	L.	L.
0.5	P.	P.	P.	-	P.
1.0	"	"	"	-	"
2.0	"	"	"	P.	"
3.0	L.P.	L.P.	L.P.	L.P.	L.P.
4.0	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.
5.0	"	"	"	"	"

RESULTS: After 48 hours' incubation at room temperature.

Plates with 0.25 ml. red cells heated.

Serum	3	4	6	7	10
0.0	L.	S.L.	L.	L.	S.L.
0.5	P.	P.	P.	P.	P.
1.0	L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.
2.0	L.	L.	L.	L.	L.
3.0	"	"	"	"	"
4.0	"	"	"	"	"
5.0	"	"	"	"	"

Plates with 0.5 ml. red cells heated.

0.0	L.	L.	L.	L.	S.L.
0.5	P.	P.	P.	P.	P.
1.0	L.P.	L.P.	L.P.	L.P.	L.P.
2.0	L.	L.R.E.P.	L.	L.	L.R.E.
3.0	"	L.	"	"	L.
4.0	"	"	"	"	"
5.0	"	"	"	"	"

Plates with 1.0 ml. red cells heated.

0.0	L.	L.	L.	L.	S.L.
0.5	P.	P.	P.	P.	P.
1.0	"	"	"	"	"
2.0	L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.
3.0	L.	"	L.	L.	"
4.0	"	L.	"	"	L.
5.0	"	"	"	"	"

EXPERIMENT 38.

To ascertain the effect on the production of Muller's phenomenon of different amounts of fresh serum added to the heated haemoglobin substrate.

MEDIA: Heated haemoglobin-agar plates with fresh added serum were prepared as follows:-

Haemoglobin	1.0	1.5	2.0
Agar (4.5%)	4.5	4.5	4.5
"Lab-Lemco" (15%)	1.0	1.0	1.0
Distilled Water	8.0	7.5	7.0
Serum	0.5	0.5	0.5

Similar sets of plates containing 1.0 and 1.5 ml. of serum were also prepared.

The mixture of haemoglobin, agar, "Lab-Lemco" and Distilled Water sufficient for a plate was heated at 75°C. for 10 minutes, cooled to 50°C. and the appropriate amount of serum added. The contents of the tube were quickly mixed and poured into a Petri dish. The haemoglobin used was a filtered solution of haemoglobin (App. 462).

INOCULA: Each plate was stab inoculated with seven strains of staphylococci from 6 hour old "Lab-Lemco" broth cultures.

INCUBATION: The plates were incubated at 37°C, for 24 hours.

READINGS: These were taken after 24 hours' incubation.

EXPERIMENT 38.

RESULTS: After 24 hours' incubation at 37°C.

Haemoglobin 1.0 ml.

Serum	A	401	406	039	061	779	935
0.5	S.L.P.	S.L.P.	S.L.P.	S.L.P.	L.P.	L.P.	L.P.
1.0	L.P.	L.P.	"	L.P.	"	"	"
2.0	"	"	L.P.	"	"	"	"

Haemoglobin 1.5 ml.

0.5	S.L.P.	S.L.P.	S.L.P.	P.	L.P.	L.P.	L.P.
1.0	L.P.	L.P.	L.P.	L.P.	"	"	"
2.0	"	"	"	"	"	"	"

Haemoglobin 2.0 ml.

0.5	P.	P.	P.	P.	P.	P.	P.
1.0	L.P.	L.P.	P.	P.	L.P.	L.P.	L.P.
2.0	"	"	L.P.	L.P.	"	"	"

In all the plates the plaques were extremely fine. The plate containing 2.0 ml. haemoglobin and 0.5 ml. serum gave no lysis but a broad zone of fine plaques. Lysis, however, did develop in this plate later.

EXPERIMENT 39.

To ascertain the effect of heating haemoglobin at different temperatures on its ability to act as a substrate for the demonstration of Muller's phenomenon, when fresh serum is added.

MEDIA: Haemoglobin heated at 65°C. for 10 minutes and at 75°C. for 10 minutes, in amounts of 0.75 ml. and 1.0 ml. was used to prepare serum-haemoglobin-plates as follows:-

Haemoglobin	0.75	0.75	0.75	1.0	1.0	1.0
"Lab-Lemco" (15%)	1.0	1.0	1.0	1.0	1.0	1.0
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5	4.5
Distilled Water	8.75	8.25	7.75	8.5	8.0	7.5
Serum	0.0	0.5	1.0	0.0	0.5	1.0

The two sets of mixtures were prepared without serum, held at 65°C. and 75°C. respectively for 10 minutes, cooled to 50°C., and after adding the serum, mixed thoroughly and poured.

INOCULA: The plates were stab inoculated from 6 hour old "Lab-Lemco" broth cultures of five strains of staphylococci, 3, 4, 6, 7 and 10.

INCUBATION: The plates were incubated at 37°C. for 24 hours.

READINGS: These were taken after 24 hours at 37°C.

EXPERIMENT 39.

RESULTS: After incubation at 37°C. for 24 hours.

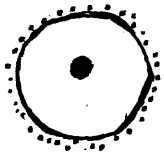
Haemoglobin heated at 65°C. for 10 minutes.

Haemoglobin (ml.)	Serum	3	4	6	7	10
0.75	0.0	L.	L.	L.	L.	-
0.75	0.5	L.P.	L.P.	L.P.	L.P.	L.P.
0.75	1.0	"	"	"	"	"
1.0	0.0	L.	L.	L.	L.	-
1.0	0.5	L.P.	L.P.	L.P.	L.P.	L.P.
1.0	1.0	"	"	"	"	"

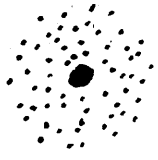
Haemoglobin heated at 75°C. for 10 minutes.

0.75	0.0	L.	L.	L.	L.	-
0.75	0.5	P.	P.	P.	P.	P.
0.75	1.0	"	"	"	"	"
1.0	0.0	L.	L.	L.	L.	-
1.0	0.5	P.	P.	P.	P.	P.
1.0	1.0	"	"	"	"	"

The plates containing haemoglobin heated at 65°C. for 10 minutes gave broader zones of lysis than the plates containing haemoglobin heated at 75°C. for 10 minutes. These latter plates, however, had a marked zone of plaques around their colonies.



Appearance around a colony in plate with haemoglobin heated at 65°C. for 10 minutes.



Appearance around a colony in plate with haemoglobin heated at 75°C. for 10 minutes.

EXPERIMENT 40.

The demonstration of Muller's phenomenon on heated 'haemoglobin' agar plates with added fresh serum.

MEDIA: The 'haemoglobin' used in these plates was prepared from packed human red cells thrice washed with saline which were frozen and then thawed, the resulting fluid containing red cell envelopes in addition to the haemoglobin. This 'haemoglobin' was used in 0.25, 0.5 and 0.75 ml. amounts, to prepare serum-haemoglobin-agar plates as follows:-

'Haemoglobin'	0.25	0.25	0.25
"Lab-Lemco" (15%)	1.0	1.0	1.0
Agar (4.5%)	4.5	4.5	4.5
Distilled Water	9.25	8.75	8.25
Serum	0.0	0.5	1.0

The haemoglobin, "Lab-Lemco", agar and distilled water mixtures were heated at 75°C. for 10 minutes, cooled to 50°C. and the appropriate amounts of serum added. The contents of the tubes were mixed and quickly poured into Petri dishes.

INOCULA: The plates were stab inoculated from 6 hour old "Lab-Lemco" broth cultures of five strains of coagulase positive staphylococci 3, 4, 6, 7 and 10.

INCUBATION: The plates were incubated at 37°C. for 24 hours.

READINGS: These were taken after 24 hours' incubation at 37°C.

EXPERIMENT 40.

RESULTS: After 24 hours' incubation at 37°C.

Haemoglobin (ml.)	Serum	3	4	6	7	10
0.25	0.0	L.	L.	L.	L.	-
"	0.5	P.	P.	P.	-	P.
"	1.0	"	"	"	P.	"
0.5	0.0	L.	L.	L.	L.	-
"	0.5	P.	P.	P.	P.	P.
"	1.0	"	"	"	"	"
0.75	0.0	L.	L.	S.L.	L.	-
"	0.5	P.	P.	P.	P.	P.
"	1.0	"	"	"	"	"

EXPERIMENT 41.

To demonstrate Muller's phenomenon on a heated plasma substrate containing fresh serum.

MEDIA: Plates containing 25% plasma which had been heated at 65°C. for 30 minutes were prepared with and without fresh serum added as follows:-

Heated Plasma (65°C./30 mins.)	3.75	3.75	3.75
Agar (4.5%)	4.5	4.5	4.5
"Lab-Lemco" (15%)	1.0	1.0	1.0
Distilled Water	5.75	5.5	5.25
Serum	0.0	0.25	0.5

INOCULA: The plates were stab inoculated from 6 hour old "Lab-Lemco" broth cultures of strains A, 039 and 935.

INCUBATION: The plates were incubated at 37°C. for 24 hours and then left at room temperature.

READINGS: These were taken after 3 days at room temperature.

EXPERIMENT 41.

RESULTS: After 24 hours' incubation at 37°C. and 72 hours at room temperature.

Amount of added serum (ml.)	A	039	935
0.0	L.*	L.*	L.*
0.25	L.R.E.P.	L.R.E.P.	L.
0.5	"	"	L.

The plate containing 0.5 ml. was photographed, (Fig.XXVIII).

* This lysis was of the indefinite type.

EXPERIMENT 42.

To ascertain the effect of different amounts of sodium chloride added to a modified Chapman's medium with regard to the production of Muller's phenomenon.

MEDIA: Tubes containing 13 ml. of a modified Chapman's medium (App.480-1) were prepared. To each tube 2 ml. plasma was added and amounts of 20% NaCl. to give final concentrations of from 0.0% to 1.6%. The tubes were then heated at 56°C. for 10 minutes and poured into Petri dishes.

Modified Chapman's Medium	13.0	13.0	13.0	13.0	13.0	13.0	13.0	13.0	13.0
Plasma	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
20% NaCl.	0.0	0.15	0.3	0.45	0.6	0.75	0.9	1.05	1.2
Final Concentration of NaCl. (%).	0.0	0.2	0.4	0.6	0.8	1.0	1.2	1.4	1.6

INOCULA: These plates were stab inoculated from 6 hour old "Lab-Lemco" broth cultures of strains A, 401, 406, 039, 061, 779 and 935.

INCUBATION: The plates were incubated at 37°C. for 48 hours.

READINGS: These were made after 24 hours' and after 48 hours' incubation at 37°C.

EXPERIMENT 42.

RESULTS: After 24 hours at 37°C.

Concentration of NaCl.	A	401	406	039	061	779	935
0.0%	L.Ppt.	L.P.	S.L.	L.P.	L.P.	L.P.	S.L.
0.2%	L.P.	"	L.P.	"	"	"	-
0.4%	L.Ppt.	"	"	"	"	S.L.	Ppt.
0.6%	"	L.R.E.	L.	L.	L.	L.	"
0.8%	"	L.Ppt.	S.L.	L.	L.^{x}	L.	"
1.0%	"	L.	-	L.^{x}	L.^{x}	S.L.	"
1.2%	L.	S.L.	-	L.	-	"	"
1.4%	"	-	-	"	-	-	"
1.6%	"	S.L.	-	"	-	-	"

After 48 hours' incubation at 37°C.

0.0%	L.	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.
0.2%	"	"	"	"	"	"	"
0.4%	"	"	"	"	"	"	"
0.6%	L.P.	L.	L.	L.	"	L.	L.
0.8%	"	L.P.	L.P.	"	"	L.P.	L.P.
1.0%	L.^{x}	L.^{x}	L.^{x}	L.^{x}	L.^{x}	L.^{x}	Ppt.
1.2%	L.	"	L.	"	L.	"	"
1.4%	"	"	"	"	"	"	"
1.6%	"	"	S.L.	"	"	L.	"

"x" signifies a "bump" on the zone of lysis.

EXPERIMENT 43.

To ascertain if Muller's phenomenon could be produced in a modified Chapman medium containing plasma heated for different times and at different temperatures.

MEDIA: Modified Chapman medium was dispensed in tubes (App.480-1) containing 13 ml. amounts. To this was added heated human plasma (heated at 56°C. for 5, 10 or 15 minutes or at 60°C. for 5, 10 or 15 minutes) in 2 ml. amounts. All tubes received 0.15 ml. of 20% NaCl. To one set of plates 0.5 ml. of fresh serum was added, the other set received 0.5 ml. of distilled water.

Modified Chapman's Medium	13.0	13.0
Plasma (heated)	2.0	2.0
NaCl. (20%)	0.15	0.15
Serum	0.0	0.5

INOCULA: These plates were stab inoculated from 6 hour old "Lab-Lemco" broth cultures of strains A, 401, 406, 039, 061, 779 and 935.

INCUBATION: Plates were incubated at 37°C. for 24 hours and then left at room temperature for 48 hours.

READINGS: These were taken after 24 hours at 37°C. and after 48 hours at room temperature.

EXPERIMENT 43.

RESULTS: After 24 hours' incubation at 37°C.

Without added serum.

Plasma heated at	A	401	406	039	061	779	935
56°C./5 mins.	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.
" 10 "	"	"	"	"	"	"	"
" 15 "	"	"	"	"	"	"	"
60°C./5 mins.	L.	L.	L.	L.	L.	L.	L.
" 10 "	"	S.L.	-	"	"	S.L.	-
" 15 "	S.L.	-	-	-	S.L.	-	-

With 0.5 ml. fresh serum added.

56°C./5 mins.	L.R.E.	L.P.	L.P.	L.R.E.	L.P.	L.R.E.	L.P.
" 10 "	L.P.	"	"	L.P.	"	L.P.	"
" 15 "	L.R.E.	"	"	"	"	"	"
60°C./5 mins.	L.	L.R.E.	L.R.E.	"	L.	L.	L.
" 10 "	"	"	L.P.	L.R.E.	"	L.P.	L.R.E.
" 15 "	"	"	L.	"	"	L.	L.

EXPERIMENT 43 (Continued).

RESULTS: After 24 hours at 37°C. and 48 hours at room temperature.

Without added serum.

Plasma heated at	A	401	406	039	061	779	935
56°C./5 mins.	L.R.E.	L.P.	L.P.	L.R.E.	L.R.E.	L.R.E.	L.P.
" 10 "	L.P.	"	"	L.P.	L.P.	L.P.	"
" 15 "	"	"	"	"	"	"	"
60°C./5 mins.	L.	L.	L.	L.	L.	L.	L.
" 10 "	"	"	"	"	"	"	"
" 15 "	"	"	"	"	"	"	"

With 0.5 ml. of fresh serum added.

56°C./5 mins.	L.	L.R.E.	L.R.E.	L.	L.	L.	L.
" 10 "	"	L.	L.	"	"	"	"
" 15 "	"	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.
60°C./5 mins.	"	L.	L.	L.	L.	L.	L.
" 10 "	"	L.P.	L.P.	L.P.	"	"	"
" 15 "	"	"	"	L.R.E.	"	"	L.P.

EXPERIMENT 44.

To ascertain the effect on the production of Muller's phenomenon by staphylococci in a heated plasma-agar medium containing 1% "Lab-Lemco", of different combinations of temperature and time employed in the heating of the plasma; and the effect of using different amounts of this heated plasma, of the addition of fresh human serum and of varied amounts of sodium chloride.

MEDIA: The heated plasma plates were made as follows:-

Agar (4.5%)	4.5	4.5	4.5	4.5	4.5	4.5
"Lab-Lemco" (15%)	1.0	1.0	1.0	1.0	1.0	1.0
Heated Plasma	1.0	2.0	3.0	1.0	2.0	3.0
NaCl. (20%)	0.0	0.0	0.0	0.0	0.0	0.0
Serum	0.0	0.0	0.0	0.5	0.5	0.5
Distilled Water	8.5	7.5	6.5	8.0	7.0	6.0

Similar sets of plates containing sodium chloride 0.2% and 0.8% (final concentrations) were prepared by the addition of 0.15 ml. and 0.6 ml. of a 20% Sodium chloride solution respectively.

The temperatures at which aliquots of plasma were heated were 53°C., 56°C. and 60°C.; the times employed at each of these temperatures were 3 minutes, 10 minutes, 15 minutes and 30 minutes.

INOCULA: These sets of plates were stab inoculated from 6 hour old "Lab-Lemco" broth cultures of A, 401, 039, 061, 779 and 935.

INCUBATION: The plates were incubated at 37°C. for 24 hours and then at room temperature for 24 hours.

READINGS: These were taken after 24 hours at 37°C. and 24 hours at room temperature.

EXPERIMENT 44.

RESULTS: After 24 hours at 37°C.

Plasma heated at 53°C. for 3 minutes.

NaCl.	Plasma	Serum	A	401	039	061	779	935
0.0%	1.0	0.0	L.	L.	L.	L.	L.	L.
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	–
0.2%	1.0	0.0	"	"	"	"	"	"
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.8%	1.0	0.0	"	"	"	"	"	"
	2.0		L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.
	3.0		"	"	"	"	"	"
0.0%	1.0	0.5	L.	L.	L.	L.	L.	L.
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.2%	1.0	0.5	"	"	"	"	"	"
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.8%	1.0	0.5	"	"	"	"	"	"
	2.0		L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.
	3.0		"	"	"	"	"	"

After 24 hours at room temperature.

0.0%	1.0	0.0	L.	L.	L.	L.	L.	L.
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.2%	1.0	0.0	"	"	"	"	"	"
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.8%	1.0	0.0	"	"	"	L.R.E.	"	L.R.E.
	2.0		L.R.E.	L.R.E.	"	"	L.R.E.	"
	3.0		"	L.P.	"	L.	L.	"
0.0%	1.0	0.5	L.	L.	L.	L.	L.	L.
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.2%	1.0	0.5	"	"	"	"	"	"
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.8%	1.0	0.5	"	"	"	"	"	"
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"

EXPERIMENT 44 (Continued).

RESULTS: After 24 hours at 37°C.

Plasma heated at 53°C. for 10 minutes.

NaCl.	Plasma	Serum	A	401	039	061	779	935
0.0%	1.0	0.0	L.	L.	L.	L.	L.	L.
	2.0		"	"	"	"	"	–
	3.0		"	"	"	"	"	L.
0.2%	1.0	0.0	"	"	"	"	"	"
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.8%	1.0	0.0	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.	"
	2.0		L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.
	3.0		"	L.R.E.	L.R.E.	L.R.E.	"	–
0.0%	1.0	0.5	L.	L.	L.	L.	L.	L.
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.2%	1.0	0.5	"	"	"	"	"	"
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.8%	1.0	0.5	"	"	"	"	"	"
	2.0		L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.
	3.0		...	...	...	...	...	...

After 24 hours at room temperature.

0.0%	1.0	0.0	L.	L.	L.	L.	L.	L.
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.2%	1.0	0.0	"	"	"	"	"	"
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.8%	1.0	0.0	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.0%	1.0	0.5	L.	L.	L.	L.	L.	L.
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.2%	1.0	0.5	"	"	"	"	"	"
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.8%	1.0	0.5	"	"	"	"	"	"
	2.0		"	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.
	3.0		...	...	...	...	...	...

EXPERIMENT 44 (Continued).

RESULTS: After 24 hours at 37°C.

Plasma heated at 53°C. for 15 minutes.

NaCl.	Plasma	Serum	A	401	039	061	779	935
0.0%	1.0	0.0	L.	L.	L.	L.	L.	L.
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.2%	1.0	0.0	"	"	"	"	"	"
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.8%	1.0	0.0	"	"	"	"	"	S.L.
	2.0		L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.	-
	3.0		"	"	"	"	"	L.R.E.
0.0%	1.0	0.5	L.	L.	L.	L.	L.	L.
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.2%	1.0	0.5	"	"	"	"	"	"
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.8%	1.0	0.5	"	"	"	"	"	"
	2.0		L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.	"
	3.0		"	"	"	"	L.	"

After 24 hours at room temperature

0.0%	1.0	0.0	L.	L.	L.	L.	L.	L.
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.2%	1.0	0.0	"	"	"	"	"	"
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.8%	1.0	0.0	L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.	L.R.E.	L.R.E.P.
	2.0		"	"	"	L.R.E.P.	L.R.E.P.	L.R.E.
	3.0		"	"	L.R.E.	L.R.E.	"	"
0.0%	1.0	0.5	L.	L.	L.	L.	L.	L.
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.2%	1.0	0.5	"	"	"	"	"	"
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.8%	1.0	0.5	"	"	"	"	"	L.R.E.
	2.0		"	"	"	"	"	L.
	3.0		"	L.R.E.	"	L.R.E.	"	"

EXPERIMENT 44 (Continued).

RESULTS: After 24 hours at 37°C.

Plasma heated at 53°C. for 30 minutes.

NaCl.	Plasma	Serum	A	401	039	061	779	935
0.0%	1.0	0.0	L.	L.	L.	L.	L.	L.
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.2%	1.0	0.0	"	"	"	"	"	"
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.8%	1.0	0.0	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.
	2.0		"	"	"	"	"	S.L.
	3.0		L.R.E.P.	"	"	L.R.E.P.	L.R.E.P.	L.R.E.
0.0%	1.0	0.5	L.	L.	L.	L.	L.	L.
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.2%	1.0	0.5	"	"	"	"	"	"
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.8%	1.0	0.5	"	"	"	"	"	"
	2.0		L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.	"
	3.0		"	"	"	"	"	L.R.E.

After 24 hours at room temperature.

0.0%	1.0	0.0	L.	L.	L.	L.	L.	L.
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.2%	1.0	0.0	"	"	"	"	"	"
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.8%	1.0	0.0	L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.
	2.0		"	"	L.R.E.	L.R.E.	L.R.E.	L.
	3.0		"	"	L.	"	"	"
0.0%	1.0	0.5	L.	L.	"	L.	L.	"
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.2%	1.0	0.5	"	"	"	"	"	"
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.8%	1.0	0.5	"	"	"	"	"	"
	2.0		L.R.E.	"	"	"	"	"
	3.0		L.	"	"	"	"	"

RESULTS: After 24 hours at 37°C.

Plasma heated at 56°C. for 3 minutes.

NaCl.	Plasma	Serum	A	401	039	061	779	935
0.0%	1.0	0.0	L.	L.	L.	L.	L.	L.
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.2%	1.0	0.0	"	"	"	"	"	"
	2.0		"	"	"	"	"	"
	3.0		L.P.	L.P.	L.P.	L.P.	L.P.	L.P.
0.8%	1.0	0.0	"	"	"	"	"	"
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.0%	1.0	0.5	L.	L.	L.	L.	L.	L.
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.2%	1.0	0.5	"	"	"	"	"	"
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.8%	1.0	0.5	"	L.R.E.	L.R.E.	"	"	L.R.E.
	2.0		L.R.E.	"	"	L.R.E.	L.R.E.	"
	3.0		"	"	"	L.R.E.P.	"	L.R.E.P.

After 24 hours at room temperature.

0.0%	1.0	0.0	L.	L.	L.	L.	-	L.
	2.0		"	"	"	"	-	"
	3.0		"	"	"	"	L.	"
0.2%	1.0	0.0	"	"	"	"	"	"
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.8%	1.0	0.0	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.
	2.0		"	"	"	"	"	"
	3.0		L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.
0.0%	1.0	0.5	L.	L.	L.	L.	L.	L.
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.2%	1.0	0.5	"	"	"	"	"	"
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.8%	1.0	0.5	"	"	"	"	"	"
	2.0		"	"	"	"	"	L.R.E.
	3.0		"	"	L.R.E.	"	L.R.E.	"

EXPERIMENT 44 (Continued).

RESULTS: After 24 hours at 37°C.

Plasma heated at 56°C. for 10 minutes.

NaCl.	Plasma	Serum	A	401	039	061	779	935
0.0%	1.0	0.0	L.	L.	L.	L.	-	L.
	2.0		"	"	"	"	L.	"
	3.0		"	"	"	"	-	"
0.2%	1.0	0.0	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.
	2.0		"	"	"	"	"	"
	3.0		L.	L.	L.	L.	L.	L.
0.8%	1.0	0.0	L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.	L.R.E.	L.R.E.P.
	2.0		L.P.	L.P.	L.P.	L.P.	L.P.	L.P.
	3.0		"	"	"	"	"	L.R.E.
0.0%	1.0	0.5	L.	L.	L.	L.	-	L.
	2.0		"	"	"	"	-	"
	3.0		"	"	"	"	-	"
0.2%	1.0	0.5	"	"	"	"	-	"
	2.0		"	"	"	"	L.	"
	3.0		"	"	"	"	"	"
0.8%	1.0	0.5	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.
	2.0		"	"	"	"	"	"
	3.0		L.R.E.P.	L.R.E.P.	L.R.E.P.	"	L.R.E.P.	L.R.E.P.

After 24 hours at room temperature.

0.0%	1.0	0.0	L.	L.	L.	L.	L.	L.
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	-	"
0.2%	1.0	"	"	"	"	"	L.	"
	2.0	0.0	"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.8%	1.0	0.0	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.
	2.0		"	"	"	"	"	L.R.E.P.
	3.0		"	"	"	"	"	"
0.0%	1.0	0.5	L.	L.	L.	L.	L.	L.
	2.0		"	"	"	"	-	"
	3.0		"	"	"	"	-	"
0.2%	1.0	0.5	"	"	"	"	-	"
	2.0		"	"	"	"	L.	"
	3.0		"	"	"	"	"	"
0.8%	1.0	0.5	"	"	"	"	"	"
	2.0		"	"	"	L.R.E	L.R.E.	L.R.E.
	3.0		L.R.E.	"	"	L.	"	"

EXPERIMENT 44 (Continued).

RESULTS: After 24 hours at 37°C.

Plasma heated at 56°C. for 15 minutes.

NaCl.	Plasma	Serum	A	401	039	061	779	935
0.0%	1.0	0.0	L.	L.	L.	L.	L.	L.
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.2%	1.0	0.0	"	"	"	"	"	"
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.8%	1.0	0.0	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.
	2.0		L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.
	3.0		"	"	"	"	"	"
0.0%	1.0	0.5	L.	L.	L.	L.	-	L.
	2.0		"	"	"	"	-	"
	3.0		"	"	"	"	-	"
0.2%	1.0	0.5	"	"	"	"	L.	"
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.8%	1.0	0.5	"	"	"	"	"	"
	2.0		L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.
	3.0		"	"	"	"	"	L.R.E.

After 24 hours at room temperature.

0.0%	1.0	0.0	L.	L.	L.	L.	-	L.
	2.0		"	"	"	"	L.	"
	3.0		"	"	"	"	-	"
0.2%	1.0	0.0	"	"	"	"	L.	"
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.8%	1.0	0.0	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.
	2.0		"	"	"	"	"	"
	3.0		L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.
0.0%	1.0	0.5	L.	L.	L.	L.	-	L.
	2.0		"	"	"	"	-	"
	3.0		"	"	"	"	-	"
0.2%	1.0	0.5	"	"	"	"	L.	"
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.8%	1.0	0.5	"	"	"	"	"	"
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	L.R.E.

EXPERIMENT 44 (Continued).

RESULTS: After 24 hours at 37°C.

Plasma heated at 56°C. for 30 minutes:

NaCl.	Plasma	Serum	A	401	039	061	779	935
0.0%	1.0	0.0	-	-	-	-	-	-
	2.0		S.L.Ppt.	L.	-	L.	-	Ppt.
	3.0		Ppt.	"	Ppt.	-	-	"
0.2%	1.0	0.0	L.	"	L.	L.	L.	L.
	2.0		L.Ppt.	"	"	"	"	"
	3.0		L.	"	"	"	"	"
0.8%	1.0	0.0	L.P.	L.P.	L.R.E.	L.R.E.P.	L.R.E.	L.R.E.P.
	2.0		L.R.E.P.	L.R.E.P.	L.R.E.P.	"	L.R.E.P.	"
	3.0		"	"	"	"	"	"
0.0%	1.0	0.5	L.	L.	L.	L.	L.	L.
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.2%	1.0	0.5	"	"	"	"	"	"
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.8%	1.0	0.5	"	"	"	"	"	"
	2.0		L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.
	3.0		"	"	"	"	"	"

After 24 hours at room temperature.

0.0%	1.0	0.0	-	-	-	-	-	-
	2.0		S.L.	S.L.	S.L.	S.L.	S.L.	S.L.
	3.0		S.L.Ppt.	"	S.L.Ppt.	"	"	S.L.Ppt.
0.2%	1.0	0.0	L.	L.	L.	L.	L.	L.
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.8%	1.0	0.0	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.
	2.0		L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.
	3.0		"	"	"	"	"	"
0.0%	1.0	0.5	L.	L.	L.	L.	L.	L.
	2.0		"	"	"	"	-	"
	3.0		"	"	"	"	-	"
0.2%	1.0	0.5	"	"	"	"	E.	"
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.8%	1.0	0.5	"	"	"	"	"	"
	2.0		"	"	"	"	"	"
	3.0		L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.

EXPERIMENT 44 (Continued).

RESULTS: After 24 hours at 37°C.

Plasma heated at 60°C. for 3 minutes.

NaCl.	Plasma	Serum	A	401	039	061	779	935
0.0%	1.0		L.	S.L.	-	-	-	-
	2.0	0.0	"	L.	L.	L.	-	L.
	3.0		"	"	"	"	-	"
0.2%	1.0		"	"	"	"	L.	"
	2.0	0.0	"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.8%	1.0		L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.
	2.0	0.0	L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.
	3.0		"	"	"	"	"	"
0.0%	1.0		L.	L.	L.	L.	L.	L.
	2.0	0.5	"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.2%	1.0		"	"	"	"	"	"
	2.0	0.5	"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.8%	1.0		L.R.E.	"	L.R.E.	"	"	"
	2.0	0.5	"	L.R.E.	"	L.R.E.	L.R.E.	L.R.E.
	3.0		"	"	"	"	"	"

After 24 hours at room temperature.

0.0%	1.0		S.L.	S.L.	-	S.L.	-	-
	2.0	0.0	L.Ppt.	L.	L.	L.	L.	L.Ppt.
	3.0		"	"	"	"	"	"
0.2%	1.0		L.	"	"	"	"	L.
	2.0	0.0	"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.8%	1.0		"	L.R.E.P.	"	L.R.E.	"	L.R.E.
	2.0	0.0	L.R.E.	"	L.R.E.	"	L.R.E.	L.R.E.P.
	3.0		L.	L.R.E.	"	"	"	L.R.E.
0.0%	1.0		"	L.	L.	L.	L.	L.
	2.0	0.5	"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.2%	1.0		"	"	"	"	"	"
	2.0	0.5	"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.8%	1.0		"	"	"	"	"	"
	2.0	0.5	"	"	"	"	"	"
	3.0		"	"	"	"	"	L.R.E.

EXPERIMENT 44 (Continued).

RESULTS: After 24 hours at 37°C.

Plasma heated at 60°C. for 10 minutes.

NaCl.	Plasma	Serum	A	401	039	061	779	935
0.0%	1.0	0.0	-	-	-	-	-	-
	2.0		S.L.	S.L.	S.L.	S.L.	S.L.	S.L.
	3.0		(...)	(...)	(...)	(...)	(...)	(...)
0.2%	1.0	0.0	L.	L.	L.	L.	-	L.
	2.0		"	"	"	"	L.	"
	3.0		"	"	"	"	-	"
0.8%	1.0	0.0	"	"	"	"	L.	"
	2.0		"	"	"	L.R.E.	"	"
	3.0		"	"	"	L.	"	"
0.0%	1.0	0.5	"	"	"	"	-	"
	2.0		"	"	"	"	-	"
	3.0		"	"	"	"	-	"
0.2%	1.0	0.5	"	"	"	"	L.	"
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.8%	1.0	0.5	L.R.E.	"	"	"	"	"
	2.0		"	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.
	3.0		"	"	"	"	"	"

After 24 hours at room temperature.

0.0%	1.0	0.0	-	-	-	-	-	-
	2.0		S.L.	S.L.	S.L.	S.L.	S.L.	S.L.
	3.0		(...)	(...)	(...)	(...)	(...)	(...)
0.2%	1.0	0.0	L.	L.	L.	L.	L.	L.
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	-	"
0.8%	1.0	0.0	"	"	"	"	L.	"
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.0%	1.0	0.5	"	"	"	"	"	"
	2.0		"	"	"	"	-	"
	3.0		"	"	"	"	L.	"
0.2%	1.0	0.5	"	"	"	"	"	"
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.8%	1.0	0.5	L.R.E.	"	"	"	"	"
	2.0		L.	L.R.E.	"	"	"	"
	3.0		L.R.E.	"	"	"	"	"

RESULTS: After 24 hours at 37°C.

Plasma heated at 60°C. for 15 minutes.

NaCl.	Plasma	Serum	A	401	039	061	779	935
0.0%	1.0	0.0	L.	S.L.	L.	S.L.	-	L.
	2.0		"	"	"	L.	-	-
	3.0		S.L.	"	"	"	-	-
0.2%	1.0	0.0	L.	L.	"	"	S.L.	L.
	2.0		"	"	"	"	-	"
	3.0		"	"	"	"	S.L.	"
0.8%	1.0	0.0	"	L.R.E.	L.R.E.	"	L.	"
	2.0		"	L.	L.	"	"	"
	3.0		"	"	"	"	"	"
0.0%	1.0	0.5	"	"	"	"	-	"
	2.0		"	"	"	"	L.	"
	3.0		"	"	"	"	"	"
0.2%	1.0	0.5	"	"	"	"	"	"
	2.0		"	"	"	"	-	"
	3.0		"	"	"	"	L.	"
0.8%	1.0	0.5	"	"	L.R.E.	"	"	"
	2.0		L.R.E.	L.R.E.	L.R.E.P.	L.R.E.	L.R.E.	L.R.E.
	3.0		L.	L.	L.	L.	L.	L.

After 24 hours at room temperature

0.0%	1.0	0.0	L.	S.L.	L.	S.L.	-	L.
	2.0		"	"	"	L.	-	-
	3.0		S.L.	"	"	"	-	-
0.2%	1.0	0.0	L.	L.	"	"	L.	L.
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.8%	1.0	0.0	"	"	"	"	"	"
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.0%	1.0	0.5	"	"	"	"	-	"
	2.0		"	"	"	"	L.	"
	3.0		"	"	"	"	"	"
0.2%	1.0	0.5	"	"	"	"	"	"
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.8%	1.0	0.5	"	"	"	"	"	"
	2.0		"	"	"	"	"	L.R.E.
	3.0		"	"	"	"	"	"

RESULTS: After 24 hours at 37°C.

Plasma heated at 60°C. for 30 minutes.

NaCl.	Plasma	Serum	A	401	039	061	779	935
	1.0		-	-	-	-	-	-
0.0%	2.0	0.0	S.L.	-	S.L.	-	-	-
	3.0		-	S.L.	"	-	-	-
	1.0		L.	L.	L.	L.	S.L.	L.
0.2%	2.0	0.0	"	"	"	"	-	-
	3.0		"	"	"	"	-	L.
	1.0		"	"	"	"	L.	"
0.8%	2.0	0.0	"	"	"	"	"	S.L.
	3.0		"	"	"	"	"	L.
	1.0		"	"	"	"	S.L.	"
0.0%	2.0	0.5	"	"	"	"	-	"
	3.0		"	"	"	"	S.L.	"
	1.0		"	"	"	"	L.	"
0.2%	2.0	0.5	"	"	"	"	"	"
	3.0		"	"	"	"	"	"
	1.0		L.R.E.	L.R.E.	L.R.E.	"	"	"
0.8%	2.0	0.5	L.	L.R.E.P.	L.R.E.P.	L.R.E.	L.R.E.	L.R.E.P.
	3.0		"	L.R.E.	L.R.E.	"	"	L.R.E.

After 24 hours at room temperature.

	1.0		-	-	-	-	-	-
0.0%	2.0	0.0	S.L.	S.L.	S.L.	L.	L.	L.
	3.0		-	"	"	-	-	-
	1.0		L.	L.	L.	L.	L.	L.
0.2%	2.0	0.0	"	"	"	"	-	-
	3.0		"	"	"	"	L.	L.
	1.0		"	"	"	"	"	"
0.8%	2.0	0.0	"	"	"	"	"	"
	3.0		"	"	"	"	"	"
	1.0		"	"	"	"	"	"
0.0%	2.0	0.5	"	"	"	"	-	"
	3.0		"	"	"	"	S.L.	"
	1.0		"	"	"	"	L.	"
0.2%	2.0	0.5	"	"	"	"	"	"
	3.0		"	"	"	"	"	"
	1.0		"	"	"	"	"	"
0.8%	2.0	0.5	"	"	"	"	"	"
	3.0		"	"	"	"	"	L.R.E.P.

EXPERIMENT 45.

To ascertain the effect on the production of Muller's phenomenon in a heated plasma-agar medium (Modified Chapman's medium) of different combinations of temperature and time employed in the heating of the plasma; and of the effect of using different amounts of this heated plasma, of the addition of fresh human serum and of varied amounts of Sodium chloride.

MEDIA: The heated plasma agar plates were made as follows:-

Chapman's Medium (Modified).	10.0	10.0	10.0	10.0	10.0	10.0
Heated Plasma	1.0	2.0	3.0	1.0	2.0	3.0
Serum	0.0	0.0	0.0	0.5	0.5	0.5
NaCl. (20%)	0.0	0.0	0.0	0.0	0.0	0.0
Distilled Water	4.0	3.0	2.0	3.5	2.5	1.5

Similar sets of plates containing sodium chloride 0.2% and 0.8% (final concentrations) were prepared by the addition of 0.15 ml. and 0.6 ml. of a 20% sodium chloride solution, respectively.

The temperatures at which aliquots of plasma were heated were 53°C., 56°C. and 60°C.; the periods of time for which plasma was heated at these temperatures were 3 minutes, 10 minutes, 15 minutes and 30 minutes.

INOCULA: These sets of plates were stab inoculated from 6 hour old "Lab-Lemco" broth cultures of A, 401, 039, 061, 779 and 935.

INCUBATION: The plates were incubated at 37°C. for 24 hours and then at room temperature for 24 hours.

READINGS: These were taken after 24 hours at 37°C. and after 24 hours at room temperature.

EXPERIMENT 45.

RESULTS: After 24 hours at 37°C.

Plasma heated at 53°C. for 3 minutes.

NaCl.	Plasma	Serum	A	401	039	061	779	935
0.0%	1.0	0.0	L.	L.	L.	L.	-	L.
	2.0		"	"	"	"	L.	-
	3.0		"	"	"	"	-	L.
0.2%	1.0	0.0	"	"	"	"	L.	"
	2.0		L.R.E.	"	"	"	"	"
	3.0		S.L.	L.R.E.	L.R.E.	L.R.E.	L.R.E.	"
0.8%	1.0	0.0	L.R.E.	L.R.E.P.	"	"	L.	Ppt.
	2.0		"	"	L.R.E.P.	L.R.E.P.	L.R.E.P.	"
	3.0		L.	L.R.E.	L.R.E.	L.R.E.	L.R.E.	"
0.0%	1.0	0.5	"	L.	L.	L.	L.	L.
	2.0		S.L.	"	"	"	"	"
	3.0		"	"	"	"	-	Ppt.
0.2%	1.0	0.5	L.	"	"	"	L.	L.
	2.0		"	"	"	"	"	"
	3.0		S.L.	"	"	"	"	"
0.8%	1.0	0.5	-	L.R.E.	L.R.E.	L.R.E.	L.	L.R.E.
	2.0		L.	"	"	"	L.R.E.	-
	3.0		"	L.R.E.P.	"	"	S.L.	Ppt.

After 24 hours at room temperature.

0.0%	1.0	0.0	L.	L.	L.	L.	L.	L.
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.2%	1.0	0.0	"	"	"	"	"	"
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	L.R.E.
0.8%	1.0	0.0	"	L.R.E.	"	L.R.E.	L.R.E.	"
	2.0		"	L.	"	L.	L.	Ppt.
	3.0		"	"	"	L.R.E.	"	"
0.0%	1.0	0.5	"	"	"	L.	"	L.
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.2%	1.0	0.5	"	"	"	"	"	"
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.8%	1.0	0.5	-	"	"	"	"	"
	2.0		L.	"	"	"	"	Ppt.
	3.0		"	"	"	"	"	

EXPERIMENT 45 (Continued).

RESULTS: After 24 hours at 37°C.

Plasma heated at 53°C. for 10 minutes.

NaCl.	Plasma	Serum	A	401	039	061	779	935
0.0%	1.0	0.0	L.	L.	L.	L.	L.	L.
	2.0		L.R.E.	"	"	"	"	Ppt.
	3.0		S.L.	"	"	"	-	"
0.2%	1.0	0.0	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.
	2.0		"	"	"	"	"	-
	3.0		"	"	L.P.	"	L.	-
0.8%	1.0	0.0	"	"	L.R.E.	"	L.R.E.	-
	2.0		L.	L.R.E.P.	L.R.E.P.	L.P.	L.	Ppt.
	3.0		"	L.P.	L.P.	"	S.L.	-
0.0%	1.0	0.5	"	L.	L.	L.	L.	L.
	2.0		"	"	"	"	"	"
	3.0		Ppt.	"	"	"	-	Ppt.
0.2%	1.0	0.5	L.	"	"	"	L.	L.
	2.0		L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.	-
	3.0		"	"	"	"	"	-
0.8%	1.0	0.5	L.	"	L.	L.	L.	L.
	2.0		"	"	L.R.E.P.	L.R.E.P.	L.R.E.	-
	3.0		"	L.P.	L.R.E.	L.P.	-	-

After 24 hours at room temperature.

0.0%	1.0	0.0	L.	L.	L.	L.	L.	L.
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.2%	1.0	0.0	"	"	"	"	"	"
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	S.L.P.
0.8%	1.0	0.0	"	"	"	"	"	L.R.E.
	2.0		"	L.R.E.	L.R.E.	L.R.E.	"	Ppt.
	3.0		"	"	L.	L.	"	L.R.E.
0.0%	1.0	0.5	"	L.	"	"	"	L.
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.2%	1.0	0.5	"	"	"	"	"	"
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	L.R.E.
0.8%	1.0	0.5	"	"	"	"	"	L.
	2.0		"	"	"	"	"	Ppt.
	3.0		"	L.R.E.	"	"	S.L.	"

EXPERIMENT 45 (Continued).

RESULTS: After 24 hours at 37°C.

Plasma heated at 53°C. for 15 minutes.

NaCl.	Plasma	Serum	A	401	039	061	779	935
0.0%	1.0	0.0	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.2%	1.0	0.0	"	"	"	"	"	"
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.8%	1.0	0.0	L.	L.R.E.P.	L.R.E.	L.R.E.	"	L.R.E.P.
	2.0		"	L.P.	L.P.	L.P.	"	L.P.
	3.0		"	"	"	"	"	Ppt.
0.0%	1.0	0.5	"	L.	L.	L.	L.	L.
	2.0		L.R.E.	L.P.	L.P.	L.P.	L.P.	L.P.
	3.0		L.Ppt.	"	L.	L.	-	S.L.
0.2%	1.0	0.5	L.	L.	"	"	L.	L.
	2.0		L.Ppt.	L.P.	L.P.	L.R.E.P.	L.P.	"
	3.0		"	"	"	L.P.	-	Ppt.
0.8%	1.0	0.5	L.R.E.	L.	L.R.E.	L.R.E.	L.	L.R.E.
	2.0		L.	L.R.E.P.	L.R.E.P.	"	L.R.E.P.	Ppt.
	3.0		"	L.R.E.	L.R.E.	"	-	"

After 24 hours at room temperature.

0.0%	1.0	0.0	L.	L.	L.	L.	L.	L.
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.2%	1.0	0.0	"	"	"	"	"	"
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.8%	1.0	0.0	"	L.R.E.	"	"	"	"
	2.0		"	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.
	3.0		"	"	"	"	"	Ppt.
0.0%	1.0	0.5	"	L.	L.	L.	L.	L.
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.2%	1.0	0.5	"	"	"	"	"	"
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.8%	1.0	0.5	"	"	"	"	"	"
	2.0		"	"	"	"	"	"
	3.0		"	L.R.E.	L.R.E.	"	"	Ppt.

EXPERIMENT 45 (Continued).

RESULTS: After 24 hours at 37°C.

Plasma heated at 53°C. for 30 minutes.

NaCl.	Plasma	Serum	A	401	039	061	779	935
0.0%	1.0	0.0	L.	L.	L.	L.	L.	L.
	2.0		"	"	"	"	"	-
	3.0		L.R.E.	L.P.	L.P.	L.P.	L.R.E.	Ppt.
0.2%	1.0	0.0	L.	L.	L.	L.	L.	"
	2.0		L.P.	L.P.	L.P.	L.P.	L.P.	"
	3.0		"	"	"	"	"	L.P.
0.8%	1.0	0.0	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.	-
	2.0		L.	L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.	-
	3.0		"	L.P.	L.P.	L.P.	-	Ppt.
0.0%	1.0	0.5	"	L.	L.	L.	L.	L.
	2.0		"	L.P.	L.P.	L.P.	L.P.	"
	3.0		"	"	"	"	"	L.P.
0.2%	1.0	0.5	"	L.	L.	L.	L.	L.
	2.0		L.R.E.	L.R.E.P.	L.R.E.	L.R.E.P.	L.R.E.P.	"
	3.0		L.	L.P.	L.P.	L.P.	L.P.	"
0.8%	1.0	0.5	"	L.	L.	L.	L.	"
	2.0		"	L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.	-
	3.0		"	"	"	"	-	-

After 24 hours at room temperature.

0.0%	1.0	0.0	L.	L.	L.	L.	L.	L.
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.2%	1.0	0.0	"	"	"	"	"	"
	2.0		"	"	"	"	"	L.R.E.
	3.0		"	"	"	"	"	L.
0.8%	1.0	0.0	"	"	"	"	"	"
	2.0		"	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.
	3.0		"	"	L.R.E.P.	"	S.L.	Ppt.
0.0%	1.0	0.5	"	L.	L.	L.	L.	L.
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.2%	1.0	0.5	"	"	"	"	"	"
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.8%	1.0	0.5	"	"	"	"	"	-
	2.0		"	"	"	"	"	S.L.P.
	3.0		"	L.R.E.	L.R.E.	L.R.E.		

RESULTS: After 24 hours at 37°C.

Plasma heated at 56°C. for 3 minutes.

NaCl.	Plasma	Serum	A	401	039	061	779	935
0.0%	1.0	0.0	L.	L.	L.	L.	S.L.	L.
	2.0		"	"	"	"	L.	"
	3.0		"	"	"	"	"	"
0.2%	1.0	0.0	"	"	"	"	"	"
	2.0		"	"	"	"	"	"
	3.0		"	L.P.	"	L.P.	"	-
0.8%	1.0	0.0	L.R.E.	S.L.	"	S.L.	S.L.	-
	2.0		L.	L.	"	"	"	-
	3.0		"	-	"	"	"	-
0.0%	1.0	0.5	"	L.	"	L.	"	L.
	2.0		"	"	"	"	L.	"
	3.0		"	"	"	"	"	"
0.2%	1.0	0.5	"	"	"	"	"	"
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.8%	1.0	0.5	"	"	"	"	"	S.L.
	2.0		"	"	"	"	S.L.	"
	3.0		"	"	L.R.E.	L.R.E.	"	-

After 24 hours at room temperature.

0.0%	1.0	0.0	L.	L.	L.	L.	L.	L.
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.2%	1.0	0.0	"	"	"	"	"	"
	2.0		"	"	"	"	"	"
	3.0		"	L.R.E.P.	"	"	"	L.R.E.P.
0.8%	1.0	0.0	L.P.	L.R.E.	"	"	"	L.
	2.0		L.	L.	"	"	"	-
	3.0		"	"	"	"	"	-
0.0%	1.0	0.5	"	"	"	"	"	L.
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.2%	1.0	0.5	"	"	"	"	"	"
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.8%	1.0	0.5	L.R.E.	L.R.E.	L.R.E.	"	L.R.E.	L.R.E.
	2.0		L.R.E.P.	L.	L.	"	L.	L.
	3.0		L.P.	L.P.	L.R.E.P	"	L.R.E.	"

EXPERIMENT 45 (Continued).

RESULTS: After 24 hours at 37°C.

Plasma heated at 56°C. for 10 minutes.

NaCl.	Plasma	Serum	A	401	039	061	779	935
0.0%	1.0	0.0	L.	L.	L.	L.	L.	L.
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.2%	1.0	0.0	"	"	"	"	"	"
	2.0		"	L.R.E.	"	"	"	L.R.E.
	3.0		"	"	L.R.E.	"	L.R.E.	L.
0.8%	1.0	0.0	"	L.R.E.P.	L.R.E.P.	L.R.E.	-	"
	2.0		"	"	"	L.R.E.P.	L.	-
	3.0		"	L.P.	L.P.	L.P.	S.L.	-
0.0%	1.0	0.5	"	L.	L.	L.	L.	L.
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.2%	1.0	0.5	"	"	"	"	"	"
	2.0		L.R.E.	L.R.E.	"	L.R.E.P.	L.R.E.P.	L.R.E.
	3.0		L.	"	L.R.E.	L.R.E.	L.R.E.	"
0.8%	1.0	0.5	"	L.	L.	L.	L.	L.
	2.0		"	L.R.E.P.	L.R.E.	L.R.E.	L.R.E.P.	-
	3.0		"	"	"	"	"	-

After 24 hours at room temperature.

0.0%	1.0	0.0	L.	L.	L.	L.	L.	L.
	2.0		"	"	"	"	"	"
	3.0		"	L.R.E.	L.R.E.	L.R.E.	"	L.R.E.
0.2%	1.0	0.0	"	L.	L.	"	"	"
	2.0		"	L.R.E.P.	L.R.E.P.	L.	L.R.E.	L.R.E.P.
	3.0		"	"	"	L.R.E.P.	"	"
0.8%	1.0	0.0	"	L.	L.	L.	L.	L.
	2.0		"	L.R.E.P.	"	"	"	L.R.E.
	3.0		"	"	L.R.E.P.	"	"	-
0.0%	1.0	0.5	"	L.	L.	"	"	L.
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.2%	1.0	0.5	"	"	"	"	"	"
	2.0		"	"	"	"	"	L.R.E.
	3.0		"	L.R.E.	"	"	L.R.E.	L.R.E.P.
0.8%	1.0	0.5	"	L.	"	"	L.	L.
	2.0		"	"	"	"	"	"
	3.0		"	L.R.E.P.	"	"	"	"

EXPERIMENT 45 (Continued).

RESULTS: After 24 hours at 37°C.

Plasma heated at 56°C. for 15 minutes.

NaCl.	Plasma	Serum	A	401	039	061	779	935
0.0%	1.0	0.0	L.	L.	L.	L.	L.	L.
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.2%	1.0	0.0	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.
	2.0		L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.
	3.0		"	L.P.	L.P.	L.P.	L.P.	L.P.
0.8%	1.0	0.0	L.	L.R.E.P.	L.R.E.	L.R.E.P.	L.	Ppt.
	2.0		"	"	L.R.E.P.	"	L.R.E.P.	"
	3.0		"	"	"	"	-	"
0.0%	1.0	0.5	"	L.	L.	L.	L.	L.
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.2%	1.0	0.5	"	"	"	L.R.E.	L.R.E.	"
	2.0		"	L.R.E.	L.R.E.P.	L.R.E.P.	"	L.R.E.P.
	3.0		"	L.P.	"	"	L.P.	L.P.
0.8%	1.0	0.5	"	L.	L.	L.	L.	L.
	2.0		"	L.R.E.P.	L.R.E.P.	L.R.E.	L.R.E.	Ppt.
	3.0		"	"	"	L.R.E.P.	S.L.P.	"

After 24 hours at room temperature.

0.0%	1.0	0.0	L.	L.	L.	L.	L.	L.
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.2%	1.0	0.0	"	"	"	"	"	"
	2.0		"	"	"	"	"	"
	3.0	"	"	L.R.E.	"	"	"	L.R.E.
0.8%	1.0	0.0	"	"	L.R.E.P.	"	"	Ppt.
	2.0		"	L.R.E.P.	"	L.R.E.P.	"	"
	3.0		"	"	"	L.	L.R.E.P.	"
0.0%	1.0	0.5	"	L.	L.	"	L.	L.
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.2%	1.0	0.5	"	"	"	"	"	"
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.8%	1.0	0.5	"	"	"	"	"	"
	2.0		"	"	"	"	"	"
	3.0		"	L.R.E.P.	L.R.E.P.	"	"	Ppt.

EXPERIMENT 45 (Continued).

RESULTS: After 24 hours at 37°C.

Plasma heated at 56°C. for 30 minutes.

NaCl.	Plasma	Serum	A	401	039	061	779	935
0.0%	1.0	0.0	L.	L.	L.	L.	L.	L.
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.2%	1.0	0.0	"	"	"	"	"	"
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.8%	1.0	0.0	"	L.R.E.	L.R.E.P.	L.R.E.P.	L.R.E.	L.R.E.P.
	2.0		"	L.R.E.P.	"	"	"	Ppt.
	3.0		"	"	"	"	L.	S.L.
0.0%	1.0	0.5	"	L.	L.	L.	"	L.
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.2%	1.0	0.5	"	"	"	"	"	"
	2.0		"	"	"	"	"	"
	3.0		"	"	"	L.R.E.	L.P.	"
0.8%	1.0	0.5	"	L.R.E.P.	L.R.E.P.	L.R.E.	L.R.E.	L.R.E.P.
	2.0		"	L.R.E.	L.R.E.	L.	L.	Ppt.
	3.0		"	L.R.E.P.	L.R.E.P.	L.R.E.P.	"	L.R.E.P.

After 24 hours at room temperature.

0.0%	1.0	0.0	L.	L.	L.	L.	L.	L.
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.2%	1.0	0.0	"	"	"	"	"	"
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.8%	1.0	0.0	"	L.R.E.P.	L.R.E.	L.R.E.P.	"	L.R.E.P.
	2.0		"	"	L.R.E.P.	L.R.E.	L.R.E.	Ppt.
	3.0		"	"	"	L.R.E.P.	L.	L.
0.0%	1.0	0.5	"	L.	L.	L.	"	"
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.2%	1.0	0.5	"	"	"	"	"	"
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.8%	1.0	0.5	"	L.R.E.P.	L.R.E.	L.R.E.	L.R.E.	L.R.E.
	2.0		"	L.	"	"	"	"
	3.0		"	L.R.E.	L.R.E.P.	L.R.E.P.	L.	L.R.E.P.

EXPERIMENT 45 (Continued).

RESULTS: After 24 hours at 37°C.

Plasma heated at 60°C. for 3 minutes.

NaCl.	Plasma	Serum	A	401	039	061	779	935
0.0%	1.0	0.0	L.	L.	L.	L.	L.	L.
	2.0		"	"	"	"	"	"
	3.0		"	L.P.	L.P.	"	"	S.L.
0.2%	1.0	0.0	"	L.	L.	"	"	"
	2.0		L.P.	L.P.	L.P.	L.P.	L.P.	-
	3.0		L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.	L.R.E.	L.R.E.
0.8%	1.0	0.0	L.	S.L.	L.	S.L.	S.L.	Ppt.
	2.0		L.P.	L.R.E.P.	L.R.E.P.	L.R.E.	L.	L.R.E.
	3.0		"	-	L.P.	L.P.	"	Ppt.
0.0%	1.0	0.5	L.	L.	L.	L.	L.	L.
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.2%	1.0	0.5	"	"	"	"	"	"
	2.0		"	L.P.	L.P.	"	"	"
	3.0		"	L.R.E.P.	L.R.E.P.	L.R.E.	"	"
0.8%	1.0	0.5	S.L.	S.L.	S.L.	S.L.	S.L.	S.L.
	2.0		-	L.R.E.	L.R.E.	L.R.E.	L.R.E.	-
	3.0		L.	L.R.E.P.	L.R.E.P.	L.	-	P.

After 24 hours at room temperature.

0.0%	1.0	0.0	L.	L.R.E.P.	L.R.E.P.	L.R.E.	L.R.E.	L.R.E.P.
	2.0		"	L.	L.	L.	L.	L.
	3.0		"	L.R.E.P.	"	"	"	"
0.2%	1.0	0.0	"	L.R.E.P.	L.R.E.P.	L.R.E.	L.R.E.	"
	2.0		L.R.E.P.	"	L.R.E.	"	L.	"
	3.0		L.	"	"	L.	"	"
0.8%	1.0	0.0	"	L.R.E.	L.	"	"	"
	2.0		"	L.R.E.P.	L.R.E.	L.R.E.	"	L.R.E.P.
	3.0		"	"	L.	L.	"	L.
0.0%	1.0	0.5	"	L.	"	"	"	"
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.2%	1.0	0.5	"	"	"	"	"	"
	2.0		"	L.R.E.	"	"	L.R.E.	"
	3.0		"	L.	"	"	L.	"
0.8%	1.0	0.5	"	"	"	"	"	"
	2.0		"	L.R.E.	"	"	"	"
	3.0		"	"	L.R.E.P.	"	"	"

EXPERIMENT 45 (Continued).

RESULTS: After 24 hours at 37°C.

Plasma heated at 60°C. for 10 minutes.

NaCl.	Plasma	Serum	A	401	039	061	779	935
	1.0		-	-	S.L.	S.L.	S.L.	S.L.
0.0%	2.0	0.0	L.	L.	L.	L.	-	L.
	3.0		"	"	"	"	-	"
	1.0		"	"	"	"	S.L.	"
0.2%	2.0	0.0	S.L.	S.L.	S.L.	S.L.	"	S.L.
	3.0		L.	-	L.	L.	-	-
	1.0		"	L.	"	"	L.	L.
0.8%	2.0	0.0	"	S.L.	"	S.L.	"	Ppt.
	3.0		"	L.	"	-	-	-
	1.0		"	"	"	L.	L.	L.
0.0%	2.0	0.5	"	"	"	"	"	"
	3.0		"	"	"	"	"	"
	1.0		"	L.P.	"	L.P.	L.R.E.	L.R.E.
0.2%	2.0	0.5	"	L.R.E.	L.R.E.	L.	L.	L.
	3.0		"	L.	L.	"	"	"
	1.0		"	"	"	"	"	"
0.8%	2.0	0.5	"	"	"	"	"	Ppt.
	3.0		"	S.L.	"	S.L.	S.L.	"

After a further 24 hours at room temperature, lysis only was detected around the growth of all six strains on every plate.

EXPERIMENT 45 (Continued).

RESULTS: After 24 hours at 37°C.

Plasma heated at 60°C. for 15 minutes.

NaCl.	Plasma	Serum	A	401	039	061	779	935
0.0%	1.0	0.0	S.L.	-	S.L.	-	S.L.	-
	2.0		L.	L.	L.	L.	L.	L.
	3.0		"	S.L.	S.L.	-	-	-
0.2%	1.0	0.0.	S.L.	-	L.	-	-	-
	2.0		L.	L.	"	L.	L.	Ppt.
	3.0		"	"	"	"	-	"
0.8%	1.0	0.0	S.L.	-	S.L.	S.L.	-	-
	2.0		L.	L.	L.	L.	L.	-
	3.0		"	S.L.	"	S.L.	-	Ppt.
0.0%	1.0	0.5	"	L.	"	L.	L.	L.
	2.0		"	L.R.E.	L.R.E.	"	L.R.E.	"
	3.0		"	L.R.E.P.	"	"	L.	-
0.2%	1.0	0.5	"	L.	L.	"	"	L.
	2.0		"	L.R.E.	"	"	"	"
	3.0		"	"	L.R.E.	"	"	S.L.P.
0.8%	1.0	0.5	"	L.	L.	"	"	L.
	2.0		"	L.R.E.	L.R.E.	"	"	"
	3.0		"	"	"	L.R.E.	-	Ppt.

After 24 hours at room temperature.

0.0%	1.0	0.0	S.L.	S.L.	S.L.	S.L.	S.L.	Ppt.
	2.0		L.	L.	L.	L.	L.	L.
	3.0		"	"	"	"	"	Ppt.
0.2%	1.0	0.0	"	"	"	"	S.L.	S.L.
	2.0		"	"	"	"	L.	L.
	3.0		"	"	"	"	"	Ppt.
0.8%	1.0	0.0	"	"	"	"	S.L.	"
	2.0		"	"	"	"	L.	L.
	3.0		"	S.L.	"	S.L.	-	Ppt.
0.0%	1.0	0.5	"	L.	"	L.	L.	L.
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	Ppt.
0.2%	1.0	0.5	"	"	"	"	"	L.
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.8%	1.0	0.5	"	"	"	"	"	"
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	Ppt.

EXPERIMENT 45 (Continued).

RESULTS: After 24 hours at 37°C.

Plasma heated at 60°C. for 30 minutes.

NaCl.	Plasma	Serum	A	401	039	061	779	935
0.0%	1.0	0.0	S.L.	S.L.	S.L.	-	-	-
	2.0		-	"	-	S.L.	-	-
	3.0		-	-	L.	L.	-	-
0.2%	1.0	0.0	S.L.	-	S.L.	S.L.	-	-
	2.0		L.	L.	L.	L.	L.	L.
	3.0		"	"	"	"	-	"
0.8%	1.0	0.0	"	"	-	"	S.L.	Ppt.
	2.0		"	"	L.	"	L.	"
	3.0		-	"	-	"	-	"
0.0%	1.0	0.5	-	"	L.	"	L.	L.
	2.0		S.L.	"	"	"	"	"
	3.0		L.	"	"	"	"	"
0.2%	1.0	0.5	"	"	"	"	"	"
	2.0		"	L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.	L.R.E.
	3.0		"	L.R.E.	L.R.E.	"	L.	-
0.8%	1.0	0.5	"	L.	L.	L.	"	L.
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"

After 24 hours at room temperature.

0.0%	1.0	0.0	S.L.	S.L.	S.L.	S.L.	S.L.	S.L.
	2.0		-	L.	L.	L.	L.	L.
	3.0		L.	"	"	"	"	"
0.2%	1.0	0.0	"	"	"	"	"	"
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.8%	1.0	0.0	"	"	"	"	"	Ppt.
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	S.L.	"
0.0%	1.0	0.5	"	"	"	"	L.	L.
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.2%	1.0	0.5	"	"	"	"	"	"
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"
0.8%	1.0	0.5	"	"	"	"	"	Ppt.
	2.0		"	"	"	"	"	"
	3.0		"	"	"	"	"	"

EXPERIMENT 46.

The use of heat-precipitated fibrinogen in a nutrient agar base, with and without the addition of serum and Sodium chloride as a substrate for the production of Muller's phenomenon.

MEDIA: Aliquots of citrated plasma were heated at 53°C., 56°C. and 60°C. for 3, 10, 15 and 30 minutes. They were centrifuged and the precipitated fibrinogen suspended in half the original volume of distilled water. 0.5 ml., 1.0 ml. and 1.5 ml. of this heat-precipitated fibrinogen was incorporated in a nutrient agar base, with and without the addition of serum and of sodium chloride as follows:-

Heat Precipitated Fibrinogen	0.5	1.0	1.5	0.5	1.0	1.5
"Lab-Lemco" (15%)	1.0	1.0	1.0	1.0	1.0	1.0
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5	4.5
Serum	0.0	0.0	0.0	0.5	0.5	0.5
Distilled Water	9.0	8.5	8.0	8.5	8.0	7.5

INOCULATION: These plates were stab-inoculated from 6 hour old "Lab-Lemco" broth cultures of Staphylococcus aureus, Strains A, 401, 039, 061, 779 and 935.

INCUBATION: The plates were incubated at 37°C. for 24 hours.

READINGS: These were taken after 24 hours at 37°C. and after 24 hours at room temperature.

EXPERIMENT 46.

RESULTS: After 24 hours at 37°C.

Heat Precipitated Fibrinogen 53°C. for 3 minutes.

NaCl.	Fibrin-ogen.	Serum	A	401	039	061	779	935
0.0%	0.5	0.0	S.L.	-	-	-	-	-
	1.0		L.	S.L.	S.L.	S.L.	S.L.	S.L.
	1.5		"	L.	L.	L.	L.	L.
0.2%	0.5	0.0	S.L.	-	-	-	-	-
	1.0		L.	L.	L.	L.	L.	L.
	1.5		"	"	"	S.L.	"	"
0.8%	0.5	0.0	L.	-	-	-	-	-
	1.0		"	-	S.L.	-	S.L.	S.L.
	1.5		"	-	"	-	"	-
0.0%	0.5	0.5	L.	S.L.	"	S.L.	"	S.L.
	1.0		"	L.	L.	L.	L.	L.
	1.5		"	"	"	"	"	"
0.2%	0.5	0.5	S.L.	S.L.	S.L.	S.L.	S.L.	S.L.
	1.0		L.	L.	L.	L.	L.	L.
	1.5		"	"	"	"	"	"
0.8%	0.5	0.5	S.L.	S.L.	S.L.	S.L.	S.L.	S.L.
	1.0		"	"	"	"	"	"
	1.5		L.	"	L.	-	L.	L.

After 24 hours at room temperature.

0.0%	0.5	0.0	S.L.	S.L.	S.L.	S.L.	S.L.	S.L.
	1.0		L.	L.	L.	L.	L.	L.
	1.5		"	"	"	"	"	"
0.2%	0.5	0.0	S.L.	S.L.	S.L.	S.L.	S.L.	S.L.
	1.0		L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.
	1.5		"	"	"	"	"	"
0.8%	0.5	0.0	L.R.E.	"	"	"	"	"
	1.0		"	"	"	"	"	"
	1.5		"	L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P
0.0%	0.5	0.5	L.	L.	L.	L.	L.	L.
	1.0		"	"	"	"	"	"
	1.5		"	"	"	"	"	"
0.2%	0.5	0.5	"	"	"	"	"	"
	1.0		"	"	"	"	"	"
	1.5		"	"	"	"	"	"
0.8%	0.5	0.5	"	"	"	"	"	"
	1.0		"	"	"	"	"	"
	1.5		"	"	"	"	"	"

EXPERIMENT 46 (Continued)

RESULTS: After 24 hours at 37°C.

Heat Precipitated Fibrinogen (53°C. for 10 minutes).

NaCl.	Fibrin-ogen	Serum	A	401	039	061	779	935
0.0%	0.5	0.0	L.	S.L.	S.L.	S.L.	S.L.	S.L.
	1.0		"	L.	L.	L.	L.	L.
	1.5		"	"	"	"	"	"
0.2%	0.5	0.0	"	"	"	"	"	"
	1.0		"	"	"	"	"	"
	1.5		"	"	"	"	"	"
0.8%	0.5	0.0	L.R.E.P.	S.L.	S.L.	S.L.	L.R.E.	S.L.
	1.0		L.P.	L.P.	L.P.	L.P.	L.P.	L.P.
	1.5		"	"	"	"	"	"
0.0%	0.5	0.5	L.	L.	L.	L.	L.	L.
	1.0		"	"	"	"	"	"
	1.5		"	"	"	"	"	"
0.2%	0.5	0.5	"	"	"	"	"	"
	1.0		"	"	"	"	"	"
	1.5		"	"	"	"	"	"
0.8%	0.5	0.5	"	"	"	"	"	"
	1.0		L.R.E.	"	"	"	"	"
	1.5		"	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.

After 24 hours at room temperature.

0.0%	0.5	0.0	L.	L.	L.	L.	L.	L.
	1.0		"	"	"	"	"	"
	1.5		"	"	"	"	"	"
0.2%	0.5	0.0	L.R.E.	L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.	L.R.E.
	1.0		"	L.R.E.	L.R.E.	L.R.E.	"	"
	1.5		"	L.R.E.P.	"	L.	L.R.E.P.	L.
0.8%	0.5	0.0	L.R.E.P.	"	L.R.E.P.	L.R.E.P.	"	L.R.E.P.
	1.0		"	"	"	"	"	"
	1.5		"	"	"	"	"	"
0.0%	0.5	0.5	L.	L.	L.	L.	L.	L.
	1.0		"	"	"	"	"	"
	1.5		"	"	"	"	"	"
0.2%	0.5	0.5	"	"	"	"	"	"
	1.0		"	"	"	"	"	"
	1.5		"	"	"	"	"	"
0.8%	0.5	0.5	"	"	"	"	"	"
	1.0		"	"	"	"	"	"
	1.5		"	"	"	"	"	"

EXPERIMENT 46 (Continued).

RESULTS: After 24 hours at 37°C.

Heat Precipitated Fibrinogen (53°C. for 15 minutes).

NaCl.	Fibrin-ogen	Serum	A	401	039	061	779	935
0.0%	0.5	0.0	L.	L.	L.	L.	S.L.	L.
	1.0		"	"	"	"	"	"
	1.5		"	"	"	"	"	"
0.2%	0.5	0.0	"	"	"	"	L.	"
	1.0		L.P.	L.P.	L.P.	L.P.	L.P.	L.P.
	1.5		"	"	"	"	"	"
0.8%	0.5	0.0	L.R.E.P.	L.R.E.P.	L.R.E.	L.R.E.P.	L.R.E.P.	L.R.E.P.
	1.0		"	"	L.R.E.P.	"	"	"
	1.5		"	"	"	"	"	"
0.0%	0.5	0.5	L.	L.R.E.	L.	L.	-	L.
	1.0		L.P.	L.P.	L.P.	L.P.	-	L.P.
	1.5		"	"	"	"	L.P.	"
0.2%	0.5	0.5	L.	L.	L.	L.	-	L.
	1.0		L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.
	1.5		"	"	"	"	"	"
0.8%	0.5	0.5	L.	L.	L.	L.	L.	L.
	1.0		"	"	"	"	"	"
	1.5		L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.

After 24 hours at room temperature.

0.0%	0.5	0.0	L.	L.	L.	L.	L.	L.
	1.0		"	"	"	"	"	"
	1.5		"	"	"	"	"	"
0.2%	0.5	0.0	L.P.	L.P.	"	L.P.	L.P.	L.P.
	1.0		"	"	L.P.	"	"	"
	1.5		"	"	"	"	"	"
0.8%	0.5	0.0	"	"	"	"	"	"
	1.0		"	"	"	"	"	"
	1.5		"	"	"	"	"	"
0.0%	0.5	0.5	L.	L.	L.	L.	-	L.
	1.0		"	"	"	"	-	"
	1.5		"	"	"	"	S.L.	"
0.2%	0.5	0.5	"	"	"	"	-	"
	1.0		"	L.R.E.	L.R.E.	L.R.E.	L.	L.R.E.
	1.5		L.R.E.	"	"	"	L.R.E.	"
0.8%	0.5	0.5	L.	L.	L.	L.	L.	L.
	1.0		"	"	"	"	"	"
	1.5		L.R.E.P.	L.R.E.P.	L.R.E.P.	L.P.	L.R.E.P.	L.P.

EXPERIMENT 46 (Continued).

RESULTS: After 24 hours at 37°C.

Heat Precipitated Fibrinogen (53°C. for 30 minutes).

NaCl.	Fibrin-ogen	Serum	A	401	039	061	779	935
0.0%	0.5	0.0	L.	L.	L.	L.	-	L.
	1.0		"	"	"	"	-	"
	1.5		"	"	"	"	-	"
0.2%	0.5	0.0	"	"	"	"	L.	"
	1.0		"	"	"	"	"	"
	1.5		"	"	"	"	"	"
0.8%	0.5	0.0	L.R.E.	L.R.E.	L.R.E.P.	L.R.E.P.	L.R.E.	L.R.E.P
	1.0		L.R.E.P.	L.R.E.P.	"	"	L.R.E.P.	"
	1.5		"	"	"	"	"	"
0.0%	0.5	0.5	L.	L.	L.	L.	-	L.
	1.0		"	L.P.	"	"	S.L.	"
	1.5		"	L.R.E.	"	"	"	"
0.2%	0.5	0.5	"	L.	"	"	-	"
	1.0		L.R.E.	L.R.E.	"	L.R.E.	S.L.	L.R.E.
	1.5		0	0	0	0	0	0
0.8%	0.5	0.5	L.	L.	L.	L.	L.	L.
	1.0		"	"	"	"	"	"
	1.5		L.R.E.	"	L.R.E.	L.R.E.	"	"

After 24 hours at room temperature. 0 = No plate.

0.0%	0.5	0.0	L.	L.	L.	L.	-	L.
	1.0		"	"	"	"	-	"
	1.5		"	"	"	"	-	"
0.2%	0.5	0.0	"	"	"	"	L.	"
	1.0		"	L.P.	"	L.P.	"	"
	1.5		L.P.	"	L.P.	"	"	"
0.8%	0.5	0.0	"	"	"	"	L.P.	L.P.
	1.0		"	"	"	"	"	"
	1.5		"	"	"	"	"	"
0.0%	0.5	0.5	L.	L.	L.	L.	-	L.
	1.0		"	"	"	"	L.	"
	1.5		"	"	"	"	"	"
0.2%	0.5	0.5	"	"	"	"	-	"
	1.0		"	"	"	"	L.	"
	1.5		0	0	0	0	0	0
0.8%	0.5	0.5	L.	L.	L.	L.	L.	L.
	1.0		"	"	"	"	"	"
	1.5		L.R.E.P.	L.R.E.	L.R.E.	L.R.E.	"	L.R.E.

0 = No plate.

EXPERIMENT 46 (Continued).

RESULTS: After 24 hours at 37°C.

Heat Precipitated Fibrinogen (56°C. for 3 minutes).

NaCl.	Fibrin-ogen	Serum	A	401	039	061	779	935
0.0%	0.5	0.0	S.L.	S.L.	S.L.	S.L.	S.L.	S.L.
	1.0		L.	L.	L.	L.	L.	L.
	1.5		"	"	"	"	"	"
0.2%	0.5	0.0	"	S.L.	S.L.	S.L.	S.L.	S.L.
	1.0		"	L.	L.	L.	L.	L.
	1.5		"	"	"	"	"	"
0.8%	0.5	0.0	L.P.	L.	L.	S.L.	L.	S.L.
	1.0		"	L.P.	L.P.	L.P.	L.P.	L.P.
	1.5		-	-	-	-	-	-
0.0%	0.5	0.5	L.	L.	L.	L.	L.	L.
	1.0		"	"	"	"	"	"
	1.5		"	"	"	"	"	"
0.2%	0.5	0.5	"	"	"	"	"	"
	1.0		"	"	"	"	"	"
	1.5		"	"	"	"	"	"
0.8%	0.5	0.5	"	"	"	"	"	"
	1.0		L.R.E.	L.	L.R.E.	"	"	"
	1.5		"	L.R.E.	"	"	L.R.E.	L.R.E.

After 24 hours at room temperature.

0.0%	0.5	0.0	L.	L.	L.	L.	L.	L.
	1.0		"	"	"	"	"	"
	1.5		"	"	"	"	"	"
0.2%	0.5	0.0	"	S.L.	"	S.L.	S.L.	S.L.
	1.0		"	L.	"	L.	L.	L.
	1.5		L.P.	L.P.	L.P.	"	"	"
0.8%	0.5	0.0	"	"	"	L.P.	L.P.	L.P.
	1.0		"	"	"	"	"	"
	1.5		-	-	-	-	-	-
0.0%	0.5	0.5	L.	L.	L.	L.	L.	L.
	1.0		"	"	"	"	"	"
	1.5		"	"	"	"	"	"
0.2%	0.5	0.5	"	"	"	"	"	"
	1.0		"	"	"	"	"	"
	1.5		"	"	"	"	"	"
0.8%	0.5	0.5	"	"	"	"	"	"
	1.0		L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.
	1.5		L.R.E.P.	"	"	L.	"	"

EXPERIMENT 46 (Continued).

RESULTS: After 24 hours at 37°C.

Heat Precipitated Fibrinogen (56°C. for 10 minutes).

NaCl.	Fibrin-ogen.	Serum	A	401	039	061	779	935
0.0%	0.5	0.0	S.L.	-	S.L.	-	-	S.L.
	1.0		"	S.L.	"	S.L.	S.L.	"
	1.5		L.	L.	L.	L.	L.	L.
0.2%	0.5	0.0	S.L.	S.L.	S.L.	S.L.	S.L.	S.L.
	1.0		L.	L.	L.	L.	L.	L.
	1.5		"	"	"	"	"	"
0.8%	0.5	0.0	"	S.L.	S.L.	-	S.L.	-
	1.0		L.P.	L.P.	L.P.	L.P.	L.P.	L.P.
	1.5		"	"	"	"	"	"
0.0%	0.5	0.5	L.	L.	L.	L.	L.	L.
	1.0		"	"	"	"	"	"
	1.5		"	"	"	"	"	"
0.2%	0.5	0.5	"	"	"	"	"	"
	1.0		"	"	"	"	"	"
	1.5		"	"	"	"	"	"
0.8%	0.5	0.5	"	"	"	"	"	"
	1.0		L.R.E.	"	"	"	"	"
	1.5		L.P.	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.

After 24 hours at room temperature.

0.0%	0.5	0.0	L.	S.L.	S.L.	S.L.	S.L.	S.L.
	1.0		"	"	L.	L.	L.	"
	1.5		"	L.	"	"	"	"
0.2%	0.5	0.0	S.L.	S.L.	S.L.	"	S.L.	L.
	1.0		L.	L.	L.	"	L.	"
	1.5		"	"	"	"	"	"
0.8%	0.5	0.0	L.P.	L.P.	L.P.	S.L.	L.P.	L.R.E.
	1.0		"	"	"	L.P.	"	L.P.
	1.5		"	"	"	"	"	"
0.0%	0.5	0.5	L.	L.	L.	L.	L.	L.
	1.0		"	"	"	"	"	"
	1.5		"	"	"	"	"	"
0.2%	0.5	0.5	"	"	"	"	"	"
	1.0		"	L.R.E.	"	"	"	"
	1.5		"	L.	"	"	"	"
0.8%	0.5	0.5	"	"	"	"	"	"
	1.0		L.R.E.P.	"	"	"	"	L.R.E.
	1.5		"	L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.

RESULTS: After 24 hours at 37°C.

Heat Precipitated Fibrinogen (56°C. for 15 minutes).

NaCl.	Fibrin-ogen.	Serum	A	401	039	061	779	935
0.0%	0.5	0.0	L.	L.	L.	L.	L.	L.
	1.0		"	"	"	"	-	"
	1.5		"	"	"	"	-	"
0.2%	0.5	0.0	"	"	"	S.L.	-	"
	1.0		"	"	"	L.	L.	"
	1.5		"	"	"	"	"	"
0.8%	0.5	0.0	"	"	"	"	-	"
	1.0		"	"	"	"	L.	"
	1.5		L.P.	L.P.	L.P.	L.P.	L.P.	L.P.
0.0%	0.5	0.5	L.	L.	L.	L.	-	L.
	1.0		"	"	"	"	L.	"
	1.5		"	"	"	"	"	"
0.2%	0.5	0.5	"	"	"	"	-	"
	1.0		L.R.E.	L.R.E.P.	L.	"	L.	L.R.E.P.
	1.5		L.P.	L.P.	"	L.P.	"	L.P.
0.8%	0.5	0.5	L.R.E.	L.	"	L.	"	L.
	1.0		"	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.
	1.5		L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.

After 24 hours at room temperature.

0.0%	0.5	0.0	L.	L.	L.	L.	-	L.
	1.0		"	"	"	"	-	"
	1.5		"	"	"	"	-	"
0.2%	0.5	0.0	"	"	"	"	-	"
	1.0		"	"	"	"	-	"
	1.5		"	"	"	"	L.	"
0.8%	0.5	0.0	"	"	"	"	S.L.	"
	1.0		L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.
	1.5		L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.
0.0%	0.5	0.5	L.	L.	L.	L.	-	L.
	1.0		"	"	"	"	L.	"
	1.5		"	"	"	"	"	"
0.2%	0.5	0.5	"	L.R.E.	L.R.E.	L.	S.L.	L.
	1.0		L.R.E.	L.R.E.P.	"	L.R.E.	"	L.R.E.P.
	1.5		L.R.E.P.	L.R.E.	"	"	"	"
0.8%	0.5	0.5	L.R.E.	"	L.	L.	L.	L.R.E.
	1.0		"	"	L.R.E.	L.R.E.	L.R.E.	L.R.E.
	1.5		L.P.	L.P.	"	L.P.	L.P.	L.P.

RESULTS: After 24 hours at 37°C.

Heated Precipitated Fibrinogen (56°C. for 30 minutes).

NaCl.	Fibrin-ogen	Serum	A	401	039	061	779	935
0.0%	0.5	0.0	S.L.	-	-	-	-	-
	1.0		L.	S.L.	S.L.	-	S.L.	S.L.
	1.5		"	"	-	-	"	"
0.2%	0.5	0.0	S.L.	-	-	-	-	-
	1.0		"	-	-	-	-	-
	1.5		L.	S.L.	L.	-	L.	L.
0.8%	0.5	0.0	L.P.	"	L.P.	S.L.	L.P.	S.L.
	1.0		"	L.P.	"	L.P.	"	L.P.
	1.5		"	"	"	"	"	"
0.0%	0.5	0.5	L.	L.	L.	L.	L.	L.
	1.0		"	"	"	"	"	"
	1.5		"	"	"	"	"	"
0.2%	0.5	0.5	"	"	"	"	"	"
	1.0		"	"	"	"	"	"
	1.5		L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.
0.8%	0.5	0.5	L.	L.	L.	L.	L.	L.
	1.0		L.R.E.	L.R.E.	"	"	"	L.R.E.
	1.5		L.R.E.P.	"	L.R.E.	L.R.E.	L.R.E.P.	"

After 24 hours at room temperature

0.0%	0.5	0.0	L.	S.L.	S.L.	-	S.L.	-
	1.0		"	L.	L.	-	L.	L.
	1.5		"	"	"	S.L.	"	"
0.2%	0.5	0.0	"	"	"	L.	"	"
	1.0		"	"	"	-	"	"
	1.5		"	"	"	L.	"	"
0.8%	0.5	0.0	L.P.	"	L.P.	S.L.	L.P.	"
	1.0		"	L.P.	"	L.P.	"	L.P.
	1.5		"	"	"	"	"	"
0.0%	0.5	0.5	L.	L.	L.	L.	L.	L.
	1.0		"	"	"	"	"	"
	1.5		"	"	"	"	"	"
0.2%	0.5	0.5	"	"	"	"	"	"
	1.0		"	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.
	1.5		L.R.E.	"	"	"	"	"
0.8%	0.5	0.5	L.	L.	L.	L.	L.	L.
	1.0		L.R.E.	L.P.	L.R.E.	"	L.R.E.P.	L.R.E.P.
	1.5		"	L.R.E.	"	L.R.E.	L.R.E.	L.R.E.

EXPERIMENT 46 (Continued).

RESULTS: After 24 hours at 37°C.

Heat Precipitated Fibrinogen (60°C. for 3 minutes.)

NaCl.	Fibrin-ogen.	Serum	A	401	039	061	779	935
0.0%	0.5	0.0	L.	S.L.	S.L.	-	-	S.L.
	1.0		"	L.	L.	L.	-	L.
	1.5		"	"	"	"	-	"
0.2%	0.5	0.0	0	0	0	0	0	0
	1.0		L.	L.	L.	L.	L.	L.
	1.5		"	"	"	"	"	"
0.8%	0.5	0.0	"	"	"	"	"	"
	1.0		L.R.E.	L.R.E.	L.R.E.	L.R.E.	"	L.R.E.
	1.5		L.R.E.P.	L.R.E.P.	L.R.E.P.	"	L.R.E.	"
0.0%	0.5	0.5	L.	L.	L.	L.	-	L.
	1.0		"	"	"	"	-	L.
	1.5		"	L.R.E.	"	"	-	"
0.2%	0.5	0.5	0	0	0	0	0	0
	1.0		L.	L.	L.	L.	-	L.
	1.5		"	L.R.E.P.	"	L.R.E.P.	L.R.E.P.	L.R.E.
0.8%	0.5	0.5	"	L.	"	L.	L.	L.
	1.0		"	"	"	"	"	"
	1.5		L.R.E.	"	"	"	"	"

After 24 hours at room temperature. 0 = No plate

0.0%	0.5	0.0	L.	L.	L.	L.	S.L.	L.
	1.0		"	"	"	"	-	"
	1.5		"	"	"	"	-	"
0.2%	0.5	0.0	0	0	0	0	0	0
	1.0		L.	L.	L.	L.	L.	L.
	1.5		"	"	"	"	"	"
0.8%	0.5	0.0	"	"	"	"	"	"
	1.0		L.P.	L.P.	L.P.	L.P.	L.P.	L.P.
	1.5		"	"	"	"	"	"
0.0%	0.5	0.5	L.	L.	L.	L.	-	L.
	1.0		"	"	"	"	L.	"
	1.5		"	"	"	"	"	"
0.2%	0.5	0.5	0	0	0	0	0	0
	1.0		L.	L.	L.	L.	L.	L.
	1.5		"	"	"	"	"	"
0.8%	0.5	0.5	"	"	"	"	"	"
	1.0		"	"	"	"	"	"
	1.5		"	"	"	"	"	"

 0 = No plate.

EXPERIMENT 46 (Continued).

RESULTS: After 24 hours at 37°C.

Heat Precipitated Fibrinogen (60°C. for 10 minutes).

NaCl.	Fibrin-ogen	Serum	A	401	039	061	779	935
0.0%	0.5	0.0	L.	-	L.	-	-	-
	1.0		"	L.	"	L.	-	L.
	1.5		"	"	"	"	-	"
0.2%	0.5	0.0	"	"	"	"	-	"
	1.0		"	"	"	"	-	"
	1.5		"	"	"	"	-	"
0.8%	0.5	0.0	"	"	"	"	-	-
	1.0		"	"	"	"	L.	L.
	1.5		"	"	"	"	"	"
0.0%	0.5	0.5	"	"	"	"	-	"
	1.0		"	"	"	"	-	"
	1.5		"	"	"	"	-	"
0.2%	0.5	0.5	"	"	"	"	-	"
	1.0		"	"	"	"	-	"
	1.5		"	"	"	"	L.	"
0.8%	0.5	0.5	"	"	"	"	"	"
	1.0		"	"	"	"	"	"
	1.5		L.R.E.	"	"	"	"	"

After 24 hours at room temperature.

0.0%	0.5	0.0	L.	L.	L.	L.	-	L.
	1.0		"	"	"	"	-	"
	1.5		"	"	"	"	-	"
0.2%	0.5	0.0	"	"	"	"	-	"
	1.0		"	"	"	"	L.	"
	1.5		"	"	"	"	"	"
0.8%	0.5	0.0	"	"	"	"	"	"
	1.0		"	"	"	"	"	"
	1.5		"	"	"	"	"	"
0.0%	0.5	0.5	"	"	"	"	-	"
	1.0		"	"	"	"	-	"
	1.5		"	"	"	"	-	"
0.2%	0.5	0.5	"	"	"	"	-	"
	1.0		"	"	"	"	L.	"
	1.5		"	"	"	"	"	"
0.8%	0.5	0.5	"	"	"	"	"	"
	1.0		"	"	"	"	"	"
	1.5		L.R.E.	"	"	"	"	L.R.E.

EXPERIMENT 46 (Continued).

RESULTS: After 24 hours at 37°C.

Heated Precipitated Fibrinogen (60°C. for 15 minutes).

NaCl.	Fibrin-ogen.	Serum	A	401	039	061	779	935
0.0%	0.5	0.0	L.	S.L.	S.L.	S.L.	-	S.L.
	1.0		"	L.	L.	L.	-	L.
	1.5		"	"	"	"	-	"
0.2%	0.5	0.0	0	0	0	0	0	0
	1.0		L.	L.	L.	L.	L.	L.
	1.5		"	"	"	"	"	"
0.8%	0.5	0.0	"	"	"	"	"	"
	1.0		"	"	"	"	"	"
	1.5		"	"	"	"	"	"
0.0%	0.5	0.5	"	"	"	"	"	"
	1.0		"	"	"	"	"	"
	1.5		"	"	"	"	"	"
0.2%	0.5	0.5	"	"	"	"	"	"
	1.0		L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.
	1.5		"	"	"	"	"	"
0.8%	0.5	0.5	"	"	L.	L.	L.R.E.	L.
	1.0		L.P.	L.P.	L.P.	L.P.	L.P.	L.P.
	1.5		L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.

After 24 hours at room temperature. 0 = No plate.

0.0%	0.5	0.0	L.	L.	L.	L.	L.	L.
	1.0		"	"	"	"	"	"
	1.5		"	"	"	"	"	"
0.2%	0.5	0.0	0	0	0	0	0	0
	1.0		L.	L.	L.	L.	L.	L.
	1.5		"	"	"	"	"	"
0.8%	0.5	0.0	"	"	"	"	"	"
	1.0		"	"	"	"	"	"
	1.5		"	"	"	"	"	"
0.0%	0.5	0.5	"	"	"	"	"	"
	1.0		L.R.E.	L.P.	"	L.R.E.	L.R.E.	L.R.E.
	1.5		L.	"	"	L.P.	"	L.
0.2%	0.5	0.5	"	L.	"	L.	L.	"
	1.0		"	"	"	"	L.R.E.	"
	1.5		L.R.E.	L.R.E.	L.R.E.	L.R.E.P.	"	L.R.E.
0.8%	0.5	0.5	L.	L.	L.	L.	L.	"
	1.0		L.R.E.	L.R.E.	L.R.E.	L.R.E.P.	L.R.E.	"
	1.5		L.R.E.P.	L.R.E.P.	L.R.E.P.	"	"	L.R.E.P.

0 = No Plate.

EXPERIMENT 46 (Continued).

RESULTS: After 24 hours at 37°C.

Heat Precipitated Fibrinogen (60°C. for 30 minutes).

NaCl.	Fibrin-ogen.	Serum	A	401	039	061	779	935
0.0%	0.5	0.0	L.	-	-	-	-	-
	1.0		"	S.L.	S.L.	-	-	-
	1.5		"	-	"	S.L.	-	-
0.2%	0.5	0.0	"	L.	"	-	-	-
	1.0		"	"	L.	L.	S.L.	-
	1.5		"	"	"	"	"	S.L.
0.8%	0.5	0.0	"	-	-	-	-	-
	1.0		"	S.L.	S.L.	L.	S.L.	-
	1.5		"	L.	"	"	L.	L.
0.0%	0.5	0.5	"	"	L.	"	"	"
	1.0		"	"	"	"	"	"
	1.5		"	"	"	"	"	"
0.2%	0.5	0.5	"	"	"	"	"	"
	1.0		"	"	"	"	"	"
	1.5		"	"	"	"	"	"
0.8%	0.5	0.5	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.
	1.0		L.P.	L.P.	L.P.	L.P.	L.P.	L.P.
	1.5		"	"	"	"	"	"

After 24 hours at room temperature.

0.0%	0.5	0.0	L.	L.	L.	S.L.	-	-
	1.0		"	"	S.L.	"	-	L.
	1.5		"	S.L.	L.	L.	S.L.	S.L.
0.2%	0.5	0.0	"	L.	"	"	-	"
	1.0		"	"	"	"	L.	L.
	1.5		"	"	"	"	"	"
0.8%	0.5	0.0	"	S.L.	"	"	"	"
	1.0		"	L.	"	"	"	"
	1.5		"	"	"	"	"	"
0.0%	0.5	0.5	"	"	"	"	"	"
	1.0		"	"	"	"	L.P.	"
	1.5		"	"	"	L.P.	"	"
0.2%	0.5	0.5	"	"	"	L.	L.	"
	1.0		L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.
	1.5		L.	"	L.	"	L.R.E.P.	L.R.E.P.
0.8%	0.5	0.5	L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.	"	"
	1.0		"	"	"	"	"	"
	1.5		"	"	"	"	"	"

EXPERIMENT 47.

RESULTS: After incubation at 37°C. for 24 hours and at room temperature for 24 hours.

Plate	1	2	3	4	5	6	7
48 hour old culture	-	-	-	P.	P.	-	-
72 " " "	P-	P.	P+	P+	P+	-	-

EXPERIMENT 48.

To ascertain the best day of harvesting the staphylococcal factor in "Lab-Lemco" broth culture.

MEDIUM: 1% "Lab-Lemco" broth. Six 250 ml. conical flasks each containing 100 ml. of 1% "Lab-Lemco" broth.

INOCULUM: 2 ml. of a 6 hour old "Lab-Lemco" broth culture of Staphylococcus aureus, Strain A.

INCUBATION: Flasks were incubated at 37°C. One flask was removed from incubator each day from the second to the seventh day after inoculation.

METHOD OF PREPARATION OF CULTURE: Culture was centrifuged and the supernatant fluid was sterilised by heating at 65°C. for 10 minutes. The sterilised culture fluids obtained from the different flasks after 1 to 6 days' incubation were incorporated in serum-milk-agar plates as follows:-

Plate	1	2	3	4	5	6	7	8	9
Milk	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75
Culture	1.0	2.0	3.0	4.0	5.0	6.0	7.0	0.0	2.0
Serum	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	0.0
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5
Distilled Water.	4.25	3.25	2.25	1.25	0.25	0.0	0.0	5.25	4.75

READINGS: Each set of plates was incubated at 37°C. for 24 hours and then left at room temperature for 24 hours. Readings were taken after 24 hours at 37°C. and after 24 hours at room temperature.

EXPERIMENT 48.

RESULTS: After 24 hours at 37°C. and after 24 hours at room temperature.

After 24 hours' incubation at 37°C.

Age of Culture	PLATES								
	1	2	3	4	5	6	7	8	9
2 day	-	P.	P.	P.	-	-	-	-	-
3 "	-	"	"	"	P.	P.	-	-	-
4 "	-	"	"	"	"	"	-	-	-
5 day	-	"	"	"	"	-	-	-	-
6 "	-	"	"	"	-	-	-	-	-
7 "	-	"	"	"	-	-	-	-	-

After 24 hours' incubation at room temperature.

2 day	-	P.	P.	P.	P.	P.	P.	-	-
3 "	-	"	"	"	"	"	"	-	-
4 "	P-	L.	L.	L.	L.	P.70%L.	P.60%L.	-	-
5 "	P.	P.50%L.	"	"	"	"	P.30%L.	-	-
6 "	"	L.	L.	L.	P.60%L.	P.	-	-	-
7 "	-	P.	P.	P.	P.	"	-	-	-

EXPERIMENT 49.

Production of the staphylococcal factor responsible for Muller's phenomenon in "Lab-Lemco" broth enriched by 10% human serum.

MEDIUM: 1% "Lab-Lemco" broth containing 10% human serum. Five 250 ml. conical flasks each containing 100 ml. of 1% "Lab-Lemco" broth with 10% human serum.

INOCULUM: 2 ml. of a 6 hour old "Lab-Lemco" broth culture of Staphylococcus aureus, Strain A.

INCUBATION: Flasks were incubated at 37°C. The first flask was withdrawn after 24 hours' incubation and one flask was withdrawn each following day.

METHOD OF PREPARATION OF STAPHYLOCOCCAL FACTOR: Culture was centrifuged and the supernatant fluid sterilised by heating at 65°C. for 10 minutes. The sterilised cultures of different ages (1 to 5 days' old) were tested by incorporation in serum-milk-agar plates as follows:-

Plate	1	2	3	4	5	6	7	8	9
Milk	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75
Serum	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	0.0
Staphylococcal Factor	1.0	2.0	3.0	4.0	5.0	6.0	7.0	0.0	2.0
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5
Distilled Water	4.25	3.25	2.25	1.25	0.25	0.0	0.0	5.25	4.75

READINGS: Each set of plates was incubated at 37°C. for 24 hours and then left at room temperature for a further 24 hours. Readings were taken after 24 hours at 37°C. and after 24 hours at room temperature.

EXPERIMENT 49.

RESULTS: After 24 hours' incubation at 37°C.

Age of Culture	PLATE								
	1	2	3	4	5	6	7	8	9
1 day	-	-	-	-	-	-	-	-	-
2 "	-	-	P.80%L.	L.	L.	P.95%L.	P.95%L.	-	-
3 "	-	-	P.	P.	-	-	-	-	-
4 "	-	-	-	-	-	-	-	-	-
5 "	-	-	-	-	-	-	-	-	-

After 24 hours incubation at room temperature.

1 day	-	-	-	-	-	-	-	-	-
2 "	-	P.	P.90%L.	P.L.	P.L.	P.	P.	-	-
3 "	-	P.90%L.	P.L.	P.80%L.	P.40%L.	"	"	-	-
4 "	-	P.	P.	-	-	-	-	-	-
5 "	-	P-	P-	-	-	-	-	-	-

EXPERIMENT 50.

An attempt to produce the staphylococcal factor in a "Lab-Lemco" broth without sodium chloride and peptone, but enriched by 10% human serum.

MEDIUM: 1% "Lab-Lemco" broth made without sodium chloride and peptone but containing 10% human serum. Five 250 ml. conical flasks each containing 100 ml. of this 1% "Lab-Lemco" broth with 10% human serum.

INOCULUM: Each flask was inoculated with 2 ml. of a 6 hour old "Lab-Lemco" broth culture of Staphylococcus aureus, Strain A.

INCUBATION: These flasks were incubated at 37°C. One flask was removed after 24 hours' incubation and one flask removed after each subsequent 24 hour period.

METHOD OF PREPARATION OF STAPHYLOCOCCAL FACTOR: The different aged cultures were centrifuged and the supernatant fluids were sterilised by heating at 65°C. for 10 minutes. Different amounts of these fluids were incorporated in serum-milk-agar plates as follows:-

Plate	1	2	3	4	5	6	7	8	9
Milk	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75
Serum	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	0.0
Staphylococcal Factor	1.0	2.0	3.0	4.0	5.0	6.0	7.0	0.0	2.0
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5
Distilled Water	4.25	3.25	2.25	1.25	0.25	0.0	0.0	5.25	4.75

READINGS: The plates were incubated at 37°C. for 24 hours and then left at room temperature for 24 hours. Readings were made after 24 hours' and 48 hours' incubation.

EXPERIMENT 50.

RESULTS: After incubation at 37°C. for 24 hours and after further incubation of these plates at room temperature, no activity was detected in any of these plates.

EXPERIMENT 51.

The testing of staphylococcal factor in serum-milk-agar plates containing different amounts of serum.

MEDIUM: 1% "Lab-Lemco" broth. Two 250 ml. flasks each containing 100 ml. of 1% "Lab-Lemco" broth were used.

INOCULUM: 2 ml. of a 6 hour old "Lab-Lemco" broth culture of Staphylococcus aureus, Strain A.

INCUBATION: One flask was incubated at 37°C. for 3 days, the other for 4 days.

METHOD OF PREPARATION OF STAPHYLOCOCCAL FACTOR: The 3 day old and 4 day old cultures were centrifuged and the supernatant fluids sterilised by heating at 65°C. for 10 minutes. These sterilised cultures were incorporated in serum-milk-agar plates as follows:-

Milk	3.75	3.75	3.75	3.75	3.75	3.75	3.75
Serum	0.5	0.5	0.5	0.5	0.5	0.0	0.5
Staphylococcal Factor	1.0	1.5	2.0	2.5	3.0	2.0	0.0
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5	4.5	4.5
Distilled Water	5.25	4.75	4.25	3.75	3.25	4.75	6.25

Similar sets of plates containing 1.0, 1.5 and 2.0 ml. of human serum were made, with corresponding decreases in the amount of distilled water added. Two control plates were also included, one without serum, and one without the sterilised staphylococcal culture.

READINGS: These sets of plates were incubated at 37°C. for 24 hours and then left at room temperature for 24 hours. Readings were made after 24 hours' and 48 hours' incubation.

EXPERIMENT 51.

RESULTS: After incubation at 37°C. for 24 hours.

Amount of Serum	Age and Amount of Culture											
	1.0 ml.		1.5 ml.		2.0 ml.		2.5 ml.		3.0 ml.		4.0 ml.	
	3 day	4 day	3 day	4 day	3 day	4 day	3 day	4 day	3 day	4 day	3 day	4 day
0.5 ml	-	-	-.	P.	P.	P.	P.	P.	P.	P.	P.	P.
1.0 "	-	-	-	-	-	-	-	P.	-	P.	P.	P.
1.5 "	-	-	-	-	-	-	-	-	-	-	-	-
2.0 "	-	-	-	-	-	-	-	-	-	-	-	-

After incubation at room temperature for 24 hours.

0.5 ml	P.	P.	P.	P.	P.	P.	P.	P.	P.	P.	P.	P.
1.0 "	-	-	-	-	-	P.	P.	P.	P.	P.	P.	P.
1.5 "	-	-	-	-	-	-	-	P.	-	P.	P.	P.
2.0 "	-	-	-	-	-	-	-	-	-	-	P.	P.

The control plates showed no change from their original appearance.

EXPERIMENT 52.

The production of the staphylococcal factor in a number of different media.

MEDIA: 100 ml. amounts of 1% "Lab-Lemco" broth, 1% "Lab-Lemco" broth containing 5% human serum, meat broth and meat broth containing 5% human serum were used in 250 ml. conical flasks.

INOCULUM: Each flask was inoculated with 2 ml. of a 6 hour old "Lab-Lemco" broth culture of Staphylococcus aureus, Strain A.

INCUBATION: The flasks of the different culture media were incubated at 37°C. for 5 days in all. 15 ml. of culture was withdrawn from the different flasks after each 24 hour period of incubation from 24 hours up to 5 days. These samples of culture were centrifuged, the supernatant fluid sterilised by heating at 65°C. for 10 minutes, and incorporated in serum-milk-agar plates as follows:-

Milk	2.0	2.0	2.0	2.0	2.0	2.0
Serum	0.5	0.5	0.5	0.5	0.5	0.0
Staphylococcal Factor	1.0	2.0	3.0	4.0	0.0	2.0
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5	4.5
Distilled Water	7.0	6.0	5.0	4.0	8.0	6.5

These serum-milk-agar plates contained 2.0 ml. of milk in place of the formerly used 3.75 ml. This smaller amount of protein substrate had been found to be just as satisfactory as the larger amount for demonstrating Muller's phenomenon.

READINGS: The sets of plates made with the different aged cultures in the four different media employed were incubated at 37°C. for 24 hours, and then left at room temperature for 24 hours. Readings were made after 24 hours' incubation.

EXPERIMENT 52.

RESULTS: After incubation of plates at 37°C. for 24 hours.

Type of Medium	Age of Culture	Amount of Culture (ml.)			
		1.0	2.0	3.0	4.0
"Lab-Lemco" Broth	1 day	-	-	-	P.
" " "	2 "	-	P.	P.	"
" " "	3 "	P.	L.	L.	L.
" " "	4 "	"	"	"	"
" " "	5 "	"	P.	P.	P.
Serum "Lab-Lemco" Broth	1 "	"	"	L.	L.
" " "	2 "	"	L.	"	"
" " "	3 "	L.	"	"	"
" " "	4 "	P.	"	"	"
" " "	5 "	"	"	"	"
Meat Broth.	1 "	-	-	P.	P.
" "	2 "	P.	P.	L.	L.
" "	3 "	L.	L.	"	"
" "	4 "	"	"	"	"
" "	5 "	"	"	"	"
Serum-Meat Broth	1 "	-	P.	P.	P.
" " "	2 "	L.	L.	L.	L.
" " "	3 "	"	"	"	"
" " "	4 "	"	"	"	"
" " "	5 "	"	"	"	"

All the control plates made without serum or culture showed no change.

Additional reading taken after 2½ hours incubation at 37°C. with 4 day old culture.

Medium	Amount of Culture (ml.)			
	1.0	2.0	3.0	4.0
"Lab-Lemco" Broth.	-	-	-	-
Serum "Lab-Lemco" Broth.	-	?P.	P.	P.
Meat Broth.	-	-	-	-
Serum-Meat Broth.	-	P.	P.	P.

EXPERIMENT 53.

The production of the staphylococcal factor in a number of different media.

MEDIA: 1% "Lab-Lemco" broth; casein digest broth; 1% peptone water (Oxoid); heart digest broth and meat broth. 100 ml. amounts of these media were used.

INOCULUM: 2 ml. of a 6 hour old "Lab-Lemco" broth culture of Staphylococcus aureus, Strain A was used to inoculate each flask of medium.

INCUBATION: The flasks were incubated at 37°C. and 15 ml. amounts of culture were withdrawn from each flask after 24, 48 and 72 hours' incubation.

METHOD OF PREPARATION OF STAPHYLOCOCCAL FACTOR: The samples of the different cultures were centrifuged, the supernatant fluid of each was sterilised by heating at 65°C. for 10 minutes and incorporated in serum-milk-agar plates as follows:-

Milk	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Serum	0.5	0.5	0.5	0.5	0.5	0.5	0.0
Staphylococcal Factor	0.5	1.0	2.0	3.0	4.0	0.0	2.0
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5	4.5	4.5
Distilled Water	7.5	7.0	6.0	5.0	4.0	8.0	6.5

READINGS: The sets of plates were incubated at 37°C. for 24 hours when readings were made.

EXPERIMENT 53.

RESULTS: After incubation at 37°C. for 24 hours.

Medium	Age of Culture	Amount of Culture (ml.).				
		0.5	1.0	2.0	3.0	4.0
Meat Broth	24 hrs.	-	P.	P.	P.60%L.	P.80%L.
" "	48 "	P.	L.	L.	L.	L.
" "	72 "	"	"	"	"	"
Casein Digest Broth	24 "	-	-	-	-	-
" " "	48 "	-	-	-	-	-
" " "	72 "	-	-	-	-	-
1% Peptone Water	24 "	-	-	-	-	-
" " "	48 "	-	-	-	-	-
" " "	72 "	-	-	-	-	-
"Lab-Lemco" Broth	24 "	-	-	P.	P-	P-
" " "	48 "	-	P.	P.50%L.	P.60%L.	P.10%L.
" " "	72 "	-	"	P.40%L.	P.70%L.	P.
Heart Digest Broth	24 "	-	-	-	-	-
" " "	48 "	-	P.	P.	P-	P-
" " "	72 "	P-	P.	P.40%L.	P.60%L.	P.

The control plates, without serum or without culture, showed no change.

EXPERIMENT 54.

Comparison of the activity of the staphylococcal factor produced in "Lab-Lemco" broth with that produced in meat broth.

MEDIA: 1% "Lab-Lemco" broth and meat broth. Six 250 ml. flasks of 60 ml. 1% "Lab-Lemco" broth and six similar flasks of 60 ml. meat broth were used.

INOCULUM: Each flask was inoculated with 2 ml. of a 6 hour old "Lab-Lemco" broth culture of Staphylococcus aureus, Strain A.

INCUBATION: The twelve flasks were incubated at 37°C. One flask of meat broth culture and one of the "Lab-Lemco" broth culture were withdrawn after 24 hours. Pairs of flasks were withdrawn from the incubator every subsequent 24 hours.

METHOD OF PREPARATION OF STAPHYLOCOCCAL FACTOR: The cultures were centrifuged, heat-killed at 65°C. for 10 minutes and incorporated in serum-milk-agar plates as follows:-

Milk	2.0	2.0	2.0	2.0	2.0	2.0
Serum	0.5	0.5	0.5	0.5	0.0	0.5
Staphylococcal Factor	1.0	2.0	3.0	4.0	4.0	0.0
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5	4.5
Distilled Water	7.0	6.0	5.0	4.0	4.5	8.0

READINGS: The plates were incubated at 37°C. for 24 hours and readings were made after 2 hours and at 24 hours.

EXPERIMENT 54.

RESULTS: After incubation at 37°C. for 2 hours and 24 hours.

Age of Culture	Amount of Culture (ml.)	Meat Broth		"Lab-Lemco" Broth	
		Period of Incubation			
		2 hrs.	24 hrs.	2 hrs.	24 hrs.
1 day	1.0	-	P.	-	-
	2.0	-	"	-	-
	3.0	-	P+	-	P-
	4.0	-	P. 10% L.	-	"
2 day	1.0	-	P+	-	-
	2.0	-	L.	-	-
	3.0	-	"	-	P-
	4.0	-	"	-	"
3 day	1.0	-	P+	-	P+
	2.0	P.	L.	-	L.
	3.0	P-	"	-	"
	4.0	"	"	-	"
4 day	1.0	-	"	-	P.
	2.0	P+	"	-	P+
	3.0	"	"	-	"
	4.0	"	"	-	"
6 day	1.0	-	"	-	P-
	2.0	P+	"	P-	P.
	3.0	"	"	"	"
	4.0	"	"	"	"
7 day	1.0	-	"	-	P-
	2.0	-	"	-	P.
	3.0	-	"	-	"
	4.0	-	"	-	"

Control plates put up with each day's cultures showed no activity.

EXPERIMENT 55.

Comparison of the activity of the staphylococcal factor produced in aliquots of meat broth which had received different inocula.

MEDIUM: Meat broth. Four 1 litre flasks each containing 200 ml. of meat broth were used.

INOCULA: One flask was inoculated with a suspension made in meat broth from a "Lab-Lemco" agar slope of Staphylococcus aureus, Strain A which had been kept in the ice-chest for a period of 6 weeks. A second flask was inoculated with a suspension of the same organism from a "Lab-Lemco" agar slope kept at room temperature for 7 days. The suspensions were made in 3 ml. of a 3 day old meat broth culture and the fourth flask with a 24 hour old "Lab-Lemco" agar slope of Staphylococcus aureus, Strain A suspended in 3 ml. of meat broth. This "Lab-Lemco" agar culture had been incubated at 37°C. for 24 hours.

INCUBATION: These flasks were incubated at 37°C. for 3 days.

METHOD OF PREPARATION OF STAPHYLOCOCCAL FACTOR: The cultures were centrifuged, sterilised by heating at 65°C. for 10 minutes and incorporated in serum-milk-agar plates as follows:-

Milk	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Serum	0.5	0.5	0.5	0.5	0.5	0.0	0.5
Staphylococcal Factor	0.5	1.0	2.0	3.0	4.0	4.0	0.0
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5	4.5	4.5
Distilled Water	7.5	7.0	6.0	5.0	4.0	4.5	8.0

READINGS: These plates were incubated at 37°C. for 24 hours when readings were made.

EXPERIMENT 55.

RESULTS: After 24 hours' incubation at 37°C.

Nature of Inoculum	Amount of Culture (ml.)				
	0.5	1.0	2.0	3.0	4.0
Ice chest (6 weeks).	-	L.	L.	L.	L.
Room Temperature (7 days' old).	-	P.	"	"	"
37°C. Culture (3 days' old).	-	P. 50% L.	"	"	"
37°C. Culture (1 day old).	-	P. 10% L.	"	"	"

The control plates showed no activity.

EXPERIMENT 56.

The influence of the age of the inoculum on the production of the staphylococcal factor in meat broth.

MEDIUM: Meat broth. Conical flasks of 250 ml. capacity, containing 100 ml. of meat broth were used.

INOCULA: One set of flasks was inoculated with a 6 hour old meat broth culture of Staphylococcus aureus, Strain A, each flask receiving 2 ml. of this culture. The remaining four sets of flasks were inoculated with a 4 day old meat broth culture of the same organism, each flask receiving 2 ml. of the culture. This culture was part of a culture 4 days' old made for the preparation of staphylococcal factor. The four sets of flasks referred to were inoculated for the purpose of this experiment over a period of 14 days.

INCUBATION: The sets of flasks were incubated at 37°C. for 4 days.

METHOD OF PREPARATION OF STAPHYLOCOCCAL FACTOR: The cultures were centrifuged after removal from the incubator, sterilised by heating at 65°C. for 10 minutes and incorporated in serum-milk-agar plates to ascertain the activity of the staphylococcal factor as follows:-

Milk	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Serum	0.5	0.5	0.5	0.5	0.5	0.0	0.5
Staphylococcal Factor	0.5	1.0	2.0	3.0	4.0	4.0	0.0
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5	4.5	4.5
Distilled Water	7.5	7.0	6.0	5.0	4.0	4.5	8.0

READINGS: These plates were incubated at 37°C. for 24 hours when readings were made.

EXPERIMENT 56.

RESULTS: After 24 hours' incubation of plates at 37°C.

Set No.	Age of Inoculum	Amount of Culture				
		0.5	1.0	2.0	3.0	4.0
1.	6 hour old	P.	P.	L.	L.	L.
2.	4 day old.	"	"	"	"	"
3.	" " "	-	P. 60% L.	P. 80% L.	P. 80% L.	P. 95% L.
4.	" " "	P.	P. 70% L.	"	L.	L.
5.	" " "	"	P.	P. 70% L.	P. 90% L.	"

Control plates showed no activity.

EXPERIMENT 57.

Investigation of the effect on the production of staphylococcal factor of altering the surface to volume ratio of the meat broth medium.

MEDIUM: Meat broth. Kolle flasks were used to contain the medium. One Kolle flask was filled with 200 ml. of meat broth and supported in the vertical position. Another Kolle flask received 60 ml. of meat broth and was left in the horizontal position.

INOCULUM: 2 ml. of a 6 hour old "Lab-Lemco" broth culture of Staphylococcus aureus, Strain A.

INCUBATION: The flasks were incubated at 37°C. for 4 days, one in the horizontal, the other in the vertical position. Samples from each flask were withdrawn every 24 hours from the 1st. to the 4th. day after inoculation.

PREPARATION OF STAPHYLOCOCCAL FACTOR: The samples of culture were centrifuged, sterilised by heating at 65°C. for 10 minutes and incorporated in serum-milk-agar plates as follows:-

Milk	2.0	2.0	2.0	2.0	2.0	2.0
Serum	0.5	0.5	0.5	0.5	0.5	0.0
Staphylococcal Factor	1.0	2.0	3.0	4.0	0.0	2.0
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5	4.5
Distilled Water	7.0	6.0	5.0	4.0	8.0	6.5

READINGS: The plates were incubated at 37°C. for 24 hours when readings were made.

EXPERIMENT 57.

RESULTS: After incubation of plates at 37°C. for 24 hours.

Age of Culture	Method of Incubation of Culture	Amount of Culture				
		0.5	1.0	2.0	3.0	4.0
1 day	Horizontal	(...)	-	-	-	-
	Vertical	"	-	-	-	-
2 day	Horizontal	(...)	P.	P. 80% L.	L.	L.
	Vertical	"	-	-	P.	P.
3 day	Horizontal	P.	L.	L.	L.	L.
	Vertical	-	P.	P. 90% L.	"	"
4 day	Horizontal	P.	L.	L.	"	"
	Vertical	"	P.	"	"	"

Control plates showed no activity.

EXPERIMENT 58.

The effect on production of staphylococcal factor of an alteration in the surface to volume ratio of meat broth medium.

MEDIUM: Meat broth. A reduction in the amount of meat broth from the usual 100 ml. to 60 ml. in the 250 ml. conical flasks was effected.

INOCULUM: 2 ml. of a 6 hour old "Lab-Lemco" broth culture of Staphylococcus aureus, Strain A was used to inoculate each flask. Five different batches of staphylococcal factor, each consisting of 12 flasks of medium were prepared in this way.

INCUBATION: The flasks were incubated at 37°C. for 4 days.

METHOD OF PREPARATION OF STAPHYLOCOCCAL FACTOR: The cultures were centrifuged and sterilised by heating at 65°C. for 10 minutes. They were tested out by incorporating them in serum-milk-agar plates as follows:-

Milk	2.0	2.0	2.0	2.0	2.0	2.0
Serum	0.5	0.5	0.5	0.5	0.0	0.5
Staphylococcal Factor	1.0	2.0	3.0	4.0	2.0	0.0
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5	4.5
Distilled Water	7.0	6.0	5.0	4.0	6.5	8.0

READINGS: The plates were incubated at 37°C. for 24 hours when readings were made.

EXPERIMENT 58.

RESULTS: After incubation of plates at 37°C. for 24 hours.

Batch Number	Amount of Culture			
	1.0	2.0	3.0	4.0
1.	P. 20% L.	P. 70% L.	P. 90% L.	P. 95% L.
2.	P.	L.	L.	L.
3.	P. 50% L.	P. 60% L.	"	"
4.	"	L.	"	"
5.	P. 95% L.	"	"	"

Control plates showed no activity.

EXPERIMENT 59.

A study of the effect of Seitz filtration on the activity of staphylococcal factor produced in meat broth.

A preparation of staphylococcal factor was produced for this experiment which when tested with 0.5 ml. serum had the following activity.

Amount of Culture	0.5	1.0	2.0	3.0	4.0
	-	P. 30% L.	L.	L.	L.

A 50 ml. sample of this sterilised culture was passed through a Seitz filter, fitted with a Ford "Sterimat" S.B. sterilising pad. The first 10 ml. of the filtrate was collected and its activity ascertained. The remainder of the filtrate was collected separately and also tested. These two filtrates were tested in serum-milk-agar plates (which contained 2.0 ml. milk and 0.5 ml. serum) in 0.5, 1.0, 2.0, 3.0 and 4.0 ml. quantities.

A mixture of the sterilised culture and human serum in the proportion of 6 parts to 1 part was made and filtered through a Seitz filter fitted with an S.B. pad. The first 10 ml. of the filtrate was collected and its activity tested; the remainder of the filtrate was also collected and tested separately. These two filtrates obtained from the mixture of staphylococcal culture and serum were tested for activity by incorporation in milk-agar plates in quantities of 0.5, 1.0, 2.0, 3.0 and 4.0 ml.

The culture, unfiltered, was retested in amounts of 0.5, 1.0, 2.0, 3.0 and 4.0 ml. in serum-milk-agar plates to act as a control for the filtered culture.

The plates were incubated at 37°C. for 24 hours when readings were made.

EXPERIMENT 59.

RESULTS: After incubation of plates at 37°C. for 24 hours.

Test Fluid	0.5	1.0	2.0	3.0	4.0
Culture.	-	P.30%L.	L.	L.	L.
First 10 ml. of Culture filtered.	-	-	-	-	-
Remainder of Filtrate.	-	P.	P.90%L.	L.	L.
Culture and Serum mixture.	-	-	L.	"	"
First 10 ml. of filtrate of serum culture mixture	-	-	P-	P.	P.
Remainder of filtrate of mixture.	-	-	P.90%L.	L.	L.

EXPERIMENT 60.

Preparation of staphylococcal factor by Elek's method.

MEDIUM: Elek's basal medium in 1% New Zealand agar (Elek, 1953). (App. 463). 20 ml. amounts of this medium were used in each Petri dish employed which were six in all.

INOCULUM: Parallel strokes 4 cms. apart were made on the surface of the medium with loopsful of a "Lab-Lemco" broth culture, 6 hours' old of Staphylococcus aureus, Strain A.

INCUBATION: The petri dishes were sealed with plasticine and incubated at 37°C. for 4 days.

PREPARATION OF STAPHYLOCOCCAL FACTOR: The petri dishes were removed from the incubator and placed in the deep freeze until the agar was frozen solid. The agar was then thawed rapidly and the liquid which exuded from it was pipetted off. The agar from each plate was placed in a strainer and the fluid which dripped from it was collected and mixed with the exuded liquid. In all about 60 ml. of fluid was obtained from the six petri dishes of medium. This fluid was filtered through a Seitz filter with a "Sterimat" S.B. (sterilising pad). This pad had been previously washed with 20 ml. of sterile distilled water, a procedure which obviated the discarding of the first 10 ml. of filtrate. The filtrate was tested in serum-milk plates as follows:-

Milk	2.0	2.0	2.0	2.0	2.0	2.0
Serum	0.5	0.5	0.5	0.5	0.5	0.0
Staphylococcal Factor	0.5	1.0	2.0	3.0	4.0	4.0
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5	4.5
Distilled Water	7.5	7.0	6.0	5.0	4.0	4.5

The filtrate was also tested out in the same amounts in serum-haemoglobin-agar plates which contained 1.5 ml. of serum and 0.75 ml. of haemoglobin solution.

READINGS: Both sets of plates were incubated at 37°C. for 24 hours when readings were taken.

EXPERIMENT 60.

RESULTS: After incubation at 37°C. for 24 hours.

Nature of Substrate	Amount of Filtrate				
	0.5	1.0	2.0	3.0	4.0
Milk	L.	L.	L.	L.	L.
Haemoglobin	-	P.	P.	P.	P.

Control plates without serum showed no activity.

COLLECTED RESULTS.

The preparation and activity of a number of batches of staphylococcal factor.

MEDIUM: Meat broth. 50 ml. amounts of this medium in 150 ml. conical flasks were used.

INOCULA: Six batches of staphylococcal factor were prepared in meat broth having received inoculum "A", the remaining four batches having received inoculum "B". Inoculum "A" was derived from a heavy growth of Staphylococcus aureus, strain A which had been obtained by centrifuging a meat broth culture of this organism some 9 months previously. It had been preserved in sterile tubes in the ice-chest for this period. It was used in the following way. A quantity of it was used to inoculate a tube of 10 ml. meat broth which was incubated at 37°C. for 24 hours. "Lab-Lemco" agar slopes were inoculated from this culture. The flasks of Batch 1 each received 1 ml. of a suspension made from this "Lab-Lemco" agar slope culture in meat broth; the flasks of batches 2 to 6 each received 1 ml. of a 72 hour old meat broth culture of this organism from the previous batch of staphylococcal factor produced.

Inoculum "B" was derived from a 12 month old waxed-culture of Staphylococcus aureus, strain A. This was used to inoculate a tube of meat broth which was incubated at 37°C. for 24 hours. 1 ml. of this culture was used to inoculate each of the flasks of Batch 7. The flasks of Batches 8, 9 and 10 each received as their inoculum 1 ml. of a 72 hour old meat broth culture of this organism, derived from the previous batch of staphylococcal factor produced, i.e. Batch 8 from Batch 7, Batch 10 from Batch 9.

INCUBATION: All flasks were incubated at 37°C. for 4 days. At the end of this period all flasks were withdrawn from the incubator, the cultures were centrifuged, sterilised by heating at 65°C. for 10 minutes and incorporated in serum-milk-agar plates as follows:-

COLLECTED RESULTS (Continued).

Milk	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Serum	0.5	0.5	0.5	0.5	0.5	0.0	0.5
Staphylococcal Factor	0.5	1.0	2.0	3.0	4.0	2.0	0.0
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5	4.5	4.5
Distilled Water	7.5	7.0	6.0	5.0	4.0	6.5	8.0

The same batch of serum was used in the testing of the activity of all these lots of staphylococcal factor.

Readings were taken after incubation of the plates at 37°C. for 24 hours.

Batch Number	Inoculum	Amount of Culture				
		0.5	1.0	2.0	3.0	4.0
1.	A.	L.	L.	L.	L.	L.
2.	"	"	"	"	"	"
3.	"	"	"	"	"	"
4.	"	"	"	"	"	"
5.	"	"	"	"	"	"
6.	"	P.	"	"	"	"
7.	B.	-	P.	P.70%L.	"	"
8.	"	-	-	P.	P.10%L.	P.20%L.
9.	"	-	P.	P.80%L.	L.	P.95%L.
10.	"	-	P.20%L.	L.	"	L.

Control plates showed no activity.

EXPERIMENT 62.

Examination of Staphylococcus aureus, Strain A on serum-milk-agar and horse-blood-agar plates.

A tube of 1% "Lab-Lemco" broth was inoculated from an agar slope culture of Staphylococcus aureus, strain A and incubated at 37°C. for a period of 21 days. Subcultures were made from it on serum-milk-agar plates (App. 476) and 10% horse-blood-agar plates (App. 456) after 6, 18 and 24 hours' incubation and after 5, 7, 8, 12, 14, 19 and 21 days' incubation.

The colonies of the staphylococcus developing on the plates were closely examined and their abilities to produce lysis and plaques on the serum-milk-agar plates and haemolysis on the horse-blood-agar plates were recorded. The results obtained from these plates after 2 days' incubation at 37°C., are recorded.

EXPERIMENT 62.

RESULTS: After 48 hours' incubation at 37°C.

Age of Culture	Serum-milk-agar	Horse-blood-agar
6 hours	= L.P.	H.
18 "	"	"
24 "	"	Type I H. " II NH.
5 days	"	" I H. " II NH.
7 "	"	" I H. " II NH.
8 "	"	" I H. " II NH.
12 "	Type I S.L. " II L.P.	" I H. " II NH.
14 "	" I S.L. " II L.P.	" I H. " II NH.
19 "	" I S.L. " II L.P.	" I H. " II NH.
21 "	" I S.L. " II L.P.	...

H = haemolytic colonies.

NH = non-haemolytic colonies.

EXPERIMENT 63.

Investigation of Colonies Type I and Type II from Horse-blood-agar plate inoculated from 5 day old "Lab-Lemco" broth culture of Staphylococcus aureus, Strain A.

The two types of colony - the large flat haemolytic designated Type I and the small raised non-haemolytic - Type II were grown in 1% "Lab-Lemco" broth for 6 hours at 37°C. and then plated out on serum-milk-agar and blood-agar plates which were incubated at 37°C. for 24 hours.

Readings were taken after 24 hours at 37°C.

EXPERIMENT 63.

RESULTS: After 24 hours' incubation at 37°C.

Strain A.	Serum-milk-agar	Horse-blood-agar
Type I	Indefinite lysis around colonies.	All colonies showed haemolysis.
Type II	Lysis and Plaques around all colonies.	No haemolysis around colonies.*

* Slight haemolysis developed after 48 hours at 37°C.

EXPERIMENT 64.

Investigations with Type I and Type II colonies on horse-blood-agar and serum-milk-agar plates.

Type I and Type II colonies were picked up from horse blood-agar plates and serum-milk-agar plates into tubes of 1% "Lab-Lemco" broth and meat broth. These tubes were incubated at 37°C. for 6 hours and were used to inoculate plates of serum-milk-agar and horse-blood-agar.

The plates were incubated at 37°C. for 24 hours when readings were taken.

The meat broth and "Lab-Lemco" broth tubes were re-incubated at 37°C. for 24 hours when the naked-eye appearance of the growth of the Type I and Type II organisms was noted.

EXPERIMENT 64.

RESULTS: After 24 hours' incubation at 37°C.

Origin of Strain	Growth in broth	Serum-milk-agar	Horse-blood-agar
Blood-agar Plate			
Type I in Lemco	Turbid : Sediment	Indef. L.	H.
" " Meat	" "	"	"
Type II in Lemco	Clear "	L.P.	N.H.
" " Meat	" "	"	"
Serum-milk-agar Plate			
Type I in Lemco	Turbid : Sediment	Indef. L.	H.
" " Meat	" "	"	"
" II " Lemco	Clear "	L.P.	N.H.
" " Meat	" "	"	"

EXPERIMENT 65.

Comparison of the abilities of Strains AI and AII to ferment "sugars" and liquefy gelatin.

A 20 hour old peptone water culture of Strain AI was used to inoculate a set of "sugar" tubes consisting of glucose, lactose, mannitol, dulcite and sucrose. A tube of litmus milk was also inoculated. A similar set of tubes received similar inocula from a 20 hour old peptone water culture of Strain AII. These were incubated at 37°C. for 5 days when readings were taken. Tubes of 15% gelatin (nutrient) received stab inocula of these two strains. They were incubated at room temperature for 5 days.

The peptone water cultures were also used to inoculate plates of serum-milk-agar and horse-blood-agar. These were incubated at 37°C. for 24 hours when readings were taken.

EXPERIMENT 65.

RESULTS: After 24 hours at 37°C.

Strain of Organism	Blood-agar plate	Serum-milk-agar plate
AI Peptone Water Culture	H.	Indef. L.
AII " " "	N.H.	L.P.

RESULTS: After 5 days at 37°C.

Medium	Strain AI	Strain AII
Glucose	A	A
Lactose	"	"
Mannitol	"	"
Dulcite	"	"
Sucrose	"	"
Litmus Milk	A.C.R.	A.C.R.
Gelatin	±	±

A = Acid.
C = Clot.
R = Reduction.

± (of gelatin) - liquefaction.

EXPERIMENT 66.

Comparison of the abilities of Staphylococcus aureus, Strains AI and AII to produce the staphylococcal factor in meat broth.

MEDIUM: Meat broth was dispensed in 50 ml. amounts in conical flasks, in all 14 such flasks were prepared, two sets of 7 flasks.

INOCULA: 6 hour old meat broth cultures of Staphylococcus aureus, Strains AI and AII were prepared from waxed "Lab-Lemco" agar slope cultures. Each flask of one set of seven was inoculated with 1 ml. of the 6 hour old meat broth culture of Strain AI; the other set was similarly inoculated from the culture of AII.

INCUBATION: The two sets of flasks were incubated at 37°C.

PREPARATION OF STAPHYLOCOCCAL FACTOR: One flask from each set was withdrawn from the 37°C. incubator after 24 hours' incubation and at 24 hour intervals up to the 8th day after inoculation (excluding Sunday). The cultures were heat-killed at 65°C. for 10 minutes and tested by incorporating them in amounts of 0.25 ml. up to 4.0 ml. in serum-milk-agar plates as follows:-

Milk	2.0	2.0	2.0	2.0	2.0	2.0
Serum	0.5	0.5	0.5	0.5	0.5	0.5
Culture	0.25	0.5	1.0	2.0	3.0	4.0
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5	4.5
Distilled Water	7.75	7.5	7.0	6.0	5.0	4.0

EXPERIMENT 66.

RESULTS: After incubation at 37°C. for 24 hours.

Age of Culture	Strain	Amount of Culture (ml.)					
		0.25	0.5	1.0	2.0	3.0	4.0
24 hrs.	AI	...	...	-	-	-	-
	AII	-	L.	L.	L.	L.	L.
2 day	AI	...	...	-	-	-	-
	AII	L.	L.	L.	L.	L.	L.
3 day	AI	...	...	-	-	-	-
	AII	L.	L.	L.	L.	L.	L.
4 day	AI	...	...	-	-	-	-
	AII	L.	L.	L.	L.	L.	L.
5 day	AI	...	...	-	-	-	-
	AII	L.	L.	L.	L.	L.	L.
7 day	AI	...	...	-	-	-	-
	AII	L.	L.	L.	L.	L.	L.
8 day	AI	...	...	-	-	-	-
	AII	L.	L.	L.	L.	L.	L.

EXPERIMENT 67.

The examination of waxed stock cultures of Staphylococcus aureus, strain AII to ascertain if non-lysis and plaque producing variants appear after sub-culture on different media.

MEDIA: Tubes of 1% "Lab-Lemco" broth, meat broth and glucose broth each containing 8 ml. of medium.

INOCULUM: Two tubes of each of these media were inoculated directly from a 5 month old waxed stock culture of Staphylococcus aureus, Strain AII.

INCUBATION: One tube of each medium was incubated at room temperature for 30 days, and one at 37°C. also for 30 days.

SUBCULTURES: These were made from each tube of medium incubated at room temperature and at 37°C. on to serum-milk-agar plates which were incubated at 37°C. for 24 hours.

READINGS: These were noted after 24 hours' incubation of the plates.

EXPERIMENT 67.

RESULTS: After incubation of plates at 37°C. for 24 hours.

Age of Culture	Room Temperature Incubation			37°C. Incubation		
	Meat Broth	"Lab-Lemco"	Glucose	Meat	"Lab-Lemco"	Glucose
6 hrs.	-	-	-	L.P.	L.P.	L.P.
30 "	L.P.	L.P.	L.P.	"	"	"
4 days	"	"	"	"	"	"
5 "	"	"	"	"	"	"
6 "	"	"	"	"	"	"
7 "	"	"	"	"	"*	"
30 "	"*	"*	"*	"*	"*	...

* In addition a few colonies showing only S.L.

EXPERIMENT 68.

Examination of Staphylococcus aureus, Strains No. 72822 and No. 73016 on serum-milk-agar and horse-blood-agar plates.

Two tubes of 1% "Lab-Lemco" broth were inoculated, one from a Dorset's egg culture of Staphylococcus aureus, Strain No. 72822, the other from a similar culture of Strain No. 73016. These tubes of broth were incubated at 37°C. for a period of 21 days. Subcultures were made from them on to serum-milk-agar plates (App. 476) and 10% horse-blood-agar after 6, 18 and 24 hours' incubation and after 5, 7, 8, 12, 14, 19 and 21 days' incubation.

The colonies of these two strains developing on the plates were closely examined and their abilities to produce Muller's phenomenon on the serum-milk-agar and haemolysis on the horse-blood-agar plates were recorded. The results obtained from these plates after 2 days' incubation at 37°C. were recorded.

EXPERIMENT 68.

RESULTS: After incubation of plates at 37°C. for 48 hours.

Age of Culture	Strain No.	Serum-milk-agar	Horse-blood-agar
6 hours.	72822	L.P.	H.
18 "	72822	"	"
24 "	"	"	"
5 days	"	"	"
7 "	"	"	"
8 "	"	"	"
12 "	"	"	"
14 "	"	* "	H. and N.H.
19 "	"	* "	" " "
21 "	"	"	" " "
6 hours	73016	L.P.	H.
18 "	"	"	"
24 "	"	"	"
5 days	"	"	"
7 "	"	"	"
8 "	"	"	"
12 "	"	"	N.H.
14 "	"	"	"
19 "	"	S.L.P.	"
21 "	"	L.P.	ø N.H.

* White and orange colonies.

ø Difference in size and colour.

H = Haemolytic colonies.

NH = Non-haemolytic colonies.

EXPERIMENT 69 (Continued).

Five plates were prepared as shown above for the testing of each batch of staphylococcal factor made from each medium used. The testing was carried out with material prepared from cultures every 24 hours for 7 days (Sundays excepted).

The pH of the culture was estimated and recorded before centrifuging.

As each culture in the different medium aged it was plated out on 10% horse blood-agar and serum-milk-agar plates to observe by the appearance of the colonies on the former and by the production of Muller's phenomenon on the latter any variation if it had occurred.

LIST OF MEDIA USED IN EXPERIMENT 69.

I.	Meat Broth (containing 1% Oxoid Peptone).
II.	1% "Lab-Lemco" broth.
III.	1% Oxoid Peptone.
IV.	1% Fairchild Peptone.
V.	1% Evan's Peptone.
VI.	Yeastrel.
VII.	Yeastrel with 1% Oxoid Peptone.
VIII.	Glucose broth.
IX.	Meat broth without Peptone.
X.	Meat broth with Evan's Peptone (1%).
XI.	1% "Lab-Lemco" broth without Peptone.
XII.	1% "Lab-Lemco" broth with 1% Evan's Peptone.
XIII.	Double Strength Bacto Broth. *
XIV.	" " " " + 0.5% NaCl.
XV.	" " " " in Meat broth.
XVI.	" " " " in Whey. "
XVII.	" " " " + 0.5% NaCl. + 1% "Lab-Lemco".
XVIII.	" " " " + 5% Human Serum.

* Bacto Heart Infusion Broth.

EXPERIMENT 69.

To ascertain the activity of staphylococcal factor produced by Staphylococcus aureus, Strain AII in 18 different media.

MEDIA: The 18 different bacteriological media used in this experiment are listed below and their method of preparation is given in Appendix 489-490.

The media used were placed in 250 ml. conical flasks in 60 ml. amounts and sterilised by autoclaving at 15 lbs. pressure/sq. in. for 15 minutes.

INOCULUM: A waxed "Lab-Lemco" agar slope culture of Staphylococcus aureus Strain AII was used to inoculate several tubes of meat broth which were incubated at 37°C. for 6 hours. 2 ml. of these 6 hour old broth cultures of Staphylococcus aureus Strain AII were used to inoculate each flask used in the experiments.

INCUBATION: The flasks of media were incubated at 37°C. for 7 days.

PREPARATION OF THE STAPHYLOCOCCAL FACTOR: 15 ml. amounts of culture were withdrawn from the flask of each different medium after periods of incubation at 37°C. The culture was centrifuged, and the supernatant fluid placed in a sterile test-tube, the top of which was heated in a bunsen flame to sterilise it. The test-tube when cold was placed in a water-bath at 65°C. for 10 minutes. This temperature and time combination was sufficient to heat-kill the Staphylococcus aureus. The heat killed staphylococcal culture was tested in serum-milk-agar plates in different amounts to find the activity of the staphylococcal factor as follows:-

Agar (4.5%)	4.5	4.5	4.5	4.5	4.5	Control 4.5
Milk	2.0	2.0	2.0	2.0	2.0	2.0
Human Serum	0.5	0.5	0.5	0.5	0.5	0.0
Staphylococcal Factor	0.5	1.0	2.0	3.0	4.0	2.0
Distilled Water	7.5	7.0	6.0	5.0	4.0	6.5

EXPERIMENT 69.

RESULTS:

Age and Amount (ml.) of heat killed culture		MEDIUM					
		I	II	III	IV	V	VI
24 hrs.	0.5	-	-	-	-	-	-
	1.0	L.	-	-	-	-	-
	2.0	"	P.	-	-	-	-
	3.0	"	P+	-	-	-	-
	4.0	"	P.	-	-	-	-
	pH.	6.1	6.4-6.7	6.7	6.7	6.7	6.7
2 days	0.5	L.	P.	-	-	-	-
	1.0	"	L.	-	-	-	-
	2.0	"	"	-	-	-	-
	3.0	"	"	P-	-	P.	-
	4.0	"	"	"	P-	"	P.
	pH.	6.4-6.7	6.7	6.7	6.7	6.7	6.7
3 days	0.5	L.	S.L.	-	-	-	-
	1.0	"	L.	-	-	-	-
	2.0	"	"	-	-	P.	-
	3.0	"	"	P.	-	"	-
	4.0	"	"	"	-	"	-
	pH.	6.7	6.7	6.7	6.7	6.7	6.7
5 days	0.5	L.	L.	-	-	-	-
	1.0	"	"	-	-	-	-
(4 days - V and VI)	2.0	"	"	-	-	P+	-
	3.0	"	"	P.	P.	"	-
	4.0	"	"	"	P-	"	-
	pH.	6.7	6.7	6.7	6.7	6.7	6.7
6 days	0.5	L.	L.	-	-	-	-
	1.0	"	"	-	-	-	-
(5 days - V and VI)	2.0	"	"	P.	-	P.	-
	3.0	"	"	"	-	P+	-
	4.0	"	"	"	P-	P.	-
	pH.	6.7	6.7	6.7	6.7	6.7	6.7
7 days	0.5	L.	L.	-	-	-	-
	1.0	"	"	-	-	-	-
	2.0	"	"	P.	"	P+	-
	3.0	"	"	"	P-	"	-
	4.0	"	"	"	P.	P.	-
	pH.	6.7	6.7	6.7	6.7	6.7	6.7

EXPERIMENT 69 (Continued).

Age and Amount (ml.) of heat killed culture		MEDIUM			
		VII	VIII	IX	X
24 hrs.	0.5	-	-	-	-
	1.0	-	-	-	-
	2.0	-	-	-	L.
	3.0	P.	-	S.L.	"
	4.0	P+	-	"	P+
	pH.	6.7	3.9	6.4	6.4
2 days.	0.5	-	-	-	-
	1.0	-	-	-	L.
	2.0	-	-	-	"
	3.0	S.L.	-	-	"
	4.0	L.	-	S.L.	"
	pH.	6.7	4.8	6.1	6.7
3 days.	0.5	-	-	-	L.
	1.0	-	-	-	"
	2.0	-	-	-	"
	3.0	S.L.	-	S.L.	"
	4.0	L.	-	"	"
	pH.	6.7	4.8	6.1	7.0
4 days.	0.5	-	-	-	S.L.
	1.0	-	-	-	L.
	2.0	-	-	-	"
	3.0	L.	-	L.	"
	4.0	"	-	"	"
	pH.	6.7	4.8	6.1	6.7
5 days.	0.5	-	-	-	S.L.
	1.0	-	-	-	L.
(6 days) - IX and X).	2.0	S.L.	-	S.L.	"
	3.0	L.	-	L.	"
	4.0	"	-	"	"
	pH.	6.7	4.8	6.1	7.0
7 days.	0.5	-	-	-	L.
	1.0	S.L.	-	-	"
	2.0	L.	-	S.L.	"
	3.0	"	-	L.	"
	4.0	"	-	"	"
	pH.	6.7	4.8	6.1	7.0

EXPERIMENT 69 (Continued).

Age and Amount (ml.) of heat killed culture		MEDIUM			
		XI	XII	XIII	XIV
24 hrs.	0.5	-	-	-	-
	1.0	-	-	-	P.
	2.0	-	P.	P.	P.90%L.
	3.0	S.L.	"	P.90%L.	L.
	4.0	L.	"	L.	"
	pH.	7.0	7.0	6.7	6.7
2 days.	0.5	-	S.L.	-	-
	1.0	-	L.	-	-
	2.0	-	"	S.L.	S.L.
	3.0	L.	"	L.	L.
	4.0	"	"	"	"
	pH.	7.0	7.0	7.0	7.0
3 days.	0.5	-	S.L.	-	-
	1.0	-	L.	-	S.L.
	2.0	P.	"	S.L.	L.
	3.0	L.	"	L.	"
	4.0	"	"	"	"
	pH.	7.0	7.0	7.0	7.0
4 days.	0.5	-	S.L.	-	-
	1.0	-	L.	-	-
	2.0	-	"	S.L.	L.
	3.0	S.L.	"	L.	"
	4.0	"	"	"	"
	pH.	7.0	7.0	7.0	7.0
6 days.	0.5	-	S.L.	-	-
	1.0	-	L.	-	S.L.
(5 days - XIII and XIV.)	2.0	S.L.	"	L.	L.
	3.0	L.	"	"	"
	4.0	"	"	"	(...)
	pH.	7.0	7.0	7.0	7.0
7 days	0.5	-	L.	-	-
	1.0	-	"	-	P-
	2.0	-	"	L.	L.
	3.0	L.	"	"	"
	4.0	"	"	"	"
	pH.	7.0	7.0	7.0	7.0

EXPERIMENT 69 (Continued).

Age and Amount (ml.) of heat killed culture		M E D I U M			
		XV	XVI	XVII	XVIII
24 hrs.	0.5	-	-	-	-
	1.0	-	-	-	S.L.
	2.0	P+	-	S.L.	L.
	3.0	P. 90% L.	-	L.	"
	4.0	"	-	"	"
	pH.	6.7	5.2-5.5	6.7-7.0	6.7-7.0
2 days.	0.5	-	-	-	-
	1.0	-	-	-	S.L.
	2.0	S.L.	-	L.	L.
	3.0	P. 90% L.	-	"	"
	4.0	"	-	"	"
	pH.	6.7	4.9-5.2	6.7-7.0	6.7-7.0
3 days.	0.5	-	-	-	-
	1.0	-	-	S.L.	L.
	2.0	S.L.	-	L.	"
	3.0	P. 90% L.	-	"	"
	4.0	"	-	"	"
	pH.	6.7	4.9-5.2	7.0	7.0-7.3
4 days.	0.5	-	-	-	-
	1.0	-	P.	S.L.	L.
	2.0	P.	90% L.	L.	"
	3.0	P. 90% L.	"	"	"
	4.0	"	(...)	"	"
	pH.	6.4-6.7	— 6.7	7.0	7.0-7.3
5 days	0.5	-	-	-	S.L.
	1.0	-	-	S.L.	L.
	2.0	L.	-	L.	"
	3.0	"	L.	"	"
	4.0	"	"	"	"
	pH.	6.7-7.0	5.2-7.0	7.0	7.0-7.3
7 days	0.5	-	(...)	-	-
	1.0	-	"	S.L.	L.
	2.0	L.	P++	L.	"
	3.0	"	"	"	"
	4.0	"	L.	"	"
	pH.	7.0	5.2-7.0	7.0	7.3

EXPERIMENT 70 (Continued).

SUMMARY OF THE COMPOSITION OF THE 40 MEDIA TESTED.

		EVAN'S PEPTONE			
	NaCl.	0.5%	1.0%	1.5%	2.0%
GROUP A. 1% "Lab-Lemco" with	0.25%	5	1	13	15
	0.5%	6	2	9	11
	0.75%	7	3	10	12
	1.0%	8	4	14	16
GROUP B. 0.5% "Lab-Lemco" with	0.25%	(...)	(...)	(...)	(...)
	0.5%	1	3	5	7
	0.75%	2	4	6	8
	1.0%	(...)	(...)	(...)	(...)
GROUP C. 1.5% "Lab-Lemco" with	0.25%	(...)	(...)	(...)	(...)
	0.5%	1	3	5	7
	0.75%	2	4	6	8
	1.0%	(...)	(...)	(...)	(...)
GROUP D. 2.0% "Lab-Lemco" with	0.25%	(...)	(...)	(...)	(...)
	0.5%	1	3	5	7
	0.75%	2	4	6	8
	1.0%	(...)	(...)	(...)	(...)

The 40 media used in this investigation were placed in one of four Groups, A, B, C or D, and the media in each group given a number. From the above Table the composition of any of these forty media can be ascertained by knowing the group letter and number of the medium.

Thus: "Group C, No. 6" refers to the medium composed of "Lab-Lemco" 1.5%, NaCl. 0.75% and Evan's Peptone 1.5%.

The activity of the staphylococcal factor obtained in these different media was ascertained in serum-milk-agar plates incubated at 37°C. for 24 hours. The results are given under the Group letter and number.

EXPERIMENT 70.

To ascertain the activity of staphylococcal factor produced in a number of different media by Staphylococcus aureus, Strain AII.

MEDIA: Forty different media were made up using different amounts of Evan's Peptone, "Lab-Lemco" and Sodium chloride. The general method of preparing these media is given in Appendix 491 . A summary of the composition of the 40 media used is given below.

For each medium used, 6 flasks of 150 ml. capacity containing 50 ml. of the particular medium was available.

INOCULUM: Each flask of medium received 2.0 ml. of a 6 hour old "Lab-Lemco" broth culture of Staphylococcus aureus, Strain AII. A waxed "Lab-Lemco" agar slope culture of this organism was opened to prepare the inocula for each batch of media used in this experiment.

INCUBATION: The flasks were incubated at 37°C.

PREPARATION OF STAPHYLOCOCCAL FACTOR: One flask of each medium was withdrawn from the 37°C. incubator at 24 hour intervals up to the 7th day after inoculation (excluding Sunday). The staphylococcal culture was centrifuged, heat killed at 65°C. for 10 minutes and tested by incorporating it in amounts of 0.5 ml. up to 4.0 ml. in serum-milk-agar plates as follows:-

					CONTROL	
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5	4.5
Milk	2.0	2.0	2.0	2.0	2.0	2.0
Human Serum	0.5	0.5	0.5	0.5	0.5	0.0
Staphylococcal Culture	0.5	1.0	2.0	3.0	4.0	2.0
Distilled Water	7.5	7.0	6.0	5.0	4.0	6.5

The pH of the culture was ascertained before it was centrifuged.

EXPERIMENT 70.

RESULTS: GROUP A.

Age of Culture	Amount (ml.)	M E D I U M			
		1	2	3	4
18 hrs.	0.5	-	-	-	-
	1.0	-	-	-	P-
	2.0	P-	P.	P.	P.
	3.0	P.	"	"	"
	4.0	"	"	"	"
	pH.	6.7	6.7	6.7	6.7
2 days.	0.5	-	-	-	-
	1.0	S.L.	L.	S.L.	S.L.
	2.0	L.	"	L.	L.
	3.0	"	"	"	"
	4.0	"	"	"	P. 95% L.
	pH.	7.0	7.0	7.0	7.0
3 days.	0.5	-	S.L.	-	P-
	1.0	S.L.	L.	L.	L.
	2.0	L.	"	"	"
	3.0	"	"	"	"
	4.0	"	"	"	P. 95% L.
	pH.	7.0	7.0	7.0	7.0
5 days.	0.5	-	S.L.	S.L.	S.L.
	1.0	L.	L.	L.	L.
	2.0	"	"	"	"
	3.0	"	"	"	"
	4.0	"	"	"	"
	pH.	7.0	7.0	7.0	7.0
6 days.	0.5	-	S.L.	S.L.	S.L.
	1.0	L.	L.	L.	L.
	2.0	"	"	"	"
	3.0	"	"	"	"
	4.0	"	"	"	P. 95% L.
	pH.	7.0	7.0	7.0	7.0
7 days.	0.5	S.L.	S.L.	S.L.	S.L.
	1.0	L.	L.	L.	L.
	2.0	"	"	"	"
	3.0	"	"	"	"
	4.0	"	"	"	P. 95% L.
	pH.	7.0	7.0	7.0	7.0

EXPERIMENT 70 (Continued).

GROUP A.

Age of Culture	Amount (ml.)	M E D I U M			
		5	6	7	8
18 hrs.	0.5	-	-	P-	-
	1.0	-	P-	"	P-
	2.0	P-	P.	P.	P.
	3.0	P.	"	"	"
	4.0	"	"	"	"
	pH.	6.7	6.7	6.7	6.7
2 days.	0.5	-	-	-	-
	1.0	-	S.L.	P-	P.
	2.0	L.	L.	L.	L.
	3.0	"	"	"	"
	4.0	"	"	"	"
	pH.	7.0	7.0	7.0	7.0
3 days.	0.5	-	-	-	-
	1.0	S.L.P.	P. 95% L.	S.L.	S.L.
	2.0	L.	L.	L.	L.
	3.0	"	"	"	"
	4.0	"	"	"	"
	pH.	7.0	7.0	7.0	7.0
5 days.	0.5	-	-	S.L.	-
	1.0	P. 50% L.	P. 95% L.	P. 95% L.	L.
	2.0	L.	L.	L.	"
	3.0	"	"	"	"
	4.0	"	"	"	"
	pH.	7.0	7.0	7.0	7.0
6 days.	0.5	-	-	-	-
	1.0	S.L.	-	P. 95% L.	L.
	2.0	L.	P+	L.	"
	3.0	"	P. 95% L.	"	"
	4.0	"	"	"	"
	pH. £	7.0	7.0	7.0	7.0
7 days	0.5	-	-	S.L.	-
	1.0	P. 50% L.	L.	P. 95% L.	L.
	2.0	L.	"	L.	"
	3.0	"	"	"	"
	4.0	"	"	"	"
	pH.	7.0	7.0	7.0	7.0

EXPERIMENT 70 (Continued).

GROUP A.

Age of Culture	Amount (ml.)	M E D I U M			
		9	10	11	12
18 hrs.	0.5	-	-	-	-
	1.0	-	-	-	-
	2.0	P.	-	P-	P-
	3.0	P+	P-	"	"
	4.0	"	"	"	"
	pH.	6.7	6.7	6.7	6.7
2 days.	0.5	P.	P-	P.	P.
	1.0	L.	P.	L.	L.
	2.0	"	P. 90% L.	"	"
	3.0	"	L.	"	"
	4.0	"	P. 90% L.	P. 95% L.	P. 95% L.
	pH.	6.7	6.7	6.7	6.7
3 days	0.5	95% L.	P-	P+	P+
	1.0	L.	P. 90% L.	L.	L.
	2.0	"	L.	"	"
	3.0	"	"	"	"
	4.0	"	P. 90% L.	P. 95% L.	P. 95% L.
	pH.	7.0	7.0	7.0	7.0
4 days	0.5	95% L.	P.	P.	P. 95% L.
	1.0	L.	L.	L.	L.
	2.0	"	"	"	"
	3.0	"	"	"	"
	4.0	"	P. 95% L.	P. 95% L.	95% L.
	pH.	7.0	7.0	7.0	7.0
5 days	0.5	95% L.	P.	90% L.	95% L.
	1.0	L.	L.	L.	L.
	2.0	"	"	"	"
	3.0	"	"	"	"
	4.0	"	P. 95% L.	P. 95% L.	P. 95% L.
	pH.	7.0	7.0	7.0	7.0
7 days.	0.5	L.	P+	P+	L.
	1.0	"	L.	L.	"
	2.0	"	"	"	"
	3.0	"	"	"	"
	4.0	"	P. 95% L.	P. 95% L.	"
	pH.	7.0	7.0	7.0	7.0

EXPERIMENT 70 (Continued).

GROUP A.

Age of Culture	Amount (ml.)	M E D I U M			
		13	14	15	16
18 hrs.	0.5	-	-	-	-
	1.0	-	-	-	-
	2.0	P.	P.	P-	P.
	3.0	P+	"	P.	P-
	4.0	"	P-	"	-
	pH.	6.7	6.7	6.7	6.7
2 days.	0.5	-	-	-	P-
	1.0	P.	P+	P. 50% L.	P+
	2.0	L.	L.	P. 90% L.	"
	3.0	"	P. 95% L.	L.	P.
	4.0	"	P+	P. 95% L.	P-
	pH.	7.0	7.0	7.0	7.0
3 days.	0.5	-	P.	P.	P.
	1.0	P. 50% L.	P. 90% L.	P. 90% L.	P. 90% L.
	2.0	L.	L.	L.	P. 95% L.
	3.0	"	P. 95% L.	"	P+
	4.0	"	P.	P. 90% L.	P-
	pH.	7.0	7.0	7.0	7.0
4 days.	0.5	-	P-	P.	P.
	1.0	P. 50% L.	P. 95% L.	P. 90% L.	L.
	2.0	L.	L.	L.	"
	3.0	P. 95% L.	P. 95% L.	"	P. 95% L.
	4.0	L.	P.	P. 95% L.	P.
	pH.	7.0	7.0	7.0	7.0
6 days	0.5	-	P.	P.	P. 50% L.
	1.0	P. 90% L.	L.	L.	L.
	2.0	L.	"	"	"
	3.0	L.	P.95% L.	"	P. 95% L.
	4.0	"	P.	P. 95% L.	P.
	pH.	7.0	7.0	7.0	7.0
7 days.	0.5	S.L.	P.	S.L.	50% L.
	1.0	L.	L.	L.	L.
	2.0	"	"	"	"
	3.0	"	"	"	P.
	4.0	P.	P.	P.	P-

EXPERIMENT 70 (Continued).

GROUP B:

Age of Culture	Amount (ml.)	M E D I U M			
		1	2	3	4
18 hrs.	0.5	-	-	-	-
	1.0	-	-	-	-
	2.0	-	-	-	-
	3.0	P.	P.	P.	P.
	4.0	P+	P+	"	"
	pH.	6.7	6.7	6.7	6.7
2 days.	0.5	P-	-	-	-
	1.0	"	-	-	P-
	2.0	P+	P+	P+	P+
	3.0	P. 90% L.	P. 90% L.	P. 90% L.	P. 90% L.
	4.0	L.	L.	"	"
	pH.	6.7	6.7	6.7	6.7
3 days.	0.5	-	-	-	-
	1.0	-	-	P-	P+
	2.0	P+	P+	P+	L.
	3.0	L.	L.	L.	"
	4.0	"	"	P. 95% L.	P. 95% L.
	pH.	7.0	7.0	7.0	7.0
4 days.	0.5	P-	-	-	-
	1.0	P.	P.	P+	P+
	2.0	L.	L.	L.	L.
	3.0	"	"	"	"
	4.0	"	"	"	P. 90% L.
	pH.	7.0	7.0	7.0	7.0
5 days.	0.5	-	-	-	-
	1.0	P.	P.	P+	P+
	2.0	L.	L.	L.	L.
	3.0	"	"	"	"
	4.0	"	"	"	P. 90% L.
	pH.	7.0	7.0	7.0	7.0
7 days.	0.5	-	-	-	-
	1.0	50% L.	P.	P+	P+
	2.0	L.	L.	L.	L.
	3.0	"	"	"	"
	4.0	"	"	P. 95% L.	P. 95% L.
	pH.	7.0	7.0	7.0	7.0

EXPERIMENT 70 (Continued).

GROUP B:

Age of Culture	Amount (ml.)	M E D I U M			
		5	6	7	8
18 hrs.	0.5	-	-	-	-
	1.0	-	-	-	-
	2.0	-	-	-	P-
	3.0	P-	P-	P.	P.
	4.0	"	"	P-	P-
	pH.	6.7	6.7	6.7	6.7
2 days.	0.5	-	-	-	-
	1.0	-	P-	-	P.
	2.0	P+	P+	P+	P+
	3.0	"	"	"	"
	4.0	P.	P.	P.	P-
	pH.	7.0	7.0	7.0	7.0
3 days.	0.5	-	-	-	-
	1.0	P-	P.	P.	P.
	2.0	P+	P+	P+	P+
	3.0	"	"	"	"
	4.0	P.	P.	P.	P-
	pH.	7.0	7.0	7.0	7.0
4 days.	0.5	-	-	-	-
	1.0	-	-	P.	P.
	2.0	P+	P+	P+	"
	3.0	P.	P.	P.	"
	4.0	"	"	"	P-
	pH.	7.0	7.0	7.0	7.0
6 days.	0.5	-	-	-	-
	1.0	-	P.	P.	P+
	2.0	P+	P+	P+	"
	3.0	P.	P.	P.	P.
	4.0	P-	P-	P-	P-
	pH.	7.0	7.0	7.0	7.0
7 days.	0.5	-	-	-	-
	1.0	P.	P.	P.	P+
	2.0	P+	P+	P+	"
	3.0	P.	P.	P.	P.
	4.0	P-	P-	P-	P-
	pH.	7.0	7.0	7.0	7.0

EXPERIMENT 70 (Continued).

GROUP C:

Age of Culture	Amount (ml.)	M E D I U M			
		1	2	3	4
18 hrs.	0.5	-	-	-	-
	1.0	-	-	-	-
	2.0	P.	P.	P.	P.
	3.0	P+	P+	P+	P+
	4.0	"	"	"	"
	pH.	6.7	6.7	6.7	6.7
2 days.	0.5	P.	P.	P.	-
	1.0	L.	L.	L.	L.
	2.0	"	"	"	"
	3.0	"	"	"	"
	4.0	"	"	"	"
	pH.	7.0	7.0	7.0	7.0
3 days.	0.5	P.	P.	(...)	-
	1.0	L.	(...)	"	L.
	2.0	"	P.	"	"
	3.0	"	"	"	"
	4.0	"	"	"	"
	pH.	7.0	7.0	7.0	7.0
5 days.	0.5	P.	P.	P.	P.
	1.0	L.	L.	L.	L.
	2.0	"	"	"	"
	3.0	"	"	"	"
	4.0	"	"	"	"
	pH.	7.0	7.0	7.0	7.0
6 days.	0.5	50% L.	50% L.	50% L.	50% L.
	1.0	L.	L.	L.	L.
	2.0	"	"	"	"
	3.0	"	"	"	"
	4.0	"	"	"	"
	pH.	7.0	7.0	7.0	7.0
7 days.	0.5	50% L.	50% L.	75% L.	50% L.
	1.0	L.	L.	L.	L.
	2.0	"	"	"	"
	3.0	"	"	"	"
	4.0	"	"	"	"
	pH.	7.0	7.0	7.0	7.0

EXPERIMENT 70 (Continued).

GROUP C:

Age of Culture	Amount (ml.)	M E D I U M			
		5	6	7	8
18 hrs.	0.5	-	-	-	-
	1.0	P.	P.	P.	P.
	2.0	P. 90% L.	P+	P+	P+
	3.0	"	"	"	"
	4.0	P+	"	"	"
	pH.	6.7	6.7	6.7	6.7
2 days.	0.5	P.	P.	P.	P.
	1.0	L.	L.	L.	L.
	2.0	"	"	"	"
	3.0	"	"	"	"
	4.0	"	P. 95% L.	P. 95% L.	P. 95% L.
	pH.	7.0	7.0	7.0	7.0
3 days.	0.5	-	-	-	-
	1.0	-	-	-	P.
	2.0	-	P+	P+	P+
	3.0	P+	"	"	"
	4.0	P.	P.	P.	P.
	pH.	7.0	7.0	7.0	7.0
5 days.	0.5	P.	P.	P.	P.
	1.0	L.	L.	L.	L.
	2.0	"	"	"	"
	3.0	"	"	"	"
	4.0	"	P. 95% L.	P. 95% L.	P. 95% L.
	pH.	7.0	7.0	7.0	7.0
6 days.	0.5	P. 75% L.	P. 50% L.	P. 50% L.	P. 75% L.
	1.0	L.	L.	L.	L.
	2.0	"	"	"	"
	3.0	"	"	"	"
	4.0	"	"	"	P. 95% L.
	pH.	7.0	7.0	7.0	7.0
7 days.	0.5	L.	P. 75% L.	L.	L.
	1.0	"	L.	"	"
	2.0	"	"	"	"
	3.0	"	"	"	"
	4.0	"	"	"	P. 95% L.
	pH.	7.0	7.0	7.0	7.0

EXPERIMENT 70 (Continued).

GROUP D:

Age of Culture	Amount (ml.)	M E D I U M			
		1	2	3	4
18 hrs.	0.5	-	-	-	-
	1.0	-	P-	P-	P-
	2.0	P+	P+	P.	P.
	3.0	"	"	P+	P+
	4.0	"	"	"	P.
	pH.	6.7	6.7	6.7	6.7
3 days.	0.5	P-	50% L.	P-	P.
	1.0	L.	L.	L.	L.
	2.0	"	"	"	"
	3.0	"	"	"	"
	4.0	"	"	"	"
	pH.	7.0	7.0	7.0	7.0
4 days.	0.5	P.	L.	90% L.	90% L.
	1.0	L.	"	L.	L.
	2.0	"	"	"	"
	3.0	"	"	"	"
	4.0	"	"	"	"
	pH.	7.0	7.0	7.0	7.0
5 days.	0.5	50% L.	50% L.	75% L.	50% L.
	1.0	90% L.	L.	L.	L.
	2.0	L.	"	"	"
	3.0	"	"	"	"
	4.0	"	"	"	"
	pH.	7.0	7.0	7.0	7.0
6 days.	0.5	P.	50% L.	50% L.	50% L.
	1.0	90% L.	L.	75% L.	L.
	2.0	L.	"	L.	"
	3.0	"	"	"	"
	4.0	"	"	"	"
	pH.	7.0	7.0	7.0	7.0
7 days.	0.5	50% L.	50% L.	50% L.	50% L.
	1.0	L.	L.	L.	L.
	2.0	"	"	"	"
	3.0	"	"	"	"
	4.0	"	"	"	"
	pH.	7.0	7.0	7.0	7.0

EXPERIMENT 70 (Continued).

GROUP D:

Age of Culture	Amount (ml.)	M E D I U M			
		5	6	7	8
24 hrs.	0.5 1.0 2.0 3.0 4.0 pH.	P. P+ 95% L. " P+ 6.7	P. P+ 95% L. " P. 6.7	P- P+ 95% L. " P+ 6.7	P- P. P+ " P- 6.7
3 days.	0.5 1.0 2.0 3.0 4.0 pH.	90% L. L. " " " 7.0	L. " " " " 7.0	L. " " " " 7.0	L. " " " " 7.0
4 days.	0.5 1.0 2.0 3.0 4.0 pH.	90% L. L. " " " 7.0	L. " " " " 7.0	L. " " " " 7.0	L. " " " " 7.0
5 days.	0.5 1.0 2.0 3.0 4.0 pH.	L. " " " " 7.0	L. " " " " 7.0	L. " " " " 7.0	L. " " " P. 95% L. 7.0
6 days.	0.5 1.0 2.0 3.0 4.0 pH.	50% L. L. " " " 7.0	90% L. L. " " P. 95% L. 7.0	L. " " " " 7.0	L. " " " P. 95% L. 7.0
7 days.	0.5 1.0 2.0 3.0 4.0 pH.	90% L. L. " " " 7.0	90% L. L. " " " 7.0	90% L. L. " " " 7.0	90% L. L. " " " 7.0

EXPERIMENT 71.

A comparison of the production of staphylococcal factor prepared in four different media.

MEDIA: Four media out of all those previously used were chosen for retesting viz. A9, B5, C5 and D7. These four media all contained "Lab-Lemco", Evan's peptone and sodium chloride, although in different amounts.

A9	1% "Lab-Lemco"	1.5% Evan's Peptone	0.5% NaCl.
B5	0.5% "Lab-Lemco"	1.5% " "	0.5% "
C5	1.5% " "	1.5% " "	0.5% "
D7	2.0% " "	2.0% " "	0.5% "

The required amounts of "Lab-Lemco", Peptone and sodium chloride were dissolved in 150 ml. of distilled water, the reaction adjusted to pH 7.4 and the medium divided equally between two flasks. Each medium was prepared in a similar way. The eight flasks of media were sterilised by autoclaving at 15 lbs./sq. in. for 15 minutes.

INOCULUM: Each flask of medium was inoculated with 2.0 ml. of a 6 hour old "Lab-Lemco" broth culture of Staphylococcus aureus, Strain AII.

INCUBATION: The flasks were incubated at 37°C. for 7 days.

PREPARATION OF THE STAPHYLOCOCCAL FACTOR: 15 ml. amounts of culture from each of the four media were withdrawn after 24 hours' incubation at 37°C., and after each subsequent 24 hours' incubation up to 7 days. The culture was centrifuged, sterilised by heating at 65°C. for 10 minutes and the activity was tested by incorporation in serum-milk-agar plates as usual.

EXPERIMENT 71.

RESULTS: After incubation of plates at 37°C. for 24 hours.

Age of Culture	Amount (ml.)	A9	B5	C5	D7
24 hrs.	0.25	-	-	-	-
	0.5	-	-	-	-
	1.0	P-	-	P-	P. 50% L.
	2.0	P+	P.	P+	L.
	3.0	"	P+	"	"
	4.0	"	P.	"	"
2 days.	0.25	-	-	-	S.L.
	0.5	P.	-	P. 90% L.	L.
	1.0	P. 90% L.	P.	L.	"
	2.0	L.	P+	"	"
	3.0	"	P. 95% L.	"	"
	4.0	"	P+	"	"
3 days.	0.25	-	-	-	P. 50% L.
	0.5	P. 50% L.	-	L.	L.
	1.0	L.	P.	"	"
	2.0	"	P+	"	"
	3.0	"	P. 95% L.	"	"
	4.0	"	L.	"	"
4 days.	0.25	-	-	-	P. 50% L.
	0.5	P.	-	L.	L.
	1.0	P. 75% L.	P.	"	"
	2.0	L.	P+	"	"
	3.0	"	P.	"	"
	4.0	"	"	"	"
6 days.	0.25	-	-	P.	P. 90% L.
	0.5	P.	-	L.	L.
	1.0	P. 75% L.	P.	"	"
	2.0	L.	P+	"	"
	3.0	"	P.	"	"
	4.0	P. 90% L.	"	"	"
7 days.	0.25	-	-	P.	P. 50% L.
	0.5	P.	-	90% L.	L.
	1.0	P. 90% L.	P.	L.	"
	2.0	L.	P+	"	"
	3.0	"	P. 95% L.	"	"
	4.0	"	P.	"	"

EXPERIMENT 72.

To compare the activity of staphylococcal factor produced by Strain AII in medium D7, one part of the culture having been heat-killed at 65°C. for 10 minutes, the other part Seitz filtered.

MEDIUM: 250 ml. of Medium D7 (App. 194) which contained 2% "Lab-Lemco", 2% Evan's Peptone, 0.5% NaCl. at pH 7.4 was prepared, sterilised by autoclaving at 15 lbs./15 minutes in 4 flasks each containing 60 ml. of medium.

INOCULUM: All four flasks were inoculated each with 2.0 ml. of a 6 hour old "Lab-Lemco" broth culture of Staphylococcus aureus, Strain AII and incubated at 37°C.

INCUBATION:

PREPARATION OF STAPHYLOCOCCAL FACTOR: After 24 hours' incubation at 37°C. the flasks were withdrawn, sufficient culture removed and prepared for testing of its activity with regard to the staphylococcal factor.

HEAT KILLED CULTURE - prepared by heating at 65°C. for 10 minutes.

FILTERED CULTURE - the culture was centrifuged. The Seitz filter pad was washed with 10 ml. distilled water and 10 ml. of uninoculated medium, then the culture was filtered through the pad and the filtrate collected and tested.

These filtrates and the heat killed cultures were tested for activity by incorporation in serum-milk-agar plates. They were made from the cultures at 24 hour intervals for 7 days (excluding Sunday).

EXPERIMENT 72.

RESULTS: After incubation at 37°C. for 24 hours.

Age of Culture		Amount of Culture in Plate (ml.)						
		0.25	0.5	1.0	2.0	3.0	4.0	pH.
24 hrs.	H-K	-	P.	P.75%L.	L.	L.	L.	6.7
	F	-	"	P.	"	"	"	"
2 days.	H-K	-	P.	L.	L.	L.	L.	7.0
	F	-	P.90%L.	"	"	"	"	"
3 days.	H-K	P.50%L.	L.	L.	L.	L.	L.	7.0
	F	"	"	"	"	"	"	"
4 days.	H-K	P.50%L.	L.	L.	L.	L.	L.	7.0
	F	P.90%L.	"	"	"	"	"	7.3
5 days.	H-K	P.75%L.	L.	L.	L.	L.	L.	7.0
	F	L.	"	"	"	"	"	7.3
7 days.	H-K	L.	L.	L.	L.	L.	L.	7.0-7.3
	F	P.90%L.	"	"	"	"	"	7.3

H-K = heat killed culture from Medium D7.

F = filtered culture from Medium D7.

EXPERIMENT 73.

To ascertain if the staphylococcal factor is produced under anaerobic conditions.

MEDIUM: The medium used in this experiment was Medium D7 (App. 194) which contained 2% "Lab-Lemco", 2% Evan's Peptone, 0.5% NaCl. at pH 7.4.

250 ml. of this medium was prepared, sterilised at 15 lbs./sq. in. for 15 minutes in the autoclave and 60 ml. amounts placed in each of three 250 ml. conical flasks.

INOCULUM: These three flasks were inoculated each with 2.0 ml. of an 18 hour old 1% "Lab-Lemco" broth culture of Staphylococcus aureus, Strain AII which had been prepared from a waxed "Lab-Lemco" agar slope culture of this organism.

INCUBATION: Each flask was then placed in a McIntosh & Fildes anaerobic jar and incubated at 37°C.

PREPARATION OF STAPHYLOCOCCAL FACTOR: One flask was removed from a McIntosh & Fildes jar after 2 days' and 4 days' and 6 days' incubation at 37°C.; the culture was heat-killed at 65°C. for 10 minutes, and then tested for activity by incorporating it in serum-milk-agar plates in amounts from 0.5 ml. to 4.0 ml. Readings were made after 24 hours' incubation at 37°C.

EXPERIMENT 73.

RESULTS: After 24 hours' incubation of plates at 37°C.

Amount of Heat-killed extract in plates (ml.)	2 days' old anaerobic culture	4 days' old anaerobic culture	6 days' old anaerobic culture
0.5	-	-	P. 50% L.
1.0	-	-	"
2.0	P+	P.	L.
3.0	"	"	"
4.0	P-	P-	"
pH.	6.7-7.0	6.7-7.0	6.7-7.0

Similar results to the above were obtained with duplicate set of serum-milk-agar plates incubated under anaerobic conditions.

EXPERIMENT 74.

To ascertain the effect of different amounts of carbon dioxide in the atmosphere on the amount of staphylococcal factor produced by Strain AII Staphylococcus aureus in Medium B5.

MEDIUM: 50 ml. amounts of Medium B5 (0.5% "Lab-Lemco", 1.5% Evan's Peptone, 0.5% NaCl. pH 7.4) was placed in each of eight flasks and autoclaved (15 lbs./15 minutes).

INOCULUM: These flasks of medium were inoculated each with 2.0 ml. of 6 hour old "Lab-Lemco" broth culture of Strain AII. Two flasks were placed in a jar with 5% CO_2 in the atmosphere; 2flasks in jar with 10% CO_2; 2 flasks with 15% CO_2. The remaining 2 flasks were left aerobically.

INCUBATION: All flasks were incubated at 37°C.

Heat-killed culture was prepared from each set of flasks after incubation for 2 days and 4 days, and tested by incorporating it in serum-milk-agar plates in amounts from 0.25 ml. up to 4.0 ml.

READINGS: These were made after 24 hours' incubation at 37°C.

EXPERIMENT 74.

RESULTS: After incubation of plates at 37°C. for 24 hours.

Age of Culture	Amount (ml.)	Aerobic Control	Percentate of Carbon Dioxide in Air		
			5	10	15
2 days.	0.25	-	-	-	-
	0.5	-	L.	L.	L.
	1.0	-	"	"	"
	2.0	P-	"	"	"
	3.0	P.	"	"	"
	4.0	"	"	"	"
	pH.	7.0	7.0	7.0	7.0
4 days.	0.25	-	90% L.	L.	90% L.
	0.5	-	L.	"	L.
	1.0	-	"	"	"
	2.0	P.	"	"	"
	3.0	"	"	"	"
	4.0	-	"	"	"
	pH.	7.3	7.3	7.3	7.3

EXPERIMENT 75.

To ascertain the activity of staphylococcal factor produced under different amounts of carbon dioxide in the atmosphere in Medium D7.

MEDIUM: 350 ml. of Medium D7 (2% "Lab-Lemco", 2% Evan's Peptone, 0.5% NaCl., pH 7.4) was made and 50 ml. distributed to each of 5 flasks which were autoclaved at 15 lbs./15 minutes.

INOCULUM: Each flask was inoculated with 2.0 ml. of a 6 hour old "Lab-Lemco" broth culture of Staphylococcus aureus, Strain AII.

INCUBATION: Four of the flasks were placed in jars which had respectively concentrations of Carbon dioxide 5%, 10%, 15% and 20%. The remaining flask was incubated aerobically. All the flasks were incubated at 37°C. for 4 days when heat-killed culture was prepared from each of these flasks, its activity being tested in serum-milk-agar plates in amounts of 0.1 ml. up to 4.0 ml.

READINGS: These were made after 24 hours' incubation at 37°C.

EXPERIMENT 75.

RESULTS: After incubation of plates at 37°C. for 24 hours.

Amount of culture (ml.)	Aerobic Control	Percentage of Carbon Dioxide in Air			
		5	10	15	20
0.1	-	90% L.	90% L.	75% L.	-
0.25	75% L.	L.	L.	L.	L.
0.5	L.	"	"	"	"
1.0	"	"	"	"	"
2.0	"	"	"	"	"
3.0	"	"	"	"	"
4.0	95% L.	"	"	"	"
pH.	7.3	7.3	7.0-7.3	7.0-7.3	7.0-7.3

EXPERIMENT 76.

To ascertain the effect on the production of staphylococcal factor of the addition of human serum (Batch 5) to Medium D7.

MEDIUM: 50 ml. amounts of Medium D7 (2% "Lab-Lemco", 2% Evan's Peptone, 0.5% NaCl. pH 7.4) were placed in each of 8 flasks and autoclaved at 15 lbs./15 minutes. 1% human serum was added to 2 flasks, 5% serum to 2 flasks and 10% serum to another 2 flasks; 2 flasks of Medium D7 without serum served as a control.

INOCULUM: Each of the eight flasks was inoculated with 2.0 ml. of 6 hour old "Lab-Lemco" broth culture of Staphylococcus aureus, Strain AII and incubated at 37°C.

A portion of the culture from each of the four sets of flasks was taken every 24 hours, heat killed and tested for activity of the staphylococcal factor by addition to serum-milk-agar plates.

EXPERIMENT 76.

RESULTS: After 24 hours' incubation of plates at 37°C.

Age of Culture	Amount (ml.)	Control	S E R U M		
			1%	5%	10%
24 hrs.	0.25	-	-	-	-
	0.5	-	-	-	-
	1.0	P. 50% L.	P. 50% L.	P. 50% L.	-
	2.0	L.	L.	P. 75% L.	P. 75% L.
	3.0	"	"	L.	L.
	4.0	"	"	"	"
2 days	0.25	-	-	-	-
	0.5	P. 50% L.	P. 50% L.	P. 50% L.	P. 50% L.
	1.0	L.	L.	90% L.	90% L.
	2.0	"	"	L.	L.
	3.0	"	"	"	"
	4.0	"	"	"	"
3 days.	0.25	-	-	-	-
	0.5	L.	L.	L.	P. 75% L.
	1.0	"	"	"	L.
	2.0	"	"	"	"
	3.0	"	"	"	"
	4.0	"	"	"	"
4 days.	0.1	-	P-	-	-
	0.25	P. 50% L.	50% L.	P-	50% L.
	0.5	L.	95% L.	L.	95% L.
	1.0	"	L.	"	L.
	2.0	"	"	"	"
	3.0	"	"	"	"
	4.0	"	"	"	"
6 days.	0.25	P. 50% L.	P. 50% L.	P. 50% L.	P. 50% L.
	0.5	L.	95% L.	L.	L.
	1.0	"	L.	"	"
	2.0	"	"	"	"
	3.0	"	"	"	"
	4.0	"	"	"	"

EXPERIMENT 77.

To ascertain the activity of staphylococcal factor produced in Medium D7 with the addition of human serum, fresh or heated.

MEDIUM: 60 ml. amounts of Medium D7 (2% "Lab-Lemco", 2% Evan's Peptone, 0.5% NaCl. at pH 7.4) were placed in flasks and autoclaved at 15 lbs./15 minutes. To two of these flasks 10% unheated human serum was added; to another two flasks, 10% human serum heated at 56°C. for 10 minutes, and to another two flasks 10% human serum heated at 65°C. for 10 minutes. Two flasks of Medium D7 without serum were also included as controls.

INOCULUM: The flasks were all inoculated with 2 ml. of a 6 hour old "Lab-Lemco" broth culture of Staphylococcus aureus,

INCUBATION: Strain AII and incubated at 37°C.

The staphylococcal cultures were heat killed and tested for activity in serum-milk-agar plates at 24 hourly intervals.

EXPERIMENT 77.

RESULTS: After incubation of plates at 37°C. for 24 hours.

Age of Culture	Amount (ml.)	Medium D7	D7 with 10% serum	D7 with 10% serum heated 56°/10 mins.	D7 with 10% serum heated 65°/10 mins.
24 hrs.	0.25	-	-	-	-
	0.5	-	-	-	-
	1.0	-	-	-	P.
	2.0	P+	P+	P.	P+
	3.0	"	"	P+	"
	4.0	P-	"	P.	P.
2 days.	0.5	P.	75% L.	-	P+
	1.0	L.	L.	95% L.	L.
	2.0	"	"	L.	"
	3.0	"	"	"	"
	4.0	"	"	"	"
3 days.	0.25	P.	S.L.	-	-
	0.5	P. 75% L.	75% L.	P+	P. 75% L.
	1.0	L.	L.	95% L.	L.
	2.0	"	"	L.	"
	3.0	"	"	"	"
	4.0	"	"	"	"
4 days.	0.25	-	-	P.	P.
	0.5	P.	P.	P. 75% L.	P. 50% L.
	1.0	90% L.	P. 90% L.	L.	L.
	2.0	L.	P. 95% L.	"	"
	3.0	"	L.	"	"
	4.0	"	"	"	"
5 days.	0.25	-	-	P.	-
	0.5	P. 90% L.	S.L.	L.	L.
	1.0	L.	95% L.	"	"
	2.0	"	L.	"	"
	3.0	"	"	"	"
	4.0	"	"	"	"
7 days.	0.25	-	-	-	-
	0.5	P. 75% L.	P.	P.	75% L.
	1.0	P. 95% L.	P. 95% L.	P. 95% L.	L.
	2.0	L.	L.	L.	"
	3.0	"	"	"	"
	4.0	"	P. 95% L.	P. 95% L.	"

EXPERIMENT 78.

To test the activity of staphylococcal factor in Medium B5 with the addition of different amounts of fresh human serum to the medium.

MEDIUM: 500 ml. of Medium B5 (containing 0.5% "Lab-Lemco", 1.5% Evan's Peptone and 0.5% NaCl. at pH 7.4) were prepared and 50 ml. amounts were placed in each of eight flasks. These flasks of medium were autoclaved (15 lbs./15 minutes) and fresh human serum added to them - to two sufficient to make final concentration 1%, to another pair to make 5% and to two more to make 10%. The remaining two flasks had no serum added and acted as controls.

INOCULATION: Each flask was inoculated with 2.0 ml. of a 6 hour old "Lab-Lemco" broth culture of Staphylococcus aureus, Strain AII.

INCUBATION: These eight flasks were incubated at 37°C. Culture was withdrawn at 24 hour intervals from each set of flasks, heat killed and the activity of the staphylococcal factor tested by incorporation in serum-milk-agar plates.

EXPERIMENT 78.

RESULTS: After incubation of plates at 37°C. for 24 hours.

Age of Culture	Amount (ml.)	MEDIUM B5 WITH			
		No serum	1% Serum	5% Serum	10% Serum
24 hrs.	0.5	-	-	-	-
	1.0	-	P-	-	-
	2.0	P-	P+	P-	-
	3.0	P.	"	P.	-
	4.0	P+	P. 90% L.	P+	-
2 days.	0.5	-	-	P-	P. 50% L.
	1.0	-	P.	L.	L.
	2.0	P+	L.	"	"
	3.0	P. 90% L.	"	"	"
	4.0	L.	"	"	"
3 days.	0.5	-	-	S.L.	P. 90% L.
	1.0	P-	90% L.	L.	L.
	2.0	P. 90% L.	L.	"	"
	3.0	"	"	"	"
	4.0	"	"	"	"
5 days.	0.5	-	-	P.	P. 90% L.
	1.0	P.	P.	L.	L.
	2.0	P. 90% L.	L.	"	"
	3.0	"	"	"	"
	4.0	L.	"	"	"
6 days.	0.5	-	-	50% L.	L.
	1.0	P.	P. 90% L.	L.	"
	2.0	P. 90% L.	L.	"	"
	3.0	"	"	"	"
	4.0	L.	"	"	"

EXPERIMENT 79.

To ascertain the effect of different amounts of inoculum on the production of staphylococcal factor by Strain AII in Medium D7.

MEDIUM: 60 ml. of Medium D7 (2% "Lab-Lemco", 2% Evan's Peptone and 0.5% NaCl. pH 7.4) was placed in each of six flasks and autoclaved at 15 lbs./15 minutes.

INOCULUM: Two flasks were inoculated each with 0.5 ml. of 6 hour old "Lab-Lemco" broth culture of AII Strain and a further 2 flasks with 2.0 ml. of the same culture. The remaining two flasks were inoculated each with the sediment obtained from centrifuging 10 ml. of a 6 hour old "Lab-Lemco" broth culture suspended in 1 ml. sterile distilled water.

INCUBATION: All the flasks were incubated at 37°C.

The cultures were heat killed and tested every 24 hours by incorporation in serum-milk-agar plates.

EXPERIMENT 79.

RESULTS: After incubation of plates at 37°C. for 24 hours.

Age of Culture	Amount (ml.)	Amount of 6 hour old Inoculum		
		0.5 ml.	2.0 ml.	10 ml.
24 hrs.	1.0	-	-	-
	2.0	-	P+	P.
	3.0	-	"	"
	4.0		P-	-
48 hrs.	0.5	P.	P.	-
	1.0	P+	L.	P+
	2.0	L.	"	95% L.
	3.0	95% L.	"	"
	4.0	"	"	"
3 days.	0.25	P.	P.	-
	0.5	90% L.	P. 75% L.	P.
	1.0	L.	L.	L.
	2.0	"	"	"
	3.0	"	"	"
	4.0	"	"	"
4 days.	0.25	-	P.	P-
	0.5	P. 90% L.	90% L.	75% L.
	1.0	L.	L.	L.
	2.0	"	"	"
	3.0	"	"	"
	4.0	"	"	"
5 days.	0.5	90% L.	90% L.	90% L.
	1.0	L.	L.	L.
	2.0	"	"	"
	3.0	"	"	"
	4.0	"	"	"
7 days.	0.5	75% L.	75% L.	75% L.
	1.0	L.	95% L.	L.
	2.0	"	L.	"
	3.0	"	"	"
	4.0	"	"	"

EXPERIMENT 80.

To ascertain the effect of altering the pH reaction on the activity of staphylococcal factor in serum-milk-agar plates.

MEDIUM: 75 ml. of Medium D7 (2% "Lab-Lemco", 2% Evan's Peptone, 0.5% NaCl. pH 7.4) was placed in each of two flasks, autoclaved at 15 lbs./15 minutes.

INOCULUM: They were inoculated with 2.0 ml. of 6 hour old "Lab-Lemco" broth culture of Staphylococcus aureus, Strain AII.

INCUBATION: The flasks were incubated at 37°C. for 4 days. After 4 days, the culture was centrifuged, divided into 10 ml. amounts in sterile test-tubes and heat killed at 65°C. for 10 minutes.

PREPARATION OF STAPHYLOCOCCAL FACTOR: The reaction of the culture which was pH 7.3 was adjusted, by the addition of NaOH or HCl to give a series of tubes of sterile staphylococcal culture with pH reaction within the range pH 9 to pH 4. These cultures were tested for the activity of the staphylococcal factor in serum-milk-agar plates. Two control plates were set up one with the culture (2.0 ml.) at pH 7.3, i.e. without addition of NaOH or HCl, but with the equivalent amount of distilled water to correspond to the amount of NaOH or HCl added to culture to alter pH to desired level. The other plate also contained 2.0 ml. of culture at pH 7.3, but no serum was used.

EXPERIMENT 80.

RESULTS: After incubation of plates at 37°C. for 24 hours.

Amount of Culture (ml.)	pH 4	pH 5.2	pH 6.1	pH 7.3	pH 8.2	pH 9.0
0.25	-	-	-	S.L.	-	-
0.5	-	-	L.	L.	L.	P+
1.0	-	-	"	"	"	P. 95% L.
2.0	-	-	"	"	"	P.
3.0	-	-	"	P+	P. 95% L.	P-
4.0	-	-	"	P.	...	...
Amount of NaOH or HCl added.	5 ml. N/5 HCl.	3.5 ml. N/5 HCl.	2 ml. N/5 HCl.	Nil	0.5 ml. N/5 NaOH.	1 ml. N/5 NaOH.
CONTROLS.						
Equivalent of 2.0 ml. at pH 7.3	L.	L.	L.	...	L.	95% L.
No serum.	-	-	-	...	...	...

EXPERIMENT 81.

To test the activity of staphylococcal factor at different levels in pH range from 5.5 to 7.9.

Seitz filtered staphylococcal culture (pH 7.3) prepared in Experiment 72 (App 208-9) was used in this Experiment. 10 ml. amounts of the filtrate was adjusted by the addition of sterile N/5 HCl or N/5 NaOH. The filtrates were then tested in the usual way to ascertain their activities by incorporation in serum-milk-agar plates.

EXPERIMENT 81.

RESULTS: After incubation of plates at 37°C. for 24 hours.

Amount of Filtrate (ml.)	pH 5.5	pH 5.8	pH 6.4	pH 6.7
0.1	-	-	-	-
0.25	-	P.	75% L.	P. 75% L.
0.5	L.	90% L.	L.	L.
1.0	"	L.	"	"
2.0	"	"	"	"
3.0	"	"	"	"
4.0	"	"	"	"
	pH 7.0	pH 7.3	pH 7.6	pH 7.9
0.1	-	-	-	-
0.25	90% L.	75% L.	75% L.	75% L.
0.5	L.	L.	L.	L.
1.0	"	"	"	"
2.0	"	"	"	"
3.0	"	"	"	"
4.0	"	"	"	"

EXPERIMENT 82.

To test the effect on the production of the staphylococcal factor in Medium D7 adjusted to different pH values.

MEDIUM: 600 ml. of Medium D7 (2% "Lab-Lemco", 2% Evan's Peptone, 0.5% NaCl) was prepared. 150 ml. amounts were adjusted to pH. 8, 7 and 6. Each 150 ml. amount was divided into 3 flasks (50 ml. in each).

INOCULUM: After sterilisation by autoclave (15lbs./15 minutes) the flasks were inoculated with 2.0 ml. of a 6 hour old "Lab-Lemco" culture of Staphylococcus aureus, Strain AII, and incubated at 37°C. At 24 hour intervals quantities of culture from each set of flasks was heat killed and tested for activity in serum-milk-agar plates whatever its pH. The culture was also tested after the pH. had been adjusted to pH. 7.0.

INCUBATION:

EXPERIMENT 82.

RESULTS: After incubation of plates at 37°C. for 24 hours.

Age of Culture	Amount (ml.)	pH. reaction of Culture Medium					
		pH. 6.0		pH. 7.0		pH. 8.0	
24 hrs.	0.5 1.0 2.0 3.0 4.0 pH.	- - - - - 6.0	- - P. " P- 7.0	- - P. " " 6.4	- P. P+ " P. 7.0	- P. P+ " P. 7.0	
48 hrs.	0.25 0.5 1.0 2.0 3.0 4.0 pH.	S.L. L. " " " " 7.0		P+ L. " " " " 7.0		- P. P.90%L. L. " " 7.0	
3 days.	0.1 0.25 0.5 1.0 pH.	- 90% L. L. " 7.3	- 90% L. L. " 7.0	P. L. " " 7.3	- L. " " 7.0	- - 75% L. 90% L. 7.3	- - 75% L. 90% L. 7.0
4 days.	0.1 0.25 0.5 1.0 pH.	- S.L. L. " 7.3	(...) " " " 7.0	- 90% L. L. " 7.3	- S.L. L. " 7.0	- - - - 7.3	- - - - 7.0
6 days.	0.1 0.25 0.5 1.0 pH.	- S.L. L. " 7.3-7.6	- S.L. L. " 7.0	- 90% L. L. " 7.3-7.6	- S.L. L. " 7.0	- - 90% L. L. 7.3-7.6	- - S.L. 90% L. 7.0
7 days.	0.25 0.5 1.0 2.0 pH.	50% L. L. " " 7.3-7.6	50% L. L. " " 7.0	90% L. L. " " 7.3-7.6	75% L. L. " " 7.0	- 90% L. L. " 7.3-7.6	- 75% L. L. " 7.0

EXPERIMENT 82 (Continued).

Age of Culture	Amount (ml.)	pH. reaction of Culture Medium					
		pH. 6.0		pH. 7.0		pH. 8.0	
8 days.	0.25 0.5 1.0 2.0 pH.	P.90%L. L. " " 7.6	P.90%L. L. " " 7.0	L. L. " " 7.6	P.90%L. L. " " 7.0	- P.75%L. L. " 7.6	- P.90%L. L. " 7.0
9 days.	0.25 0.5 1.0 2.0 pH.	P.50%L. L. " (...) 7.6	P.50%L. L. " (...) 7.0	L. " " (...) 7.6	- L. " (...) 7.0	- P.75%L. L. " 7.6	- P. L. " 7.0
10 days.	0.25 0.5 1.0 2.0 pH.	L. " " (...) 7.6	P.75%L. L. " (...) 7.0	L. " " (...) 7.6	P.50%L. L. " (...) 7.0	- P.90%L. L. " 7.6	- P.50%L. L. " 7.0
11 days.	0.1 0.25 0.5 1.0 2.0 pH.	- 95% L. L. " (...) 7.6		- 90% L. L. " (...) 7.6		- - L. " " 7.6	
13 days.	0.1 0.25 0.5 1.0 2.0 pH.	- P.75%L. L. " (...) 7.6		- 90% L. L. " (...) 7.6		- - 75% L. L. " 7.6	
15 days.	0.25 0.5 1.0 pH.	90% L. L. " 7.6		P.90%L. L. " 7.6		S.L. P.75%L. L. 7.6	

EXPERIMENT 83.

The testing of staphylococcal factor produced in Medium D7 with two different specimens of serum.

MEDIUM: 350 ml. of Medium D7 (2% "Lab-Lemco", 2% Evan's Peptone, 0.5% NaCl. at pH 7.4) was divided into six equal parts in six flasks and sterilised at 15 lbs./ 15 minutes.

INOCULUM: Each flask was inoculated with 2.0 ml. of a 6 hour old 1% "Lab-Lemco" broth culture of Staphylococcus aureus, Strain AII and incubated at 37°C. for 4 days.

INCUBATION:

The flasks were then withdrawn from the incubator, the culture centrifuged, the supernatant then Seitz filtered. (The Seitz filter pad was first washed by passing through it distilled water, 10 ml. and then 10 ml. of the culture. This filtrate was discarded. The remainder of the culture was then Seitz filtered and the filtrate kept in a sterile bottle at room temperature.

The activity of the staphylococcal factor in this filtrate was tested out with two different batches of pooled sera used in 0.5 ml. amounts, in serum-milk-agar plates.

EXPERIMENT 83.

RESULTS: After 24 hours' incubation of plates at 37°C.

Amount of Filtrate (ml.)	0.1	0.25	0.5	1.0	2.0	3.0	4.0
Serum "4"	-	S.L.	L.	L.	L.	95% L.	95% L.
Serum "5"	-	95% L.	"	"	"	L.	L.

EXPERIMENT 84.

Comparison of the activities of staphylococcal factor produced by a number of strains of Staphylococcus aureus in Medium D7.

MEDIUM: 750 ml. of Medium D7 (p. 194) which contained 2% "Lab-Lemco", 2% Evan's Peptone and 0.5% Sodium chloride was prepared and 60 ml. was placed in each of 12 flasks.

INOCULA: 6 hour old "Lab-Lemco" broth cultures of Staphylococcus aureus, Strains AII, 73016'W', 73016'O', 72822'W', 72822'O' and 72822'P' (prepared from waxed agar slope cultures of these organisms) were used to inoculate the flasks of medium two flasks being inoculated with the culture of one strain.

INCUBATION: The flasks were incubated at 37°C.

PREPARATION OF THE STAPHYLOCOCCAL FACTOR: About 12 ml. of culture was removed from each flask after 24 hours' incubation at 37°C., heat-killed at 65°C. for 10 minutes and the activity of the staphylococcal factor ascertained by incorporating the heat-killed culture in serum-milk-agar plates in amounts from 0.1 up to 4.0 ml., as has been described before (p. 231). A similar procedure was adopted with samples of these cultures after each subsequent 24 hours' incubation of the medium up to 11 days from the time of inoculation.

READINGS: The plates were incubated at 37°C. for 24 hours when readings were taken.

EXPERIMENT 84.

RESULTS: After incubation of plates at 37°C. for 24 hours.

Age of Culture	Amount (ml.)	Strains of Staphylococci					
		AII	73016'W'	73016'O'	72822'W'	72822'O'	72822'P'
24 hrs.	0.5	-	P-	-	-	-	-
	1.0	-	P.	-	-	-	-
	2.0	P.	P+	-	-	-	-
	3.0	"	"	-	P-	-	-
	4.0	"	"	-	"	-	-
2 days.	0.5	75% L.	P.	(...)	(...)	(...)	(...)
	1.0	L.	P.90%L.	-	L.	P.	-
	2.0	"	L.	P-	"	P+	-
	3.0	"	"	"	"	"	-
	4.0	"	"	"	"	"	-
3 days.	0.5	L.	P.90%L.	P.	P.	P-	(...)
	1.0	"	L.	L.	L.	P.	-
	2.0	"	"	"	"	P+	-
	3.0	"	"	"	"	P.	-
	4.0	"	"	"	"	P-	-
4 days.	0.25	P.	-	-	P.	-	-
	0.5	L.	P.	-	P.95%L.	P.	-
	1.0	"	L.	P.	L.	"	-
	2.0	"	"	P+	"	L.	P.
	3.0	"	"	"	"	"	P-
	4.0	"	"	P-	"	P.95%L.	"
5 days.	0.25	S.L.	-	-	S.L.	-	-
	0.5	L.	P.	P.	L.	P-	-
	1.0	"	P.90%L.	"	"	P+	P-
	2.0	(...)	(...)	"	(...)	(...)	P.
	3.0	"	"	(...)	"	"	"
8 days.	0.1	-	P-	-	-	-	-
	0.25	P.90%L.	P.	-	75% L.	-	-
	0.5	L.	"	P.	L.	P-	75% L.
	1.0	"	P.90%L.	"	"	P++	P.95%L.
	2.0	(...)	(...)	"	(...)	(...)	L.
	3.0	"	"	(...)	"	"	"
	4.0						

EXPERIMENT 84 (Continued).

Age of Culture	Amount (ml.)	Strains of Staphylococci					
		AII	73016'W'	73016'O'	72822'W'	72822'O'	72822'P'
9 days.	0.1	-	-	-	P.	-	-
	0.25	S.L.	-	P-	75% L.	-	-
	0.5	L.	P.	P.	L.	75% L.	75% L.
	1.0	"	L.	P++	"	95% L.	L.
	2.0	(...)	(...)	P.95%L.	(...)	L.	"
10 days.	0.25	S.L.	P.	-	75% L.	P-	-
	0.5	L.	P.	P.	L.	P.	P++
	1.0	"	L.	P++	"	L.	L.
	2.0	(...)	(...)	L.	(...)	"	"
11 days.	0.25	S.L.	-	-	75% L.	-	-
	0.5	L.	P.	P.	L.	P.	75% L.
	1.0	"	L.	P++	"	P.90%L.	L.
	2.0	(...)	(...)	P.95%L.	(...)	L.	"

EXPERIMENT 85.

Comparison of the activities of staphylococcal factor produced by a number of strains of Staphylococcus aureus in Medium D7.

MEDIUM: 750 ml. of Medium D7 (P. 194) which contained 2% "Lab-Lemco", 2% Evan's Peptone and 0.5% Sodium chloride was prepared and distributed in 60 ml. amounts in each of 12 flasks.

INOCULA: 6 hour old "Lab-Lemco" broth cultures of Strains AII, 72493, 72871, 73008 and 72959 were used to inoculate the flasks of medium, each culture being used to inoculate two flasks.

INCUBATION: The flasks were incubated at 37°C.

PREPARATION OF THE STAPHYLOCOCCAL FACTOR: About 12 ml. of culture of each strain was withdrawn from the appropriate flasks after 24 hours' incubation, heat-killed at 65°C. for 10 minutes and the activity of the heat-killed culture ascertained by incorporating different amounts of it in serum-milk-agar plates as previously described (P. 231). Samples of these cultures were treated in a similar way after each subsequent 24 hours' incubation up to 7 days after inoculation of the medium.

READINGS: The plates were incubated at 37°C. for 24 hours when readings were taken.

EXPERIMENT 85.

RESULTS: After incubation of plates at 37°C. for 24 hours.

Age of Culture	Amount (ml.)	Strains of Staphylococci				
		AII	72493	72871	73008	72959
24 hrs.	1.0	S.L.	–	–	–	–
	2.0	P++	–	–	–	–
	3.0	"	–	–	–	–
	4.0	P.	–	–	–	–
2 days.	0.25	75% L.	(...)	(...)	(...)	(...)
	0.5	L.	"	"	"	"
	1.0	"	P.	P.	–	–
	2.0	"	P++	P++	–	–
	3.0	(...)	"	"	–	–
	4.0	"	P.	P.	–	–
3 days.	0.1	–	(...)	(...)	(...)	(...)
	0.25	L.	"	"	"	"
	0.5	"	P–	–	"	"
	1.0	"	P.	P–	–	–
	2.0	(...)	P+	P+	–	–
	3.0	"	P.	P.	P–	P.
	4.0	"	"	"	"	"
4 days.	0.1	–	(...)	(...)	(...)	(...)
	0.25	L.	"	"	"	"
	0.5	"	–	–	"	"
	1.0	"	P+	P+	–	–
	2.0	(...)	L.	L.	–	–
	3.0	"	"	"	–	–
	4.0	"	"	"	–	P.
5 days.	0.1	–	(...)	(...)	(...)	(...)
	0.25	L.	"	"	"	"
	0.5	"	P.	P.	"	"
	1.0	"	P+	P+	–	P–
	2.0	"	L.	L.	–	P.
	3.0	(...)	"	P.95%L.	–	P–
	4.0	"	"	(...)	–	"

EXPERIMENT 85 (Continued).

Age of Culture	Amount (ml.)	Strains of Staphylococci				
		AII	72493	72871	73008	72959
7 days.	0.25	L.	(...)	(...)	(...)	(...)
	0.5	"	"	"	"	"
	1.0	"	P+	"	-	P.
	2.0	"	P.	"	-	P+
	3.0	(...)	P-	"	-	P.
	4.0	"	-	"	-	"

EXPERIMENT 86.

Comparison of the activity of heat-killed cultures obtained when Staphylococcus aureus, Strains A, AI and AII are grown in flasks of Medium D7.

MEDIUM: 850 ml. of Medium D7 (2% "Lab-Lemco" broth, 2% Evan's Peptone, 0.5% NaCl., 7.4 pH.) prepared and distributed in 12 flasks of 250 ml. capacity.

INOCULA: Four flasks of medium each received 2.0 ml. of a 6 hour old "Lab-Lemco" broth culture of Staphylococcus aureus, Strain A; four flasks each received 2.0 ml. of a 6 hour old "Lab-Lemco" broth culture of Strain AI, and a third set of four flasks received 2.0 ml. of a 6 hour old "Lab-Lemco" broth culture of Strain AII.

INCUBATION: The flasks were incubated at 37°C.

At intervals of 24 hours, 30 ml. of culture was removed from flasks of each type of culture, centrifuged heat-killed at 65°C. for 10 minutes and tested for activity in serum-milk-agar plates. Serum-milk-agar plates were also inoculated from each culture to permit record of what type of colonies were produced. All plates were incubated at 37°C. for 24 hours.

EXPERIMENT 86.

RESULTS: After incubation of plates at 37°C. for 24 hours.

Age of Culture	Amount (ml.)	Culture A	Culture AI	Culture AII
20 hrs.	1.0 2.0 3.0 4.0 pH.	P- " " P. 6.4-6.7	- - - - 6.4-6.7	P- P+ P. P- 6.7
48 hrs.	1.0 2.0 3.0 4.0 pH.	- P. " " 6.7	- - - - 6.7	90% L. L. " " 6.7
3 days.	1.0 2.0 3.0 4.0 pH.	P- P. P+ L. 6.7-7.0	- - - - 6.7	0.1 - 0.25 - 0.5 - 1.0 L. 7.0
4 days.	1.0 2.0 3.0 4.0 pH.	P- P+ 95% L. L. 7.0	- - - - 7.0	0.25 - 0.5 P- 1.0 L. 2.0 " 7.0
5 days.	0.5 1.0 2.0 3.0 pH.	S.L. P+ " P. 7.3-7.6	1.0 - 2.0 - 3.0 - 4.0 - 7.0	0.25 - 0.5 S.L. 1.0 L. 2.0 " 7.3
7 days.	0.5 1.0 2.0 3.0 pH.	S.L. P+ L. " 7.3-7.6	1.0 - 2.0 - 3.0 - 4.0 - 7.0	0.25 - 0.5 75%L. 1.0 L. 2.0 " 7.3

EXPERIMENT 86 (Continued).

CULTURE A: Two types of colony observed on each of the 6 days this was plated.

(1) Majority of colonies surrounded by lysis and plaques.

(2) A few colonies with only slight indefinite lysis surrounding.

CULTURE AI: All colonies on each of the plates of medium used were surrounded by slight indefinite lysis.

CULTURE AII: All colonies on each of the plates of medium used were surrounded by lysis and plaques.

EXPERIMENT 87.

To ascertain if staphylococcal factor produced in meat broth culture can be concentrated without loss of activity.

Several batches of staphylococcal factor produced by growing Staphylococcus aureus, Strain A in meat broth were pooled to give a total volume of 1,500 ml. and this was used for experiments on concentration.

The activity of this pooled staphylococcal factor was tested in standard serum-milk-agar plates and the result obtained is given in "RESULTS" as 'Result 1'.

The 1,500 ml. of staphylococcal factor was concentrated by evaporation under pressure at a temperature of 25°C. - 30°C., to a volume of 180 ml.

The activity of this concentrated staphylococcal factor (i.e. conc. x8.3) was tested in standard serum-milk-agar plates and the result obtained is given in "RESULTS" as 'Result 2'.

This 180 ml. of concentrated staphylococcal factor was further concentrated by freeze-drying to a volume of 55 ml., it not being possible in the circumstances to remove all the water from the preparation. A deposit appeared in this 55 ml. amount of concentrated factor. It was removed by centrifugation, tested and found to be inactive in standard serum-milk-agar plates. The supernatant, however, when tested out in standard serum-milk-agar plates was active, the result obtained being given in 'Result 3'.

Thus, it was possible by a concentration of 27 times to prepare staphylococcal factor which could produce plaques of clearing with 0.0312 ml. amount.

EXPERIMENT 87.

RESULTS:

Result 1: Activity of original pooled staphylococcal factor in standard serum-milk-agar plates.

After 24 hours' incubation at 37°C.

Amount of Staphylococcal Factor (ml.)				
0.5	1.0	2.0	3.0	4.0
-	P+	P. 30% L.	P. 50% L.	P. 75% L.

Result 2: Activity of concentrated staphylococcal factor (conc. x8.3) in standard serum-milk-agar plates.

After 24 hours' incubation at 37°C.

Amount of Staphylococcal Factor (conc. x8.3) (ml.)				
0.1	0.2	0.3	0.5	1.0
P+	L.	L.	L.	P. 90% L.

Result 3: Activity of twice concentrated staphylococcal factor (conc x27) in standard serum-milk-agar plates, after 24 hours' incubation at 37°C.

Amount of twice concentrated staphylococcal factor (ml.)						
0.5	0.1	0.25	0.125	0.0625	0.0312	0.0156
Dil. 1/1		1/2	1/4	1/8	1/16	1/32
P++	L.	L.	L.	L.	P+	-

EXPERIMENT 88 (Continued).

20 ml. of the concentrated culture were freeze-dried, and the resultant material was brown and crystalline in appearance but which on exposure to the air became sticky. This freeze-dried deposit was redissolved in half its original volume of distilled water and tested in 0.2 ml. amount and serial doubling dilutions of that amount down to 0.00625 ml. by incorporation in standard serum-milk-agar plates. The result obtained after incubation of the plates at 37°C. for 24 hours is given as Result 5.

EXPERIMENT 88.

The concentration of staphylococcal factor (Batch 2) prepared in Medium D7.

A batch of staphylococcal factor (330 ml. volume) was prepared in Medium D7 and its activity was ascertained by testing in standard serum-milk-agar plates. The result obtained is given as Result 1.

The 330 ml. original volume was concentrated by evaporation under pressure at a temperature of 25°C. - 30°C. to 28 ml. This concentrated staphylococcal factor was tested for activity in standard serum-milk-agar plates and the result obtained is given as Result 2.

2.0 ml. of the concentrated staphylococcal factor had added to it 2.0 ml. of saturated ammonium sulphate, left at room temperature for 3½ hours and then centrifuged. The deposit which had collected was dissolved in 2.0 ml. of distilled water and dialysed overnight in running water as was also the supernatant collected from this deposit.

After dialysis the volumes of the redissolved deposit and of the supernatant were measured, the degree of dilution resulting from the dialysis noted, and amounts of both fluids tested for activity in standard serum-milk-agar plates. The result obtained is given as Result 3.

0.1 gm. of animal charcoal was added to 1 ml. of concentrated staphylococcal factor and left overnight at room temperature. The mixture was centrifuged, the supernatant fluid withdrawn and the charcoal resuspended in 1.0 ml. of distilled water.

The supernatant and the resuspended deposit were tested out in standard serum-milk-agar plates in amounts from 0.25 ml. to 0.016 ml. The result obtained is given as Result 4.

EXPERIMENT 88.

RESULTS:

RESULT 1: The activity of staphylococcal factor (Batch 2) tested in standard serum-milk-agar plates after 24 hours' incubation at 37°C.

Amount of Staphylococcal Factor (ml.)						
0.1	0.25	0.5	1.0	2.0	3.0	4.0
-	L.	L.	L.	L.	L.	L.

RESULT 2: The activity of the concentrated staphylococcal factor (conc. about 11 times) in standard serum-milk-agar plates after 24 hours at 37°C.

Amount of Staphylococcal Factor (conc.) (ml.)					
0.25	0.125	0.06	0.03	0.016	0.008
L.	L.	L.	L.	P++	-

RESULT 3: The activity of the supernatant and of the redissolved deposit obtained on adding saturated Ammonium sulphate to staphylococcal factor (concentrated) tested in standard serum-milk-agar plates. After 24 hours' incubation at 37°C.

S U P E R N A T A N T				
Amount in Plate	0.5	0.25	0.125	0.0625
Equivalent of concentrated Staph. Factor.	0.09	0.046	0.023	0.012
Result	L.	L.	90% L.	-

The redissolved deposit tested under the same conditions in amounts equivalent to concentrated factor of 0.016 ml. to 0.25 ml. showed no activity.

RESULT 4: The activity of the staphylococcal factor and of suspended animal charcoal with which the staphylococcal factor was treated, tested in standard serum-milk-agar plates, after 24 hours at 37°C.

Amounts (ml.)	Supernatant	Resuspended Deposit
0.25	L.	-
0.125	"	-
0.06	"	-
0.03	"	-
0.016	-	-

RESULT 5: The activity of the staphylococcal factor after redissolving freeze-dried staphylococcal factor in half its volume of distilled water ascertained in standard serum-milk-agar plates, after 24 hours' incubation at 37°C.

Amount of Resuspended Deposit Freeze-dried	Equivalent of Concentrated Staphylococcal Factor.	Result after 24 hours at 37°C.
0.2	0.4	L.
0.1	0.2	"
0.05	0.1	"
0.025	0.05	"
0.0125	0.025	75% L.
0.00625	0.0125	-

∴ complete lysis should be given by $\frac{1}{5.3}$ ml. of the redissolved powder, i.e. by 0.19 ml. and clearing of the standard serum-milk-agar plate by $\frac{0.5}{5.3}$ = 0.09 ml.

The brown powder was redissolved in distilled water to give a 2% solution (i.e. 0.1 gm. in 5.0 ml.) and used in standard serum-milk-agar plates. The activity of the 2% solution of staphylococcal factor is given as Result 4.

An attempt was made to purify further, the staphylococcal factor by treating 0.2 gm. of the powdered preparation with 10 ml. of acetone, and leaving them in the ice-chest for 2 hours. After this the supernatant which consisted of the acetone and a white suspension was removed leaving the brown powder. This latter was washed 3 times with fresh acetone, each washing occupying 10 minutes. The brown precipitate was dried and dissolved in 5 ml. of distilled water. The activity of this preparation was tested in standard serum-milk-agar and the result is given as Result 5.

EXPERIMENT 89.

The Concentration of staphylococcal factor (Batch 4) prepared in Medium D7.

Staphylococcal factor, designated Batch 4 was prepared in Medium D7 and its activity tested in standard serum-milk-agar plates. The result obtained is given as Result 1.

732 ml. of Batch 4 staphylococcal factor was available for concentration by evaporation under pressure. The final volume after concentration by this method was 58 ml. This 12.6 times concentrated staphylococcal factor was tested for activity in standard serum-milk-agar plates and the result obtained is given as Result 2.

This concentrated staphylococcal factor was mixed with 3 other preparations of similarly concentrated staphylococcal factor and the resultant batch had added to it an equal volume of saturated Ammonium sulphate. After 1 hour at room temperature the staphylococcal factor-Ammonium sulphate mixture was centrifuged, the supernatant fluid collected and dialysed overnight against running water. The activity of the dialysed supernatant was tested in standard serum-milk-agar plates and the result obtained is given as Result 3.

550 ml. of dialysed supernatant fluid was available for further evaporation under pressure and then freeze-drying. A brown powder was obtained, of total weight 2.09 gm. from this 550 ml. volume of fluid.

2.09 gm. was obtained from 550 ml.

$\therefore$ 0.1 gm. " " " $\frac{550}{2.09} \times .1 = 26.4$ ml.

0.1 gm. of powder was dissolved in 5 ml., i.e. a final concentration, compared with the dialysed supernatant fluid of 1 in 5.3.

1 ml. of dialysed supernatant was active in a standard serum-milk-agar plate giving complete lysis.

EXPERIMENT 89.

RESULTS: After 24 hours at 37°C.

RESULT 1: The activity of the staphylococcal factor tested on standard serum-milk-agar plates, after 24 hours at 37°C.

Amount of Staphylococcal Factor (ml.)			
1.0	0.5	0.25	0.125
L.	L.	-	-

RESULT 2: The activity of concentrated staphylococcal factor (conc. x12.6) tested on standard serum-milk-agar plates, after 24 hours at 37°C.

Amount of Staphylococcal Factor (conc.) (ml.)				
0.2	0.1	0.05	0.025	0.0125
L.	L.	L.	-	-

RESULT 3: The activity of dialysed supernatant collected after Ammonium sulphate precipitation of staphylococcal factor, tested on serum-milk-agar plates, after 24 hours' incubation at 37°C.

Amount of Staphylococcal Factor as dialysed supernatant (ml.)				
2.0	1.0	0.5	0.25	0.1
L.	L.	S.L.	-	-

RESULT 4: The activity of a 2% solution of powdered staphylococcal factor tested in standard serum-milk-agar plates, after 24 hours at 37°C.

Amount of 2% Solution of Powdered Staphylococcal Factor.				
0.5	0.3	0.2	0.1	0.05
L.	L.	L.	75% L.	-

RESULT 5: The activity of a 2% solution of staphylococcal factor, after thrice washing the powdered factor with acetone, in standard serum-milk-agar plates, after 24 hours at 37°C.

Amount of 2% Solution of Staphylococcal Factor (ml.).			
0.2	0.1	0.05	0.025
L.	L.	75% L.	-

EXPERIMENT 90.

The concentration of staphylococcal factor (Batch 6) prepared in Medium D7.

Two litres of staphylococcal factor were prepared in Medium D7 and its activity tested using standard serum-milk-agar plates. The result obtained is given as Result 1.

The two litres of staphylococcal factor were concentrated by evaporation under pressure at a temperature of 25°C. - 30°C., the volume of the concentrate was 165 ml., i.e. a concentration of 12.2 times had been obtained. The activity of this concentrate was ascertained by testing it in standard serum-milk-agar plates. The result is given as Result 2.

To 164 ml. of this concentrated staphylococcal factor was added 164 ml. of saturated ammonium sulphate and the mixture was left in the ice-chest for 2 hours before being centrifuged. The supernatant was dialysed overnight and the final volume of the staphylococcal factor was then 975 ml. representing a dilution of the concentrated staphylococcal factor of 1 in 3.2. The activity of the supernatant was ascertained by testing in standard serum-milk-agar plates. The result obtained is given as Result 3.

The 975 ml. of dialysed supernatant containing staphylococcal factor was freeze-dried and a total weight of residue, which on rubbing with pestle in mortar became a fine brown powder, was 10.82 gms.

A 1% solution of this powder was prepared and tested in standard serum-milk-agar plates. The result obtained is given as Result 4.

EXPERIMENT 90.

RESULTS:

RESULT 1: The activity of the staphylococcal factor tested on standard serum-milk-agar plates after 24 hours at 37°C.

Amount of Staphylococcal Factor (ml.).			
1.0	0.5	0.25	0.125
L.	L.	75% L.	-

RESULT 2: The activity of concentrated staphylococcal factor tested on standard serum-milk-agar plates after 24 hours at 37°C.

Amount of Staphylococcal Factor (Concentrated x12.2) (ml.).			
0.1	0.05	0.025	0.0125
L.	L.	75% L.	-

RESULT 3: The activity of dialysed supernatant after ammonium sulphate precipitation tested in standard serum-milk-agar plates, after 24 hours at 37°C. (Dilution 1 in 3.2).

Amount of Staphylococcal Factor (ml.).			
0.4	0.2	0.1	0.05
L.	L.	75% L.	-

EXPERIMENT 90 (Continued).

RESULT 4: The activity of a 1% solution of staphylococcal factor tested on standard serum-milk-agar plates after 24 hours and 48 hours at 37°C.

Incubation	Amount of 1% solution of Staphylococcal Factor (ml.).			
	0.4	0.2	0.1	0.05
24 hours.	L.	75% L.	-	-
48 "	"	L.	P+	P-

EXPERIMENT 91.

To test the activity of a 1% preparation of staphylococcal factor with different amounts of serum.

MEDIA: Serum-milk-agar plates were prepared as follows:-

Milk	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5	4.5	4.5
1% Staphylococcal Factor	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Serum.	0.1	0.25	0.5	1.0	2.0	3.0	4.0
Distilled Water	8.2	8.05	7.8	7.3	6.3	5.3	4.3

Similar sets of plates were prepared containing 0.4 ml. and 0.8 ml. of 1% Staphylococcal factor.

INCUBATION: Plates were incubated at 37°C. for 24 hours.

READINGS: These were made after 24 hours at 37°C.

EXPERIMENT 91.

RESULTS: After 24 hours at 37°C.

1% Staphylococcal Factor (ml.).	Amount of Serum (ml.)						
	0.1	0.25	0.5	1.0	2.0	3.0	4.0
0.2	-	-	75% L.	P.	-	-	-
0.4	-	75% L.	90% L.	L.	-	-	-
0.8	-	"	L.	"	L.	P.	-

Plaques were seen at $2\frac{1}{2}$ and $3\frac{1}{2}$ hours in all the plates which showed lysis after 24 hours' incubation at 37°C.

EXPERIMENT 92.

To test the activity of concentrated staphylococcal factor on serum-milk-agar plates containing different amounts of horse serum.

MEDIA: Serum-milk-agar plates containing 0.5, 1.0 or 2.0 ml. of horse-serum were prepared as follows:-

Milk	2.0	2.0	2.0
Agar (4.5%)	4.5	4.5	4.5
Horse Serum	0.5	1.0	2.0
Distilled Water	8.0	7.5	6.5

On the surface of each plate six unglazed porcelain cups were placed containing two drops of undiluted and a dilution of concentrated staphylococcal factor, $\frac{1}{2}$, $\frac{1}{4}$, 1/8, 1/16 and 1/32 respectively.

INCUBATION: The plates were incubated at 37°C. for 48 hours.

READINGS: These were taken after 24 hours and 48 hours.

EXPERIMENT 92.

RESULTS: After 24 hours' incubation at 37°C.

Serum (ml.)	Dilutions of Concentrated Staphylococcal Factor					
	1/2	1/4	1/8	1/16	1/32	Un-diluted
0.5	L.	L.	L.	L.	L.	L.
1.0	"	"	"	"	"	L.P.
2.0	L.P.	"	"	"	"	"

After 48 hours' incubation at 37°C.

0.5	L.	L.	L.	L.	L.	L.
1.0	L.R.E.	"	"	"	"	L.P.R.E.
2.0	L.	"	"	"	"	L.P.

With decreasing amounts of concentrated staphylococcal factor there was a decrease in the breadth of the zones of lysis.

Control cups containing distilled water showed no change around them on these serum-milk-agar plates.

EXPERIMENT 93.

The effect of filtration on the activity of a 1% solution of staphylococcal factor tested on serum-milk-agar plates.

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50 ml. of a 1% solution of staphylococcal factor was prepared and tested out in amounts of from 0.1 ml. up to 1.0 ml. by inclusion in standard serum-milk-agar plates. The remainder of the preparation was filtered through a small Seitz filter (diameter 3 cms.) fitted with an S.B. pad (sterilising) according to the following scheme:-

Added to filter	Filtrate collected
(a) 10 ml. "Lab-Lemco" broth.	-
(b) 5 ml. of 1% Staphylococcal Factor.	5 ml. "Lab-Lemco" broth (discarded).
(c) 35 ml. of 1% Staphylococcal Factor.	6 ml. of "Lab-Lemco" broth and Staphylococcal Factor (discarded).
(d) -	11 ml. of 1% Staphylococcal Factor - Filtrate I.
(e) -	8 ml. of 1% Staphylococcal Factor - Filtrate II.
(f) -	8 ml. of 1% Staphylococcal Factor - Filtrate III.
(g) 10 ml. of 1% Staphylococcal Factor.	8 ml. of 1% Staphylococcal Factor - Filtrate IV.
(h) -	5 ml. of 1% Staphylococcal Factor - Filtrate V.

These five filtrates, viz. I, II, III, IV and V were tested out in standard serum-milk-agar plates in amounts of 0.1, 0.25, 0.5 and 1.0 ml.

INCUBATION: All the plates were incubated at 37°C. for 24 hours.

READINGS: These were made after 24 hours at 37°C.

EXPERIMENT 93.

RESULT: After 24 hours at 37°C.

Amount of Staphylococcal Factor (ml.)	Control (unfiltered) *	Filtrates				
		I	II	III	IV	V
0.1	-	-	-	-	-	-
0.25	50% L.	-	75% L.	75% L.	75% L.	75% L.
0.5	90% L.	50% L.	90% L.	90% L.	90% L.	90% L.
1.0	L.	75% L.	L.	L.	L.	L.

* The control plates contained "Merthiolate" 1/100,000 (final concentration).

EXPERIMENT 94.

To ascertain the effect on the production of Muller's phenomenon by the staphylococcal factor of different concentrations of milk in serum-milk-agar medium.

MEDIA: Serum-milk-agar plates were prepared containing different amounts of milk as follows:-

Milk	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0	5.5	6.0
Human Serum	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Staphylococcal Factor.	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5
Distilled Water.	7.5	7.0	6.5	6.0	5.5	5.0	4.5	4.0	3.5	3.0	2.5	2.0

INCUBATION: The plates were incubated at 37°C. for 24 hours and then left at room temperature for 48 hours.

READINGS: These were taken after 6 hours and 24 hours at 37°C. and after 48 hours at room temperature.

EXPERIMENT 94.

RESULTS:

Amount of Milk	After 6 hours at 37°C.	After 24 hours at 37°C.	After 48 hours at Room Temperature.
0.5	P++	L.	L.
1.0	"	P. 90% L.	"
1.5	"	P. 75% L.	"
2.0	"	"	P. 90% L.
2.5	"	"	"
3.0	"	P. 60% L.	P. 75% L.
3.5	P.	P++	"
4.0	"	P+	"
4.5	-	P.	"
5.0	-	"	P. 60% L.
5.5	-	"	"
6.0	-	"	P. 50% L.

EXPERIMENT 95.

To ascertain the effects of temperature on the production of Muller's phenomenon by staphylococcal factor in serum-milk-agar plates.

MEDIA: Four sets of serum-milk-agar plates and milk-agar control plates were made up as follows:-

			Control
Milk	2.0	2.0	2.0
Staphylococcal Factor	2.0	4.0	4.0
Serum	0.5	0.5	0.0
Agar (4.5%)	4.5	4.5	4.5
Distilled Water	6.0	4.0	4.5

INCUBATION: A set of plates and control plate were placed at the following temperatures: 4°C., 22°C., 30°C. and 37°C.

READINGS: The plates were examined after 5 hours, 24 hours and 48 hours at these different temperatures.

EXPERIMENT 95.

RESULTS:

Temperature	Amount of Staphylococcal Factor		Time of Reading
	2.0 ml.	4.0 ml.	
4°C.	– P. L.	– P. L.	5 hours 24 " 48 "
22°C.	P++ L. "	P++ L. "	5 hours 24 " 48 "
30°C.	P. 30% L. L. "	P. 30% L. L. "	5 hours 24 " 48 "
37°C.	P. 50% L. L. "	P. 50% L. L. "	5 hours 24 " 48 "

Plaques were first seen in plate incubated at 37°C. after $1\frac{3}{4}$ hours; at 30°C. after $2\frac{3}{4}$ hours and at 22°C. after $3\frac{1}{2}$ hours. No change was detected in any of the control plates, irrespective of the temperature at which they were placed.

EXPERIMENT 96.

To ascertain if Muller's phenomenon is produced in serum-milk-agar plates by the staphylococcal factor under anaerobic conditions.

MEDIA: Two sets of serum-milk-agar plates were prepared as follows:-

Milk	2.0	2.0	2.0	2.0
Serum	0.5	1.0	2.0	3.0
Staphylococcal Factor	4.0	4.0	4.0	4.0
Agar (4.5%)	4.5	4.5	4.5	4.5
Distilled Water	4.0	3.5	2.5	1.5

INCUBATION: One set of plates was incubated at 37°C. for 24 hours in a McIntosh and Fildes anaerobic jar; the other set was also incubated at 37°C. for 24 hours under aerobic conditions.

READINGS: These were taken after 24 hours' incubation at 37°C.

EXPERIMENT 96.

RESULTS: After 24 hours at 37°C.

Amount of Serum	Aerobic Plates	Anaerobic Plates
0.5 ml.	L.	L.
1.0	"	"
2.0	"	"
3.0	"	"

EXPERIMENT 97.

To ascertain the effect on the production of Muller's phenomenon by staphylococcal factor of different agar concentrations in the serum-milk-agar medium.

MEDIA: Serum-milk-agar plates were prepared with agar concentrations from 3.0% to 0.6% as follows:-

Milk	2.0	2.0	2.0	2.0	2.0
Serum	0.5	0.5	0.5	0.5	0.5
Staphylococcal Factor	1.0	1.0	1.0	1.0	1.0
Agar (6%)	7.5	6.0	4.5	3.0	1.5
Distilled Water	4.0	5.5	7.0	8.5	10.0
Final Agar Concentration	3.0%	2.4%	1.8%	1.2%	0.6%

Similar series of plates containing 1.0 ml., 2.0 ml., 3.0 ml. and 4.0 ml. of serum were prepared.

INCUBATION: These plates were incubated at 37°C. for 24 hours and then left at room temperature for 24 hours.

READINGS: These were taken after 24 hours and 48 hours' incubation at 37°C.

EXPERIMENT 97.

RESULTS: After 24 hours' incubation at 37°C.

Agar Concentration	Amount of Serum (ml.)				
	0.5	1.0	2.0	3.0	4.0
3.0%	L.	L.	P. (fine Network)	-	-
2.4%	"	"	" "	-	-
1.8%	"	"	P. 50% L.	-	-
1.2%	"	"	"	-	-
0.6%	"	"	"	-	-

After 48 hours' incubation at 37°C.

3.0%	L.	L.	P. (fine Network)	P.	-
2.4%	"	"	" "	"	-
1.8%	"	"	P. 80% L.	P-	-
1.2%	"	"	"	-	-
0.6%	"	"	"	-	-

EXPERIMENT 98.

To ascertain the effect on the production of Muller's phonomenon by staphylococcal factor of different agar concentrations in the serum-milk-agar medium.

MEDIA: Serum-milk-agar plates were prepared with agar concentrations from 6.0% to 1.5% as follows:-

Milk	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Serum	0.5	1.0	0.5	1.0	2.0	4.0	0.5	1.0	2.0	4.0	0.5	1.0	2.0	4.0		
Staphylococcal Factor	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0		
Agar (10%)	9.0	9.0	6.0	6.0	6.0	6.0	3.75	3.75	3.75	3.75	*5.0	*5.0	*5.0	*5.0		
Distilled Water	1.5	1.0	4.5	4.0	3.0	1.0	6.75	6.25	5.75	3.25	5.5	5.0	4.0	2.0		
Final Agar Concentration. (%)	6.0	6.0	4.0	4.0	4.0	4.0	2.5	2.5	2.5	2.5	1.5	1.5	1.5	1.5		

* 4.5% Agar used in preparating these plates.

INCUBATION: These plates were incubated at 37°C. for 24 hours.

READINGS: These were taken after 5½ hours and after 24 hours at 37°C.

EXPERIMENT 98.

RESULTS: After 5½ hours at 37°C.

Agar Concentration	Amount of Serum (ml.).			
	0.5	1.0	2.0	4.0
6.0%	-	-	...	...
4.0%	-	-	-	-
2.5%	P++ (fine)	P++	-	-
1.5%	P++	P.	-	-

After 24 hours at 37°C.

6.0%	-	-	...	...
4.0%	-	-	-	-
2.5%	P++ (fine)	P++	-	-
1.5%	P. 90% L.	P. 70% L.	-	-

EXPERIMENT 99.

To ascertain the effect of heating staphylococcal factor on its ability to produce Muller's phenomenon in serum-milk-agar plates.

Portions of staphylococcal factor were heated at 55°C., 65°C., 80°C., 90°C. and 100°C. for periods of 10, 30 and 60 minutes; portions were also heated at 100°C. for 2, 2½, 3, 3½ and 4 hours. These differently heated samples were incorporated in amounts of 1.0, 2.0, 3.0 and 4.0 ml. in serum-milk-agar plates as follows:-

Milk	2.0	2.0	2.0	2.0
Agar (4.5%)	4.5	4.5	4.5	4.5
Serum	0.5	0.5	0.5	0.5
Staphylococcal Factor	1.0	2.0	3.0	4.0
Distilled Water	7.0	6.0	5.0	4.0

INCUBATION: Plates were incubated at 37°C. for 48 hours.

READINGS: These were made after 24 hours and 48 hours at 37°C.

EXPERIMENT 99.

RESULTS: After 24 hours at 37°C.

Time & Temperature of heating.	Amount of Staphylococcal Factor (ml.)			
	1.0	2.0	3.0	4.0
Unheated	P.	P. 90% L.	L.	L.
55/10 mins.	"	P. 70% L.	P. 80% L.	P. 90% L.
55/30 "	"	P. 50% L.	P. 60% L.	P. 80% L.
55/60 "	"	"	P. 70% L.	P. 90% L.
65/10 mins.	P.	P. 50% L.	P. 70% L.	P. 80% L.
65/30 "	"	"	P. 60% L.	"
65/60 "	"	"	"	P. 70% L.
80/10 mins.	P.	P. 20% L.	P. 50% L.	P. 50% L.
80/30 "	"	P.	P. 20% L.	P. 20% L.
80/60 "	-	"	P. 10% L.	P. 20% L.
90/10 mins.	P.	P. 20% L.	P. 50% L.	P. 50% L.
90/30 "	P+	P. 10% L.	P. 20% L.	P. 20% L.
90/60 "	-	P.	"	"
100/10 mins.	P.	P.	P. 10% L.	P. 10% L.
100/30 "	P-	"	"	"
100/60 "	-	P-	P-	P.
2 hours/100°C.	-	-	-	-
2½ " "	-	-	-	-
3 " "	-	-	-	-
3½ " "	-	-	-	-
4 " "	-	-	-	-

EXPERIMENT 99 (Continued).

RESULTS: After 48 hours at 37°C.

Time & Temperature of Heating.	Amount of Staphylococcal Factor (ml.)			
	1.0	2.0	3.0	4.0
55/10 mins.	P.	P. 90% L.	P. 90% L.	P. 90% L.
55/30 "	"	P. 60% L.	P. 80% L.	"
55/60 "	"	"	P. 70% L.	P. 80% L.
65/10 mins.	P.	P. 50% L.	P. 70% L.	P. 80% L.
65/30 "	"	P. 60% L.	"	"
65/60 "	"	P. 50% L.	"	"
80/10 mins.	P.	P. 20% L.	P. 50% L.	P. 60% L.
80/30 "	"	P. 10% L.	P. 20% L.	P. 30% L.
80/60 "	"	P.	P. 10% L.	"
90/10 mins.	P.	P. 20% L.	P. 60% L.	P. 70% L.
90/30 "	"	P. 10% L.	(...)	P. 40% L.
90/60 "	"	P.	P. 30% L.	"
100/10 mins.	P.	P. 10% L.	P. 30% L.	P. 40% L.
100/30 "	"	P.	P. 10% L.	P. 20% L.
100/60 "	P-	"	P.	P.
2 hours/100°C.	-	-	-	-
2½ " "	-	-	-	-
3 " "	-	-	-	-
3½ " "	-	-	-	-
4 " "	-	-	-	-

EXPERIMENT 100.

To ascertain the effect of different amounts of serum on the production of Muller's phenomenon by staphylococcal factor in serum-milk-agar plates.

MEDIA: Serum-milk-agar plates containing different amounts of serum were prepared as follows:-

Milk	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5
Staphylococcal Factor	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Distilled Water	7.5	7.4	7.25	7.0	6.5	5.5	4.5	3.5
Serum	0.0	0.1	0.25	0.5	1.0	2.0	3.0	4.0

INCUBATION: The plates were incubated at 37°C. for 24 hours.

READINGS: These were made after 24 hours at 37°C.

EXPERIMENT 100.

RESULTS: After 24 hours at 37°C.

Amount of Serum	Appearance
0.0 ml.	-
0.1	P.
0.25	"
0.5	P. 50% L.
1.0	L.
2.0	"
3.0	P. 40% L.
4.0	-

EXPERIMENT 101.

To compare the production of Muller's phenomenon by staphylococcal factor in serum-haemoglobin-agar and serum-milk-agar plates with increasing amounts of serum.

MEDIA: Serum-haemoglobin-agar and serum-milk-agar plates each containing amounts of serum from 0.5 ml. up to 4.0 ml. were prepared as follows:-

Haemoglobin	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Serum	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5
Staphylococcal Factor	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Distilled Water	4.5	4.0	3.5	3.0	2.5	2.0	1.5	1.0

and

Milk	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Serum	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5
Staphylococcal Factor	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Distilled Water	4.0	3.5	3.0	2.5	2.0	1.5	1.0	0.5

INCUBATION: The plates were incubated at 37°C. for 48 hours.

READINGS: These were taken after 24 hours and 48 hours at 37°C.

EXPERIMENT 101.

RESULTS: After 24 hours at 37°C.

Amount of Serum (ml.)	Serum-haemoglobin-agar plates	Serum-milk-agar plates
0.5	P. 95% L.	L.
1.0	P. 90% L.	"
1.5	P. 80% L.	"
2.0	"	P. 90% L.
2.5	P. 50% L.	"
3.0	P.	P.
3.5	-	-
4.0	-	-

After 48 hours at 37°C.

0.5	L.	L.
1.0	P. 95% L.	"
1.5	P. 80% L.	"
2.0	"	"
2.5	P. 50% L.	"
3.0	"	P. 50% L.
3.5	-	-
4.0	-	-

EXPERIMENT 102.

To ascertain the effect on the production of Muller's phenomenon by staphylococcal factor of pre-heating for different times and temperatures the serum incorporated in the serum-milk-agar plates.

MEDIUM: Serum, pre-heated at 55°C., 60°C. and 65°C. for 15 minutes or 30 minutes, was incorporated in serum-milk-agar plates made as follows:-

Milk	2.0
Staphylococcal Factor	4.0
Serum	0.5
Agar (4.5%)	4.5
Distilled Water	4.0

A control plate containing 0.5 ml. unheated serum was also made.

INCUBATION: Plates were incubated at 37°C. for 24 hours and then left at room temperature.

READINGS: These were made after 24 hours at 37°C. and after being at room temperature for 24 and 72 hours.

EXPERIMENT 102.

RESULTS: After 24 hours at 37°C. and after further 24 hours and 72 hours at room temperature.

Serum heated at	After 24 hours at 37°C.	After 24 hours at room temperature	After 72 hours at room temperature
55°C./15 mins.	L.	L.	L.
55°C./30 mins.	P.	P.	P.
60°C./15 mins.	-	-	-
60°C./30 mins.	-	-	-
65°C./15 mins.	-	-	-
65°C./30 mins.	-	-	-

CONTROL: The unheated specimen of the same serum tested at the same time under the same conditions, after incubation at 37°C. gave plaques in 2 hours and lysis in 18 hours.

EXPERIMENT 103.

To ascertain if serum from naturally shed blood has the same activity as serum prepared by recalcification of plasma with regard to the production of Muller's phenomenon in serum-milk-agar plates containing staphylococcal factor.

MEDIA: Serum-milk-agar plates (and Plasma-milk-agar plates for comparison) were prepared as follows:-

Milk	2.0	2.0	Milk	2.0	2.0
Serum*	0.5	1.0	Plasma	0.5	1.0
Agar (4.5%)	4.5	4.5	Agar (4.5%)	4.5	4.5
Staphylococcal Factor	2.0	2.0	Staphylococcal Factor	2.0	2.0
Distilled Water	6.0	5.5	Distilled Water	6.0	5.5

* Naturally shed blood serum or serum prepared from plasma.

INCUBATION: All plates were incubated at 37°C. for 24 hours and then left at room temperature for 24 hours.

READINGS: These were made after 24 hours at room temperature, following the 24 hours' incubation at 37°C.

EXPERIMENT 103.

RESULTS: After 24 hours' at room temperature, following 24 hours incubation at 37°C.

Nature of Serum	0.5 ml.	1.0 ml.
From naturally clotted blood.	P. 80% L.	P. 90% L.
From recalcified Plasma.	P. 70% L.	"
Plasma.	"	"

EXPERIMENT 104.

To ascertain if meat broth is inhibitory to plaque production by the staphylococcal factor in serum-milk-agar plates.

MEDIA: Two sets of serum-milk-agar plates were made containing increasing amounts of staphylococcal factor from 1.0 ml. up to 7.0 ml., the diluent used to keep the volume at 15 ml. being in one set distilled water, and in the other, meat broth. One plate was made with 8.0 ml. staphylococcal factor without diluent.

Milk	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5
Serum	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Staphylococcal Factor	1.0	2.0	3.0	4.0	5.0	6.0	7.0	8.0
Distilled Water or Meat Broth.	7.0	6.0	5.0	4.0	3.0	2.0	1.0	0.0

INCUBATION: The two sets of plates were incubated at 37°C. for 24 hours and then left at room temperature for 24 hours.

READINGS: These were made after 24 hours at 37°C. and after 24 hours at room temperature.

EXPERIMENT 104.

RESULTS: After 24 hours at 37°C. and after further 24 hours at room temperature.

Amount of Meat Broth in plate (ml.).	After 24 hours' incubation at 37°C.	After 24 hours at room temperature.
7.0	P.	P. (P. 1 mm. diameter)
6.0	P. 10% L. (P. 1-2 mm. diameter).	P. 80% L. (P. up to 4 mm. diameter)
5.0	P. 20% L. (P. 1-3 mm. diameter)	L.
4.0	P. 20% L. (P. 1-5 mm. diameter)	P. 95% L. (trace of large P.)
3.0	P. (approximately 60 in number)	P. 90% L. (P. up to 6 mm. diameter)
2.0	P. (approximately 30 in number)	P. (P. up to 7 mm. in diameter)
1.0	P. (approximately 17 in number)	P. (P. up to 7 mm. in diameter.

Amount of Distilled Water in plate (ml.)	After 24 hours' incubation at 37°C.	After 24 hours at room temperature.
7.0	P.	P. 10% L.
6.0	L.	L.
5.0	"	"
4.0	"	"
3.0	"	"
2.0	"	"
1.0	P. 10% L. (P. 1-5 mm. diameter)	P. 70% L. (P. 1-7 mm. diameter)

EXPERIMENT 104 (Continued).

The plate containing 8.0 ml. of staphylococcal factor alone showed only about 7 plaques after 24 hours' incubation at 37°C., while after a further 24 hours at room temperature a much larger number of plaques were to be seen. These plaques were up to 7 mm. in diameter.

EXPERIMENT 105.

To ascertain the effect of peptone on the production of Muller's phenomenon by staphylococcal factor in serum-milk-agar plates.

MEDIUM: A series of serum-milk-agar plates containing different concentrations of peptone (Oxoid) were prepared as follows:-

Milk	2.0	2.0	2.0	2.0	2.0
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5
Staphylococcal Factor	1.0	1.0	1.0	1.0	1.0
Serum	0.5	0.5	0.5	0.5	0.5
Distilled Water	7.0	6.75	6.5	6.25	6.0
Peptone (Oxoid) (15%)	0.0	0.25	0.5	0.75	1.0
Final Concentration of Peptone.	0.0%	0.25%	0.5%	0.75%	1.0%

INCUBATION: Plates were incubated at 37°C. for 24 hours.

READINGS: These were made after 24 hours' incubation of plates at 37°C.

EXPERIMENT 105.

RESULTS: After 24 hours at 37°C.

Concentration of Peptone	0.0%	0.25%	0.5%	0.75%	1.0%
Result	P. 95% L.	P.	P-	-	-

The plate containing 0.25% peptone showed plaques some of which were very small, while others were of usual size. This plate was photographed and is reproduced as Fig. XXXVI.

EXPERIMENT 106.

To compare the effect of the addition of 1% "Lab-Lemco" broth and 1% "Lab-Lemco" in distilled water on the production of Muller's phenomenon by staphylococcal factor in serum-milk-agar plates.

MEDIA: Two sets of serum-milk-agar plates were made up as follows, one containing 1% "Lab-Lemco" in distilled water, the other 1% "Lab-Lemco" broth*.

Milk	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Staphylococcal Factor	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5	4.5	4.5
1% "Lab-Lemco" Broth or 1% "Lab-Lemco" in Distilled Water	0.0	1.0	2.0	3.0	4.0	5.0	6.0
Serum	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Distilled Water	6.0	5.0	4.0	3.0	2.0	1.0	0.0

INCUBATION: These plates were incubated at 37°C. for 24 hours and then left at room temperature.

READINGS: These were taken after 24 hours at 37°C. and after 48 hours at room temperature.

* The 1% "Lab-Lemco" broth contained 1% peptone (Oxoid) and 0.5% NaCl.

EXPERIMENT 106.

RESULTS: After 24 hours at 37°C.

Amount of 1% "Lab-Lemco" in distilled water or as broth	ml.					
	1.0	2.0	3.0	4.0	5.0	6.0
1% "Lab-Lemco" in distilled water	L.	L.	L.	L.	L.	L.
1% "Lab-Lemco" broth.	"	"	P. (large & small.)	P.	P-	-

After 48 hours at room temperature.

1% "Lab-Lemco" in distilled water	L.	L.	L.	L.	L.	L.
1% "Lab-Lemco" broth.	"	"	"	P. 95% L. (P. very large)	P. 80% L. (P. very large)	(P. large & small)

The control plate without added "Lab-Lemco" either in distilled water or as broth gave Lysis within 24 hours' incubation at 37°C.

EXPERIMENT 107.

To ascertain the effect of the addition of Sodium chloride to serum-milk-agar plates on the production of Muller's phenomenon using staphylococcal factor.

MEDIA: Serum-milk-agar plates with different amounts of Sodium chloride were prepared as follows:-

Milk	2.0	2.0	2.0	2.0	2.0	2.0	Control 2.0
Distilled Water	7.0	6.75	6.5	6.25	6.0	5.0	7.5
Serum	0.5	0.5	0.5	0.5	0.5	0.5	0.0
Staphylococcal Factor	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5	4.5	4.5
NaCl. (15%)	0.0	0.25	0.5	0.75	1.0	2.0	0.0
Final percentage NaCl.	0.0	0.25	0.5	0.75	1.0	2.0	0.0

INCUBATION: Plates were incubated at 37°C. for 24 hours, thereafter left at room temperature.

READINGS: These were noted after 24 hours' incubation at 37°C., and after a period of 24 hours at room temperature.

EXPERIMENT 107.

RESULTS: After 24 hours' incubation at 37°C. and further 24 hours at room temperature.

NaCl.	0.0%	0.25%	0.5%	0.75%	1.0%	2.0%
24 hours	L.	P. 50% L. (large & small)	P. (large & small)	-	-	-
48 "	"	P. 70% L. (large)	P. (large)	P. (large & small)	-	-

Control plate (without serum) showed No change.

There was complete lysis in all test plates except those with 1% and 2% Sodium chloride after 7 days at room temperature.

EXPERIMENT 108.

To ascertain if Acriflavine or "Merthiolate" inhibits the production of Muller's phenomenon by staphylococcal factor in serum-milk-agar plates.

MEDIA: Seven serum-milk-agar plates were made as follows:-

Milk	2.0 ml.
Staphylococcal Factor	2.0
Serum	0.5
Agar (4.5%)	4.5
Distilled Water	6.0

To three plates there was added 0.75 ml., 0.375 ml. or 0.15 ml. of a 1/500 solution of Acriflavine solution to give final concentrations of Acriflavine of 1/10,000, 1/20,000 and 1/50,000 respectively.

To another three plates there was added 0.15 ml. of a 1/100 solution or 0.75 ml. and 0.30 ml. of 1/1,000 solution of "Merthiolate" to give final concentrations of "Merthiolate" of 1/10,000, 1/20,000 and 1/50,000 respectively.

The remaining plate without any added antiseptic acted as a control plate.

INCUBATION: The plates were incubated at 37°C. for 24 hours and then left at room temperature.

READINGS: These were taken after 24 hours at 37°C. and after 48 hours at room temperature.

EXPERIMENT 108.

RESULTS:

	After 24 hours at 37°C.	After 48 hours at Room Temperature
Control	P. 70% L.	L.
Acriflavine		
1/10,000	-	P. 90% L.
1/20,000	P.	L.
1/50,000	P. 70% L.	"
"Merthiolate"		
1/10,000	P. 70% L.	L.
1/20,000	"	"
1/50,000	"	"

EXPERIMENT 109.

To ascertain if soya-bean-trypsin-inhibitor inhibits the production of Muller's phenomenon by staphylococcal factor in serum-milk-agar plates.

MEDIUM: A serum-milk-agar plate was prepared as follows:-

Milk	2.0 ml.
Agar (4.5%)	4.5
Staphylococcal Factor	2.0
Serum	0.5
Distilled Water	6.0

Merthiolate was incorporated in this plate to give a final concentration of 1/30,000.

Three porous test cups were placed on the surface of the medium, one containing 2 drops soya-bean-trypsin-inhibitor made up in distilled water (5 mg./ml.), the second also containing 2 drops of soya-bean-trypsin-inhibitor (2.5 mg./ml.) and the third - as control - containing only 2 drops of distilled water.

INCUBATION: The plate was placed at 37°C. for 24 hours.

READING: This was taken after 24 hours at 37°C.

EXPERIMENT 109.

RESULT: After 24 hours at 37°C.

The plate showed lysis and plaques throughout the serum-milk-agar medium except for a zone around each of the cups containing the soya-bean-trypsin-inhibitor, in which there was no evidence of Muller's phenomenon - the medium here being unaltered. There was no inhibition of the phenomenon around the control cup containing distilled water.

The plate was photographed and is reproduced as Fig. XXXIX.

EXPERIMENT 110.

To compare the effect on the production of Muller's phenomenon in serum-milk-agar plates by staphylococcal factor of holding mixtures of serum and staphylococcal factor at 37°C. for different periods of time with mixtures of serum, staphylococcal factor and milk also held at 37°C. for the same similar periods of time, before being added to agar and plates poured.

MEDIA: A series of tubes each containing 4.0 ml. of staphylococcal factor, 0.5 ml. serum and 2.0 milk were incubated at 37°C. for 15, 30, 45 and 60 minutes before being added to 4.5 ml. of 4.5% agar and 4.0 ml. distilled water and poured into plates. In addition a control plate with the same ingredients was poured immediately.

A series of tubes each containing 4.0 ml. staphylococcal factor, 0.5 ml. serum were incubated at 37°C. for 15, 30, 45 and 60 minutes before being added to mixtures of 4.5 ml. of 4.5% agar, 4.0 ml. distilled water and 2.0 ml. milk and poured into plates. In addition a control plate with the same ingredients was poured immediately.

INCUBATION: Plates were incubated at 37°C.

READINGS: The first appearance of Muller's phenomenon in the different plates was noted.

EXPERIMENT 110.

RESULTS:

Plaques were visible in all plates, including the control plates after 1 hour and 45 minutes at 37°C.

EXPERIMENT 111.

To ascertain the effect of different amounts of haemoglobin solution on the production of Müller's phenomenon in serum-haemoglobin-agar by staphylococcal factor.

MEDIA: Serum-haemoglobin-agar plates were made up as follows:-

Haemoglobin	0.5	1.0	1.5	2.0	3.0	4.0	5.0
Serum	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5	4.5	4.5
Staphylococcal Factor	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Distilled water	4.5	4.0	3.5	3.0	2.0	1.0	0.0

INCUBATION: The plates were incubated at 37°C. for 24 hours and then left at room temperature.

READINGS: These were made after 24 hours at 37°C. and after 24 hours at room temperature.

EXPERIMENT 111.

RESULTS: After 24 hours at 37°C. and 24 hours at room temperature.

Amount of Haemoglobin	After 24 hours at 37°C.	After 24 hours at room temperature
0.5 ml.	L.	L.
1.0	"	"
1.5	"	"
2.0	P. 95% L.	"
3.0	P++	P++
4.0	"	P. 80% L.
5.0	"	"

EXPERIMENT 112.

To ascertain the effect of adding different amounts of staphylococcal factor to serum-haemoglobin-agar plates on the production of Muller's phenomenon.

MEDIA: Serum-haemoglobin-agar plates were made up as follows:-

Haemoglobin	1.5	1.5	1.5	1.5	1.5	1.5
Serum	1.5	1.5	1.5	1.5	1.5	1.5
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5	4.5
Staphylococcal Factor	1.0	2.0	3.0	4.0	5.0	6.0
Distilled Water	6.5	5.5	4.5	3.5	2.5	1.5

INCUBATION: The plates were incubated at 37°C. for 24 hours and then left at room temperature.

READINGS: These were made after 24 hours at 37°C. and 24 hours at room temperature.

EXPERIMENT 112.

RESULTS: After 24 hours at 37°C. and 24 hours at room temperature.

Amount of Staphylococcal Factor	After 24 hours at 37°C.	After 24 hours at room temperature
1.0 ml.	-	L.
2.0	P. 95% L.	"
3.0	L.	"
4.0	"	"
5.0	P. 90% L.	P. 95% L.
6.0	"	"

EXPERIMENT 113.

To ascertain the effect of temperature on the production of Muller's phenomenon by staphylococcal factor in serum-haemoglobin-agar plates.

MEDIUM: Four plates of serum-haemoglobin-agar and/four control haemoglobin-agar plates were prepared as follows:-

Haemoglobin	1.5	1.5
Serum	1.5	0.0
Staphylococcal Factor	4.0	4.0
Agar (4.5%)	4.5	4.5
Distilled Water	3.5	5.0

INCUBATION: One plate of serum-haemoglobin-agar and control plate of haemoglobin-agar were placed at each of the following temperatures (1) 4°C., (2) 22°C., (3) 30°C. and (4) 37°C.

READINGS: The plates were examined up to 7 days after placing at the temperature specified.

EXPERIMENT 113.

RESULTS:

Temperature	Serum-haemoglobin-agar plate.	Control plate
4°C.	- (at 7 days)	-
22°C.	P. (18 hours)	-
30°C.	" "	-
37°C.	" "	-

EXPERIMENT 114.

To ascertain if Muller's phenomenon can be produced anaerobically by staphylococcal factor in serum-haemoglobin-agar plates.

MEDIUM: Two sets of serum-haemoglobin-agar plates were prepared as follows:-

Haemoglobin	1.5	1.5	1.5	1.5	1.5
Serum	1.5	2.0	3.0	4.0	5.0
Staphylococcal Factor	4.0	4.0	4.0	4.0	4.0
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5
Distilled Water	3.5	3.0	2.0	1.0	0.0

INCUBATION: These plates were incubated at 37°C. for 24 hours, one set in a McIntosh & Fildes' anaerobic jar, the other set, as control, in the incubator under aerobic conditions.

READINGS: These were taken after 24 hours' incubation at 37°C.

EXPERIMENT 114.

RESULTS: After 24 hours' incubation at 37°C.

Amount of Serum	Incubated Anaerobically	Control Incubated Aerobically
1.5 ml.	P. 40% L.	P. 40% L.
2.0	P. 50% L.	"
3.0	P. 60% L.	P. 50% L.
4.0	"	P. 60% L.
5.0	P. 50% L.	P. 50% L.

EXPERIMENT 115.

To ascertain the effect on the production of Muller's phenomenon by staphylococcal factor of the addition of different amounts of serum to serum-haemoglobin-agar plates.

MEDIUM: Serum-haemoglobin-agar plates containing different amounts of serum and a control plate without serum were prepared as follows:-

Haemoglobin	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5
Staphylococcal Factor	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Serum	0.0	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0
Distilled Water	5.0	4.5	4.0	3.5	3.0	2.5	2.0	1.5	1.0	0.5	0.0

INCUBATION: These plates were incubated at 37°C. for 48 hours.

READINGS: These were made after 24 hours' and 48 hours' incubation at 37°C.

EXPERIMENT 115.

RESULTS: After 24 hours' and 48 hours' incubation at 37°C.

Amount of Serum (ml.)	After 24 hours' Incubation	After 48 hours' Incubation
0.0	-	-
0.5	P-	P.
1.0	P.	P+
1.5	P. 40% L.	P. 50% L.
2.0	P. 50% L.	"
2.5	"	P. 60% L.
3.0	P. 60% L.	P. 80% L.
3.5	L.	L.
4.0	"	"
4.5	P. 50% L.	P. 50% L.
5.0	P.	P+

The plate with 2.0 ml. of serum was photographed and is reproduced as Fig. XXXVII.

EXPERIMENT 116.

To ascertain if Muller's phenomenon can be produced by staphylococcal factor in a heated red-cell ("chocolate") agar medium containing fresh serum.

MEDIUM: A series of tubes containing a mixture of agar, distilled water and human red cells was heated at 75°C. for 10 minutes. These tubes were then cooled to 50°C. and different amounts of serum and of staphylococcal factor were added to them. They were then poured into plates. The composition of these different heated red cell-serum agar mixtures is given as follows:-

Human Red Cells	0.5	0.5	0.5	0.5
Agar (4.5%)	4.5	4.5	4.5	4.5
Distilled Water	9.0	8.5	8.0	5.0

After heating at 75°C. for 10 minutes, cooled to 50°C. and serum and staphylococcal factor added.

Serum	0.0	0.5	1.0	4.0
Staphylococcal Factor	1.0	1.0	1.0	1.0

Similar series of plates were made with 2.0 ml., 3.0 ml. and 4.0 ml. of staphylococcal factor.

INCUBATION: The plates were incubated at 37°C. for 24 hours.

READINGS: These were made after 24 hours at 37°C.

EXPERIMENT 116.

RESULTS: After 24 hours at 37°C.

Amount of Staphylococcal Factor	Amount of Serum (ml.)			
	0.0	0.5	1.0	4.0
1.0	-	P.(faint)	P.(faint)	-
2.0	-	P.	P.	P.
3.0	-	"	"	P.80%L.
4.0	-	"	"	P.95%L.

EXPERIMENT 117.

To ascertain if Muller's phenomenon can be produced by staphylococcal factor in a heated-haemoglobin-agar medium containing fresh serum.

MEDIUM: Three tubes each containing a mixture of haemoglobin solution, agar and distilled water were heated at 75°C. for 10 minutes, and after cooling to 50°C. had added to them staphylococcal factor and different amounts of serum, viz. 0.5 ml., 1.0 ml. and 2.0 ml. respectively. These mixtures were then poured into plates. The composition of these mixtures is here detailed:-

Haemoglobin	1.0	1.0	1.0
Agar (4.5%)	4.5	4.5	4.5
Distilled Water	5.0	4.5	3.5

The above were heated at 75°C. for 10 minutes, cooled to 50°C. when serum and staphylococcal factor were added as follows:-

Staphylococcal Factor	4.0	4.0	4.0
Serum	0.5	1.0	2.0

INCUBATION: The plates were incubated at 37°C. for 24 hours.

READINGS: These were taken after 24 hours at 37°C.

EXPERIMENT 117.

RESULTS: After 24 hours at 37°C.

Amount of Serum	After 24 hours' Incubation at 37°C.
0.5 ml.	P-
1.0	P.
2.0	P.

The plate containing 2.0 ml. serum was photographed and is reproduced as Fig. XXXVIII.

EXPERIMENT 118.

To ascertain the effect on the production of Muller's phenomenon by strains of staphylococci of varied amounts of pooled human serum in serum-milk-agar plates.

MEDIUM: Serum-milk-agar plates were prepared with a pooled human serum "IN" in amounts from 0.1 ml. up to 7.0 ml. as follows:-

Milk	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5
"Lab-Lemco" (15%)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Serum	0.0	0.1	0.25	0.5	1.0	2.0	3.0	4.0	5.0	6.0	7.0
Distilled Water	7.5	7.4	7.25	7.0	6.5	5.5	4.5	3.5	2.5	1.5	0.5

INOCULATION: These plates were stab-inoculated from 6 hour old "Lab-Lemco" broth cultures of Staphylococcus aureus, Strains A, 401, 406, 039, 061, 779 and 935.

INCUBATION: These plates were incubated at 37°C. for 24 hours and then left at room temperature for 24 hours.

READINGS: These were made after 24 hours at 37°C. and 24 hours at room temperature.

EXPERIMENT 118.

RESULTS: After 24 hours at 37°C.

Amount of Serum (ml.)	A	401	406	039	061	779	935
0.0 *	S.L.	S.L.	S.L.	S.L.	S.L.	S.L.	S.L.
0.1	L.P.	P.	P.	L.P.	P.	P.	L.P.
0.25	"	"	"	P.	"	"	"
0.5	"	L.P.	L.P.	L.P.	L.P.	L.P.	"
1.0	"	"	"	"	"	"	"
2.0	"	"	"	"	"	"	"
3.0	L.R.E.	L.R.E.	L.R.E.	L.R.E.	"	L.R.E.	L.R.E.
4.0	L.	L.	L.	L.	L.	L.	L.
5.0	"	"	"	"	"	"	"
6.0	"	"	"	"	"	"	"
7.0	"	"	"	"	"	"	"

After 24 hours at room temperature.

0.0 *	L.	L.	L.	L.	L.	L.	L.
0.1	L.P.	P.	P.	L.P.	P.	L.P.	L.P.
0.25	"	L.P.	L.P.	"	L.P.	"	"
0.5	"	"	"	"	"	"	"
1.0	L.R.E.P.	L.R.E.P.	L.R.E.P.	L.R.E.P.	"	L.R.E.P.	"
2.0	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.P.	L.R.E.	L.R.E.
3.0	L.	L.	L.	L.	L.R.E.	L.	L.
4.0	"	"	"	"	L.	"	"
5.0	"	"	"	"	"	"	"
6.0	"	"	"	"	"	"	"
7.0	"	"	"	"	"	"	"

* The lysis occurring in this plate without serum was of the indefinite type.

EXPERIMENT 119.

To ascertain the ability of different amounts of serum "13" to produce Muller's phenomenon in serum-milk-agar plates containing staphylococcal factor.

MEDIA: Serum-milk-agar plates containing amounts of serum from 0.1 ml. up to 5.0 ml. were prepared as follows:-

Milk	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5
Staphylococcal Factor	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Serum "13"	0.0	0.1	0.25	0.5	1.0	2.0	3.0	4.0	5.0
Distilled Water	7.5	7.4	7.25	7.0	6.5	5.5	4.5	3.5	2.5

INCUBATION: Plates were incubated at 37°C. for 24 hours.

READINGS: These were made after 24 hours at 37°C.

EXPERIMENT 119.

RESULTS: After 24 hours at 37°C.

Amount of Serum (ml.)								
0.0	0.1	0.25	0.5	1.0	2.0	3.0	4.0	5.0
-	P.	P.	P. 90% L.	L.	L.	P. 20% L.	-	-

The plates containing 0.1/ml. and 3.0 ml. amounts of serum were photographed and are reproduced as Fig. XL and Fig. XLI.

EXPERIMENT 120.

To titrate three samples of pooled serum with the same batch of staphylococcal factor.

Amounts of the three pooled sera "PI", "P2" and "P3", 0.5 ml., 1.0 ml., 2.0 ml., 3.0 ml. and 4.0 ml. were tested with 1.0 ml. 2.0 ml., 3.0 ml. and 4.0 ml. of a batch of staphylococcal factor as follows:-

Milk	2.0	2.0	2.0	2.0	2.0
Serum "PI"	0.5	1.0	2.0	3.0	4.0
Staphylococcal Factor	1.0	1.0	1.0	1.0	1.0
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5
Distilled Water	7.0	6.5	5.5	4.5	3.5

Similar plates of Serum "PI" with 2.0 ml., 3.0 ml. and 4.0 ml. of staphylococcal factor respectively were prepared.

INCUBATION: The plates were incubated at 37°C. for 24 hours, then left at room temperature for 24 hours.

READINGS: These were made after 24 hours at 37°C. and 24 hours at room temperature.

EXPERIMENT 120.

RESULTS: After 24 hours at 37°C.

Serum "PI".

Amount of Staphylococcal Factor	Amount of Serum (ml.)					
	0.5		1.0	2.0	3.0	4.0
1.0	L.	P.	70% L.	-	-	-
2.0	"	L.		P. 80% L.	-	-
3.0	"	"		L.	P. 40% L.	-
4.0	"	"		"	L.	-

Serum "P2"

1.0	L.	P.	90% L.	-	-	-
2.0	"	L.		L.	-	-
3.0	"	"		"	P. 90% L.	-
4.0	"	"		"	L.	-

Serum "P3"

1.0	P. 90% L.	P.	10% L.	-	-	-
2.0	L.	L.		P. 10% L.	-	-
3.0	"	"		P. 30% L.	-	-
4.0	"	"		L.	P.	-

After 24 hours at room temperature. Serum "PI".

1.0	L.	L.	-	-	-
2.0	"	"	P. 80% L. (small)	-	-
3.0	"	"	L.	P. 95% L. (normal)	-
4.0	"	"	"	L.	P. 10% L. (large)

Serum "P2"

1.0	L.	L.	-	-	-
2.0	"	"	L.	P. 10% L.	-
3.0	"	"	"	L.	P. 10% L. (normal)
4.0	"	"	"	"	P. 95% L. (large)

EXPERIMENT 120 (Continued).

Serum "P3".

Amount of Staphylococcal Factor	Amount of Serum (ml.)				
	0.5	1.0	2.0	3.0	4.0
1.0	P. 70% L. (small)	P. 40% L. (small)	-	-	-
2.0	L.	L.	P. 80% L.	-	-
3.0	"	"	P. 90% L. (normal)	P. 70% L.	-
4.0	"	"	L.	P. 90% L. (normal)	-

EXPERIMENT 121.

To test the ability of eight sera to produce Muller's phenomenon when tested with the same batch of staphylococcal factor in milk-agar plates by "checkerboard titration".

MEDIUM: Eight different individual sera "B5", "B9", "B317", "A51", "B335", "A15", "A333" and "B38" were tested in amounts of 0.5, 1.0, 2.0, 3.0 and 4.0 ml. with 1.0, 2.0, 3.0 and 4.0 ml. of staphylococcal factor as follows:-

Milk	2.0	2.0	2.0	2.0
Serum	0.5	0.5	0.5	0.5
Staphylococcal factor	1.0	2.0	3.0	4.0
Agar (4.5%)	4.5	4.5	4.5	4.5
Distilled Water	7.0	6.0	5.0	4.0

and with 1.0 ml., 2.0 ml., 3.0 ml. and 4.0 ml. of serum.

INCUBATION: The plates were incubated at 37°C. for 24 hours and then left at room temperature for 24 hours.

READINGS: These were made after 24 hours at 37°C. and 24 hours at room temperature.

EXPERIMENT 121.

RESULTS: After 24 hours at room temperature only are recorded here.

Serum "B5".

Amount of Staphylococcal Factor.	Amount of Serum (ml.)				
	0.5	1.0	2.0	3.0	4.0
1.0	L.	P. 80% L.	-	-	-
2.0	"	L.	-	-	-
3.0	"	"	L.	P. 30% L.	-
4.0	"	"	"	L.	P.

Serum "B9"

1.0	L.	L.	P.	-	-
2.0	"	"	L.	P. 60% L.	-
3.0	"	"	"	L.	P. 10% L.
4.0	"	"	"	"	L.

Serum "B317"

1.0	P. 50% L.	L.	-	-	-
2.0	L.	"	P. 50% L.	-	-
3.0	"	"	L.	P.	-
4.0	"	"	"	P. 90% L.	-

Serum "A51"

1.0	L.	P. 70% L.	-	-	-
2.0	"	L.	L.	-	-
3.0	"	"	"	L.	-
4.0	"	"	"	"	P. 70% L.

Serum "B335"

1.0	L.	L.	L.	-	-
2.0	"	"	"	L.	L.
3.0	"	"	"	"	"
4.0	"	"	"	"	"

Serum "A15"

1.0	L.	L.	-	-	-
2.0	"	"	-	-	-
3.0	"	"	L.	-	-
4.0	"	"	"	L.	-

EXPERIMENT 121 (Continued)

Serum "A333"

Amount of Staphylococcal Factor.	Amount of Serum (ml.)				
	0.5	1.0	2.0	3.0	4.0
1.0	P. 10% L.	-	-	-	-
2.0	L.	L.	-	-	-
3.0	"	"	-	-	-
4.0	"	"	P. 10% L.	-	-

Serum "B38"

1.0	L.	P. 70% L.	-	-	-
2.0	"	L.	L.	-	-
3.0	"	"	"	P. 10% L.	-
4.0	"	"	"	P. 90% L.	-

EXPERIMENT 122.

To titrate a number of pathological sera with the same staphylococcal factor in serum-milk-agar plates.

MEDIUM: Small quantities of sera from patients in hospital with different complaints were available. Amounts of these sera from 0.1 ml. up to 2.0 ml. (usually) were tested with 1.5 ml. of staphylococcal factor in serum-milk-agar plates as follows:-

Milk	2.0	2.0	2.0	2.0	2.0
Serum	0.1	0.5	1.0	1.5	2.0
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5
Staphylococcal Factor	1.5	1.5	1.5	1.5	1.5
Distilled Water	6.9	6.5	6.0	5.5	5.0

Occasionally a plate with 3.0 ml. of serum is added if available.

INCUBATION: The plates were incubated at 37°C. for 24 hours and then left at room temperature for 24 hours.

READINGS: These were made after 24 hours at 37°C. and 24 hours at room temperature.

EXPERIMENT 122.

RESULTS: Those taken after 24 hours at room temperature are given here.

Condition of Patient	Amount of Serum (ml.)					
	0.1	0.5	1.0	1.5	2.0	3.0
Diabetes.	-	L.	L.	L.	-	(...)
Haemochromatosis.	P. 40% L.	"	"	"	L.	P. 40% L.
Malignant Disease.	-	"	"	"	"	(...)
Nephritis (after A.C.T.H. therapy)	P.	P. 90% L.	"	"	-	(...)
Luminal Poisoning.	-	P. 50% L.	"	(...)	-	-
Ureteric transplantation.	-	L.	"	L.	L.	L.
Gastric Ulcer	P. 50% L.	"	"	"	"	(...)
Thromophlebitis.	P.	"	"	"	80% L.	"
On "Tromexan"	P.	"	"	"	L.	"
Thrombophlebitis.	"	P.	-	-	-	"
On "Cortisone"	"	"	P.	(...)	P.	"

EXPERIMENT 123.

To ascertain the effect on the production of Muller's phenomenon by living staphylococci of the incorporation of plasma in place of serum in serum-milk-agar plates.

MEDIUM: Plasma-milk-agar plates were prepared as follows:-

Milk	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Plasma	0.0	0.5	1.0	2.0	3.0	4.0	5.0	6.0
"Lab-Lemco" (15%)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5
Distilled Water	7.5	7.0	6.5	5.5	4.5	3.5	2.5	1.5

These plates were made in duplicate.

INOCULATION: One set was inoculated from 6 hour old "Lab-Lemco" broth cultures of 5 strains of Staphylococcus aureus, A, 401, 039, 061 and 935. The other set was uninoculated.

INCUBATION: Both sets of plates, inoculated and uninoculated were incubated at 37°C. for 24 hours and then left at room temperature for 24 hours.

READINGS: These were made after 24 hours at 37°C. and after 24 hours at room temperature.

EXPERIMENT 123.

RESULTS: After 24 hours at 37°C. and 24 hours at room temperature.

Amount of Plasma	Time of Incubation (hrs.)	Strains of Staphylococci				
		A	401	039	061	935
0.0	24	S.L.	-	S.L.	-	-
	48	"	-	"	-	-
0.5	24	L.P.	L.P.	L.P.	L.P.	L.P.
	48	"	"	"	"	"
1.0	24	"	"	"	"	"
	48	"	"	"	"	"
2.0	24	"	"	"	"	"
	48	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.
3.0	24	L.P.	L.P.	L.P.	L.P.	L.P.
	48	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.
4.0	24	L.P.	L.P.	L.P.	L.P.	L.P.
	48	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.
5.0	24	L.P.	L.	L.	L.P.	L.
	48	L.	"	"	L.	"
6.0	24	L.R.E.P.	L.R.E.P.	"	"	"
	48	L.	L.	"	"	"

There was a lessening of opacity with the increase of plasma in the plates; the uninoculated set of plates incubated under the same conditions acted as controls.

EXPERIMENT 124.

To ascertain if Muller's phenomenon can be produced by staphylococcal factor in a serum-milk-agar plate containing serum from placental blood in place of adult human serum.

MEDIUM: Serum-milk-agar plates were prepared with amounts of placental blood serum from 0.5 ml. up to 3.0 ml. Two such sera were available.

Milk	2.0	2.0	2.0	2.0
Placental Serum	0.5	1.0	2.0	3.0
Agar (4.5%)	4.5	4.5	4.5	4.5
Staphylococcal Factor	2.0	2.0	2.0	2.0
Distilled Water	6.0	5.5	4.5	3.5

INCUBATION: The plates were incubated at 37°C. for 24 hours.

READINGS: These were made after 5 hours and 18 hours.

EXPERIMENT 124.

RESULTS: After 5 hours and 18 hours at 37°C.

After Incubation at 37°C.	Serum	Amount of Serum (ml.)			
		0.5	1.0	2.0	3.0
5 hours	I	P.	P. 40% L.	P. 40% L.	P.
"	II	"	"	"	(...)
18 hours	I	P. 95% L.	L.	L.	L.
"	II	"	"	"	(...)

After 24 hours' incubation at 37°C. there was complete (100%) Lysis of all serum-milk-agar plates with Serum I and Serum II.

EXPERIMENT 125.

To ascertain the effects of spreading serum over the surface of a milk-agar plate containing staphylococcal factor and of spreading staphylococcal factor over the surface of a serum-milk-agar plate (not containing staphylococcal factor).

MEDIA: Two plates of each of the following two media were prepared:-

Staphylococcal Factor	0.0	2.0
Milk	2.0	2.0
Serum	0.5	0.0
Agar (4.5%)	4.5	4.5
Distilled Water	8.0	6.5

PREPARATION: One plate of serum-milk-agar had 1.0 ml. of staphylococcal factor spread over its surface, the other 0.2 ml. of staphylococcal factor over its surface. One plate of milk-agar (containing the staphylococcal factor) had 0.5 ml. of serum spread over its surface, and the other plate 0.2 ml. of serum spread over its surface.

INCUBATION: Plates were incubated at 37°C. for 24 hours.

READINGS: These were taken after 24 hours at 37°C.

EXPERIMENT 125.

RESULTS: After 24 hours at 37°C.

Type of Plate	Material spread on surface.	Amount of spread material	Result
Serum-milk-agar	Staphylococcal Factor	1.0 ml. 0.2 ml.	P++(faint) -
Milk-agar containing Staphylococcal Factor	Serum.	0.5 ml. 0.2 ml.	P++(faint) "

EXPERIMENT 126.

To ascertain the effects of drops of dilutions of serum spread over the surface of a milk-agar plate containing staphylococcal factor, and of drops of dilutions of staphylococcal factor spread over the surface of a serum-milk-agar plate.

MEDIA: Two milk-agar plates containing different amounts of staphylococcal factor and two serum-milk-agar plates with different amounts of serum were prepared as follows:-

Milk	2.0	2.0	2.0	2.0
Serum	0.0	0.0	0.5	1.0
Staphylococcal Factor	2.0	4.0	0.0	0.0
(agar (4.5%)	4.5	4.5	4.5	4.5
Distilled Water	6.5	4.5	9.0	8.5

Drops of neat serum, and dilutions 1/2, 1/4, 1/8, 1/16 and 1/32 of serum were placed on the surface of the two milk-agar plates. Drops of staphylococcal factor - neat, 1/2, 1/4, 1/8, 1/16 and 1/32 were placed on the surface of the two serum-milk-agar plates.

INCUBATION: The plates were incubated at 37°C. for 24 hours and then left at room temperature.

READINGS: These were made after 24 hours at 37°C. and 24 hours and 48 hours at room temperature.

EXPERIMENT 126.

RESULTS: After 24 hours at 37°C. and after 24 and 48 hours at room temperature.

Milk-Agar Plates.	Incubation	Dilutions of serum added					
		Neat	1/2	1/4	1/8	1/16	1/32
With Staphylococcal Factor. 2.0 ml.	24 hrs./37°C.	P.	P.	P.	-	-	-
	24 hrs./R.T.	"	"	"	P.	-	-
	48 "	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.
With Staphylococcal Factor. 4.0 ml.	24 hrs./37°C.	P.	P.	P.	-	-	-
	24 hrs./R.T.	"	"	"	P.	-	-
	48 "	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.

Serum-Milk-Agar Plates	Incubation	Dilutions of Staphylococcal Factor added.					
		Neat	1/2	1/4	1/8	1/16	1/32
With 0.5 ml. Serum.	24 hrs./37°C.	-	-	-	-	-	-
	24 hrs./R.T.	-	-	-	-	-	-
	48 "	-	-	-	-	-	-
With 1.0 ml. Serum.	24 hrs./37°C.	-	-	-	-	-	-
	24 hrs./R.T.	-	-	-	-	-	-
	48 "	-	-	-	-	-	-

EXPERIMENT 127.

To compare the results obtained by titrating a serum on sets of milk-agar discs containing different amounts of two preparations of staphylococcal factor.

MEDIA: Milk-agar plates containing staphylococcal factor '(a)' were prepared as follows:-

Milk	2.0	2.0	2.0	2.0	2.0
Staphylococcal Factor	0.0	0.5	1.0	2.0	4.0
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5
Distilled Water	8.5	8.0	7.5	6.5	4.5

A similar series of plates were made containing staphylococcal preparation '(b)'.

These plates of milk-agar medium were dried in the incubator (37°C.) for 30 minutes and then, by means of a sterile brass cutter, discs 16 mm. in diameter were prepared. Dilutions of serum made in 0.1 ml. amounts were placed on the surface of the two sets of discs. These discs were incubated in Petri plates at 37°C. for 24 hours, after which they were transferred to the surface of a black plastic sheet to facilitate examination.

In the results presented here, not only are the amounts of staphylococcal factor and of serum present in the discs given, but also the equivalent amounts of these substances which would require to be incorporated in the 15 ml. volumes of medium customarily used for plates.

EXPERIMENT 127.

RESULTS: After 24 hours at 37°C.

Amount of Staphylococcal Factor (a)		Amount of Serum (ml.) - In plate						
		3.5	1.75	0.0875	0.44	0.22	0.11	0.055
In Plate	In disc	Amount of Serum (ml.) - In disc						
		0.1	0.05	0.025	0.0125	0.00625	0.003	0.0015
0.5	0.0135	-	-	-	-	P.	-	-
1.0	0.0270	-	-	-	P.	"	-	-
2.0	0.0540	P.	P.10%L.	P.	"	"	P. (faint)	-
4.0	0.1080	"	P.60%L.	P.80%L.	"	"	"	-

Amount of Staphylococcal Factor (b)								
In Plate	In disc							
0.5	0.0135	-	P.	P.	P.	-	-	-
1.0	0.0270	-	P.60%L.	P.30%L.	"	P.	P.	P-
2.0	0.0540	L.	L.	L.	P.20%L.	P-	P-	"
4.0	0.1080	"	"	"	P.80%L.	P.30%L.	P.10%L.	P.

EXPERIMENT 128.

To compare a titration of staphylococcal factor carried out on serum-milk-agar discs with a titration carried out by the usual method of incorporating serum and staphylococcal factor in a milk-agar base.

MEDIA: The staphylococcal factor was tested in amounts of 0.1 ml. up to 3.5 ml. in a series of serum-milk-agar plates containing 0.44 ml. serum, as follows:-

Milk	2.0	2.0	2.0	2.0	2.0	2.0
Serum	0.44	0.44	0.44	0.44	0.44	0.44
Staphylococcal Factor	0.1	0.2	0.44	0.875	1.75	3.5
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5	4.5
Distilled Water	7.96	7.86	7.62	7.185	6.31	4.56

Two other sets of plates were prepared with 0.875 ml. and 3.5 ml. serum respectively, each with the above range of staphylococcal factor (0.1 to 3.5 ml.).

In addition a series of serum-milk-agar plates containing serum in amounts of 0.11, 0.22, 0.44, 0.875, 1.75 and 3.5 ml. were prepared as those above except that they did not contain any staphylococcal factor. These plates were dried at 37°C. for 30 minutes, and 16 mm. diameter discs were cut with a sterile brass cutter. Dilutions of staphylococcal factor in 0.1 ml. amounts from 0.1 ml. to 0.0015 ml. were placed on the discs of serum-milk-agar prepared with different amounts of serum. These discs were incubated in Petri plates at 37°C. for 24 hours when readings were taken.

EXPERIMENT 128.

RESULTS: After 24 hours at 37°C. "Plate Titration".

Amount of Serum (ml.)	Amount of Staphylococcal Factor (ml.)					
	0.1	0.2	0.44	0.875	1.75	3.5
0.44	-	-	-	L.	L.	L.
0.875	-	-	-	-	"	"
3.5	-	-	-	-	-	-

RESULTS: After 24 hours at 37°C. "Disc Titration".

Amount of Serum		Staphylococcal Factor (0.1 ml. amounts) - On discs					
In Plates	In Discs	0.003	0.00625	0.0125	0.025	0.05	0.1
		Equivalent in Plate					
		0.11	0.22	0.44	0.875	1.75	3.5
0.11	0.003	-	-	-	-	P.	P.
0.22	0.00625	-	-	P.	P.	"	P.50% L.
0.44	0.0125	-	-	-	"	L.	L.
0.875	0.025	-	-	-	"	P.80% L.	"
1.75	0.05	-	-	-	-	"	"
3.5	0.10	-	-	-	-	P.	P.90% L.

EXPERIMENT 129.

The titration of specimens of serum by the test-tube method.

MEDIUM: A milk-agar medium containing staphylococcal factor (prepared from concentrated material) and "merthiolate" was prepared as follows:-

Milk	2.0
Agar (1%)	4.5
1% Staphylococcal Factor	1.5
"Merthiolate" (1/1000)	0.5
Distilled Water	6.5

Doubling dilutions of the sera to be tested were made in distilled water in 0.4 ml. amounts from 1/1 up to 1/250 in 2" x 3/8" tubes. These serum dilutions were warmed to 50°C. in a water-bath and 1.0 ml. of the milk-agar medium containing the staphylococcal factor, was added to each tube. The contents of each tube were thoroughly mixed and after having been in the ice-chest until the agar had set, the tubes were incubated at 37°C. for 48 hours.

A control tube containing 0.4 ml. of distilled water (in place of the serum dilution) with the 1.0 ml. of milk-agar mixture was set up and incubated along with the rest of the tubes in the test.

All tubes were closed with rubber bungs to prevent evaporation and drying.

READINGS: These were taken after 48 hours at 37°C.

EXPERIMENT 129.

RESULTS: After incubation at 37°C. for 48 hours.

Amount of Serum		Serum Number						
In tube	Equivalent in usual 15 ml. plate	1	2	3	4	5	6	7
0.4	4.3 ml.	-	-	-	-	-	-	-
0.2	2.15	L.	P.10%L.	-	L.	-	L.	L.
0.1	1.075	"	L.	P.	"	P.	"	"
0.05	0.5375	"	P.90%L.	P.10%L.	"	L.	"	"
0.025	0.2688	P.95%L.	P.80%L.	"	"	"	P.80%L.	"
0.0125	0.1344	P.20%L.	P.50%L.	P.	P.95%L.	P.95%L.	P.60%L.	"
0.0062	0.0672	P.	P.	-	P.50%L.	P.50%L.	P.	P.95%L.
0.0031	0.0336	-	-	-	-	P. (faint)	-	P.
0.00155	0.0168	-	-	-	-	-	-	P.
		8	9	10	11	12	13	14
0.4	4.3 ml.	-	-	-	-	-	-	-
0.2	2.15	L.	P.	-	P.	P.	-	-
0.1	1.075	"	L.	-	L.	L.	L.	L.
0.05	0.5375	"	"	L.	"	"	"	"
0.025	0.2688	"	"	"	"	"	"	"
0.0125	0.1344	"	"	"	"	"	"	"
0.0062	0.0672	P.80%L.	P.90%L.	"	P.30%L.	P.70%L.	P.50%L.	-
0.0031	0.0336	P.	P.	P.10%L.	P.	P.	P.	-
0.00155	0.0168	P-	-	-	-	-	-	-

Control tubes:- No change.

EXPERIMENT 130.

To compare the effect of heated serum with unheated serum in the production of Muller's phenomenon in serum-milk-agar plates containing staphylococcal factor.

MEDIA: Serum-milk-agar plates containing staphylococcal factor were prepared as follows:-

Milk	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Serum	0.5	1.0	2.0	3.0	4.0	5.0	6.0
Staphylococcal Factor	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5	4.5	4.5
Distilled Water	6.0	5.5	4.5	3.5	2.5	1.5	0.5

Similar series of plates were prepared using serum heated at 55°C., 60°C. and 65°C. for 15 minutes and 30 minutes in place of unheated serum.

INCUBATION: The plates were incubated at 37°C. for 48 hours.

READINGS: These were taken after 48 hours at 37°C.

EXPERIMENT 130.

RESULTS: After 48 hours at 37°C.

Amount of Serum	Unheated Serum	Heated Serum					
		55°C./15 mins.	55°/30	60°/15	60°/30	65°/15	65°/30
0.5	L.	P. 75% L.	P.75%L	-	-	-	-
1.0	P++	P+	P+	-	-	-	-
2.0	-	-	-	-	-	-	-
3.0	-	-	-	-	-	-	-
4.0	-	-	-	-	-	-	-
5.0	-	-	-	-	-	-	-
6.0	-	-	-	-	-	-	-

EXPERIMENT 131.

To compare the effect of horse serum with heated horse serum on the production of Muller's phenomenon in serum-milk-agar plates containing staphylococcal factor.

MEDIA: The serum-milk-agar plates made with horse serum in place of the usual human serum were prepared as follows:-

Milk	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5
Staphylococcal Factor	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Horse Serum	0.0	0.1	0.25	0.5	1.0	2.0	3.0	4.0
Distilled Water	7.5	7.4	7.25	7.0	6.5	5.5	4.5	3.5

Similar plates were prepared with horse-serum heated at 56°C. for 5, 15 or 30 minutes or 65°C. for 5, 15 or 30 minutes.

INCUBATION: The plates were incubated at 37°C. for 24 hours.

READINGS: These were made after 24 hours at 37°C.

EXPERIMENT 131.

RESULTS: After 24 hours at 37°C.

Temperature and Time of Heating	Amount of Serum (ml.)							
	0	0.1	0.25	0.5	1.0	2.0	3.0	4.0
Unheated Serum	-	L.	L.	L.	L.	P+	P-	-
Heated 56°C./5 mins.	-	-	S.L.	"	"	P.	-	-
" 56°C./15 "	-	-	-	-	-	-	-	-
" 56°C./30 "	-	-	-	-	-	-	-	-
" 65°C./5 "	-	-	-	-	-	-	-	-
" 65°C./15 "	-	-	-	-	-	-	-	-
" 65°C./30 "	-	-	-	-	-	-	-	-

EXPERIMENT 132.

To ascertain if serum heated with "Lab-Lemco" retains its ability to produce Muller's phenomenon when incorporated in serum-milk-agar plates on which strains of coagulase-positive staphylococci are grown.

MEDIA: Aliquots of serum were heated at 65°C. for 5, 10, 20 and 30 minutes and incorporated in serum-milk-agar plates as follows:-

Milk	2.0	2.0
Agar (4.5%)	4.5	4.5
"Lab-Lemco" (15%)	1.0	1.0
Distilled Water	7.0	6.5
Serum (heated at 65°C.	0.5	1.0

Serum, "Lab-Lemco" (15%) and distilled water were placed as test-tubes as follows:-

Serum	0.5	1.0
"Lab-Lemco" (15%)	1.0	1.0
Distilled Water	7.0	6.5

Five tubes of each of these two mixtures were heated at 65°C. for 5, 10, 20 and 30 minutes respectively and then added to tubes of milk-agar made with 2.0 ml. of milk and 4.5 ml. of 4.5% Agar.

CONTROL PLATES: Serum-milk-agar plates containing unheated serum in 0.5 ml. and 1.0 ml. amounts were prepared and in addition a milk-agar plate without serum.

INOCULATION: Each plate was stab-inoculated from 6 hour old "Lab-Lemco" broth cultures of strains A, 401, 039, 779 and 935.

INCUBATION: The plates were incubated at 37°C. for 24 hours and then left at room temperature.

READINGS: These were taken after 24 hours at 37°C. and after 24 hours at room temperature.

EXPERIMENT 132.

RESULTS: After 24 hours at 37°C. and 24 hours at room temperature.

Serum heated alone:

Temperature and Time.	Amount of Serum	A	401	039	779	935
65°C./5 mins.	0.5	L.	-	L.	L.	S.L.
	1.0	"	-	S.L.	S.L.	S.L.
65°C./10 "	0.5	"	-	S.L.	L.	"
	1.0	"	-	"	"	L.
65°C./20 "	0.5	"	-	L.	"	"
	1.0	"	-	"	S.L.	"
65°C./30 "	0.5	"	-	S.L.	L.	S.L.
	1.0	"	-	L.	"	L.

The lysis noted in the above table is of the "indefinite" type produced by staphylococci either in the absence of fresh serum or in the presence of inactivated serum.

Serum Heated with "Lab-Lemco":

65°C./5 mins.	0.5	L.P.	L.P.	L.P.	L.P.	L.P.
	1.0	"	"	"	"	"
65°C./10 "	0.5	"	"	"	"	"
	1.0	"	"	"	"	"
65°C./20 "	0.5	"	"	"	"	"
	1.0	"	"	"	"	"
65°C./30 "	0.5	"	"	"	"	"
	1.0	L.	"	"	"	"

The lysis is of the definite clear cut total type associated with Muller's phenomenon. The plaques are less numerous than those in the control plates.

Control Plates:

No Serum	-	L.	-	L.	L.	S.L.
Unheated Serum	0.5	L.P.	L.P.	L.P.	L.P.	L.P.
" "	1.0	"	"	"	L.R.E.P.	L.R.E.P.

EXPERIMENT 133.

To ascertain if the heating of serum and staphylococcal factor together has any protective action on the serum factor necessary for Muller's phenomenon.

MEDIA: 1.0 ml. of serum and 4.0 ml. of staphylococcal factor were heated together at 56°C. and at 65°C. for 5, 10, 20 and 30 minutes before adding them to milk-agar medium.

As controls, 1.0 ml. serum and 4.0 ml. of staphylococcal factor were heated separately at 56°C. and 65°C. for 5, 10, 20 and 30 minutes.

In addition 1.0 ml. of serum was heated with 1.0 ml., 2.0 ml., 3.0 ml. and 4.0 ml. of meat broth at 56°C. and 65°C. for 5, 10, 20 and 30 minutes before being added to the milk-agar medium containing 4.0 ml. of unheated staphylococcal factor.

These serum-milk-agar plates with staphylococcal factor had the usual volume of 15 ml. containing in addition to the amounts of serum staphylococcal factor and meat broth 2.0 ml. of milk, 4.5 ml. of 4.5% Agar and distilled water q.s. 15 ml.

INCUBATION: The plates were incubated at 37°C.

READINGS: These were made after 24 hours at 37°C.

EXPERIMENT 133.

RESULTS: After 24 hours at 37°C.

	Temperature at which heated	Time of Heating (mins.)			
		5	10	20	30
Staphylococcal Factor and Serum. (Heated together.)	56°C.	L.	L.	L.	L.
	65°C.	"	P+	P-	P-
Staphylococcal Factor and Serum. (Heated separately.)	56°C.	L.	L.	L.	L.
	65°C.	-	-	-	-
Serum and 1.0 ml. of Meat Broth.	56°C.	L.	L.	L.	L.
	65°C.	P-	-	-	-
Serum and 2.0 ml. of Meat Broth.	56°C.	L.	L.	L.	L.
	65°C.	P-	P.	-	-
Serum and 3.0 ml. of Meat Broth.	56°C.	L.	L.	L.	L.
	65°C.	"	"	"	"
Serum and 4.0 ml. of Meat broth.	56°C.	L.	L.	L.	L.
	65°C.	"	"	"	P.50%L.

EXPERIMENT 134.

To ascertain if "Merthiolate" increases the heat resistance of the factor in serum necessary for Muller's phenomenon.

MEDIA: Serum-milk-agar plates were prepared with unheated serum, and serum heated at 56°C., 60°C. and 70°C. for 15 minutes; plates were also prepared with unheated serum containing 0.01% "Merthiolate", serum heated with 0.01% "Merthiolate" at 56°C., 60°C. and 70°C. for 15 minutes.

These plates were prepared as follows:-

Milk	2.0	2.0
Agar (4.5%)	4.5	4.5
Staphylococcal Factor	2.0	2.0
Distilled Water	7.5	7.0
Serum (With and without "Merthiolate"; heated and unheated).	0.5	1.0

INCUBATION: The plates were incubated at 37°C. for 24 hours.

READINGS: These were made after 24 hours at 37°C.

EXPERIMENT 134.

RESULTS: After 24 hours at 37°C.

	Serum		Serum and "Merthiolate"	
	0.5	1.0	0.5	1.0
Unheated	P.	P. 10% L.	P.	P. 20% L.
Heated at 56°C./15 mins.	P. 10% L.	P.	-	-
" " 60°C./15 "	-	-	-	-
" " 70°C./15 "	-	-	-	-

EXPERIMENT 135.

To compare the effect on the production of Muller's phenomenon by staphylococci of fresh serum and serum heated at different temperatures, incorporated in a heated-red-cell-("chocolate") agar medium.

MEDIA: Mixtures of washed human red cells, 15% "Lab-Lemco", 4.5% Agar and Distilled Water were heated at 75°C. for 10 minutes and then cooled to 50°C. They were then added to tubes of 0.5 ml., 1.0 ml. or 2.0 ml. amounts of fresh serum, or heated serum (heated at 56°C., 60°C., 70°C. and 75°C. for 10 minutes) and mixed thoroughly. The compositions of these plates were:-

Human red cells	1.0	1.0	1.0
"Lab-Lemco" (15%)	1.0	1.0	1.0
Agar (4.5%)	4.5	4.5	4.5
Distilled Water	8.0	7.5	6.5

The above heated at 75°C. for 10 minutes when serum, fresh or heated was added.

Serum	0.5	1.0	2.0

These mixtures were poured into plates, allowed to set and then stab-inoculated with 6 hour old "Lab-Lemco broth cultures of Staphylococcus aureus, Straina, 3, 4, 6, 7, 9 and 10.
A control plate without serum was also included.

INCUBATION: The plates were incubated at 37°C. for 24 hours.

READINGS: These were made after 24 hours at 37°C.

EXPERIMENT 135.

RESULTS: After 24 hours at 37°C.

Serum		Strains of Staphylococcus aureus					
		3	4	6	7	9	10
None. *		L.	L.	L.	L.	L.	-
Unheated	0.5 ml.	P.	P.	P.	P.	P.	P.
	1.0	"	"	"	"	"	"
	2.0	"	"	"	"	"	"
Heated 56°C./10 mins.	0.5 ml.	P.	P.	P.	P.	P.	P.
	1.0	"	"	"	"	"	"
	2.0	"	"	"	"	"	"
Heated 60°C./10 mins.	0.5 ml.	-	-	P-	-	-	-
	1.0	-	P-	"	-	P-	-
	2.0	-	"	P.	P-	"	P-
Heated 65°C./10 mins.	0.5 ml.	-	-	-	-	-	-
	1.0	-	-	-	-	-	-
	2.0	-	-	-	-	-	-
Heated 70°C./10 mins.	0.5 ml.	L.	L.	L.	L.	L.	-
	1.0	"	"	"	"	"	-
	2.0	"	"	"	"	"	-
Heated 75°C./10 mins.	0.5 ml.	L.	L.	L.	L.	L.	-
	1.0	"	"	"	"	"	-
	2.0	"	"	"	"	"	-

* The lysis in this plate without serum was of the indefinite type.

EXPERIMENT 136.

To ascertain if Muller's phenomenon can be produced by staphylococcal factor in a serum-milk-agar plate containing sheep serum in place of human serum.

MEDIUM: Plates of serum-milk-agar containing amounts of sheep serum from 0.5 ml. up to 4.0 ml. were prepared as follows:-

Milk	2.0	2.0	2.0	2.0	2.0	2.0
Staphylococcal Factor	1.0	1.0	1.0	1.0	1.0	1.0
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5	4.5
Sheep Serum	0.0	0.5	1.0	2.0	3.0	4.0
Distilled Water	7.5	7.0	6.5	5.5	4.5	3.5

INCUBATION: The plates were incubated at 37°C. for 24 hours.

READINGS: These were taken after 24 hours at 37°C.

EXPERIMENT 136.

RESULTS: After 24 hours at 37°C.

Amount of Serum (ml.)	0.0	0.5	1.0	2.0	3.0	4.0
After 24 hours at 37°C.	-	L.	L.	L.	P. 40% L.	P.

The plate containing 3.0 ml. of sheep serum was photographed after 24 hours' incubation at 37°C. and is reproduced as Fig. XLIV.

EXPERIMENT 137.

To Ascertain if Muller's phenomenon can be produced by staphylococcal factor in a serum-milk-agar plate containing guinea-pig serum in place of human serum.

MEDIUM: Plates of serum-milk-agar containing guinea-pig serum in amounts from 0.5 ml. up to 3.0 ml. were prepared as follows:-

Milk	2.0	2.0	2.0	2.0	2.0
Staphylococcal Factor	1.0	1.0	1.0	1.0	1.0
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5
Guinea-pig serum	0.0	0.5	1.0	2.0	3.0
Distilled Water	7.5	7.0	6.5	5.5	4.5

INCUBATION: Plates were incubated at 37°C. for 24 hours.

READINGS: These were made after 24 hours at 37°C.

EXPERIMENT 137.

RESULTS: After 24 hours at 37°C.

Amount of Serum (ml.)				
0.0	0.5	1.0	2.0	3.0
-	L.	L.	L.	L.

The plate containing 3.0 ml. serum was found on examination after 4 hours' incubation to show numerous plaques of clearing. This plate was photographed and is reproduced as Fig. XLII.

After a further 20 hours' incubation at 37°C. all plaques had disappeared and the plate was complete lysed as recorded in results above.

EXPERIMENT 138.

To ascertain if Muller's phenomenon can be produced by staphylococcal factor in a serum-milk-agar plate containing dog plasma in place of human serum.

MEDIUM: Plates of milk-agar containing dog plasma in amounts from 0.1 ml. up to 2.0 ml. were prepared as follows:-

Milk	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Staphylococcal Factor	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5	4.5	4.5
Dog Plasma	0.1	0.2	0.3	0.4	0.5	1.0	2.0
Distilled Water	7.4	7.3	7.2	7.1	7.0	6.5	5.5

And in addition similar plates with dog plasma in amounts from 0.1 ml. up to 0.5 ml. with only 0.25 ml. of staphylococcal factor were also prepared.

INCUBATION: Plates were incubated at 37°C. for 24 hours.

READINGS: They were made after 24 hours' incubation of plates at 37°C.

EXPERIMENT 138.

RESULTS: After 24 hours' incubation at 37°C.

Plates prepared with 1.0 ml. of Staphylococcal Factor.

Amount of Dog Plasma (ml.)						
0.1	0.2	0.3	0.4	0.5	1.0	2.0
L.	L.	L.	L.	L.	L.	L.

Plates prepared with 0.25 ml. of Staphylococcal Factor.

Amount of Dog Plasma (ml.)				
0.1	0.2	0.3	0.4	0.5
S.L.	S.L.	P.S.L.	P.S.L.	P.S.L.

The plate containing 0.5 ml. of dog plasma was photographed after 24 hours' incubation at 37°C. and is reproduced as Fig. XLV.

EXPERIMENT 139.

To compare the production of Muller's phenomenon by staphylococci in serum-milk-agar plates made with human serum and with rabbit serum.

MEDIA: Serum-milk-agar plates were made with human serum and a similar batch of plates was made with rabbit serum as follows:-

Milk	2.0	2.0	2.0	2.0	2.0	2.0
Serum	0.0	0.5	1.0	2.0	3.0	4.0
"Lab-Lemco" (15%)	1.0	1.0	1.0	1.0	1.0	1.0
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5	4.5
Distilled Water	7.5	7.0	6.5	5.5	4.5	3.5

A control plate without serum was also included.

INOCULUM: The batches of plates made with human serum and with rabbit serum were stab inoculated from 6 hour old "Lab-Lemco" broth cultures of strains of staphylococci, A, 401, 406, 039 and 935.

INCUBATION: The plates were incubated at 37°C. for 24 hours and then left at room temperature for 24 hours.

READINGS: These were taken after 24 hours at 37°C. and after a further 24 hours at room temperature.

EXPERIMENT 139.

RESULTS: After 24 hours' incubation at 37°C.

Human Serum (ml.).	A	401	406	039	935
0.5	L.P.	P.	P.	L.P.	P.
1.0	"	L.P.	L.P.	"	L.P.
2.0	"	"	"	"	"
3.0	"	"	"	"	L.R.E.P.
4.0	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.
Rabbit Serum					
0.5	L.P.	P.	S.L.	S.L.	S.L.
1.0	L.P.	"	"	"	"
2.0	"	L.P.	"	"	P.
3.0	"	"	"	"	L.P.

RESULTS: After a further 24 hours at room temperature.

Controls without serum, human or rabbit.	S.L.*	S.L.*	S.L.*	S.L.*	-
Human Serum (ml.).					
0.5	L.P.	L.P.	L.P.	L.P.	L.P.
1.0	"	"	"	"	"
2.0	L.R.E.	L.R.E.	L.R.E.	L.R.E.	L.R.E.
3.0	L.	L.	L.	L.	L.
4.0	"	"	"	"	"

* The lysis noted here was of the indefinite type.

EXPERIMENT 139 (Continued).

Rabbit Serum (ml.)	A	401	406	039	935
0.5	L.P.	P.	S.L.P.	S.L.	P.
1.0	"	L.P.	P.	L.	L.P.
2.0	"	"	L.P.	S.L.	"
3.0	"	"	L.	"	"

EXPERIMENT 140.

To ascertain if Muller's phenomenon can be produced by staphylococcal factor in a serum-milk-agar plate containing horse serum in place of human serum.

MEDIA: Serum-milk-agar plates containing horse serum in amounts from 0.1 ml. up to 4.0 ml. were prepared as follows:-

Milk	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Staphylococcal Factor	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5
Horse Serum	0.0	0.1	0.25	0.5	1.0	2.0	3.0	4.0
Distilled Water	8.0	7.9	7.75	7.5	7.0	6.0	5.0	4.0

INCUBATION: These plates were incubated at 37°C. for 24 hours and then left at room temperature for 24 hours.

READINGS: These were made after 24 hours at 37°C. and after a further 24 hours at room temperature.

EXPERIMENT 140.

RESULTS: After 24 hours at 37°C. and after a further 24 hours at room temperature.

	Amount of Horse Serum (ml.)							
	0.0	0.1	0.25	0.5	1.0	2.0	3.0	4.0
After 24 hours at 37°C.	-	P.	L.	L.	L.	L.	P. 20% L. (P. large)	P.
After 24 hours at room temperature.	-	L.	L.	L.	L.	L.	P. 60% L. (P. large)	P.

amounts from 0.1 ml. up to 4.0 ml.
Those plates which contained heated haemoglobin were prepared by heating the requisite amounts of haemoglobin, agar, distilled water and "Lab-Lemco" in tubes at 75°C. for 10 minutes. These tubes were cooled to 50°C., 1 ml. of 15% "Lab-Lemco" added and the varied amounts of horse serum as indicated above. The contents of the tubes were thoroughly mixed and poured into plates.

INOCULATION: The (b) set of plates, i.e. those containing "Lab-Lemco" were stab-inoculated from 6 hour old "Lab-Lemco" broth cultures of five strains of staphylococci, viz.: A, 406, 061, 779 and 935.

INCUBATION: All plates were incubated at 37°C. for 48 hours.

READINGS: These were taken after 24 hours and 48 hours at 37°C. in the case of the plates containing the staphylococcal factor and after 48 hours at 37°C. in the case of the plates with the live organisms.

EXPERIMENT 141.

To ascertain if Muller's phenomenon can be produced (a) by staphylococcal factor, or (b) by living staphylococci on serum-haemoglobin-agar or serum-heated-haemoglobin-agar plates which contained horse serum in place of human serum.

MEDIA: (a) The media containing staphylococcal factor and amounts of horse serum from 0.1 ml. up to 4.0 ml. were prepared as follows:-

Haemoglobin (fresh or heated)	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75
Staphylococcal Factor.	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5
Horse Serum	0.0	0.1	0.25	0.5	1.0	2.0	3.0	4.0
Distilled Water	8.75	8.65	8.5	8.25	7.75	6.75	5.75	4.75

Those plates which were prepared with heated haemoglobin (as above) had their haemoglobin, agar and distilled water heated at 75°C. for 10 minutes in test tubes. The tubes were cooled to 50°C. and the requisite amounts of staphylococcal factor and horse serum added. The contents of the tubes were thoroughly mixed and poured into plates.

(b) The media prepared for live organisms (i.e. without staphylococcal factor) each contained 1.0 ml. of 15% "Lab-Lemco" and also horse serum in

EXPERIMENT 141.

RESULTS: After 24 hours and 48 hours at 37°C.

(a) Plates with staphylococcal factor.

	Hours at 37°C.	Amount of Horse Serum (ml.)							
		0.0	0.1	0.25	0.5	1.0	2.0	3.0	4.0
Serum-Haemoglobin-Agar	24	-	-	-	S.L.	L.	P.	P.	P.
	48	-	-	S.L.	"	"	"	"	"
Serum-Heated-Haemoglobin-Agar	24	-	-	-	P-	P-	P.	P.	P.
	48	-	-	-	"	P.	"	P.20%L.	P.50%L.

RESULTS: After 48 hours at 37°C.

(b) Plates with Live Organisms.

Serum-Haemoglobin-Agar Plates:

Amount of Serum	A	406	061	779	935
0.0*	L.	L.	L.	L.	L.
0.1	"	"	"	"	"
0.25	"	"	"	"	"
0.5	"	"	"	"	"
1.0	"	"	"	S.L.	"
2.0	"	"	"	L.	"
3.0	"	S.L.	"	"	"
4.0	"	L.	"	"	L.P.

* The lysis here was of the indefinite type.

EXPERIMENT 141 (Continued).

Serum-Heated-Haemoglobin-Agar Plates:

Amount of Serum	A	406	061	779	935
0.0 *	-	L.	L.	L.	L.
0.1	S.L.	S.L.	-	S.L.	"
0.25	L.	-	S.L.	"	L.P.
0.5	"	-	L.P.	P.	"
1.0	L.P.	P.	P.	"	"
2.0	"	"	L.P.	"	"
3.0	"	"	P.	"	"
4.0	"	"	"	"	"

* The lysis here was of the indefinite type.

EXPERIMENT 142 (Continued).

Milk	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5
Staphylococcal Factor	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Acriflavine (1/5000)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Distilled Water	6.4	6.25	6.0	5.5	4.5	3.5	2.5	1.5
Supernatant Deposit or Serum.	0.1	0.25	0.5	1.0	2.0	3.0	4.0	5.0

INCUBATION: These plates were incubated at 37°C. for 24 hours and then left at room temperature for 48 hours.

READINGS: These were taken after 24 hours at 37°C. and 48 hours at room temperature.

EXPERIMENT 142.

To ascertain if the active principle in serum necessary for the production of Muller's phenomenon can be precipitated by ammonium sulphate.

Amounts of ammonium sulphate were weighed out and added to volumes of serum to give final concentrations of the salt from 10% up to 30% w/v.

Serum (ml.)	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0
$(NH_4)_2SO_4$. (gm.)	2.0	3.0	3.4	3.8	4.2	4.6	5.0	6.0
Final Concentration of $(NH_4)_2SO_4$.	10%	15%	17%	19%	21%	23%	25%	30%

These mixtures were left at room temperature for 2 hours. Precipitates appeared in all the tubes except that with 10% ammonium sulphate and after centrifugation these precipitates were resuspended in distilled water, the volume being made up to 20.0 ml. The resuspended deposits, the supernatant fluids from these deposits and the 10% ammonium sulphate-serum mixture were placed in 'Visking' cellophane tubing and dialysed against running water.

These supernatants and deposits were incorporated in milk-agar plates containing staphylococcal factor, in amounts from 0.1 ml. up to 5.0 ml., allowance being made for any increase in volume which had occurred during dialysis. In addition fresh serum was also used in the same amounts in milk-agar plates with staphylococcal factor. Acriflavine (1/5000) in 1 ml. amounts was added to each plate to ensure that no growth took place during the period of incubation of the plates.

EXPERIMENT 142.

RESULTS: After 24 hours at 37°C.

Material tested	Amounts (ml.)							
	0.1	0.25	0.5	1.0	2.0	3.0	4.0	5.0
Fresh serum	-	P.	L.	L.	P.	-	-	-
10% $(NH_4)_2SO_4$ - Serum	-	P.50%L.	L.	P.60%L.	-	-	-	-
Supernatant 15% $(NH_4)_2SO_4$	-	P-	P.40%L.	-	-	-	(...)	(...)
Deposit	-	-	P.	L.	L.	L.	(...)	(...)
Supernatant 17% $(NH_4)_2SO_4$	-	-	-	-	-	-	-	-
Deposit	-	-	S.L.	L.	L.	L.	90% L.	-
Supernatant 19% $(NH_4)_2SO_4$	-	-	-	-	-	-	-	-
Deposit	-	-	S.L.	L.	L.	L.	-	-
Supernatant 21% $(NH_4)_2SO_4$	-	-	-	-	-	-	-	-
Deposit	-	-	S.L.	L.	P.	-	-	-
Supernatant 23% $(NH_4)_2SO_4$	-	-	-	-	-	-	-	-
Deposit	-	P.	L.	L.	P.50%L.	-	-	-
Supernatant 25% $(NH_4)_2SO_4$	-	-	-	-	-	-	-	-
Deposit	-	S.L.	L.	L.	P.30%L.	-	-	-
Supernatant 30% $(NH_4)_2SO_4$	(...)	(...)	(...)	(...)	(...)	(...)	(...)	(...)
Deposit	-	S.L.	L.	P.90%L.	-	-	-	-

EXPERIMENT 142 (Continued).

RESULTS: After 48 hours at room temperature.

Material tested	Amounts (ml.)							
	0.1	0.25	0.5	1.0	2.0	3.0	4.0	5.0
Fresh serum	-	S.L.	L.	L.	P.	-	-	-
10% $(NH_4)_2SO_4$ - Serum.	-	L.	L.	L.	-	-	-	-
Supernatant 15% $(NH_4)_2SO_4$	-	P.	P.40%L.	-	-	-	(...)	(...)
Deposit	-	-	P.	L.	L.	L.	(...)	(...)
Supernatant 17% $(NH_4)_2SO_4$	-	P-	P.20%L.	P.20%L.	-	-	-	-
Deposit	-	-	L.	L.	L.	L.	L.	-
Supernatant 19% $(NH_4)_2SO_4$	-	P.	P.	-	-	-	-	-
Deposit	-	-	L.	L.	L.	L.	-	-
Supernatant 21% $(NH_4)_2SO_4$	-	-	P.	-	-	-	-	-
Deposit	-	-	L.	L.	L.	-	-	-
Supernatant 23% $(NH_4)_2SO_4$	-	-	-	-	-	-	-	-
Deposit	-	L.	L.	L.	L.	-	-	-
Supernatant 25% $(NH_4)_2SO_4$	-	-	-	-	-	-	-	-
Deposit	-	S.L.	L.	L.	P.	P-	-	-
Supernatant 30% $(NH_4)_2SO_4$	(...)	(...)	(...)	(...)	(...)	(...)	(...)	(...)
Deposit	-	S.L.	L.	L.	P-	-	-	-

EXPERIMENT 143.

To ascertain if the active principle in serum necessary for Muller's phenomenon can be precipitated by sodium sulphate.

2.7 gm. sodium sulphate was mixed with 20.0 ml. of serum and allowed to stand in the 37°C. incubator for 3 hours. The precipitate which formed was collected after centrifugation and re-suspended in 20.0 ml. of distilled water. This resuspended deposit and the supernatant were dialysed against running water overnight and tested in amounts of 0.1 ml. to 5.0 ml. in milk-agar plates containing staphylococcal factor. A set of plates containing fresh serum was also prepared.

Milk	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Staphylococcal Factor	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5
Acriflavine (1/5,000)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Distilled Water	6.5	6.4	6.25	6.0	5.5	4.5	3.5	2.5	1.5
Supernatant or Deposit or Serum.	0.0	0.1	0.25	0.5	1.0	2.0	3.0	4.0	5.0

INCUBATION: The plates were incubated at 37°C. for 24 hours and then left at room temperature.

READINGS: These were made after 24 hours at 37°C. and after 2 days at room temperature.

EXPERIMENT 143.

RESULTS: After 24 hours at 37°C.

Material tested	Amounts (ml.)								
	0.0	0.1	0.25	0.5	1.0	2.0	3.0	4.0	5.0
Fresh Serum	-	S.L.	L.	L.	L.	P.	P-	-	-
Sodium sulphate									
Supernatant		-	P-	P-	-	-	-	-	-
Deposit		-	-	L.	L.	L.	L.	-	-

RESULTS: After 2 days at room temperature.

Fresh Serum	-	L.	L.	L.	L.	P.70%L.	P.20%L.	-	-
Supernatant		-	P.	P.60%L.	P.20%L.	P.	-	-	-
Deposit		-	L.	L.	L.	L.	L.	L.	-

EXPERIMENT 144.

To ascertain if the effect of sodium chloride on the active factor in serum necessary for the production of Muller's phenomenon can be reversed.

MEDIA: Sodium chloride-serum mixtures were prepared as follows (and in addition a control without sodium chloride):-

	Control					
NaCl. (15% in Distilled Water)	0.0	1.0	2.0	3.0	4.0	8.0
Serum	2.0	2.0	2.0	2.0	2.0	2.0

These tubes were left at room temperature for one hour and amounts from each incorporated in milk-agar plates as follows:-

	Control					
Milk	2.0	2.0	2.0	2.0	2.0	2.0
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5	4.5
Staphylococcal Factor	1.0	1.0	1.0	1.0	1.0	1.0
Distilled Water	7.0	6.75	6.5	6.25	6.0	5.0
NaCl.-serum mixture	0.5	0.75	1.0	1.25	1.5	2.5
Final Concentration of NaCl. in plate	0.0%	0.25%	0.5%	0.75%	1.0%	2.0%

The remainder of the sodium chloride-serum mixtures were dialysed through "Visking" in running water overnight. They were then tested as above in amounts comparable with those used in the test plates above, after having made adjustment for the increase in volume which occurred during dialysis.

EXPERIMENT 144.

RESULTS: After 24 hours at 37°C.

Concentration of Sodium chloride in mixture	Mixtures before dialysis	Mixtures after dialysis
0.0%	L.	L.
0.25%	P. 80% L.	"
0.5%	P.	"
0.75%	P- (faint)	"
1.0%	-	"
2.0	-	"

In another experiment in which 3.0 ml. of staphylococcal factor was used in place of 1.0 ml. as in above experiment a similar result was obtained. Plaques of clearing were visible in all plates with 3.0 ml. of staphylococcal factor and the dialysed sodium chloride-serum mixtures after only 2 hours' incubation at 37°C.

EXPERIMENT 145.

To ascertain if the factor in serum necessary for the production of Muller's phenomenon can be precipitated by sodium chloride.

Amounts of 1.0, 1.5, 2.0 and 2.5 gm. of sodium chloride were added to 10 ml. volumes of human serum, giving concentrations of sodium chloride in serum of 10%, 15%, 20% and 25% respectively. These serum-sodium chloride mixtures were left at room temperature for 1 hour after which there appeared precipitates in the 20% and 25% sodium chloride containing mixtures. These latter were centrifuged and their precipitates resuspended in 10 ml. distilled water.

The serum-sodium chloride mixtures containing 10% and 15% sodium chloride, the supernatants and resuspended precipitates from the 20% and 25% sodium chloride mixtures were dialysed overnight in running water to remove the sodium chloride. The activity of sodium chloride treated samples of serum was tested by incorporating them in milk-agar-staphylococcal factor plates in volumes of from 0.1 to 3.0 ml., allowance being made for any increase in volume which may have occurred during dialysis.

Milk	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5	4.5	4.5
Staphylococcal Factor	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Distilled Water	7.5	7.4	7.25	7.0	6.5	5.5	4.5
Serum, Supernatant or Deposit.	0.0	0.1	0.25	0.5	1.0	2.0	3.0

("Merthiolate", final concentration 1/25,000 was incorporated in all these plates.)

INCUBATION: Plates were incubated at 37°C. for 24 hours.

READINGS: Made after 24 hours at 37°C.

EXPERIMENT 145.

RESULTS: After 24 hours at 37°C.

	0.0	0.1	0.25	0.5	1.0	2.0	3.0
Serum	-	P. (faint)	L.	L.	P.50%L.	-	-
Mixture with 10% Sodium chloride		P. (faint)	P.	L.	L.	P.	-
Mixture with 15% Sodium chloride		P.	L.	L.	L.	P.	-
Supernatant from mixture with 20% Sodium chloride		P. (faint)	L.	L.	L.	P.	-
Deposit from mixture with 20% Sodium chloride		-	-	P. (faint)	P.	P.	P.90%L.
Supernatant from mixture with 25% Sodium chloride		-	P.	L.	L.	-	-
Deposit from mixture with 25% Sodium chloride		P.	P.	P.20%L.	P.90%L.	L.	L.

EXPERIMENT 146.

To compare the plaques produced by different strains of staphylococci in (1) serum-milk-agar plates containing different amounts of serum, and (2) serum-milk-agar plates made with two different sera.

MEDIA: Serum-milk-agar plates were prepared with different amounts of serum as follows:-

Milk	2.0	2.0	2.0	2.0	2.0	2.0
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5	4.5
"Lab-Lemco" (15%)	1.0	1.0	1.0	1.0	1.0	1.0
Distilled Water	7.5	7.35	7.25	7.0	6.75	6.5
Serum	0.0	0.15	0.25	0.5	0.75	1.0

Two sets of serum-milk-agar plates were prepared one with "serum 13", the other with "serum 17".

INOCULATION: All the plates were stab-inoculated with Staphylococcus aureus, Strains A, 401, 406, 039 061, 779 and 935 from 6 hour old "Lab-Lemco" broth cultures.

INCUBATION: The plates were incubated at 37°C. for 24 hours.

READINGS: These were made after 24 hours at 37°C.

EXPERIMENT 146.

RESULTS: After 24 hours at 37°C.

Amount of Serum	A	401	406	039	061	779	935
"Serum 13"							
0.0	S.L.	-	-	Ppt.	Sl.Ppt.	Sl.Ppt.	Ppt.
0.15	L.P.	P.	P.	L.P.	Faint P.	L.P.	Faint P.
0.25	"	"	"	"	"	"	"
0.5	"	L.P.	L.P.	"	L.P.	"	L.P.
0.75	"	"	"	"	"	"	"
1.0	"	"	"	"	"	"	"
"Serum 17"							
0.0	S.L.	-	Ppt.	Ppt.	Sl.Ppt.	S.L.	Ppt.
0.15	L.P.	L.P.	L.P.	L.P.	Faint P.	L.P.	L.P.
0.25	"	"	"	"	L.P.	"	"
0.5	"	"	"	"	"	"	"
0.75	"	"	"	"	"	"	"
1.0	"	"	"	"	"	"	"

With "Serum 13", the plaques which appeared around all the strains in the plates containing 0.15 and 0.25 ml. of serum, were very fine, about $\frac{1}{2}$ mm. in diameter, while the plaques in plates with 0.5, 0.75 and 1.0 ml. serum were 1 to 2.5 mm. in diameter - small, medium and large.

With "Serum 17" there were plaques both big and small in all the plates. There were a few plaques in the plates with 0.15 and 0.25 ml. of this serum which were bigger than about 1.0 to 1.5 mm. in diameter, the majority being from 0.5 to 1.0 mm. in diameter. In the plate with the 1.0 ml. of "Serum 17" most of the plaques were 1.0 to 2.0 mm. although there were a number of the smaller kind.

Within limits, an increase in the amount of serum in a series of plates results in the appearance of larger plaques. These larger plaques are always accompanied by a number of smaller plaques.

EXPERIMENT 147.

To compare the size and pattern of plaques produced by two strains of staphylococci on serum-milk-agar plates containing different amounts of serum.

MEDIA: Serum-milk-agar plates were prepared in duplicate with different amounts of serum as follows:-

Milk	2.0	2.0
Agar (4.5%)	4.5	4.5
"Lab-Lemco" (15%)	1.0	1.0
Distilled Water	7.35	6.5
Serum (No. 17).	0.15	1.0

INOCULUM: Each plate was stab inoculated 4 times from 6 hour old "Lab-Lemco" broth cultures of strain A and strain 061.

INCUBATION: The four plates were incubated at 37°C. for 24 hours and then left at room temperature.

READINGS: These were made after 24 hours at 37°C. and then after 3 days at room temperature.

EXPERIMENT 147.

RESULTS: After 24 hours at 37°C.

Amount of Serum (ml.)	Stab Inoculation	Strains of Staphylococci	
		A	061
0.15	1	L.P.	L.P.
	2	"	"
	3	"	"
	4	"	"
1.0	1	L.P.	L.P.
	2	"	"
	3	"	"
	4	"	"

The patterns of lysis and plaques around the four stab-inoculations of Strain A in the plate containing 0.15 ml. of serum were similar but they differed markedly from the patterns around the four stab-inoculations of Strain A in the plate containing 1.0 ml. of serum. A similar finding was made with the patterns of lysis and plaques produced by Strain 061. The patterns produced by Strain A on both serum-milk-agar plates, however, differed markedly from the patterns produced by Strain 061 on the corresponding plates of serum-milk-agar.

After 3 days at room temperature.

Around each of the four stab-inoculations of Strain A on the plate with 0.15 ml. of serum there was a broad zone of lysis with a few plaques whereas around each of the four stab-inoculations of Strain 061 there was a broad zone of plaques with a narrow zone of lysis. In the plates with 1.0 ml. of serum, however, the pattern around each stab-inoculation of Strain A was very similar to that around the stab-inoculations of Strain 061, viz. a broad zone of lysis with a rough edge and few isolated plaques in the medium beyond the lysis.

EXPERIMENT 148.

To ascertain if the addition of animal charcoal to a serum-milk-agar plate would increase the number of alter the plaque production by strains of staphylococci.

MEDIA: Serum-milk-agar plates were prepared as follows:-

Milk	3.0	3.0	3.0	3.0	3.0	3.0
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5	4.5
Charcoal Suspension (0.1%)	0.0	0.0	0.0	0.5	0.5	0.5
"Lab-Lemco" (15%)	1.0	1.0	1.0	1.0	1.0	1.0
Serum	0.25	0.5	1.0	0.25	0.5	1.0
Distilled Water	7.25	7.0	6.5	6.75	6.5	6.0

The charcoal suspension was prepared as a 0.1% suspension in sterile distilled water and heated at 100°C. for 10 minutes before being used in the above plates.

INOCULATION: These plates were stab inoculated from 6 hour old "Lab-Lemco" broth cultures of Staphylococcus aureus, Strains A, 401, 406, 039, 061, 779 and 935.

INCUBATION: The plates were inoculated at 37°C. for 24 hours.

READINGS: These were made after 24 hours at 37°C.

EXPERIMENT 148.

RESULTS: After 24 hours at 37°C.

Without Charcoal	A	401	406	039	061	779	935
Serum 0.25	S.L.	-	-	S.L.	-	L.	S.L.
0.5	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.
1.0	L.	"	L.R.E.	L.R.E.P.	L.	L.	L.R.E.
With Charcoal							
Serum 0.25	L.	-	-	S.L.	-	-	L.
0.5	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.	L.P.
1.0	L.	"	L.R.E.	L.R.E.	L.	L.	L.R.E.P.

EXPERIMENT 149.

To observe the production of plaques by Staphylococcus aureus, Strain A in a series of serum-milk-agar plates containing different amounts of serum.

MEDIA: Serum-milk-agar plates containing amounts of serum from 0.5 ml. up to 7.0 ml. were prepared as follows:-

Milk	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Agar (4.5%)	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5
"Lab-Lemco" (15%)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Serum	0.5	1.0	2.0	3.0	4.0	5.0	6.0	7.0
Distilled Water	7.0	6.5	5.5	4.5	3.5	2.5	1.5	0.5

Six sets of these plates were made.

INOCULATION: These six sets of eight plates were stab inoculated from a 6 hour old "Lab-Lemco" broth culture of Staphylococcus aureus, Strain A, with intervals of 2 hours, 2 hours, 2 hours, 1 hour and 4 hours between the first and second, second and third, etc. inoculations respectively.

INCUBATION: Plates were incubated at 37°C. for 48 hours.

READINGS: These were made hourly for 8 hours, and at various other intervals. It was possible by having inoculated the six sets of plates at the different intervals to obtain hourly readings (almost), of the development of Muller's phenomenon on these plates in the different amounts of serum.

EXPERIMENT 149.

RESULTS: Hourly readings from the different sets of plates containing amounts of serum from 0.5 ml. to 7.0 ml.

Time of Incubation	Amount of Serum (ml.)							
	0.5	1.0	2.0	3.0	4.0	5.0	6.0	7.0
1 hour.	-	-	-	-	-	-	-	-
2 hours.	-	-	-	-	-	-	-	-
3 "	-	-	-	L.	-	-	-	-
4 "	-	L.	L.	"	L.	L.	L.	L.
5 "	-	P.	"	"	"	"	"	"
6 "	-	"	L.P.	"	"	"	"	"
7 "	L.	"	"	P.	"	"	"	"
8 "								
9 "	L.P.	L.P.	L.P.	L.P.	L.P.	L.R.E.	L.	L.
10 "								
11 "	L.P.	L.P.	L.P.	L.P.	L.P.	L.R.E.	L.	L.
12 "	"	"	"	"	"	"	"	"
13 "	"	"	"	"	"	"	"	"
16 "	L.P.	L.P.	L.P.	L.P.	L.R.E.	L.R.E.	L.	L.
17 "	"	"	"	"	L.P.	"	"	"
18 "	"	"	"	"	"	"	"	"
19 "	"	"	"	"	"	"	"	"
20 "	"	"	"	"	"	"	"	"
21 "	"	"	"	"	"	"	"	"
22 "	"	"	"	"	"	"	"	"
23 "	"	"	"	"	"	"	"	"
24 "	"	"	"	"	"	"	"	"

EXPERIMENT 149 (Continued).

Time of Incubation	Amount of Serum (ml.)							
	0.5	1.0	2.0	3.0	4.0	5.0	6.0	7.0
25 hours	L.P.	L.P.	L.P.	L.P.	L.P.	L.R.E.	L.	L.
26 "	"	"	"	"	"	L.	"	"
27 "	"	"	"	"	L.R.E.	L.R.E.	"	"
28 "	"	"	"	"	"	L.	"	"
29 "								
30 "	L.P.	L.P.	L.P.	L.R.E.	L.R.E.	L.	L.	L.
36 "	L.P.	L.P.	L.P.	L.R.E.	L.R.E.	L.	L.	L.
40 "	L.P.	L.P.	L.P.	L.R.E.	L.R.E.	L.	L.	L.
41 "	"	"	"	"	"	"	"	"
43 "	L.P.	L.P.	L.P.	L.R.E.	L.R.E.	L.	L.	L.
45 "	L.P.	L.P.	L.P.	L.R.E.	L.R.E.	L.	L.	L.
47 "	L.P.	L.P.	L.P.	L.R.E.	L.R.E.	L.	L.	L.

TABLES.

T A B L E I .

DIVISION OF STAPHYLOCOCCI INTO COAGULASE POSITIVE AND COAGULASE NEGATIVE STRAINS.

HOSPITAL OF ORIGIN	COAGULASE POSITIVE	COAGULASE NEGATIVE	TOTAL
GENERAL HOSPITAL	27	2	29
ROYAL VICTORIA INFIRMARY.	103	27	130
PRINCESS MARY MATERNITY HOSPITAL	62	12	74
TOTALS.	192	41	233

TABLE II.

SOURCES OF STRAINS OF STAPHYLOCOCCI EXAMINED.

NUMBERS	HOSPITAL NUMBERS	SOURCE OF STRAINS
1 - 29	15650 to 16329	Pathological Specimens - General Hospital.
30 - 159	34191 to 35015	Pathological Specimens - Royal Victoria Infirmary.
160 - 233	P342 to P247	Routine swabs and specimens taken during survey - Princess Mary Maternity Hospital.

TABLE III.

EXAMINATION OF 233 STRAINS OF STAPHYLOCOCCI TO ASCERTAIN THEIR ABILITIES TO PRODUCE MULLER'S PHENOMENON ON SERUM-MILK-AGAR AND SERUM-HAEMOGLOBIN-AGAR PLATES.

NUMBER	HOSPITAL NUMBER	ORIGIN OF STRAIN	COAGULASE TEST.	RESULT OF GROWTH ON			
				S.M.A.	M.A.	S.Hb.A.	Hb.A.
1	15650	Swab	+ve	L.P.	L.	L.P.	Ppt.
2	15676	Throat	+	"	S.L.	"	"
3	15714	Burn	+	"	"	"	"
4	15719	Wound	+	"	L.	"	"
5	15726	Scald	+	"	S.L.	"	"
6	15797	Sputum	+	"	L.	"	"
7	15813	Wound	+	"	S.L.	"	S.Ppt.
8	15827	Wound	+	"	"	"	"
9	15831	Ear	+	"	"	"	Ppt.
10	15845	Burn	+	"	"	"	S.Ppt.
11	15846	Burn	+	"	L.	"	Ppt.
12	15864	Pus	+	"	S.L.	"	"
13	15865	Wound	+	"	"	"	S.Ppt.
14	15866	Pus	+	"	"	"	-
15	15886	Wound	+	"	L.	"	Ppt.
16	15888	Eye	+	"	S.L.	"	"
17	15889	Septic finger	+	"	"	"	"
18	15908	Pus	+	"	"	"	"
19	15975	Abscess	+	"	"	"	-
20	15976	Pus	+	"	L.	"	Ppt.

TABLE III (Continued).

NUMBER	HOSPITAL NUMBER	ORIGIN OF STRAIN	COAGULASE TEST.	RESULT OF GROWTH ON			
				S.M.A.	M.A.	S.Hb.A.	HbA.
21	15977	Wound	+ve	L.P.	L.	L.P.	Ppt.
22	16050	Wound	+	"	L.	"	"
23	16101	Pus	+	"	S.L.	"	"
24	16160	Pus	+	"	L.	"	S.Ppt.
25	16165	Wound	+	"	S.L.	"	Ppt.
26	16170	Pus	+	"	L.	"	-
27	16271	Pus	+	"	L.	"	Ppt.
28	16309	Pus	-ve	-	S.L.	-	-
29	16329	Blister	-ve	-	-	-	-
30	34191	Burns	+ve	L.P.	S.L.	L.P.	Ppt.
31	34216	Adenitis	+	"	"	"	-
32	34219	Osteitis	+	"	"	"	-
33	34221	Cataract	+	-	"	S.L.	-
34	34222	Cataract	+	L.P.	L.Ppt.	L.P.	-
35	34223	Abscess	+	"	"	"	-
36	34247	Abscess	+	"	"	"	Ppt.
37	34249	Prostate	+	"	-	"	"
38	34336	Eye	+	"	L.	"	"
39	34339	Boil	+	"	L.Ppt.	"	"
40	34358	Abscess	+	"	L.	"	"

TABLE III (Continued).

NUMBER	HOSPITAL NUMBER	ORIGIN OF STRAIN	COAGULASE TEST.	RESULT OF GROWTH ON			
				S.M.A.	M.A.	S.Hb.A.	HbA.
41	34379	Eye	+ve	L.P.	S.L.	L.P.	S.Ppt.
42	34434	Pyrexia	-ve	-	-	-	Ppt.
43	34435	Pyrexia	-	-	-	-	-
44	34440	...	-	-	-	L.	-
45	34452	Sinus	+ve	L.P.	S.L.	L.P.	-
46	34471	Ulcer	+	L.	"	L.	Ppt.
47	34480	Urine	+	L.P.	L.	L.P.	"
48	34502	Hip joint	+	"	-	"	"
49	34508	Pyrexia	+	"	-	"	-
50	34515	Dacryocystitis	+	"	-	"	Ppt.
51	34534	Pustule	+	"	S.L.	"	-
52	34538	Pyrexia	+	"	"	"	S.Ppt.
53	34557	Pustule	+	"	"	"	Ppt.
54	34566	Pneumonia	+	"	"	"	"
55	34572	Burn	+	-	-	-	"
56	34607	Abscess	+	L.P.	-	L.P.	"
57	34610	Wound	+	"	S.L.	"	"
58	34613	Burn	+	"	-	"	"
59	34619	Eye	+	S.L.	S.Ppt.	"	-
60	34629	Cataract	-	L.	L.	-	-

TABLE III (Continued).

NUMBER	HOSPITAL NUMBER	ORIGIN OF STRAIN	COAGULASE TEST.	RESULT OF GROWTH ON			
				S.M.A.	M.A.	S.Hb.A.	HbA.
61	34634	...	-ve	-	-	-	Ppt.
62	34635A	...	+ve	L.P.	-	L.P.	S.Ppt.
63	34635B	...	-ve	L.	L.	L.	-
64	34636	...	-	L.	L.	L.	-
65	34637	Milk	-	L.	L.	-	-
66	34647	Pyrexia	-	L.	L.	-	-
67	34655	Cataract	-	-	-	-	Ppt.
68	34690	Urine	-	-	-	-	-
69	34729	...	+ve	L.P.	S.L.	L.P.	Ppt.
70	34754	Prostate	-ve	L.	L.	L.	-
71	34757A	...	-	-	L.	-	-
72	34757B	...	-	-	-	-	-
73	34771	Faeces	+ve	L.P.	S.L.	L.P.	Ppt.
74	34775	Pyrexia	-ve	L.	L.	-	-
75	34822	Abscess	+ve	S.L.	S.L.	-	-
76	34823	Abscess	+	L.P.	"	L.P.	-
77	34824	Sinusitis	+	"	"	"	-
78	34826	Wound	+	"	"	"	-
79	34830	Dacryocystitis	+	"	L.	"	Ppt.
80	34835	Pus	+	"	S.L.	"	"

TABLE III (Continued).

NUMBER	HOSPITAL NUMBER	ORIGIN OF STRAIN	COAGULASE TEST.	RESULT OF GROWTH ON			
				S.M.A.	M.A.	S.Hb.A.	HbA.
81	34850	Hydronephrosis	+ve	L.P.	S.L.	L.P.	Ppt.
82	34862	Boil	+	"	L.	"	"
83	34867	Faeces	+	"	L.	"	"
84	34878	Pneumonia	+	"	S.L.	"	"
85	34886	Abscess	+	"	"	"	"
86	34916	Adenitis	+	"	"	"	S.Ppt.
87	34935	Ulcer	+	"	-	"	"
88	34937	Abscess	+	"	S.L.	"	"
89	34940	...	-ve	-	L.	S.L.	-
90	34947	Wound	+ve	L.P.	L.	L.P.	S.Ppt.
91	34949	Pyrexia	-ve	-	L.	-	-
92	34953	Adenitis	+ve	L.P.	-	L.P.	S.Ppt.
93	34958	Wound	+	L.P.	L.	"	"
94	34845	...	-ve	-	-	-	Ppt.
95	34869	Eye	-	-	-	-	"
96	34903	Appendix	-	-	-	-	"
97	34936	Impetigo	+ve	L.P.	-	L.P.	-
98	35001	Osteitis	+	"	L.	"	S.Ppt.
99	35003	Bladder	-ve	L.	L.	L.	-
100	35015	Pus	+ve	L.P.	L.	L.P.	-

TABLE III (Continued).

NUMBER	HOSPITAL NUMBER	ORIGIN OF STRAIN	COAGULASE TEST.	RESULT OF GROWTH ON			
				S.M.A.	M.A.	S.Hb.A.	HbA.
101	35028	Milk	+ve	L.P.	-	L.P.	Ppt.
102	35033	Eye	+	"	-	"	-
103	35035	Meningitis	+	-	-	-	-
104	35039	Osteitis	+	L.P.	S.L.	L.P.	-
105	35061	Pustule	+	"	"	"	-
106	35105	Cataract	-ve	L.	L.	-	-
107	35107	Cataract	-	-	S.L.	S.L.	-
108	35109	Cataract	-	L.	L.	-	-
109	35110	Cataract	-	L.	S.L.	-	-
110	35111	Cataract	-	L.	L.	-	-
111	35958	...	+ve	L.P.	S.L.	L.P.	-
112	36688	Cataract	+	"	-	"	-
113	36707	Meningitis	+	"	-	"	-
114	36720	Urine	+	"	L.	"	-
115	36721	Urine	+	"	S.L.	"	Ppt.
116	36727	Urine	+	"	"	"	"
117	36737	Sputum	+	"	L.	"	-
118	36778	Urine	+	"	L.	"	Ppt.
119	36779	...	+	"	S.L.	"	-
120	36817	Pus	+	"	"	"	Ppt.

TABLE III (Continued).

NUMBER	HOSPITAL NUMBER	ORIGIN OF STRAIN	COAGULASE TEST.	RESULT OF GROWTH ON			
				S.M.A.	M.A.	S.Hb.A.	HbA.
121	36819	Sputum	+ve	L.P.	S.L.	L.P.	Ppt.
122	36841	...	+	"	L.	"	"
123	36848	Pus	+	"	-	"	"
124	36857	Urine	+	"	S.L.	"	-
125	36868	...	+	"	-	"	Ppt.
126	36883	Pyrexia	+	"	S.L.	"	"
127	36884	Urine	+	"	L.	"	"
128	35189	Graft	+	"	L.	"	"
129	35235	Pus	+	"	L.	"	"
130	35237	Eye	+	"	...	"	...
131	35240	Burn	+	L.	-	-	-
132	35241	Wound	+	L.P.	S.L.	L.P.	-
133	35304	Sinus	+	"	L.	"	-
134	35358	Pustule	+	"	L.	"	-
135	35375	Tonsillitis	+	"	Ppt.L.	"	-
136	35378	...	+	"	L.	"	-
137	35508	Abscess	+	"	S.L.	"	-
138	35579	Abscess	+	"	L.	"	-
139	35590	Sinusitis	+	"	L.	"	Ppt.
140	35614	Urine	+	"	S.L.	"	-

TABLE III (Continued).

NUMBER	HOSPITAL NUMBER	ORIGIN OF STRAIN	COAGULASE TEST.	RESULT OF GROWTH ON			
				S.M.A.	M.A.	S.Hb.A.	HbA.
141	35628	Abscess	+	L.P.	S.Ppt.	L.P.	Ppt.
142	35630	Conjunctivitis	+	"	S.L.	"	"
143	35667	Cellulitis	+	"	-	"	-
144	35737	Boil	+	"	L.	"	Ppt.
145	35756	Wound	-ve	-	S.L.	-	"
146	35778	Throat	+ve	L.P.	S.L.Ppt.	L.P.	S.Ppt.
147	35779	Abscess	+	"	S.L.	"	"
148	35817	Eye	+	"	Ppt.	"	Ppt.
149	35819	Urine	+	"	"	"	"
150	35841	Cataract	+	"	"	"	"
151	35848	Abscess	+	"	S.L.	"	"
152	35857	Faeces	+	"	S.L.Ppt.	"	"
153	35868	Faeces	+	"	S.L.	"	"
154	35883	Wound	+	"	L.	"	"
155	35884	Abscess	+	"	L.	"	"
156	35208(ii)	Milk	+	"	-	"	"
157	35039	Osteitis	+	"	S.L.	"	"
158	35001	Osteitis	+	"	L.	"	"
159	35015	Pus	+	"	L.	"	"
160	P342	Maternity Hospital Service	+	"	S.L.Ppt.	"	S.L.

TABLE III (Continued).

NUMBER	HOSPITAL NUMBER	ORIGIN OF STRAIN	COAGULASE TEST.	RESULT OF GROWTH ON			
				S.M.A.	M.A.	S.Hb.A.	HbA.
161	P344	Maternity Hospital Survey	+ve	L.P.	L.	L.P.	Ppt.
162	P346	"	+	"	L.	"	S.L.
163	P347	"	-ve	L.	-	-	-
164	P348	"	+ve	L.P.	L.	L.P.	Ppt.
165	P349	"	+	"	S.L.	"	S.L.Ppt.
166	P354	"	+	"	L.	"	L.
167	P355	"	+	"	S.L.	"	S.Ppt.
168	P359	"	+	"	L.	"	Ppt.
169	P360	"	+	"	L.	"	"
170	P361	"	+	"	S.L.	"	"
171	P362	"	+	"	L.	"	"
172	P365	"	+	"	S.L.	"	"
173	P366	"	+	"	L.	"	S.L.Ppt.
174	P368	"	+	"	S.L.	"	-
175	P369	"	+	"	"	"	-
176	P370	"	+	"	L.	"	-
177	P371	"	+	"	S.L.	"	-
178	P373	"	+	"	"	"	S.Ppt.
179	P374	"	-ve	-	-	-	-

TABLE III (Continued).

NUMBER	HOSPITAL NUMBER	ORIGIN OF STRAIN	COAGULASE TEST.	RESULT OF GROWTH ON			
				S.M.A.	M.A.	S.Hb.A.	HbA.
180	P380	Maternity Hospital Survey.	+ve	L.P.	S.L.Ppt.	L.P.	Ppt.
181	P381	"	+	"	S.L.	"	S.L.
182	P383	"	+	"	L.	"	Ppt.
183	P385	"	+	"	S.L.	"	-
184	P386	"	+	"	L.	"	-
185	P387	"	+	"	S.L.	"	Ppt.
186	P390	"	+	"	S.L.Ppt.	"	-
187	P394A	"	-ve	-	S.L.	-	-
188	P394B	"	+ve	L.P.	"	L.P.	-
189	P398	"	+	"	"	"	-
190	P350	"	-ve	L.	L.	-	-
191	P351A	"	-	L.	L.	S.L.	-
192	P351B	"	+ve	L.P.	-	L.P.	Ppt.
193	P358	"	-ve	S.L.	L.	S.L.	-
194	P375	"	+ve	L.P.	S.L.Ppt.	L.P.	-
195	P377	"	-ve	L.	L.Ppt.	L.	-
196	P379	"	+ve	L.P.	L.	L.P.	-
197	P382	"	-ve	L.	L.	L.	-
198	P393	"	-	S.L.	L.	S.L.	-
199	P395	"	-	"	S.L.	-	-

TABLE III (Continued).

NUMBER	HOSPITAL NUMBER	ORIGIN OF STRAIN	COAGULASE TEST	RESULT OF GROWTH ON			
				S.M.A.	M.A.	S.Hb.A.	HbA.
200	P397	Maternity Hospital Survey	-ve	S.L.	L.	-	-
201	P401	"	+ve	L.P.	S.L.	L.P.	-
202	P402	"	+	"	"	"	Ppt.
203	P406	"	+	"	"	"	-
204	P410(i)	"	+	"	L.	"	Ppt.
205	P414(ii)	"	+	"	S.L.	"	"
206	P415	"	+	"	L.	"	"
207	P416	"	+	"	S.L.	"	"
208	P417	"	+	"	L.	"	"
209	P418	"	+	"	L.	"	"
210	P419	"	+	"	S.L.	"	"
211	P420	"	+	"	"	"	"
212	P421	"	+	"	"	"	"
213	P422	"	+	"	"	"	-
214	P423(i)	"	+	"	"	"	S.Ppt.
215	P423(ii)	"	+	"	L.Ppt.	"	-
216	P424	"	+	"	L.	"	Ppt.
217	P426(i)	"	+	"	L.	"	"
218	P426(ii)	"	+	"	L.	"	"
219	P430	"	+	"	L.	"	"

TABLE III (Continued).

NUMBER	HOSPITAL NUMBER	ORIGIN OF STRAIN	COAGULASE TEST.	RESULT OF GROWTH ON			
				S.M.A.	M.A.	S.Hb.A.	HbA.
220	P432	Maternity Hospital Survey.	+ve	L.P.	S.L.	L.P.	Ppt.
221	P434	"	+	"	"	"	"
222	P435	"	+	"	"	"	"
223	P436	"	+	"	"	"	"
224	P437	"	+	"	L.	"	"
225	P438	"	+	"	S.L.	"	"
226	P441	"	+	"	L.	"	-
227	P446	"	+	"	S.L.	"	Ppt.
228	P458	"	+	"	"	"	-
229	P456	"	+	"	Ppt.	"	Ppt.
230	P440	"	+	"	-	"	-
231	P447	"	+	"	S.L.	"	-
232	P449	"	+	"	-	"	Ppt.
233	P247	"	-ve	-	S.L.	-	-

S.M.A. = Serum-milk-agar.
M.A. = Milk-agar.
S.Hb.A. = Serum-haemoglobin-agar.
HbA. = Haemoglobin-agar.

TABLE IV.

THE ABILITY OF STRAINS OF STAPHYLOCOCCI TO PRODUCE MULLER'S PHENOMENON IN SERUM-MILK-AGAR AND SERUM-HAEMOGLOBIN-AGAR PLATES.

STRAINS OF STAPHYLOCOCCI.	NUMBER	Producing Muller's Phenomenon on	
		Serum-Milk-Agar	Serum-Haemoglobin-Agar.
COAGULASE POSITIVE	192	185 (95.8%)	186 (96.4%).
COAGULASE NEGATIVE	41	0	0
TOTAL	233	185	186

TABLE V.

EXAMINATION OF 116 STRAINS OF COAGULASE POSITIVE STAPHYLOCOCCUS AUREUS TO ASCERTAIN THEIR ABILITY TO PRODUCE MULLER'S PHENOMENON ON SERUM-MILK-AGAR.

NUMBER	HOSPITAL NUMBER	ORIGIN OF STRAIN	MULLER'S PHENOMENON ON S.M.A.		APPEARANCE ON MILK-AGAR
			STAB.	STREAK	
1	71060	Blood Culture	L.P.	L.P.	Ppt.
2	71064	Wound	"	"	"
3	71090	Nasal swab	-	-	"
4	71095	Dacryocystitis	L.P.	L.P.	S.L.
5	71096	Sputum	P.	"	"
6	71102	Adenitis	L.P.	"	Ppt.
7	71155	Boil	"	"	"
8	71156	Nasal swab	"	"	"
9	71185	Throat swab	"	"	Ppt.S.L.
10	71204	Conjunctiva	"	"	Ppt.
11	71205	Conjunctiva	"	P.	"
12	71256	Abscess	"	L.P.	-
13	71258	Pus	"	"	S.L.
14	71273	3rd. Degree Burn	-	-	"
15	71284	Pus	L.P.	L.P.	Ppt.
16	71334	Osteitis	"	"	S.L.
17	71340	Pus	"	"	Ppt.S.L.
18	71341	Pus	"	"	Ppt.
19	71344	Boil	"	"	"
20	71345	Abscess	"	"	"
21	71372	Sputum	"	"	"

T A B L E V (Continued).

NUMBER	HOSPITAL NUMBER	ORIGIN OF STRAIN	MULLER'S PHENOMENON ON S.M.A.		APPEARANCE ON MILK-AGAR
			STAB	STREAK	
22	71428	Sputum	L.P.	L.P.	-
23	71429	Boil	"	"	S.L.
24	71443	Dacryocystitis	"	"	Ppt.
25	71465	Dacryocystitis	"	"	S.L.
26	71487	Sputum	"	"	"
27	71504	Osteitis	"	"	Ppt.
28	71512	Pus	"	"	"
29	71517	Wound	"	"	"
30	71533	Nasal swab	"	"	"
31	71547	Blood culture	"	"	S.L.
32	71577	Urine	"	"	Ppt.
33	71602	Pus	"	"	-
34	71603	Pus	"	"	Ppt.
35	71624	Pus	"	"	S.L.
36	71625	Pus	"	"	"
37	71637	Abscess	"	"	"
38	71662	Conjunctivitis	"	"	Ppt.
39	71663	Conjunctivitis	"	"	"
40	71689	Pus	"	"	S.L.
41	71692	Osteitis	"	"	"
42	71706	Wound	-	-	Ppt.S.L.
43	71709	Wound	-	-	S.L.
44	71710	Wound	-	-	"
45	71713	Abscess	L.P.	L.P.	Ppt.
46	71728	Pus	"	"	S.L.
47	71893	Sputum	"	"	Ppt.

T A B L E V (Continued).

NUMBER	HOSPITAL NUMBER	ORIGIN OF STRAIN	MULLER'S PHENOMENON ON S.M.A.		APPEARANCE ON MILK-AGAR
			STAB	STREAK	
48	71936	Faeces	L.P.	L.P.	Ppt.
49	71938	Pus	"	"	"
50	71939	Pus	"	"	"
51	71955	Pus	"	"	"
52	71960	Swab	"	"	S.L.
53	71961	Nasal swab	"	"	Ppt.S.L.
54	71979	Pus	"	"	Ppt.
55	72005	Pus	"	"	S.L.
56	72019	Pus	"	"	Ppt.
57	72023	Pus	"	"	"
58	72030	Pus	"	"	S.L.
59	72041	Pus	-	-	"
60	72042	Pus	L.P.	L.P.	Ppt.
61	72074	Lung	"	"	"
62	72086	Abscess	"	"	"
63	72104	Pus	"	"	"
64	72130	Swab	"	"	S.L.
65	72133	Pus	"	"	Ppt.
66	72136	Carbuncle	"	"	S.L.
67	72160	Pus	"	"	"
68	72211	Swab	"	"	Ppt.
69	72212	Swab	"	"	"
70	72239	Synovial fluid	"	"	"
71	72246	Pus	"	"	S.L.
72	72251	Eye swab	"	"	Ppt.S.L.

T A B L E V (Continued).

NUMBER	HOSPITAL NUMBER	ORIGIN OF STRAIN	MULLER'S PHENOMENON ON S.M.A.		APPEARANCE ON MILK-AGAR
			STAB	STREAK	
73	72279	Nasal swab	L.P.	L.P.	Ppt.
74	72285	Abscess	"	"	"
75	72298	Pus	"	"	S.L.
76	72333	Pus	-	-	Ppt.
77	72339	Pus	L.P.	L.P.	"
78	72445	Pus	"	"	"
79	72754	Pleural cavity	"	"	"
80	72979	Pus	"	"	"
81	72808	Sputum	P.	"	"
82	72822	Swab	L.P.	"	S.L.
83	72864	Swab	"	"	Ppt.
84	72871	Pus	"	"	S.L.
85	72886	Ulcer	"	"	"
86	72892	Abscess	"	"	Ppt.
87	72907	Urine	-	-	"
88	72932	Skin-graft	L.	L.	"
89	72943	Wound	L.P.	L.P.	"
90	72958	Abscess	"	"	"
91	72959	Pus	"	"	Ppt.S.L.
92	72970	Nasal swab	"	"	S.L.
93	72971	Nasal swab	"	"	Ppt.
94	72976	Cellulitis	"	"	S.L.
95	72997	Abscess	P.	"	"
96	73008	Pus	L.P.	"	"
97	73016	Pus	"	"	"
98	73030	Cellulitis	"	"	Ppt.

T A B L E V (Continued).

NUMBER	HOSPITAL NUMBER	ORIGIN OF STRAIN	MULLER'S PHENOMENON ON S.M.A.		APPEARANCE ON MILK-AGAR
			STAB	STREAK	
99	73031	Swab	L.P.	L.P.	Ppt.
100	73061	Swab	"	"	"
101	73105	Pus	-	-	S.L.
102	73157	Vesicle	L.P.	L.P.	Ppt.
103	73158	Vesicle	"	"	"
104	73175	Pus	"	"	"
105	73192	Pus	"	"	Ppt.S.L.
106	73193	Pus	"	"	S.L.
107	73194	Marrow	"	"	Ppt.
108	73203	Pus	"	"	S.L.
109	73206	Pus	"	"	Ppt.
110	73209	Sputum	"	"	S.L.
111	73210	Osteitis	"	"	"
112	73230	Wound	"	"	Ppt.
113	73236	Boil	"	"	"
114	73237	Nasal swab	"	"	Ppt.S.L.
115	73249	Urine	"	"	S.L.
116	73250	Swab	"	"	Ppt.

TABLE VI.

SUMMARY OF FINDINGS REPORTED IN TABLE V.

NUMBER OF STRAINS OF COAGULASE POSITIVE *STAPHYLOCOCCUS AUREUS* EXAMINED.	116
NUMBER PRODUCING MULLER'S PHENOMENON ON SERUM-MILK-AGAR.	
IN STAB INOCULUM.	106
IN STREAK INOCULUM.	106
NUMBER OF STRAINS NOT PRODUCING THE PHENOMENON.	10
PERCENTAGE OF STRAINS PRODUCING MULLER'S PHENOMENON.	91.3

T A B L E VII (a) .

NATURE OF BACTERIOLOGICAL SPECIMENS FROM WHICH STRAINS OF STAPHYLOCOCCUS AUREUS WERE ISOLATED.

SPECIMEN	NUMBER
Pus.	30
Sputum.	21
Urine.	27
Gastric Aspiration.	1
Swabs.	128
TOTAL	207

T A B L E VII (b) .

ORIGIN OF SWABS	NUMBER
Pus swabs.	38
Vaginal swabs.	18
Nasal swabs.	12
Eye swabs.	10
Wound swabs.	8
Postmortem swabs.	5
Miscellaneous sites.	16
Throat swabs.	6
Not known.	15
TOTAL	128

TABLE VIII .

NATURE OF DISCARDED SPECIMENS.

NATURE OF SPECIMENS	NUMBER
Sputum.	6
Urine.	1
Faeces.	1
Purulent Fluid.	1
Swabs.	11
Total number of specimens discarded.	20

ORIGIN OF SWABS DISCARDED	NUMBER
Pus.	3
Nasal.	2
Throat.	2
Skin graft.	2
Wound.	1
Vaginal.	1
Total.	11

TABLE IX.

EXAMINATION OF 207 BACTERIOLOGICAL SPECIMENS CONTAINING COAGULASE POSITIVE STAPHYLOCOCCUS AUREUS.

NUMBER	HOSPITAL NUMBER	NATURE OF SPECIMEN	MULLER'S PHENOMENON ON S.M.A.	GROWTH OF STAPH. AUREUS ON		GROWTH OF OTHER BACTERIA.
				S.M.A.	B.A.	
1	88004	Pus	+	+	+	Coliforms.
2	88512	Wound pus	+	+	+	Micrococci
3	88516	Sputum	+	+	-	Micrococci
4	88518	Sputum	+	+	-	Yeasts
5	88536	Wound pus	+	+	+	Nil
6	88541	Urine	+	+	+	Ps. pyocyanea
7	88544	Vaginal swab	+	+	+	Coliforms
8	88545	Vaginal swab	+	+	+	Nil
9	88546	Urine	+	+	+	Streptococci
10	88553	Pus	+	+	+	Nil
11	88557	Wound pus	+	+	+	Nil
12	88561	Sputum	+	+	+	Nil
13	88592	Pus	+	+	+	Nil
14	88606	Pus swab	+	+	+	Nil
15	88607	Swab	+	+	+	Coliforms
16	88614	Pus swab	+	+	+	Ps. pyocyanea
17	88669	Bronchial swab	+	+	+	Nil.
18	88670	Tracheal swab	+	+	+	Nil.
19	88671	Lung swab	+	+	+	Mixed flora
20	88673	Lung swab	+	+	+	Mixed flora

TABLE IX (Continued).

NUMBER	HOSPITAL NUMBER	NATURE OF SPECIMEN	MULLER'S PHENOMENON ON S.M.A.	GROWTH OF STAPH. AUREUS ON		GROWTH OF OTHER BACTERIA
				S.M.A.	B.A.	
21	88684	Pus swab	+	+	+	Nil
22	88686	Pus	+	+	+	Nil
23	88665	Pus	+	+	+	Nil
24	88715	Fistula swab	+	+	+	Bact. aerogenes
25	88722	Pus	+	+	+	Nil
26	88732	Swab	+	+	+	Nil
27	88750	Pus	+	+	+	Nil
28	88844	Wound swab	+	+	+	Nil
29	88849	Pus swab	+	+	+	Nil
30	88863	Abscess swab	+	+	+	Nil
31	88869	Vaginal swab	+	+	+	Nil
32	88862	Pus	+	+	+	Nil
33	88917	Mouth swab	+	+	+	Mixed flora
34	88965	Pus swab	+	+	+	Nil
35	88969	Eye swab (pus)	+	+	+	Coliforms (on Blood-Agar only)
36	88971	Pus	+	+	+	Nil
37	88907	Sputum	+	+	+	Nil
38	88959	Swab	+	+	+	Micrococci
39	88991	Sputum	+	+	-	Mixed flora

TABLE IX (Continued).

NUMBER	HOSPITAL NUMBER	NATURE OF SPECIMEN	MULLER'S PHENOMENON ON S.M.A.	GROWTH OF STAPH. AUREUS ON		GROWTH OF OTHER BACTERIA
				S.M.A.	B.A.	
40	88997	Pus swab	+	+	+	Streptococci
41	89014	Swab	+	+	-	Mixed flora
42	89030	Pus	+	+	+	Nil
43	89061	Swab	+	+	+	Mixed flora
44	89065	Swab	+	+	+	Nil
45	89073	Sputum	+	+	+	Nil
46	89076	Sputum	+	+	+	Micrococci
47	89018	Sputum	+	+	-	Mixed flora
48	89120	Urine	+	+	+	Coliforms
49	89125	Pus	+	+	+	Nil
50	89128	Swab	+	+	+	Mixed flora
51	89170	Gastric aspiration	+	+	+	Nil
52	89182	Pus swab	-	+	+	Streptococci
53	89192	Pus swab	+	+	+	Micrococci
54	89191	Pus swab	+	+	+	Proteus sp.
55	89218	Pus swab	+	+	+	Nil
56	89228	Vaginal swab	+	+	+	Coliforms
57	89236	Nasal swab	+	+	+	Nil
58	89237	Throat swab	+	+	+	Micrococci
59	89253	Pus swab	+	+	+	Nil

TABLE IX (Continued).

NUMBER	HOSPITAL NUMBER	NATURE OF SPECIMEN	MULLER'S PHENOMENON ON S.M.A.	GROWTH OF STAPH. AUREUS ON		GROWTH OF OTHER BACTERIA
				S.M.A.	B.A.	
60	89277	Nasal swab	+	+	+	Nil
61	89278	Nasal swab	+	+	+	Nil
62	89263	Throat swab	+	+	+	Micrococci
63	89291	Sputum	+	+	+	Nil
64	89335	Eye swab	+	+	+	Micrococci
65	89336	Swab	+	+	-	Micrococci
66	89350	Wound swab	+	+	+	Nil
67	89351	Pus swab	+	+	+	Micrococci
68	89371	Cough swab	-	+	+	Mixed flora
69	89392	Swab	+	+	+	Nil
70	89407	Sputum	+	+	+	Mixed flora
71	89408	Sputum	+	+	+	Micrococci
72	89409	Swab	+	+	+	Nil
73	89412	Swab	+	+	+	Nil
74	89430	Throat swab	+	+	+	Nil
75	89456	Swab	+	+	+	Nil
76	89466	Pus	-	+	+	Mixed flora
77	89482	Eye swab	+	+	+	Nil
78	89486	Eye swab	+	+	+	Nil
79	89495	Swab	+	+	+	Nil

TABLE IX (Continued).

NUMBER	HOSPITAL NUMBER	NATURE OF SPECIMEN	MULLER'S PHENOMENON ON S.M.A.	GROWTH OF STAPH. AUREUS ON		GROWTH OF OTHER BACTERIA
				S.M.A.	B.A.	
80	89503	Sputum	+	+	+	Mixed flora
81	89508	Sputum	+	+	+	Mixed flora
82	89528	Urine	+	+	+	Nil
83	89545	Sputum	+	+	+	Neisseriae
84	89560	Eye swab	+	+	+	Nil
85	89529	Vaginal swab	+	+	+	Nil
86	89578	Eye swab	+	+	+	Coliforms
87	89611	Swab	+	+	+	Micrococci
88	89627	Vaginal swab	+	+	+	Coliforms
89	89643	Ulcer swab	+	+	+	Micrococci
90	89656	Urine	+	+	-	Proteus sp.
91	89668	Urine	+	+	+	Nil
92	89670	Nasal swab	+	+	+	Bact. aerogenes
93	89689	Urine	+	+	+	Nil
94	89690	Vaginal swab	+	+	+	Nil
95	89697	Nasal swab	+	+	+	Micrococci
96	89706	Pus swab	+	+	+	Mixed flora
97	90078	Urine	+	+	+	Proteus sp.
98	90130	Pus swab	+	+	+	Nil

TABLE IX (Continued).

NUMBER	HOSPITAL NUMBER	NATURE OF SPECIMEN	MULLER'S PHENOMENON ON S.M.A.	GROWTH OF STAPH. AUREUS ON		GROWTH OF OTHER BACTERIA
				S.M.A.	B.A.	
99	90134	Pus swab	+	+	+	Nil
100	90137	Nasal swab	+	+	+	Coliforms
101	90140	Pus	+	+	+	Nil
102	90167	Vaginal swab	+	+	+	Mixed flora
103	90174	Swab	+	+	+	Nil
104	90186	Eye swab	+	+	+	Micrococci
105	90195	Urine	+	+	+	Streptococci
106	90214	Eye swab	+	+	+	Nil
107	90215	Urine	+	+	+	Nil
108	90216	Vaginal swab	+	+	+	Nil
109	90217	Throat swab	+	+	+	Yeasts
110	90238	Pus swab	+	+	+	Nil
111	90265	Sputum	+	+	+	Mixed flora
112	90270	Vaginal swab	+	+	+	Nil
113	90275	Urine	+	+	+	Mixed flora
114	90325	Vaginal swab	+	+	+	Nil
115	90336	Nasal swab	+	+	+	Micrococci
116	90337	Nasal swab	+	+	+	Mixed flora
117	90342	Pus swab	+	+	+	Nil
118	90370	Throat swab	+	+	+	Streptococci

TABLE IX (Continued).

NUMBER	HOSPITAL NUMBER	NATURE OF SPECIMEN	MULLER'S PHENOMENON ON S.M.A.	GROWTH OF STAPH. AUREUS ON		GROWTH OF OTHER BACTERIA
				S.M.A.	B.A.	
119	90389	Pus swab	-	+	+	Mixed flora
120	90395	Urine	+	+	+	Mixed flora
121	90396	Vaginal swab	+	+	+	Nil
122	90410	Wound swab	+	+	+	Nil
123	90456	Pus	+	+	+	Nil
124	90478	Nasal swab	+	+	+	Mixed flora
125	90483	Sputum	+	+	+	Streptococci
126	90497	Nasal swab	+	+	+	Nil
127	90507	Vaginal swab	+	+	+	Nil
128	90552	Pus	+	+	+	Streptococci
129	90698	Cough swab	+	+	+	Yeasts
130	90714	Urine	+	+	+	Mixed flora
131	90717	Vaginal swab	+	+	+	Coliforms
132	90742	Pus swab	+	+	+	Nil
133	90743	Urine	+	+	+	Mixed flora
134	90754	Wound swab	+	+	+	Nil
135	90770	Pus	+	+	+	Nil
136	90771	Urine	+	+	+	Coliforms
137	90772	Vaginal swab	+	+	-	Coliforms
138	90796	Urine	+	+	-	Coliforms
139	90800	Pus	+	+	+	Nil

TABLE IX (Continued).

NUMBER	HOSPITAL NUMBER	NATURE OF SPECIMEN	MULLER'S PHENOMENON ON S.M.A.	GROWTH OF STAPH. AUREUS ON		GROWTH OF OTHER BACTERIA
				S.M.A.	B.A.	
140	90827	Pus swab	+	+	+	Nil
141	90838	Pus swab	+	+	+	Nil
142	90839	Throat swab	+	+	+	Micrococci
143	90849	Pus	+	+	+	Nil
144	90850	Pus swab	+	+	+	Mixed flora
145	90912	Abscess swab	+	+	+	Micrococci
146	90913	Abscess swab	+	+	+	Nil
147	90928	Nasal swab	+	+	+	Nil
148	90929	Pus swab	+	+	+	Nil
149	90935	Mouth swab	+	+	+	Nil
150	90942	Vaginal swab	-	+	+	Nil
151	90947	Ear swab	+	+	+	Nil
152	90965	Vaginal swab	+	+	+	Mixed flora
153	90970	Sputum	+	+	+	Coliforms
154	90974	Vaginal swab	+	+	+	Coliforms
155	90978	Pus swab	+	+	+	Nil
156	90987	Pus swab	+	+	+	Streptococci
157	90988	Wound swab	+	+	+	Micrococci
158	91014	Sputum	+	+	+	Mixed flora
159	91026	Swab	+	+	+	Nil

TABLE IX (Continued).

NUMBER	HOSPITAL NUMBER	NATURE OF SPECIMEN	MULLER'S PHENOMENON ON S.M.A.	GROWTH OF STAPH. AUREUS ON		GROWTH OF OTHER BACTERIA
				S.M.A.	B.A.	
160	91044	Pus	+	+	+	Nil
161	91051	Urine	+	+	+	Micrococci
162	91066	Swab	+	+	+	Mixed flora
163	91077	Pus	+	+	+	Nil
164	91083	Pus swab	+	+	+	Nil
165	91120	Urine	+	+	+	Nil
166	91126	Nasal swab	+	+	+	Micrococci
167	91140	Eye swab	+	+	+	Nil
168	91156	Urine	+	+	+	Streptococci
169	91160	Swab	+	+	+	Nil
170	91169	Sputum	+	+	+	Mixed flora
171	91183	Urine	+	+	+	Bact. aerogenes
172	91096	Pus swab	+	+	+	Mixed flora
173	91234	Swab	+	+	+	Nil
174	91242	Swab	+	+	+	Mixed flora
175	91309	Urine	+	+	-	Nil
176	91275	Urine	+	+	+	Nil
177	91293	Pus	+	+	+	Nil
178	91301	Wound swab	+	+	+	Nil
179	91343	Pus swab	+	+	+	Nil

TABLE IX (Continued).

NUMBER	HOSPITAL NUMBER	NATURE OF SPECIMEN	MULLER'S PHENOMENON ON S.M.A.	GROWTH OF STAPH. AUREUS ON		GROWTH OF OTHER BACTERIA
				S.M.A.	B.A.	
180	91352	Urine	+	+	+	Nil
181	91362	Pus swab	+	+	+	Nil
182	91372	Pus	+	+	+	Nil
183	91364	Ear swab	+	+	-	Micrococci
184	91433	Urine	+	+	+	Micrococci
185	91461	Pus swab	+	+	+	Ps. pyocyanea
186	91462	Pus swab	+	+	-	Coliforms
187	91498	Urine	+	+	+	Mixed flora
188	91512	Eye swab	+	+	+	Mixed flora
189	91513	Eye swab	+	+	+	Micrococci
190	91583	Swab	+	+	+	Nil
191	91587	Wound swab	+	+	+	Mixed flora
192	91593	Nasal swab	+	+	+	Diphtheroid
193	91595	Swab	+	+	+	Nil
194	91598	Swab	+	+	+	Bact. aerogenes
195	91605	Swab	+	+	+	Nil
196	91653	Pus swab	+	+	+	Nil
197	91689	Urine	+	+	+	Nil
198	91698	Swab	+	+	+	Nil
199	91703	Pus	+	+	+	Nil

TABLE IX (Continued).

NUMBER	HOSPITAL NUMBER	NATURE OF SPECIMEN	MULLER'S PHENOMENON ON S.M.A.	GROWTH OF STAPH. AUREUS ON		GROWTH OF OTHER BACTERIA
				S.M.A.	B.A.	
200	91731	Urine	+	+	+	Micrococci
201	91732	Sputum	+	+	+	Micrococci
202	91739	Swab	+	+	+	Nil
203	91742	Pus	+	+	+	Nil
204	91777	Pus	+	+	+	Micrococci
205	91807	Sputum	+	+	+	Nil
206	91811	Pus	+	+	-	Ps. pyocyanea
207	91825	Pus	+	+	+	Nil

TABLE X.

SUMMARY OF THE FINDINGS REPORTED IN TABLE IX.

TOTAL NUMBER OF SPECIMENS.	207
NUMBER OF SPECIMENS FROM WHICH STAPHYLOCOCCUS AUREUS WAS ISOLATED.	
on Serum-Milk-Agar plates.	207
on Blood-Agar plates.	194
NUMBER OF STRAINS OF STAPHYLOCOCCUS AUREUS WHICH PRODUCED MULLER'S PHENOMENON ON SERUM-MILK-AGAR PLATES.	202
PERCENTAGE OF STRAINS OF STAPHYLOCOCCUS AUREUS WHICH PRODUCED MULLER'S PHENOMENON.	97.6
NUMBER OF STRAINS OF STAPHYLOCOCCUS AUREUS ISOLATED IN PURE CULTURE ON SERUM-MILK-AGAR PLATES.	104

TABLE XI.

THE PRODUCTION OF MULLER'S PHENOMENON BY 46 STRAINS OF COAGULASE POSITIVE STAPHYLOCOCCI INOCULATED DIRECTLY FROM SPECIMENS ONTO 3 SETS OF SERUM-MILK-AGAR PLATES

HOSPITAL NUMBER OF SPECIMEN	OCCURRENCE OF MULLER'S PHENOMENON IN SERUM-MILK-AGAR PLATES MADE WITH		
	TEST SERUM	SERUM 4	SERUM 7
90827	+	+	+
90838	+	+	+
90839	+	+	+
90849	+	+	+
90850	+	+	+
90912	...	+	+
90913	+	+	+
90928	+	+	+
90929	+	+	+
90935	+	+	+
90942	-	-	-
90947	+	+	+
90965	+	+	+
90970	...	+	+
90974	...	+	+
90978	+	+	+
90987	+	+	+
90988	...	+	+
91014	+	+	+
91026	+	+	+
91044	+	+	+

T A B L E XI (Continued).

HOSPITAL NUMBER OF SPECIMEN	OCCURRENCE OF MULLER'S PHENOMENON IN SERUM-MILK-AGAR PLATES MADE WITH		
	TEST SERUM	SERUM 4	SERUM 7
91051	+	+	+
91066	+	+	+
91077	+	+	+
91083	+	+	+
91120	+	+	+
91126	+	+	+
91140	+	+	+
91156	+	+	+
91160	+	+	+
91169	+	+	+
91183	+	+	+
91096	+	+	+
91235	+	+	+
91242	+	+	+
91309	+	+	+
91275	+	+	+
91293	+	+	+
91301	+	+	+
91343	+	+	+
91352	+	+	+
91362	+	+	+
91372	+	+	+
91364	+	+	(•••)
91433	+	+	+
91462	+	+	+

TABLE XII.

BACTERIOPHAGE TYPING OF STRAINS OF *STAPHYLOCOCCUS AUREUS* WHICH DID NOT PRODUCE MULLER'S PHENOMENON ON SERUM-MILK-AGAR PLATES.

HOSPITAL NUMBER	PHAGE TYPE	GROUP
89182	75/77/B1	3
89371	29/52/53/54	1 + 3
89466	6/7/47/75/77	3
90389	6/7/47/54/75/77	3
90942	6/7/54/75/77/B1	3

T A B L E XIII.

THE RESULTS OF BIOCHEMICAL INVESTIGATIONS ON CERTAIN STRAINS OF STAPHYLOCOCCUS AUREUS AND THEIR VARIANTS.

STRAIN NUMBER	LACTOSE	GLUCOSE	SUCROSE	MALTOSE	DULCITE	MANNITE	UREA	LITMUS MILK	DIGESTION OF SERUM	MULLER'S PHENOM.
89065 Yellow	A	A	A	A	-	A	+ve	A.C.R.	-ve/21	+ve
89065 White	"	"	"	"	"	"	"	"	"	"
88592 L.P.	"	"	"	"	"	"	"	"	"	"
88592 N.L.P.	"	"	"	"	"	"	"	"	"	-ve
91343 L.P.	"	"	"	"	"	"	"	"	"	+ve
91343 N.L.P.	"	"	"	"	"	"	"	"	"	-ve
91587 L.P.	"	"	"	"	"	"	"	"	"	+ve
91587 N.L.P.	"	"	"	"	"	"	"	"	"	-ve

-ve/21 = Negative result at 21 days.

L.P. = Lysis and Plaques producing strain.

N.L.P. = Non Lysis and Plaques " "

A = Acid. C = Clot. R = Reduction

TABLE XIV.

A COMPARISON OF THE ANTIBIOTIC SENSITIVITY PATTERN OF STRAINS OF STAPHYLOCOCCI AND THEIR VARIANTS WITH THOSE OF THE ORIGINAL STRAIN ISOLATED IN THE ROYAL VICTORIA INFIRMARY.

STRAIN NUMBER	PENICILLIN		STREPTO-MYCIN.		CHLORAM-PHENICOL		TETRA-CYCLINE		ERYTHRO-MYCIN.	NEO-MYCIN	OLEAND-MYCIN		NOVO-BIOCIN	
	1 *	10	10	1000	20	200	4	200	1	1	2	10	2	10
89065														
Original	R	R	R	S	S	S	R	R	S	S	S	S	-	S
Yellow	R	R	R	S	S	S	R	R	S	S	S	S	-	S
White	R	R	R	R	S	S	R	S	S	S	S	S	S	S
88592														
Original	R	R	R	R	R	S	S	S	S	S	S	S	S	S
L.P.	R	R	R	R	R	S	S	S	S	S	S	S	S	S
N.L.P.	R	R	R	R	R	S	S	S	S	S	S	S	S	S
91343														
Original	R	R	R	R	S	S	S	S	S	S	S	S	S	S
L.P.	R	R	R	R	S	S	S	S	S	S	S	S	S	S
N.L.P.	R	R	R	R	S	S	S	S	S	S	S	S	S	S
91587														
Original	R	R	R	S	S	S	R	S	S	S	S	S	S	S
L.P.	R	R	R	S	S	S	R	S	S	S	S	S	S	S
N.L.P.	R	R	R	S	S	S	R	S	S	S	S	S	S	S

L.P. = Strain producing Lysis and Plaques. S = Sensitive.
N.L.P. = Strain not producing Lysis and Plaques. R = Resistant.

* Penicillin in units per ml.; others in μg. per ml.

TABLE XV.

THE PHAGE PATTERNS OF LYSIS AND PLAQUE PRODUCING STRAINS OF STAPHYLOCOCCI AND THEIR NON LYSIS AND PLAQUE PRODUCING VARIANTS COMPARED WITH THE PHAGE PATTERNS OBTAINED ON THE ORIGINAL ISOLATION OF THE PARENT STRAINS.

STRAIN	CULTURE	PHAGE PATTERN
88592	Parent	6/53/54
	L.P.	7/53/54/73/75/Bl.
	N.L.P.	6/7/53/54/73/Bl.
91343	Parent	7/47/54/75/77/Bl.
	L.P.	7/47/75/77/Bl.
	N.L.P.	6/7/47/75/77/Bl/44.
91587	Parent.	29/53/75/77/Bl.
	L.P.	75/77/Bl.
	N.L.P.	75/77/Bl.

TABLE XVI.

THE ACTION OF THREE STRAINS OF STAPHYLOCOCCUS AUREUS AND THEIR VARIANTS ON DIFFERENT PROTEIN SUBSTRATES

Strain Number	Heated Plasma Agar I. 15% Heated Plasma (56°C./20 mins.).	Heated Plasma Agar II. 25% Heated Plasma (65°C./30 mins.).	Heated Plasma Agar II and Fresh Serum. 25% Heated Plasma (65°C./30 mins.) with Fresh Serum.	Heated Haemoglobin (75°C./10 mins.)	Heated Haemoglobin (75°C./10 mins.) with Fresh Serum.
88592					
(L.P.)	L.	-	L.P.	-	P.
(N.L.P.)	-	-	-	-	-
91343					
(L.P.)	L.	-	L.P.	-	P.
(N.L.P.)	Ppt.	-	-	-	-
91587					
(L.P.)	L.	-	L.P.	S.L.	L.P.
(N.L.P.)	Ppt.	Ppt.	Ppt.	S.L.	-

(L.P.) = Lysis and Plaques produced by that strain in serum-milk-agar.

(N.L.P.) = No Lysis and Plaques produced by that strain in serum-milk-agar.

TABLE XVII.

EXAMINATION OF 207 STRAINS OF COAGULASE POSITIVE STAPHYLOCOCCUS AUREUS ON DIFFERENT PROTEIN SUBSTRATES.

NUMBER	HOSPITAL NUMBER	MILK-AGAR	15% HEATED PLASMA (56°C./20 mins.) *	25% HEATED PLASMA (65°C./30 mins.) **	25% HEATED PLASMA (65°C./30 mins.) WITH FRESH SERUM. ***	HEATED HAEMOGLOBIN	HEATED HAEMOGLOBIN WITH FRESH SERUM.
1	88004	S.L.	L.	-	L.	S.L.	P.
2	88512	"	L.	-	L.	...	...
3	88516	"	L.	-	L.P.	L.	L.P.
4	88518	"	L.	-	L.	L.	P.
5	88536	"	L.	-	L.	L.	L.P.
6	88541	S.Ppt.	L.	-	L.	-	P.
7	88544	Ppt.	L.	S.L.	L.P.	S.L.	P.
8	88545	"	L.	-	"	-	P.
9	88546	-	L.	S.L.	"	-	P.
10	88533	S.L.	L.	"	"	L.	L.P.
11	88557	...	...	...	...	...	...
12	88561	Ppt.	L.	S.L.	L.	S.L.	L.P.
13	88592	S.L.	L.	-	L.P.	-	P.
14	88606	S.Ppt.	L.	-	"	-	P.
15	88607	-	L.	S.L.	L.	S.L.	P.
16	88614	S.L.	L.	"	L.	L.	L.P.
17	88669	Ppt.	L.	"	L.	-	P.
18	88670	"	L.	"	L.	S.L.	L.P.
19	88671	S.L.Ppt.	L.	"	L.	"	"

TABLE XVII (Continued).

NUMBER	HOSPITAL NUMBER	MILK-AGAR	15% HEATED PLASMA (56°C./20 mins.) *	25% HEATED PLASMA (65°C./30 mins.) **	25% HEATED PLASMA (65°C./30 mins) WITH FRESH SERUM ***	HEATED HAEMOGLOBIN	HEATED HAEMOGLOBIN WITH FRESH SERUM.
20	88673	Ppt.	L.	S.L.	L.	L.	P.
21	88684	S.L.	L.	"	L.	L.	L.P.
22	88686	-	L.	"	L.	L.	S.L.P.
23	88665	L.	L.	"	L.	L.	P.
24	88715	S.L.Ppt.	L.	"	L.	-	P.
25	88722	S.L.	L.	-	L.	L.	P.
26	88732	"	L.	-	L.	L.	P.
27	88750	S.L.Ppt.	L.	-	L.	L.	P.
28	88840	-	L.	-	L.P.	L.	P.
29	88849	S.L.	L.	-	L.	L.	L.P.
30	88863	"	L.	-	L.	L.	P.
31	88869	S.L.Ppt.	L.	-	L.P.	L.	P.
32	88882	S.L.	L.	-	"	L.	P.
33	88917	...	...	...	...	...	...
34	88965	-	L.	-	L.	L.	P.
35	88969	-	L.	-	L.	L.	P.
36	88971	S.L.	L.	-	L.	L.	P.
37	88907	"	L.	-	L.	-	P.
38	88959	-	L.	-	L.P.	S.L.	P.

TABLE XVII (Continued).

NUMBER	HOSPITAL NUMBER	MILK-AGAR	15% HEATED PLASMA (56°C./20 mins.) *	25% HEATED PLASMA (65°C./30 mins.) **	25% HEATED PLASMA (65°C./30 mins) WITH FRESH SERUM ***	HEATED HAEMOGLOBIN	HEATED HAEMOGLOBIN WITH FRESH SERUM.
39	88991	S.L.Ppt.	...	-	L.P.	L.	P.
40	88997	"	L.	-	L.	L.	P.
41	89014	S.L.	L.	-	L.P.	L.	P.
42	89030	"	L.	-	L.	L.	L.P.
43	89061	Ppt.	L.	-	L.	L.	"
44	89065 Yellow	-	L.	-	L.P.	L.	"
	White	S.L.Ppt.	L.	-	"	-	P.
45	89073	S.L.	L.	S.L.	L.	L.	P.
46	89076	Ppt.	L.	-	L.P.	-	P.
47	89018	S.L.	L.	-	L.	L.	L.P.
48	89120	"	L.	-	L.	L.	P.
49	89125	"	L.	S.L.	L.	L.	P.
50	89128	Ppt.	L.	-	L.	-	P.
51	89170	"	L.	-	L.	L.	S.L.P.
52	89182	"	Ppt.	Ppt.	Ppt.	-	-
53	89192	"	L.	-	L.	S.L.	P.
54	89191	"	L.	-	L.P.	L.	P.
55	89218	"	L.	-	L.	S.L.	P.
56	89228	"	L.	-	L.	-	P.

TABLE XVII (Continued).

* ** ***

NUMBER	HOSPITAL NUMBER	MILK-AGAR	15% HEATED PLASMA (56°C./20 mins.)	25% HEATED PLASMA (65°C./30 mins.)	25% HEATED PLASMA (65°C./30 mins.) WITH FRESH SERUM	HEATED HAEMOGLOBIN	HEATED HAEMOGLOBIN WITH FRESH SERUM.
57	89236	S.L.Ppt.	L.	-	L.	-	S.L.P.
58	89237	Ppt.	L.	-	L.	S.L.	"
59	89253	S.L.Ppt.	L.	-	L.	L.	P.
60	89277	Ppt.	L.	-	L.	-	P.
61	89278	S.L.	L.	-	L.	-	P.
62	89263	S.Ppt.	L.	-	L.P.	S.L.	P.
63	89291	L.	L.	S.L.	L.	...	...
64	89335	S.L.	L.	"	L.	L.	L.P.
65	89336	Ppt.	L.	"	L.	L.	"
66	89350	"	L.	-	L.	-	P.
67	89351	S.L.Ppt.	L.	S.L.	L.	-	P.
68	89371	S.L.Ppt.	S.L.Ppt.	Ppt.	S.L.Ppt.	L.	S.L.
69	89392	Ppt.	L.	-	L.	-	P.
70	89407	S.L.	L.	-	L.	-	P.
71	89408	S.L.Ppt.	L.	S.L.	L.P.	L.	L.P.
72	89409	"	L.	"	"	S.L.	"
73	89412	Ppt.	L.	"	"	-	P.
74	89430	"	L.	-	L.	-	P.
75	89456	S.L.	L.	-	L.P.	-	P.
76	89466	S.L.Ppt.	S.Ppt.	Ppt.	Ppt.	-	-

TABLE XVII (Continued).

NUMBER	HOSPITAL NUMBER	MILK-AGAR	15% HEATED PLASMA (56°C./20 mins.) *	25% HEATED PLASMA (65°C./30 mins.) **	25% HEATED PLASMA (65°C./30 mins.) WITH FRESH SERUM ***	HEATED HAEMOGLOBIN	HEATED HAEMOGLOBIN AND FRESH SERUM.
77	89482	S.L.Ppt.	L.	-	L.P.	-	P.
78	89486	Ppt.	L.	-	L.	S.L.	L.P.
79	89495	S.L.	...	-	L.	"	P.
80	89503	S.L.Ppt.	L.	S.L.	L.	-	P.
81	89508	Ppt.	L.	-	L.	-	P.
82	89528	"	L.	-	L.P.	-	P.
83	89545	"	L.	-	"	S.L.	L.P.
84	89560	S.L.	L.	-	"	-	P.
85	89529	Ppt.	L.	-	L.	-	P.
86	89578	"	L.	-	L.P.	-	P.
87	89611	S.L.	L.	-	L.P.	S.L.	P.
88	89627	Ppt.	L.	-	"	-	P.
89	89643	S.L.	L.	-	L.	L.	P.
90	89656	Ppt.	L.	-	L.	S.L.	P.
91	89668	"	L.	-	L.	-	P.
92	89670	-	L.	-	L.	-	P.
93	89689	Ppt.	L.	-	L.	S.L.	P.
94	89690	-	L.	S.L.	L.	"	P.
95	89697	S.L.	L.	-	L.P.	-	P.
96	89706	"	L.	S.L.	L.	L.	P.

TABLE XVII (Continued).

* ** ***

NUMBER	HOSPITAL NUMBER	MILK-AGAR	15% HEATED PLASMA (56°C./20 mins.)	25% HEATED PLASMA (65°C./30 mins.)	25% HEATED PLASMA (65°C./30 mins.) WITH FRESH SERUM	HEATED HAEMOGLOBIN	HEATED HAEMOGLOBIN WITH FRESH SERUM.
97	90078	Ppt.	L.	-	L.P.	S.L.	P.
98	90130	"	L.	-	L.	-	P.
99	90134	S.L.Ppt.	L.	-	L.	L.	P.
100	90137	Ppt.	L.	-	L.P.	S.L.	P.
101	90140	"	L.	S.L.	"	L.	P.
102	90167	"	L.	-	"	-	P.
103	90174	-	L.	-	L.	-	P.
104	90186	-	L.	-	L.P.	-	P.
105	90195	Ppt.	L.	-	L.	-	P.
106	90214	S.L.Ppt.	L.	-	L.P.	L.	P.
107	90215	Ppt.	L.	-	"	-	P.
108	90216	"	L.	-	"	-	P.
109	90217	"	L.	-	"	-	P.
110	90238	S.L.	L.	-	"	L.	P.
111	90265	"	L.	-	"	L.	P.
112	90270	S.Ppt.	L.	-	"	S.L.	P.
113	90275	Ppt.	L.	-	"	-	P.
114	90325	"	L.	-	"	S.L.	P.
115	90336	"	L.	-	"	-	P.
116	90337	"	L.	-	"	-	P.

TABLE XVII (Continued).

NUMBER	HOSPITAL NUMBER	MILK-AGAR	15% HEATED PLASMA (56°C./20 mins.) *	25% HEATED PLASMA (65°C./30 mins.) **	25% HEATED PLASMA (65°C./30 mins.) WITH FRESH SERUM ***	HEATED HAEMOGLOBIN	HEATED HAEMOGLOBIN WITH FRESH SERUM
117	90342	S.L.Ppt.	L.	-	L.	L.	P.
118	90370	"	L.	-	L.	L.	L.P.
119	90389	"	Ppt.	Ppt.	Ppt.	-	-
120	90395	Ppt.	L.	-	L.P.	S.L.	P.
121	90396	"	L.	S.L.	L.	"	P.
122	90410	"	L.	-	L.	-	P.
123	90456	...	...	...	...	-	P.
124	90478	-	L.	-	L.	S.L.	P.
125	90483	Ppt.	L.	-	L.	"	P.
126	90497	S.Ppt.	L.	-	L.	L.	P.
127	90507	Ppt.	L.	-	L.P.	-	P.
128	90552	S.L.Ppt.	L.	-	"	-	P.
129	90698	-	L.	-	L.	S.L.	P.
130	90714	S.Ppt.	L.	-	L.	-	P.
131	90717	"	L.	-	L.P.	-	P.
132	90742	"	L.	-	"	L.	L.P.
133	90743	Ppt.	L.	-	L.	-	P.
134	90754	L.	L.	-	L.	L.	P.
135	90770	S.L.Ppt.	L.	-	L.P.	L.	P.

TABLE XVII (Continued).

NUMBER	HOSPITAL NUMBER	MILK-AGAR	15% HEATED PLASMA (56°C./20 mins.) *	25% HEATED PLASMA (65°C./30 mins.) **	25% HEATED PLASMA (65°C./30 mins.) WITH FRESH SERUM ***	HEATED HAEMOGLOBIN	HEATED HAEMOGLOBIN WITH FRESH SERUM.
136	90771	-	L.	-	L.	-	P.
137	90772	S.Ppt.	L.	-	L.P.	-	P.
138	90796	"	L.	-	L.	-	P.
139	90800	-	L.	-	L.	...	...
140	90827	S.Ppt.	L.	-	L.P.	-	P.
141	90838	S.L.	L.	-	L.	S.L.	L.P.
142	90839	Ppt.	L.	-	L.	-	P.
143	90849	"	L.	-	L.	-	P.
144	90850	"	L.	-	L.	-	P.
145	90912	"	L.	-	L.	L.	P.
146	90913	"	L.	-	L.	L.	P.
147	90928	"	L.	-	L.	S.L.	P.
148	90929	S.L.	L.	-	L.	"	P.
149	90935	Ppt.	L.	-	L.	-	P.
150	90942	"	S.L.Ppt.	-	-	-	-
151	90947	"	L.	-	L.	L.	P.
152	90965	"	L.	-	L.P.	S.L.	P.
153	90970	"	L.	-	"	"	P.
154	90974	"	L.	-	"	-	P.

TABLE XVII (Continued).

NUMBER	HOSPITAL NUMBER	MILK-AGAR	15% HEATED PLASMA (56°C./20 mins.) *	25% HEATED PLASMA (65°C./30 mins.) **	25% HEATED PLASMA (65°C./30 mins.) WITH FRESH SERUM ***	HEATED HAEMOGLOBIN	HEATED HAEMOGLOBIN WITH FRESH SERUM.
155	90978	Ppt.	L.	-	L.	-	P.
156	90987	"	L.	-	L.	-	P.
157	90988	"	L.	-	L.	L.	P.
158	91014	"	L.	-	L.P.	S.L.	P.
159	91026	S.L.Ppt.	L.	S.L.	L.	-	P.
160	91044	"	L.	-	L.	L.	P.
161	91051	Ppt.	L.	-	L.P.	-	P.
162	91066	"	L.	-	"	L.	P.
163	91077	S.L.Ppt.	L.	S.L.	"	L.	P.
164	91083	Ppt.	L.	-	L.	L.	P.
165	91120	S.L.Ppt.	L.	-	L.	-	P.
166	91126	-	L.	-	L.	S.L.	P.
167	91140	S.L.Ppt.	L.	-	L.P.	"	P.
168	91156	Ppt.	L.	-	"	"	P.
169	91160	"	L.	-	L.	"	P.
170	91169	S.L.Ppt.	L.	-	L.	L.	P.
171	91183	"	L.	-	L.	-	P.
172	91096	Ppt.	L.	-	L.	-	P.
173	91235	"	L.	-	L.	-	P.
174	91242	"	L.	S,L.	L.	...	...

TABLE XVII (Continued).

NUMBER	HOSPITAL NUMBER	MILK-AGAR	15% HEATED PLASMA (56°C./20 mins.) *	25% HEATED PLASMA (65°C./30 mins.) **	25% HEATED PLASMA (65°C./30 mins.) WITH FRESH SERUM ***	HEATED HAEMOGLOBIN	HEATED HAEMOGLOBIN WITH FRESH SERUM.
175	91309	S.L.Ppt.	L.	-	L.P.	L.	P.
176	91275	"	L.	-	L.	-	P.
177	91293	Ppt.	L.	S.Ppt.	L.	-	L.P.
178	91301	S.L.Ppt.	L.	-	L.P.	-	P.
179	91343	Ppt.	L.	-	"	-	L.P.
180	91352	S.L.Ppt.	L.	-	L.	S.L.	P.
181	91362	"	L.	S.L.	L.	"	S.L.P.
182	91372	"	L.	"	L.	L.	P.
183	91364	"	L.	"	L.P.	S.L.	L.P.
184	91433	Ppt.	L.	-	"	-	P.
185	91461	"	L.	-	L.	-	P.
186	91462	S.L.Ppt.	L.	S.L.	L.P.	L.	L.P.
187	91498	Ppt.	L.	-	L.	-	P.
188	91512	"	L.	S.L.	L.	-	P.
189	91513	"	L.	-	L.	-	P.
190	91583	S.L.	L.	-	L.P.	L.	L.P.
191	91587	Ppt.	L.	-	"	S.L.	"
192	91593	S.Ppt.	L.	-	"	L.	P.
193	91595	Ppt.	L.	-	L.	-	L.P.
194	91598	"	L.	L.	-	-	P.

TABLE XVII (Continued).

NUMBER	HOSPITAL NUMBER	MILK-AGAR	15% HEATED PLASMA (56°C./20 mins.) *	25% HEATED PLASMA (65°C./30 mins.) **	25% HEATED PLASMA (65°C./30 mins.) WITH FRESH SERUM ***	HEATED HAEMOGLOBIN	HEATED HAEMOGLOBIN WITH FRESH SERUM
195	91605	Ppt.	L.	-	L.	L.	P.
196	91653	"	L.	-	L.	L.	P.
197	91689	"	L.	-	L.	S.L.	P.
198	91698	"	L.	-	L.	L.	L.P.
199	91703	"	L.	-	L.P.	L.	"
200	91731	"	L.	-	L.	-	P.
201	91732	"	L.	-	L.P.	-	P.
202	91739	S.L.Ppt.	L.	S.L.	"	L.	P.
203	91742	Ppt.	L.	-	L.	L.	P.
204	91777	"	L.	-	L.P.	L.	P.
205	91807	"	L.	-	L.	-	P.
206	91811	"	L.	-	L.	-	P.
207	91825	"	L.	-	L.	S.L.	P.

* = Heated-Plasma-Agar I.
** = Heated-Plasma-Agar II.
*** = Heated-Plasma Agar II + Fresh Serum.

T A B L E XVIII .

THE RESULTS OBTAINED BY GROWING 202 STRAINS OF COAGULASE POSITIVE STAPHYLOCOCCI ON HEATED PLASMA AGAR PLATES. (PLASMA HEATED AT 56°C. FOR 20 MINUTES.)

STAPHYLOCOCCI	NUMBER	RESULT ON HEATED PLASMA	
		Fibrinolysis	Precipitation
Strains producing Muller's phenomenon.	197	197	-
Strains not producing Muller's phenomenon.	5	-	5
TOTAL	202	197	5

TABLE XIX.

THE RESULTS OBTAINED BY GROWING 204 STRAINS OF COAGULASE POSITIVE STAPHYLOCOCCI ON HEATED PLASMA AGAR PLATES (PLASMA HEATED AT 65°C. for 30 MINUTES)

STAPHYLOCOCCI	NUMBER	RESULT ON HEATED PLASMA AGAR PLATES		
		Precipitation	Slight lysis	No change
Strains producing Muller's phenomenon.	199	1	36	162
Strains not producing Muller's phenomenon.	5	4	-	1
TOTAL	204	5	36	163

TABLE XX.

THE RESULTS OBTAINED BY GROWING 204 STRAINS OF COAGULASE POSITIVE STAPHYLOCOCCI ON HEATED PLASMA AGAR PLATES (PLASMA HEATED AT 65°C. FOR 30 MINUTES) WHICH CONTAINED ADDED FRESH SERUM.

STAPHYLOCOCCI	NUMBER	RESULTS ON HEATED PLASMA AGAR PLATES WITH FRESH SERUM.			
		Lysis only	Plaques & Lysis	Precipitation	No change
Strains producing Muller's phenomenon.	199	124	75	-	-
Strains not producing Muller's phenomenon.	5	-	-	4	1
TOTAL.	204	124	75	4	1

TABLE XXI.

PRODUCTION OF MULLER'S PHENOMENON ON A HEATED HAEMOGLOBIN SUBSTRATE CONTAINING FRESH HUMAN SERUM.

TOTAL NUMBER OF STRAINS OF *STAPHYLOCOCCUS AUREUS* GROWN.	201
NUMBER OF STRAINS PRODUCING MULLER'S PHENOMENON.	196
NUMBER OF STRAINS NOT PRODUCING MULLER'S PHENOMENON.	5
PERCENTAGE OF STRAINS PRODUCING MULLER'S PHENOMENON ON THIS SUBSTRATE.	97.6

TABLE XXII.

GELATINASE ACTIVITY OF 207 STRAINS OF STAPHYLOCOCCUS AUREUS.

\- = gelled. + = not-gelled.

	TIME AT 37°C.	4 days.		7 days.		10 days.		14 days.	
	TIME IN ICE-CHEST	30 min	3 hrs	30 min	3 hrs	30 min	3 hrs	30 min	3 hrs
1	88004	+	-	+	-	+	-	+	-
2	88512	...	...	...	...	...	...	...	...
3	88516	-	-	-	-	+	-	+	-
4	88518	+	-	+	+				
5	88536	+	+						
6	88541	+	-	+	+				
7	88544	-	-	+	-	+	-	+	-
8	88545	-	-	+	-	+	-	+	-
9	88546	-	-	+	-	+	-	+	-
10	88553	+	+						
11	88557	...	...	...	...	...	...	...	...
12	88561	+	+						
13	88592	-	-	+	-	+	-	+	-
14	88606	-	-	+	+				
15	88607	-	-	+	-	+	-	+	-
16	88614	+	+						
17	88665	+	-	+	-	+	+		
18	88669	+	+						
19	88670	+	+						
20	88671	+	+						
21	88673	+	+						

TABLE XXII (Continued).

	TIME AT 37°C.	4 days.		7 days.		10 days.		14 days.	
	TIME IN ICE-CHEST	30 min	3 hrs	30 min	3 hrs	30 min	3 hrs	30 min	3 hrs
22	88684	-	-	-	-	-	-	-	-
23	88686	+	+						
24	88715	+	+						
25	88722	+	-	+	-	+	-	+	-
26	88732	+	+						
27	88750	+	-	+	+				
28	88840	+	+						
29	88849	+	-	+	-	+	-	+	-
30	88863	+	-	+	-	+	-	+	-
31	88869	+	-	+	-	+	+		
32	88882	+	-	+	-	+	+		
33	88907	-	-	-	-	+	-	+	-
34	88917	...	...	...	...	...	...	...	...
35	88959	-	-	-	-	+	-	+	-
36	88965	-	-	-	-	+	-	+	-
37	88969	+	-	+	-	+	-	+	-
38	88971	+	-	+	-	+	+		
39	88991	+	-	+	-	+	+		
40	88997	+	-	+	-	+	+		
41	89014	+	-	+	+				
42	89018	+	+						
43	89030	+	-	+	+				
44	89061	+	+						
45	89065 WHITE	+	+						
	YELLOW	-	-	+	+				