The University of Glasgow

# C O A G U L A S E

## The production and mode of action of the plasma-clotting enzyme of *Staphylococcus aureus*

by

ROBERT B. MORRISON, B.Sc.
(Carnegie Senior Scholar)

Thesis submitted for the Degree of Ph.D.
in the Faculty of Science.

Department of Bacteriology October, 1952.



ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

# INDEX

## SECTION I

**The production of coagulase in laboratory media; isolation of variants of Staphylococcus aureus giving high and low yields of coagulase.**



(Experimental ..... p.53 et seq.).

## SECTION II

## The production of coagulase in chemically defined media.

(Experimental ..... p.79 et seq.)

## SECTION III

### The mode of action of coagulase



(Experimental ... p.145 et seq.)

## PREFACE

Any work on the synthesis of bacterial enzymes is faced at the outset by the truth embodied in the aphorism of Maurice Nicolle in his teaching at the Pasteur Institute: "The bacterial cell is a mosaic of antigens and enzymes." The pattern is so intricate even within a single species that any one investigator can hope to throw only a little light on a minute corner of this mosaic. In the work that forms the subject matter of this thesis interest is concentrated on a single enzyme, coagulase, of a single bacterial species, Staphylococcus aureus.

There are many reasons for choosing to investigate this particular enzyme. Most bacterial enzymes are common to a large number of species - coagulase is unique. It is produced only by Staph. aureus. Most bacterial enzymes act as small links in long chains of metabolic reactions - coagulase appears to have only one function, the clotting of animal plasma. Most bacterial enzymes are elaborated in an active form by the organism - coagulase is produced in an inactive state and requires for activation the intervention of a factor present in the plasma of animals. Finally, an additional interest attaches to this enzyme from the increasing emphasis now being placed on its rôle in establishing staphylococcal infections.

For these reasons it was decided to investigate conditions governing the formation and mode of action of coagulase, the plasma-clotting enzyme of Staph. aureus. The investigations fell into three main divisions: (1) factors affecting coagulase production in laboratory media, (2) factors affecting coagulase production in chemically defined media, and (3) the nature of the reaction by which coagulase is converted to its active form.

The work on coagulase production in chemically defined media and in laboratory media was done partly in collaboration with other members of the Department of Bacteriology. The work on the conversion of coagulase to active coagulase was done entirely in collaboration. Throughout the text bibliographic references indicate what was joint work.

## Introduction

# INTRODUCTION

## The discovery of coagulase

The earliest mention in the literature of the ability of Staph. aureus to cause blood to clot was in part an erroneous finding. When Loeb (1903) published the results of a number of experiments in which he tested the power of broth cultures of different organisms to clot goose plasma (spontaneously a very slow-clotting plasma) he reported that Bacillus typhosus, Corynebacterium diphtheriae, C. xerosis, streptococci and tubercle bacilli were without effect, that Bacterium pyocyaneum, Bacterium prodigiosum and Bacterium coli had some clotting power, and that Staph. aureus had very marked clotting power. He believed that the differences between Staph. aureus and Bact. pyocyaneum, Bact. prodigiosum and Bact. coli were differences of degree and did not realise that the ability of Staph. aureus to cause coagulation of blood was of a quite distinctive nature.

Loeb's erroneous results are now ascribed to the fact that he worked with goose plasma containing no anti-clotting agent. Goose and other fowl plasmas are known to remain fluid for a considerable time if kept in clean vessels free from extraneous particles which act as foci for clot formation. The bacterial cells may, in some cases, have acted as foci in Loeb's experiments. It is also possible that

the products of metabolism (formed during 24 hours' growth of the organism) could have caused a change in pH leading to coagulation. An instance of non-specific clotting of blood is that due to utilisation of the anti-clotting agent by bacteria. Thus the utilisation of citrate by bacteria present in citrated blood ultimately leads to clot formation (Lominski, Conway, Harper & Rennie, 1947; Harper & Conway, 1948).

Clotting of blood by Staph. aureus is quite distinct from the example mentioned above. It is a unique property of Staph. aureus and its discovery should properly be attributed to Much (1908). In a most thorough and extensive investigation of the clotting of blood by Staph. aureus he discovered a series of facts about the nature of the process which are substantially accepted to-day although some of them have been challenged by different workers during the past forty-four years. One feels, indeed, that a great deal more credit is due to Much as the discoverer of coagulase than to Loeb.

His attention was directed to the phenomenon by an observation that when Staph. aureus was added to human blood it caused it to clot, whereas streptococci, pneumococci, B. typhosus and B. coli did not. He worked with citrated blood and showed that although the speed of clotting was related to the amount of Staph. aureus added, the clot was

not due to the supply of calcium by the organisms or to the removal of citrate. With different strains of Staph. aureus the clotting power was found to vary, but with Staph. albus and with other bacterial species no clot formed. Much recognised that the mechanism of clotting by Staph. aureus did not involve thrombin. He showed that fibrinogen, which could be clotted by thrombin, was not clotted by Staph. aureus. The implications of this finding were not appreciated until 1944 when Smith & Hale (1944) showed that a co-factor was required. He also found that the product of Staph. aureus causing clot was neither prothrombin nor thrombokinase since, firstly, citrate did not impede the action of Staph. aureus and, secondly, purified fibrinogen + thrombokinase + Staph. aureus did not give a clot. Finally, he noted the presence of a fibrinolytic product of Staph. aureus and of clot inhibitors in plasma. His findings are summarised below.

1. The blood-clotting property of Staph. aureus is unique and is associated with the pathogenic (aureus) strains and not with the non-pathogenic (albus) strains.
2. Strains differ in regard to this property.
3. The blood-clotting mechanism of Staph. aureus is different from the normal clotting mechanism of blood.
4. The clotting agent is not prothrombin, thrombokinase or thrombin.

5. The clotting agent will not clot purified fibrinogen.
6. A fibrinolytic agent is sometimes associated with the clotting agent.
7. Clot inhibitors may be present in plasma.

Much did not, and obviously could not, fully interpret the results of his work, but from the perspective of present knowledge it is clear that he established most of the salient characters of the blood-clotting property of Staph. aureus. Many of the investigations of this subject during the past forty years have consisted of a verification and expansion of Much's findings. The growth of our knowledge of coagulase since these first experiments of Much forms the subject matter of the following pages.

Intra- or extra-cellular nature of coagulase

Kleinschmidt (1909) attempted to separate a clotting agent from cultures of staphylococci by Berkefeld filtration and concluded that the ability to clot plasma was associated with living cells since he found no activity in the filtrate. Gratia (1921) found activity in the filtrate from a culture of Staph. aureus, but since the cells had been lysed by bacteriophage the question of whether coagulase was confined to the cells during life or liberated into the medium remained unresolved. Gratia did not specify the type of filter used and, as will be seen later, this

point is of some importance. Gross (1927) found coagulase activity in cultures which had been passed through Seitz filters and confirmed this result four years later (Gross, 1931). Kemkes (1928), however, was unable to detect any activity in the liquid passing through Chamberland filters and concluded from this, and from the results of experiments using heat-killed staphylococci, that the coagulating property was associated with live staphylococci only. Gengou (1933) was able to demonstrate slight activity in filtrates of broth cultures with Seitz or Chamberland filters, but found that most of the coagulase in the original cultures had been lost in filtration. He noticed, however, that if defibrinated plasma was added to the culture medium most of the coagulase could be filtered without loss.

More definite evidence that coagulase was filterable, at least through Berkefeld filters, was provided by Vanbreuseghem (1934a) who found that coagulase would pass through a Berkefeld but not a Chamberland filter, and by Walston (1935) who found that it would pass through a Berkefeld but not through a Seitz filter. A possible explanation of the inability of some earlier workers to detect coagulase in Berkefeld filtrates is provided by Fisher (1936a) who found that the amount of coagulase passing through a filter differed from strain to strain and was apparently not related to the activity of the whole culture.

At this period the most that could be said for the results of filtration experiments was that they had shown that it was possible to filter coagulase through Berkefeld filters. A clear answer to the problem of whether coagulase was an intra-cellular or extra-cellular product of staphylococci had not been provided. Most of the experiments had been concerned with the qualitative demonstration of the presence of coagulase in filtrates and in those cases where activity was found in the filtrates it might have been due to coagulase liberated by cell autolysis.

Finally Smith & Hale (1944) proved conclusively that coagulase was an extra-cellular product of staphylococci. Using a Gradocol membrane of specific pore size (0.76 $\mu$) they were able to obtain a sterile filtrate as active as the original culture (see p.19). The discovery reported in this same paper that coagulase is produced by staphylococci in an inactive form which differs in properties from the active material formed in the presence of plasma explains why Gengou (1933) and later Lominski (1944) found the filterability of coagulase greatly increased by the addition of plasma.

Further work on the filtration of coagulase by Lominski & Milne (1947) showed that the amount passing the filter was greatly affected by the pH of the liquid, and Hale (1949) found not only that the pH affected the yield

but also that the type of filter largely determined whether coagulase was filterable or not. There was no loss on filtering through Berkefeld (V) candles and slight loss with sintered glass, but Seitz, Chamberland and Mandler filters completely retained coagulase.

To sum up, coagulase is an extra-cellular product of staphylococci, but its filterability largely depends on the conditions under which filtration is carried out.

Heat stability of coagulase

The first experiments on the effect of heat on coagulase were made by Gonzenbach & Uemura (1916). They found that the coagulating action of staphylococci was not dependent on the presence of living cells since cultures killed by heating were still capable of clotting plasma; prolonged heating (1½ hours at 90°C.), however, destroyed coagulase activity. However, four years later Gratia (1920b) found that suspensions of organisms from solid cultures lost their power of clotting plasma after being killed by heat. Kemkes (1928) also found that killing by heating destroyed coagulase and, as mentioned earlier (see p.8) used this result together with the results of his filtration experiments as a basis for asserting that the power to clot plasma is associated only with living staphylococci. This assertion was disproved by Gross (1931) when he demonstrated

that coagulase could survive heating for 1 hour at 90°C., a temperature incompatible with the survival of cells. Further proof of the relatively high thermostability of crude coagulase was provided by the experiments of Gengou (1933) in which coagulase survived heating for 20 minutes at 100°C. The disagreement about the heat-stability of coagulase was probably due to the fact that in some of the tests suspensions of organisms from solid cultures which contained little or no coagulase were used. Suspensions of organisms from solid media contain so little coagulase that clot generally forms only after growth has taken place and heating of the suspensions would prevent this growth.

Numerous workers since Gengou have confirmed his finding that coagulase is very heat-stable. Walston (1935) found no loss of activity after heating broth cultures for 15 minutes at 100°C. and very little loss after 30 minutes at 100°C. Fisher (1936a) found that the resistance of coagulase in broth cultures to heat depended on the strain, activity being completely lost after 5 minutes at 60°C. in some and retained after 30 minutes at 100°C. in others. Strain differences in thermostability have not, so far, been confirmed. Smith & Hale (1944) found that coagulase activity largely survived heating for 30 minutes at 100°C.; there was some loss, however, after 30 minutes at 80°C. and rapid inactivation of coagulase filtrates at 120°C. Walker,

Schaffer & Derow (1947) were also unable to secure complete inactivation even after 30 minutes at 100°C. Thus the relatively thermostable nature of coagulase appears to have been well established by a number of workers.

The problem of the heat-stability of coagulase assumed a new aspect with the work of Tager (1948b). He found that stability depends to a large extent on the protective action of materials present in culture media. Working with a purified preparation of coagulase dissolved in distilled water he found that 75% of the activity was lost after 30 minutes at 56°C. and over 90% after 30 minutes at 65°C.; over 99% of the coagulase was inactivated by heating for 15 minutes at 100°C. Even at the relatively low temperature of 37°C. a solution of purified coagulase in saline or distilled water rapidly lost activity although the same coagulase dissolved in 2% peptone showed no decrease in potency.

From Tager's results it would seem that the conception of coagulase as remarkably stable at temperatures of 100°C. or more requires to be revised; coagulase, like the majority of enzymes, is inactivated by heating to 100°C. but heat inactivation can be prevented by one or more constituents of common culture media such as peptone water.

Effect of pH on stability of coagulase

The first observation on the effect of pH on coagulase was made by Vanbreuseghem (1934c). He obtained the surprising result that there was a definite lowering of activity in a coagulase preparation kept at pH 7.5 for 48 hours as compared with a control maintained at pH 7.6 at the same temperature. Apart from this isolated observation and the report by Lominski & Milne (1947) the only other work on the effect of pH on the stability of coagulase appears to be that of Tager (1948b) which he carried out on a purified preparation. He found that coagulase was most stable between pH 4.5 and 7.0, and that there was rather greater loss between pH 2.5 and 4.0. On the alkaline side there was marked deterioration with complete loss of activity at pH 11.2 after 22 hours at 37°C. Although little work has been done on the stability of coagulase at different levels of pH some confirmation for Tager's finding is provided by the fact that coagulase can be purified and concentrated by a process involving acid precipitation at pH 4.0 and this treatment does not seem to cause great loss of activity (Walston, 1935; Tager, 1948b; Walker, Berow & Schaffer, 1948): Lominski & Milne (1947) obtained results which are in apparent contradiction to those of Tager. They found that crude coagulase survived heating for 45 minutes at 65°C. much better at a slightly alkaline than a slightly acid pH.

The key to the contradiction may lie in the different temperatures used, and the fact that Lominski and Milne worked with coagulase in culture media for their tests whereas Tager used pure coagulase dissolved in buffer. It is perhaps significant that Lominski and Milne found that coagulase became much more stable after filtration, some inactivating material having been held back by the filters.

Chemical reactivity of coagulase

Very little is known of the effect of different chemical reagents on coagulase and such knowledge as exists is mainly concerned with the inhibition or non-inhibition of coagulating activity. Tager (1943b) found that reducing substances did not interfere with the action of coagulase and that sodium thioglycollate was able to retard the deterioration normally observed when a solution of a purified preparation of coagulase was kept at 37°C. for 24 hours. If, however, Tager had used higher molar concentrations of reducing agents he would have found marked inhibition of coagulase activity (Lominski, 1943). Oxidising agents, on the other hand, were found to cause a rapid loss of activity. Finally, ascorbic acid caused a marked loss of activity which was prevented by the addition of cysteine; this destructive effect of ascorbic acid might be due to the formation of hydrogen peroxide in the course of its

breakdown (Barron, De Meio & Klemperer, 1936).

Penicillin appears to interfere with the action of coagulase (Mason, 1945; Agnew, Kaplan & Spink, 1947; Miale, 1949) although Walker, Berow & Schaffer (1948) did not find this to be so. Agnew et al. found that streptomycin was inhibitory and this was confirmed by Walker et al. who found that sulphathiazole, sulphadiazine and a number of fungal antibiotics do not interfere with the action of coagulase, but that propylene glycol and sodium azide are inhibitory.

Coagulase is not inhibited by hirudin, citrate and sodium fluoride (Much, 1908) oxalate (Gonzenbach & Uemura, 1916) cobra venom, chlorazol fast pink, hydroquinone and low oxygen tension (Walston, 1935) dilute phenol, potassium permanganate and trypaflavine solutions (Gengou, 1933) and heparin (Rigden, 1942). Farkas (1947) found inhibition by bromine and iodine at concentrations of 0.3% and 0.8% respectively.

It can thus be seen that such information as has been acquired from the use of oxidising and reducing agents, antibiotics and so forth consists of a number of detached observations which add little to our knowledge of coagulase. Except in the experiments of Tager (1948b) and Walker et al. (1948) the work was carried out with coagulase containing culture material; with such material experiments of this

kind could only be expected to yield information on interference or non-interference with the clotting process.

Purification of Coagulase

The first attempt to concentrate coagulase was made by Walston (1935). Using cell-free filtrates of staphylococcal cultures he found that coagulase could be precipitated by ethyl alcohol, ammonium sulphate and acetic acid. The precipitate did not represent a much purer material than the original culture, and in the most concentrated preparations that he could make there was little or no enhancement of activity; presumably coagulase had been lost or rendered insoluble by the treatment. Fisher (1936a) confirmed the finding that coagulase precipitated at an acid pH. Smith & Hale (1944) concentrated the Gradocol membrane filtrates from staphylococcal cultures by evaporating some of the water under reduced pressure at 60 - 70°C., and obtained a ten-fold increase in activity. In none of these experiments was there much purification of the product.

The results of the first attempts to achieve a highly concentrated and purified coagulase were published almost simultaneously by Walker, Derow & Schaffer (1948) and Tager (1948b). Walker et al. took as their starting material digest broth cultures of Staph. aureus from which coagulable proteins had been removed by heating. Coagulase was

separated and purified by a two-stage acid precipitation and freeze-dried to give a powder with a very high activity. This powder showed a coagulase activity per mg. of nitrogen about 1000 times greater than that of the original material. The only criterion of purity which they used was enhancement of activity per mg. of nitrogen; this was so considerable, however, that separation of coagulase from extraneous material must have taken place.

Tager's method of purifying coagulase (Tager, 1948b) was more complex than that of Walker et al. It was based on a comparison of the effectiveness of ethyl alcohol, acid and ammonium sulphate as precipitants, and involved acid and alcohol precipitation of coagulase with ammonium sulphate precipitation of impurities. About half the coagulase activity of the broth culture was lost during the seven stages of the separation, but since most of this could be accounted for in the various fractions discarded, and was not due to destruction of coagulase, Tager suggested that higher yields could be obtained by improvement of details. He also pointed out the desirability of applying the methods of Cohn et al. (1946) for fractionation of plasma and Pillemer's (1946) method for purification of bacterial toxins.

Tager's (1948b) purified preparation was a freeze-dried powder giving water clear to slightly opalescent solutions. This powder had a very high activity and con-

centrated solutions could clot plasma at a dilution of one part in several million. It seems reasonable to expect that elaborations and improvements of Tager's (1948b) method may yield coagulase of comparable purity to the crystalline enzymes. Although Tager (1948b) did not claim to have isolated a pure coagulase his very potent product is presumably the purest material so far made. It is from his preparation that most of the existing and admittedly inadequate information on the nature of coagulase has been derived.

The Nature of Coagulase

The first indication that coagulase was a substance of large molecular size was the finding that coagulase was not dialysable (Cross, 1928): oxalated rabbit plasma suspended in a collophane bag in a broth culture of Staph. aureus did not clot. A similar result was obtained by Vanbreuseghem (1932) who found that when oxalated plasma inoculated with Staph. aureus and contained in a collodion bag was immersed in sterile oxalated plasma the inoculated plasma clotted but the sterile plasma did not. Gengou (1933) confirmed this point and found in addition that coagulase could be exposed to dialysis for several weeks in a current of physiological saline without loss of activity.

Only one investigator (Walston, 1935) found coagulase able to pass through a dialysing membrane; he found that partly purified coagulase passed through the membrane, but that the coagulase in the unpurified culture filtrates did not. In view of Tager's finding that a much purer coagulase was not dialysable Walston's isolated result must be attributed to some accident of experiment such as a faulty membrane.

Smith & Hale (1944), having found that crude coagulase could pass through 0.76 $\mu$ membranes, but that less and less got through as the pore size decreased, suggested that coagulase was "particulate" in that it seemed to be associated with particles of fairly uniform size. This may have been so in their impure preparation, but experiments carried out on purified coagulase by Tager (1948b) did not confirm this view. Tager found that ultra-centrifugation at 39,000 r.p.m. for 3 hours did not give the sedimentation to be expected if coagulase were of fairly large particle size. Determination of activity at various levels in the centrifuge tube led him to conclude that in the ultra-centrifuge "the behaviour of coagulase is far more comparable to that of smaller molecules as of the order of proteins." (p.496).

Evidence for the protein structure of coagulase is partly based on the effect of digestive enzymes upon it, which has been investigated on several occasions. Thus,

Walston (1935) found that trypsin inactivated coagulase and Walker, Schaffer & Derow (1947) found that both trypsin and pepsin completely destroyed its activity. These authors pointed out that although their results indicated that coagulase contained peptide linkages its heat stability made it difficult to believe that coagulase activity depended on an intact protein structure. The discovery of Tager (1948b) that heat stability was a property of crude but not of purified coagulase removed this difficulty.

A possible source of error in the interpretation of these experiments lies in the fact that the pepsin and trypsin used were relatively crude preparations which, it is known, may contain other enzymes such as lipases and amylases (Sumner & Somers, 1947). In order to eliminate the chance that the destruction of coagulase might be due to extraneous enzymes Tager (1948b) used crystalline trypsin and chymotrypsin in addition to a number of other proteolytic enzymes. Both trypsin and chymotrypsin caused a rapid and almost complete loss of coagulase activity. The fraction of plasma containing the precursor of a fibrin-digesting enzyme together with its activator, streptokinase, caused only slightly less rapid and complete destruction. A curious finding which Tager (1948b) did not discuss was that the plasma fraction in absence of its activator was nearly as effective. A possible explanation is that the purified

coagulase contained staphylococcal fibrinolysin which is known to be an enzyme activator of identical properties to streptokinase. The presence of this activator in Tager's preparation in sufficient quantity to cause almost complete destruction of coagulase within ten minutes does not, however, seem very likely. The other enzymes used, crude papain and calf thymus peptidase, caused no destruction of coagulase. Recently Lominski, Smith & Morrison (1952) found that coagulase produced by one variant of a strain is rapidly inactivated by an enzyme produced by another variant of the same strain.

Tager (1948b) investigated a number of other characters of coagulase and his findings are summarised below. Purified coagulase was precipitated quantitatively at pH 3.8 - 4.0. Tests for carbohydrates were negative or doubtfully positive. The purified material contained the amino acid tyrosine. The nitrogen content of 14 - 16% was compatible with a protein composition. Electrophoresis revealed the presence of two mobile components indicating that absolute purity had not been achieved.

To sum up, the inadequate data outlined above, and the results of heat-stability experiments with purified coagulase, point to its protein nature. A tentative description of coagulase would be as follows:

Coagulase is a protein with an iso-electric point

of approximately pH 4.0: it is digested by a number of proteolytic enzymes with loss of activity: it is rapidly inactivated in watery solutions at temperatures above 56°C. and is unstable in dilute solutions unless protected by peptone: it is inhibited or destroyed by oxidising agents and a number of other substances of unrelated structure. It is also destroyed by an enzyme produced by staphylococci themselves.

This meagre list of properties emphasises that the one distinctive character of coagulase so far firmly established is its ability to clot blood plasma.

Coagulase as an in-vitro criterion of pathogenicity

The first account of the clotting of plasma by Staph. aureus (Loeb, 1903) gave no indication that the clotting mechanism of this species is specific and distinctive. Much (1908) found, however, that not only was this property of clotting plasma peculiar to staphylococci, but that it seemed to be limited to the pathogenic aureus group. Many years were to elapse before any attempt was made to use coagulase production as a criterion of pathogenicity or to investigate the part which it played in establishing staphylococcal infections. Thus twenty years later the authoritative textbook of Topley & Wilson (1929) did not

mention coagulase production as a characteristic property of Staph. aureus. Yet the ability to produce coagulase is now regarded as almost synonymous with pathogenicity. This emergence of coagulase as the most reliable single character for distinguishing between pathogenic and non-pathogenic staphylococci is largely due to the failure of other criteria to give a clear distinction between the two groups.

The earliest attempts at classification of the staphylococci were based mainly on the colour of the pigment produced. Thus the staphylococci were divided into the golden and white varieties by Rosenbach (1884) and an additional yellow variety was included shortly afterwards. The first of these varieties, Staph. aureus, included the pathogenic organisms causing suppurative lesions in man and animals; the second, Staph. albus, included the weakly pathogenic organisms found in the skin, hair, etc.; the third, Staph. citreus, consisted of non-pathogenic saprophytes. While it is true that the majority of pathogenic staphylococci are of the Staph. aureus species, there is a considerable range of pigment production among strains isolated from lesions. The difficulty in classifying borderline strains with pale or creamy pigment has resulted in a decrease in the emphasis placed on pigment in identifying pathogenic staphylococci. It has been shown, also, that aureus strains can give rise to albus variants

(Bigger, Boland & O'Meara, 1927) and that the two types are interconvertible (Geisse, 1914; Pinner & Veldrich, 1932).

The defects of the classification of staphylococci by the colour of the colony led to attempts to devise a system based on the presence or absence of two properties - haemolysin production and agglutinability by an immune serum (Neisser & Wechsberg, 1901; Kutscher & Konrich, 1904; Veiel, 1904). Otto (1903) and Baranyi (1926) found that haemolysin production was confined to strains from pathological sources, but Julianelle (1922) believed that all strains of staphylococci were capable of producing haemolysin. This observation has not been substantiated by subsequent workers. With regard to agglutinability, Otto (1903), Kutscher & Konrich (1904), Veiel (1904) and Klopstock & Bockenheimer (1904) all found evidence to support the belief that staphylococci might be divided fairly sharply into pathogenic and non-pathogenic types on the basis of their agglutinability by an anti-serum prepared against a strain of known pathological origin. The use of both these properties as a means of classifying staphylococci was proposed (Kutscher & Konrich, 1904); strains producing haemolysin and agglutinable by an anti-serum prepared against a pathogenic strain were to be regarded as pathogenic, whereas strains negative in both respects were to be regarded as non-pathogenic.

Another attempt at classification was made by Gordon (1903/4) based on the action of staphylococci on a wide range of sugars and other organic substrates. He found that of the 41 strains examined there were 23 different patterns of results. However, the six aureus strains tested gave almost identical results and all fermented mannitol and liquefied gelatin, whereas few albus strains showed these properties. But an extension of this work by Dudgeon (1908) showed that if a sufficiently large number of sugars was tested it was unusual to find two strains of staphylococci giving identical results. Thus no sharp division in biochemical activities corresponding to a sharp separation into pathogenic and non-pathogenic staphylococci emerged from this work. A subsequent extension of this type of investigation by Winslow, Rothberg & Parsons (1920) and Dudgeon & Simpson (1928) led them to conclude that Staphylococci consisted of one large group of organisms showing a wide range of biochemical activities. At one end there was the deeply pigmented, haemolytic, mannitol-fermenting, gelatin-liquefying, precipitin-producing, pathogenic Staph. aureus, and at the other end the white, non-haemolytic, mannitol-negative, gelatin-non-liquefying, feebly or non-pathogenic Staph. albus without precipitin formation in the subjects from which it had been isolated. In passing from the pathogenic to the non-pathogenic there was not a clear cut separation of characters;

it was not a case of black or white but of all shades of grey.

One difficulty of basing any classification on pathogenicity is the great variability of this property, and the different senses in which the term is used. With the staphylococci additional confusion is introduced by the use of the source of strain as a measure of pathogenicity; in most of the early work a strain was regarded as pathogenic or non-pathogenic according to whether it had been isolated from a suppurative lesion or healthy tissues. It is known, however, that pathogenic staphylococci are found on various tissues unaccompanied by lesions (Kemres, 1928). It is also thought that strains may exist in an avirulent state on tissues and only acquire virulence under suitable conditions. Indeed Pinner & Voldrich (1932) state that they succeeded in converting an avirulent Staph. albus to a virulent Staph. aureus by growing it for a long time in Staph. albus anti-serum.

The claim by the earlier workers that agglutination tests could be used to distinguish between pathogens and saprophytes has not been borne out by later work. Julianelle (1922), using agglutinin-absorption methods, found no correspondence between serological grouping and pathogenicity and Hine (1933) found only a rough correspondence between serological grouping, pigmentation and mannitol fermentation.

Julianelle carried out few virulence tests and Hine none at all so that the qualifications made in the previous paragraph apply to this work. There seems, however, to be some correlation between pathogenicity and the ability to stimulate precipitin formation and several workers have found this correspondence (Dudgeon & Simpson, 1928; Burky, 1933; Cowan, 1938).

Of the different methods for distinguishing between pathogenic and non-pathogenic staphylococci outlined above, haemolysin production has best stood the test of time. The failure of earlier workers to name the species of red corpuscle against which a haemolysin acted and their unawareness of the existence of different haemolysins acting differently on the red cells of different species may have prevented the recognition of the close relationship between $\alpha$-haemolysin and pathogenicity. Lately, however, there has been a tendency to restore $\alpha$-haemolysin to a key position (Marks, 1952). But, whatever the reason, coagulase production has tended more and more to be accepted as the most constant and easily demonstrable character of pathogenic staphylococci. An account is given in the following pages of the emergence of the coagulase test as the chief means of recognising pathogens.

For several years after Much (1908), work on coagulase was concerned mainly with the nature of coagulase and

conditions governing its production rather than the association between coagulase and pathogenicity. Gonzenbach & Uemura (1916), however, confirmed Much's finding that oxalated plasma from men and animals suffering from staphylococcal infections tended to clot within a few hours of withdrawal; they also found that five strains of Staph. aureus of different origin were able to clot plasma.

The first real attempt to correlate coagulase production with pathogenicity was made by Darenyi (1926). He collected a number of strains of staphylococci (110), including strains from pus, from the healthy skin and from the environment and tested them for their ability to haemolyse rabbit red cells, to clot milk and to clot plasma. Although all his strains of pathological origin produced haemolysin, a fairly high proportion of other strains did so too (20 to 70 per cent, depending on the source); a similar result was obtained with milk clotting. But with coagulase production he found complete correspondence between pathogenicity as measured by the source of the strain and the result of the test. None of the 30 strains from the environment or healthy skin clotted citrated rabbit blood, whereas it was clotted by all the 30 strains from pus, whether aureus or albus. Darenyi then compared the pathogenicity of the following three groups:- (1) Coagulase +ve, haemolysin +ve, milk clot +ve; (2) coagulase -ve, haemolysin +ve, milk clot +ve;

(3) coagulase -ve, haemolysin -ve, milk clot -ve. On subcutaneous injection into rabbits, group (1) gave a rapid and severe infection, group (2) a slower and less severe infection, and group (3) no infection. He concluded from his results that coagulase production was the character to which most importance should be given in deciding whether a strain was markedly pathogenic or not. His experiments are of interest not only because Baranyi was the first to study the association between coagulase and pathogenicity on a fairly large scale, but also because he carried out animal virulence tests on his strains instead of relying on their origin in classifying them.

The importance of coagulase was confirmed within a short time by two workers. Gross (1927), in an investigation in which gelatin liquefaction, coagulase production and haemolysin production were compared, came to the conclusion that these three properties were most closely associated with organisms from pathological sources and in doubtful cases allowed a decision about pathogenicity to be made within a short time. Kemkes (1928) found that the coagulase reaction was given only by pathogenic staphylococci and not by non-pathogens or other bacterial species and that the results ran parallel with haemolysin and animal virulence tests. Although a number of strains isolated from the skin and healthy mucous membranes were coagulase positive, animal

inoculation proved that they were pyogenic thus emphasising once more the danger of classifying strains on the basis of their origins. Kendres concluded that not only was the coagulase test a valid means of excluding non-pathogens, but that it enabled a definite decision on pathogenicity to be made. Cross (1931) examined 250 strains isolated from infected and healthy tissues in respect of coagulase production, haemolysin production and the ability to produce necrosis when injected intracutaneously into rabbits. He found marked necrosis with strains having high haemolysin and coagulase production, reddening and swelling without marked necrosis in strains with low haemolysin and coagulase production, and no pathological changes with strains which were haemolysin and coagulase negative. His classification, although crude, indicated the close relationship between coagulase production and pathogenicity.

An attempt to evaluate the relationship of coagulase and haemolysin production to pathogenicity was carried out on a very large scale by Chapman, Berens, Peters & Curcio (1934), who used 5000 strains from a variety of sources. Out of these 5000 strains they selected 690 which showed golden pigment (aureus type) and were either haemolytic or coagulase positive or both. Eighty-eight per cent were coagulase positive and 51 per cent haemolysin positive.

Having first screened the 5000 strains, Chapman et al. (1934) selected one group whose strains were coagulase positive and haemolysin negative and another group in which the strains were haemolysin positive and coagulase negative and carried out animal virulence tests with about 7% of the strains. The results indicated that either coagulase or haemolysin production implied pathogenicity in aureus strains, but with albus strains coagulase production was more closely related to pathogenicity than haemolysin production (14/16 coagulase +ve haemolysin -ve strains killed a rabbit within 10 days, but only 4/17 coagulase -ve haemolysin +ve strains did so). Since only a small proportion (about 7%) of the strains with the selected properties were tested in animals, too much weight should not be given to the results, but they indicate that coagulase production may be a better guide to pathogenicity than haemolysin production.

Another comparison of a number of different characters of staphylococci including coagulase was made by Cruikshank (1937). In addition to confirming previous observations that coagulase and haemolysin production were not necessarily associated characters of a strain, he showed that coagulase and haemolysin could be distinguished by their different heat resistance and the preferential absorption of haemolysin by rabbit red cells.

Cruikshank divided a number of strains of pathological

and non-pathological origin into three groups:-
(1) pathogenic aureus strains, (2) albus variants of the pathogenic aureus strains, and (3) non-pathogenic albus strains. Although a higher proportion of strains of pathological than of non-pathological origin fermented mannitol and lactose, clotted milk, and liquefied gelatin, a sufficient number of saprophytic strains possessed these properties to make their use for differentiation unreliable: 40/40 of the strains of pathological origin produced coagulase and 38/40 haemolysin; all of the 20 saprophytic strains were negative in both respects. Pathogenicity tests on rabbits gave 21/24 positive results for the strains of pathological origin and 0/12 for the saprophytic strains. He concluded that coagulase production was the most constant property associated with pathogenicity and because of its constancy and ease of demonstration recommended its use for differentiating pathogenic from non-pathogenic staphylococci.

In an extensive investigation of 243 strains Chapman, Berens, Wilson & Curcio (1938) reached the same conclusion as Cruikshank. They compared their strains for coagulase production, haemolysin production on rabbit blood agar, pigmentation, source of strains, growth on crystal violet agar (Chapman & Berens, 1938), growth on brom-thymol blue agar and phenol red mannitol agar (Chapman, Lieb & Curcio, 1938), and rabbit pathogenicity. Their conclusions were

that it was possible, provided certain details of technique were observed, to correlate in-vitro tests, the source of the strains and their pathogenicity towards rabbits, and that the coagulase test was the most reliable single criterion.

An observation which they made provides a possible explanation of discrepancies which other workers had found between in-vitro tests and animal pathogenicity. Chapman et al. (1938) discovered that a number of their stock strains of undoubted pathological origin had dissociated into variants of widely differing pathogenicity and that a decrease in pathogenicity was associated with the loss of such characters as haemolysin production, pigmentation, etc. with coagulase production as one of the last to disappear. They emphasised the need for selecting haemolysin-positive colonies, since this was one of the first characters to be lost, before applying pathogenicity tests. In addition to accepting coagulase production as the most reliable differentiating test, they concluded that the extent of haemolysis on rabbit blood agar when taken in conjunction with coagulase production and pigmentation of a strain, or variants of a strain, provided an indication of the degree of pathogenicity.

To quote only a few of a very large number of authors, Burky (1933), Flaum (1938), Moss, Squires & Pitt (1941), Fairbrother (1940) and Dienst (1942) produced additional

evidence of the close association of coagulase production and pathogenicity in staphylococci. It thus would seem to have been soundly established by numerous workers that coagulase production is the most constant and reliable single criterion that can be used in distinguishing between pathogenic and non-pathogenic staphylococci.

There appears to be a very close association between coagulase and ∝-haemolysin production and in a recent paper Marks (1962) suggests that ∝-haemolysin production is an even better indication of pathogenicity than coagulase. He also suggests that the claims of other workers that they had found coagulase-positive, haemolysin-negative strains which were pathogenic were often due to the source of the strain being used as a criterion of pathogenicity and that such strains might well be adventitiously present in lesions. Unfortunately the experimental evidence which he brings forward to support his belief in the superiority of ∝-haemolysin to coagulase production as a criterion of pathogenicity is open to exactly the same criticism.

The rôle of coagulase in staphylococcal infections

Although much work has been devoted to the study of coagulase production by pathogenic staphylococci a great deal less attention has been given to the part which it plays in staphylococcal infections. The first suggestions

were based on its in-vitro behaviour, and Gross (1933), Gengou (1933) and Cruikshank (1937) thought that it might be responsible for the formation of thrombi. Gross (1933) found that the injection of a cellfree filtrate of staphylococcal cultures into a tied-off segment of the jugular vein of rabbits caused intravascular clotting and the formation of thrombi. Kellaway, Burnet & Williams (1930) and Fisher (1936a), however, could not obtain intravascular clotting and Fisher did not find any significant amount of thrombosis in 27 autopsies of cases of staphylococcal septicaemia.

A striking feature of staphylococcal infections as opposed to streptococcal infections is their tendency to produce localised lesions consisting of the traditional "good and laudable pus". An attractive deduction to be drawn from the in-vitro clotting of plasma is that the production of coagulase leads to the enclosure of the infecting organisms in a fibrin clot which prevents spread through a vascular system. Following the work of Menkin (1933; 1934) Menkin & Walston (1934/35) took up this conception and after experimental investigation rejected it. Menkin had shown that Staph. aureus produced a powerfully injurious reaction in tissues around the site of inoculation. Injuries to the capillaries increased their permeability and allowed

fibrinogen to pass through into the inflamed area and within one hour of injection the draining lymphatics could be occluded by the formation of fibrinous thrombi. Berkefeld filtrates of Staph. aureus cultures produced the same result. It was found that when the dye, trypan blue, was injected into such an area it failed to diffuse into the lymphatic vessels in marked contrast to the results obtained when streptococci were the organisms used. It was concluded that the staphylococcus was primarily a non-invading organism owing to its intense local effects which caused it to be circumscribed quickly by the mechanical barrier of a fibrinous mesh and by the thrombosis of the lymphatics.

In order to determine whether coagulase played a significant part in this localising process Menkin & Walston made a preparation by acid precipitation of filtered broth cultures of Staph. aureus. They found that this acid-precipitated coagulase retained its clotting power but did not fix trypan blue in tissues. They also found that broth-culture filtrates which showed no coagulating powers were still able to fix trypan blue and that filtrates with marked coagulating powers were unable to fix the dye. From this they concluded that coagulase played no rôle in inducing prompt mechanical obstruction to lymph flow and that this obstruction was due to the powerfully necrotising action of other products of Staph. aureus.

Cruikshank (1937) put forward a hypothetical scheme in explanation of the course followed by staphylococcal infections. The sequence of events postulated was as follows:- (1) Implantation of staphylococci; (2) inflammatory reaction with stagnation of blood flow; (3) concentration of coagulase built up with resultant thrombosis in the small blood vessels; (4) gradual solution of the thrombus by fibrinolysin produced by the staphylococci; and (5) dissemination of small infective emboli leading to the formation of pyaemic abscesses in the lungs, kidneys, etc.

This scheme attempted to account for the fact that although staphylococcal infections tend to be localised, dispersion throughout the host may ensue in the shape of pyaemic abscesses of different organs. The assumption that fibrinolysin played a part in staphylococcal infection was no doubt prompted by the frequency with which it is found along with coagulase. In fact, so close is the association that Gengou (1933) regarded the formation of a clot and its subsequent lysis as different stages of the same process.

The understanding of the part played by coagulase in staphylococcal infections was greatly enlarged by the work of Hale & Smith (1945) who showed that coagulase inhibited the phagocytosis of staphylococci in vitro. When a mixture of staphylococci and coliforms is exposed to phagocytes in coagulable plasma only coliforms are phagocytosed. When

non-coagulable plasma is used phagocytosis of staphylococci takes place unless coagulase activator (see p.46) is added. They suggested that the formation of a fibrin envelope round the organisms may prevent opsonisation or nullify the changes to the cell surface accompanying opsonisation, and that agglutination of staphylococci followed by the formation of compact masses embedded in a fibrin matrix might form a mechanical obstacle to phagocytosis.

In more general terms Smith & Hale interpreted their results in the following way: they considered that coagulase confers on staphylococci a resistance against the host's first line of defence, the phagocytes, and enables the infecting organisms to elaborate their toxic products. They also drew the conclusion that susceptibility of a species to infection by staphylococci is related to the coagulability of the plasma.

An in-vivo attempt to demonstrate the validity of the above hypotheses was carried out by Smith, Hale & Smith (1947). They found that the minimum lethal dose in terms of the number of cocci inoculated intracardially into guinea pigs was four to eight times greater with strains which did not clot guinea-pig plasma than with strains which did. The distribution of the lesions in the internal organs of the two sets of animals indicated that invasiveness was also dependent on coagulase production.

In a second set of experiments Hale & Smith attempted to demonstrate *in vivo* the finding that the phagocytosis of staphylococci in a menstruum containing incoagulable plasma is inhibited by rendering it coagulable. The animals used in this series of experiments were mice, whose plasma is not clotted by coagulase. One set of animals was inoculated intraperitoneally with a coagulase-positive strain of *Staph. aureus* in a coagulable menstruum and another set with the same strain suspended in an incoagulable menstruum. It was found that the cocci injected in the coagulable menstruum showed an initial decrease in number followed by a rapid increase so that by the end of 24 hours the peritoneum contained a very large number of organisms both free and intracellular. The cocci injected in an incoagulable menstruum progressively decreased in number so that by the end of 24 hours they were virtually absent from the peritoneum.

A similar set of experiments was carried out with a view to finding out whether there was any difference in virulence between *Staph. aureus* pre-treated with human or rabbit plasma and the same strain when pre-treated with fibrinogen-free serum. The results, though not clear-cut, indicated that a coagulable menstruum enhanced the virulence of the organisms.

In the same study Smith, Hale & Smith investigated the relative importance of coagulase and $\alpha$ -haemolysin in

staphylococcal infections. They compared the infections produced in guinea-pigs by strains which were able to clot guinea-pig plasma (g.p. coagulase +ve) with strains which could not (g.p. coagulase -ve). It was found that strains producing ∝-haemolysin which were g.p. coagulase -ve produced less marked pathological changes at the site of inoculation, the testes, than strains which were g.p. coagulase +ve and produced no ∝-haemolysin. Intradermal inoculation of guinea pigs produced some surprising results. G.p. coagulase +ve strains with little or no ∝-toxigenicity gave lesions comparable to those obtained with highly ∝-toxigenic strains which were g.p. coagulase -ve. One would expect that a relatively large inoculation of a toxigenic organism into a dense tissue like the skin should produce necrosis, but not that the g.p. coagulase +ve strains producing little ∝-toxin should do so. In this connection the findings of Chapman, Berens, Wilson & Curcio (1939), which were described in an earlier section, may be remembered. They showed that variants of staphylococci arise in laboratory cultures which lack one or more of the characters of the parent pathogenic strain and that ∝-haemolysin production is more easily lost than coagulase production. This raises the possibility that the strains used by Smith, Hale & Smith were ∝-haemolysin -ve variants, and that animal inoculation restored ∝-toxigenicity; unfortunately no

mention is made of tests on organisms recovered from the lesions produced in the experimental animals.

The work of Smith, Hale & Smith has been described at some length since it is the main contribution to the understanding of the part played by coagulase in staphylococcal infections. These authors did not claim that coagulase was the only or even the most important factor in the infective process, but they undoubtedly demonstrated its importance. Allowing for the criticism that they used experimental animals not normally susceptible to infection with Staph. aureus, there seems to be a good deal of evidence for the view put forward in this and a previous paper (Hale & Smith, 1945) that the main rôle of coagulase in infection is the provision of a protective barrier against phagocytosis by virtue of its action on fibrinogen. It is also possible that the occurrence of lesions in other parts of the body, through the transport of infective emboli, is determined by coagulase.

The only subsequent work which throws light on the rôle of coagulase in infection is that of Lominski (1949). Lominski & Roberts (1946) had shown that human plasma contained a substance which inhibited the clotting action of coagulase and that this inhibitory substance was present in greater amount in the blood of healthy individuals than in patients with major staphylococcal infections. Lominski

found that rabbits which had been given a single intraperitoneal injection of an inhibitory human serum survived intravenous inoculation with a virulent strain of Staph. aureus, whereas rabbits without the protective dose of human serum died. The protection depended on the coagulase-inhibitory and not the anti-$\alpha$-haemolysin titre of the four sera tested. Lominski points out that although variation in the amount of activator in the blood of different species might account for variation in species susceptibility to staphylococcal infection, it cannot account for differences in susceptibility within a single species such as man where the level of activator is uniformly high.

Mode of action of coagulase

This aspect of the subject will be treated in greater detail in a later section, but an outline of the development of our knowledge of the clotting process is given below.

It will be recalled that the classical experiments of Much (1908) showed that the clotting of blood by Staph. aureus differed from the normal clotting process. Coagulase was neither prothrombin, thrombokinase nor thrombin; it acted in the absence of free calcium ions and did not clot purified fibrinogen. In addition, some factor which was lost in the purification of fibrinogen seemed to be necessary for staphylococcal clotting.

Much's experiments were crude in the sense that live staphylococci were used and some of his negative results might be attributable to lack of growth: nevertheless his findings remain substantially true.

There was a lapse of some years before this problem again attracted attention, but from 1919 to 1921 Gratia published a series of papers on the action of staphylococci on plasma. In his first paper Gratia (1919a) found the behaviour of staphylococci analogous to other bacterial species in rendering oxalated rabbit plasma more easily clottable on re-calcifying. In his next paper, however (Gratia, 1919b), he altered his views; he distinguished between thrombin and coagulase by showing that staphylococci could clot plasma in the presence of hirudin which interfered with the action of thrombin.

The conception that staphylococci clotted blood by a mechanism independent of thrombin of which the exponents were Much and Gratia was challenged by Wolf (1919a, 1919b) who suggested that the action of staphylococci was merely the kind of thromboplastic activity shown by other bacterial species and required the agency of thrombin. Wolf was probably right in saying that with the crude methods of purification used it was unlikely that Gratia's preparation had been completely deprived of the generators of thrombin - prothrombin and thrombokinase. However, Gratia (1920a,

1920b, 1920c) after a series of complex experiments involving fibrinogen believed to be free from prothrombin, thrombokinase and thrombin and the use of differential inhibition of coagulase and thrombin re-affirmed his belief in the distinctive nature of the two clotting mechanisms.

The problem remained in statu quo until it engaged the attention of Gengou (1933) who made a comprehensive study of the mode of action of coagulase. His starting point was the conflicting evidence on whether staphylococci clot or do not clot purified fibrinogen. Gengou's first step was to demonstrate that coagulase could be produced in the absence of fibrinogen. He next showed that staphylococci gave no clot either when grown in a medium containing fibrinogen or when fibrinogen was added to a broth culture. Clot formed when plasma was added even when the plasma had been freed from fibrinogen and the precursors of thrombin. In fact, Gengou demonstrated that the clotting of fibrinogen by staphylococci required the intervention of some other factor present in plasma. His interpretation was wrong; he thought that when no clot formed this was due to lysis. The action of the defibrinated plasma was believed to be due to its containing lysis inhibitors and, in fact, he showed that serum albumin and globulin were lysis inhibitors.

Vanbreuseghem (1934b) took up the question of the relation of coagulation to lysis. He used the plasma of

guinea-pigs which is not normally clotted by staphylococci and found that although it did not normally clot at 37°C., clot formed at temperatures below 20°C. In order to clot at 37°C. a weak preparation of coagulase had to be used or human serum albumin or globulin added. He assumed, along lines of reasoning similar to those of Gengou, that lysis was slowed down at temperatures below 20°C. and inhibited by albumin or globulin. His interpretation was wrong since guinea-pig plasma which has remained unclotted after a prolonged period at 37°C. will clot if the temperature is reduced to 20°C. (Smith & Hale, 1944). Smith & Hale provided a partial explanation of this anomalous behaviour of guinea-pig plasma (see p.46) but despite further work by Tager & Hales (1948b) this complex problem remains unresolved.

There is no doubt that coagulase and fibrinolysin are frequently associated; Fisher (1936b) found that 24 of 26 coagulase +ve strains produced fibrinolysin, Christie & Wilson (1941) 92 of 99 and Chapman (1942) 62 of 66. The fact that there is a small proportion of coagulase +ve strains which produce no fibrinolysin seems to rule out the possibility that coagulase and fibrinolysin are identical, and a clear separation of the two activities was shown by Gerheim, Ferguson, Travis, Johnston & Boyles (1948). They found that the relative proportions of coagulase and

fibrinolysin in the alcohol precipitates from broth cultures of Staph. aureus differed from strain to strain.

Little knowledge of the mode of action of coagulase had emerged from the work just described except in the negative sense that coagulase appeared to act in a different manner from the normal clotting mechanism of blood. Smith & Hale (1944) at last were able to bring some order into this confused subject. They showed that coagulase by itself is inactive but that by reacting with a factor present in some plasmas and deficient or lacking in others it is converted to active coagulase, a thrombin-like substance. The reaction of coagulase with activator to give active coagulase was shown to be analogous to the conversion of prothrombin to thrombin by thrombokinase except that calcium ions are not required. They found that plasmas not normally clotted by coagulase (guinea-pig, mouse and fowl) could be made easily clottable by the addition of a small quantity of activator in the form of human testis extract. A purified fibrinogen solution which remained unclotted in the presence of coagulase gave a rapid clot on the addition of activator. In this same paper the theory that the peculiar behaviour of guinea-pig plasma was due to the fibrinolytic properties of coagulase was disposed of. Smith & Hale found that a mixture of guinea-pig plasma and coagulase which had remained fluid for 24 hours at 37°C. clotted when placed at 20°C. and

believed that the explanation lay in the rate of destruction of active coagulase exceeding the rate of formation at the higher temperature.

Thus Smith & Hale showed that coagulase as produced by staphylococci is inactive, that it is converted to its active form by reaction with an activator present in some plasmas but deficient or totally lacking in others, and that the non-coagulability of the plasmas of some species is due to deficiency of activator or conditions which prevent the accumulation of sufficient active coagulase. This discovery of the existence of two forms of coagulase explains the discrepant results obtained by some earlier workers in regard to the physical properties of coagulase. Coagulase prepared in a medium containing plasma was really active coagulase which is more filterable and more easily destroyed by heating than the inactive coagulase formed in media without plasma.

The above account covers only the period up to the time of Smith & Hale (1944). Later developments are discussed in Section III which describes experiments undertaken with the object of elucidating the activator/coagulase reaction.

Antigenicity of coagulase

Much (1908) mentioned the presence of clot inhibitors in human plasmas and several others have since confirmed this observation (Gross, 1933; Walston, 1935; Smith & Hale, 1944; Lominski & Roberts, 1946; Tager & Hales, 1948a). The clot inhibitors were found only in occasional specimens of plasma and were usually effective to a very low titre. Other workers (Kemkes, 1928; Sudhues & Schimrigk, 1933) were unable to demonstrate any retardation of clotting in plasmas of patients with staphylococcal infections even when the sera showed a high anti-haemolysin titre. Cruikshank (1937) found that the plasmas of two patients with staphylococcal infections gave normal clotting times and that four patients, after a course of staphylococcal toxoid showed a rise in anti-haemolysin titre but no increased resistance to clotting by coagulase. Gross (1933), on the other hand, found that anti-staphylococcal rabbit sera were capable of inhibiting the clotting action of coagulase, but only to a low titre.

Various attempts to immunise animals to coagulase have met with slight success. Gross (1931), Sudhues & Schimrigk (1933), Walston (1935) and Smith & Hale (1944) were unable to obtain any marked antibody formation in experimental animals. Thus it seemed that although coagulase inhibition could occasionally be found in human plasmas the antigenicity of

coagulase remained in doubt. The inhibitors had not been found to be significantly higher in the plasmas of patients suffering from staphylococcal infections, and animal experiments showed that coagulase had little if any ability to elicit antibodies.

A new aspect of the problem emerged, however, in the results of an investigation of the occurrence, nature, and mode of action of inhibitors carried out by Lominski & Roberts (1946). They demonstrated the presence of an inhibitory substance in the sera of a large proportion of people (212 of 348). The infrequency with which clot inhibitors had been found by previous workers was explained by two findings; firstly, coagulase was shown to have a greater affinity for fibrinogen than for inhibitor, and secondly, the use of live staphylococci or highly potent preparations of coagulase caused the inhibitory effect to be swamped. The rarity of clot inhibition by plasma is therefore to be expected and in fact Lominski & Roberts found that only a small proportion of the plasmas examined inhibited clotting. The incidence of low- or non-inhibitory sera was greater in patients suffering from acute major staphylococcal infections than in healthy people. They showed that inhibitor possessed a number of antibody characteristics but did not definitely accept it until successful immunisation in man or animals had been achieved.

Such conclusive evidence of antibody formation in response to coagulase was provided by Tager & Hales (1948c). They were able by the use of a highly purified and potent coagulase in the presence of staphylococcal ∝-haemolysin as a potentiating agent to antibody formation, to achieve a definitely increased inhibitory titre in the sera of immunised animals. Complement-fixation tests and agglutination of collodion particles coated with coagulase provided additional evidence of antibody formation.

Further work by Rammelkamp and his associates seems to have established beyond doubt that coagulase is antigenic but only in respect of certain animals. Rammelkamp, Badger, Dingle, Feller & Hodges (1950) were unable to immunise rabbits, ducks, chickens and geese to coagulase, but with monkeys they found a striking rise in anti-coagulase activity after a course of injections of either coagulase or active coagulase. Further experiments seemed to show that not only was coagulase antigenic but that several coagulases could be distinguished serologically (Rammelkamp, Hezebicks & Dingle, 1950). Coagulase was prepared from three strains of staphylococci and a large number of human sera were tested for inhibitory action against each coagulase. The inhibitory titre of the sera was found to depend on the coagulase used in the test. Neutralisation experiments with human sera and the sera from immunised monkeys confirmed the distinct anti-

genic nature of the three coagulases. Despite some cross-reaction it was found possible to absorb, specifically, the material inhibitory to one of the three coagulases without altering the inhibitory titre to another. The existence of specific coagulases of Staph. aureus might be used to explain the apparent absence of antibodies to coagulase in the sera of patients recovering from staphylococcal infections on the assumption that the antibodies present were not specific to the coagulase used in testing. It seems unlikely, however, that the correlation observed by Lominski & Roberts between susceptibility to staphylococcal infections and lack of inhibitor in the patients' sera can be accounted for in this way. This year Duthie & Lorenz (1952) produced coagulase antibody formation even in rabbits by the injection of coagulase adsorbed on aluminium phosphate; like previous workers, they did not get a marked antibody response when coagulase was injected by itself.

Although the ability of coagulase to elicit antibodies and the existence of antigenically distinct coagulases can be regarded as established, there are a number of problems still unresolved. If inhibitor is a coagulase-antibody, why is antigenicity so difficult to demonstrate in experimental immunisation? If inhibitor is not an antibody, to what category of substance does it belong? These questions still await a convincing answer.

## SECTION I

**The production of coagulase in laboratory media; isolation of variants of Staphylococcus aureus giving high and low yields of coagulase.**



(Experimental ..... p.53 et seq.).

**The production of coagulase in laboratory media: isolation of variants of STAPH. AUREUS giving high and low yields of coagulase.**

In order to study the influence of nutrients on the synthesis of coagulase by Staph. aureus it is important to have strains which give high and regular yields of coagulase. But as Tager & Hales (1947) pointed out, under prolonged cultivation a strain may undergo profound changes in its capacity to produce coagulase. Laboratory strains often deteriorate and less often suddenly improve, and the variability cannot be accounted for by changes in the conditions of culture since it occurs even when these conditions are carefully controlled and the same batch of medium or a chemically defined medium is used.

The difficulties which this irregular behaviour caused in work on the synthesis of coagulase led to the investigation of a number of laboratory cultures of Staph. aureus described below. It was felt that a knowledge of the conditions giving rise to fluctuations in the coagulase-producing powers of a strain might explain inconsistent results and perhaps enable consistency to be achieved.

One of the first points to be investigated was whether all the organisms of a culture of Staph. aureus produced the same amount of coagulase. It soon became evident that wide variations existed and in the following

pages a description is given of some of the characters of high- and low-coagulase-producing variants and of methods for isolating them.

## Materials and Methods

Strains. Ten strains of coagulase positive Staph. aureus were used; they were selected because they gave high yields of coagulase. Two were one-year-old laboratory strains and the other eight were recently isolated.

Media. Three media were used: (1) horse-heart extract broth, (2) "Lab-lemco" broth agar, and (3) horse-heart digest-broth agar with 5% of oxalated horse blood. Medium No.3 was used either unheated or heated to 80°C. for 10 minutes; the pH of the three media was between 7.2 and 7.5.

Plasma. Human plasma containing 0.6% sodium acid citrate and 0.4% glucose was used, care being taken to use the same batch when coagulase production of variants was compared.

Estimation of coagulase. Four ml. of broth in 4 x 1/2 in. test-tubes were inoculated and incubated at 37.5°C. for four days. These details are important because it was found that the degree of aeration and the surface to volume ratio of the cultures influenced coagulase production. Two methods of coagulase estimation were used: (1) a clotting-time measurement, and (2) titration.

(1) Clotting-time measurement. 0.5 ml. of culture

was added to 0.5 ml. of a 1 in 5 dilution of plasma in 0.85% saline in a 4 x 1/2 in. test-tube. The tube was gently rocked through 45° from the vertical and the time taken for clot to form was noted.

(2) Titration. To 0.5 ml. of serial doubling dilutions of the culture in 0.85% saline were added 0.5 ml. of a 1 in 5 dilution of plasma in merthiolated saline, the final concentration of merthiolate being 1 in 1000. The highest dilution of the cultures to show clot after 24 hours at 37.5°C. was noted and recorded as the coagulase titre.

## Isolation of variants

The method was originally devised in order to find out whether the individual organisms in a culture of Staph. aureus were identical in regard to coagulase production. Accordingly, broth cultures were plated, and from a number of colonies, usually 24, broth cultures were again made. After 4 days' incubation these were tested for coagulase by measurement of clotting times. When differences in clotting speed were found the cultures with the highest and lowest clotting times were again plated, colonies picked into broth and the broth--plate--broth cycle repeated several times with the aim of selecting the best and the worst coagulase producers. At first the colonies were picked at random but it was soon realised that the fast-

clotting and the slow-clotting variants could be recognised by their characteristic growth in broth and colonial morphology, particularly on blood agar and heated-blood agar. The following association of coagulase production and growth characters was found: the fast-clotting variants gave granular growth in broth and colonies of varying degree of roughness, whereas the slow-clotting variants showed uniform turbidity in broth and gave smooth colonies, typical of Staph.aureus. Consequently growth characters became the guide in the selection of variants.

At this stage it was recalled that Bigger, Boland & O'Meara (1927) had found that rough colonies of Staph. aureus may readily be obtained by plating old broth cultures. Since the fast-clotting variants were of the rough type and since it was noticed that rough variants could be detected in young broth cultures by the granular appearance of the broth and the presence of pellicle, the following method for the isolation of rough variants was evolved. Young broth cultures showing granularity, or broth cultures which had been allowed to stand for 1 - 8 weeks, were plated and checked for the presence of rough (R or fast-clotting) and smooth (S or slow-clotting) variants. These colonies served as starting material for broth cultures with which the experiments on coagulase production were carried out.

# RESULTS

## Description of variants

The experiments demonstrated the existence in the 10 strains of variants differing considerably in coagulase production and growth characters; a description of their distinguishing features is given below.

The fast-clotting variants giving high yields of coagulase showed a variable degree of roughness in their growth; in 24-hour broth cultures the liquid might be almost clear or more or less turbid but it always contained coarse granules visible to the naked eye. There was always a pellicle with early ring formation and a heavy deposit which was viscid on shaking and had an irregular edge (Fig.1, p.60). On solid media the colonial morphology showed a gradation in R characters. On blood agar or heated-blood agar the fast-clotting variants of some strains were smaller, more opaque and more convex than the typical smooth colony of Staph. aureus (Fig.5, p.64); it was only when picked into broth that they revealed their rough character. When touched with a wire these colonies were coherent and could be shifted as a whole on the surface or lifted entire on the loop; they often had an irregular outline and dry appearance. On heated-blood agar the rough characters were more marked. Finally, four of the strains frequently gave typical R colonies (Figs.2 and 3,

pp.61,62) resembling those described by Bigger, Boland & O'Meara. The rough characters became less marked with age and after 48 hours colonies of the fast-clotting and slow-clotting variants might be indistinguishable. Haemolysis did not appear tobe correlated with R--S variation. On occasion, after 48 hours' incubation, a slight clearing of heated-blood agar around the colonies was noted. Figs.2-5 (pp.61-64) show the different colonial appearances associated with the fast-clotting and slow-clotting variants.

The slow-clotting variants giving low yields of coagulase showed growth of the smooth S type generally associated with Staph. aureus. In 24--48 hours' broth cultures there was uniform turbidity with a little smooth sediment which could easily be shaken up; no pellicle or ring formation in these young cultures was observed (Fig.1,p.60). On solid media 24-hour colonies were circular, slightly convex, smooth, glistening and entire (Figs.4 and 5, pp.63,64). The clearing of heated-blood agar occasionally noted around 48-hour colonies of fast-clotting variants was much more frequent and extensive with the smooth variants.

From one strain a smooth variant was isolated which produced no coagulase after 72 hours' growth in broth. The results of phage-typing described below show that it was a variant and not an accidental contaminant.

The characters of the fast-clotting and slow-clotting

variants and the association of R and S variation with coagulase production are summarised in Tables 1 and 2 (pp.65, 66).

The question arose whether the difference in coagulase production between the two variants was due to a difference in growth. The results shown in Table 3 (p.67) indicate that the amount of coagulase produced by the R as compared with the S variant is not proportionate to the difference in their growth.

On prolonged incubation the cell count of S variants sometimes rose and approached that of the R, but coagulase production lagged behind. Whenever older cultures of S variants showed a rise in coagulase content, the broth had become granular and R variants could be found on plating.

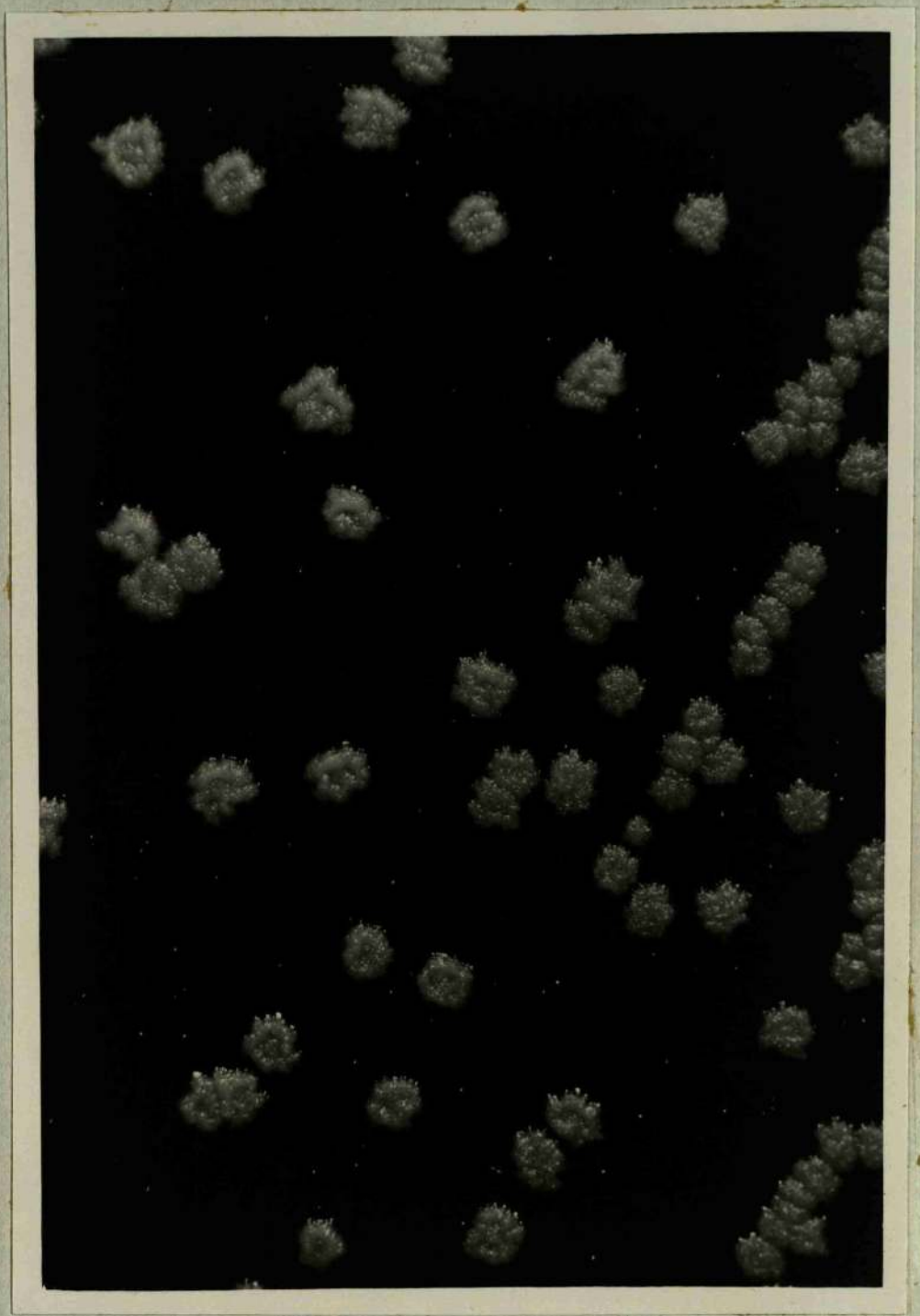
## Stability of variants

R variants produced S variants and vice versa, the rate of conversion differing from strain to strain. It was always found that after one or more plate--broth--plate cycles, colonies of the opposite type appeared on the plate. With different strains, the number of such colonies ranged from 1 - 2 per plate to about 20% of the colonies present. On the whole, there seemed to be a greater tendency for S to be formed from R variants than the reverse. In three strains (C, W and M) the tendency seemed to be in the opposite direction, and smooth colonies picked into broth

Figure 1.

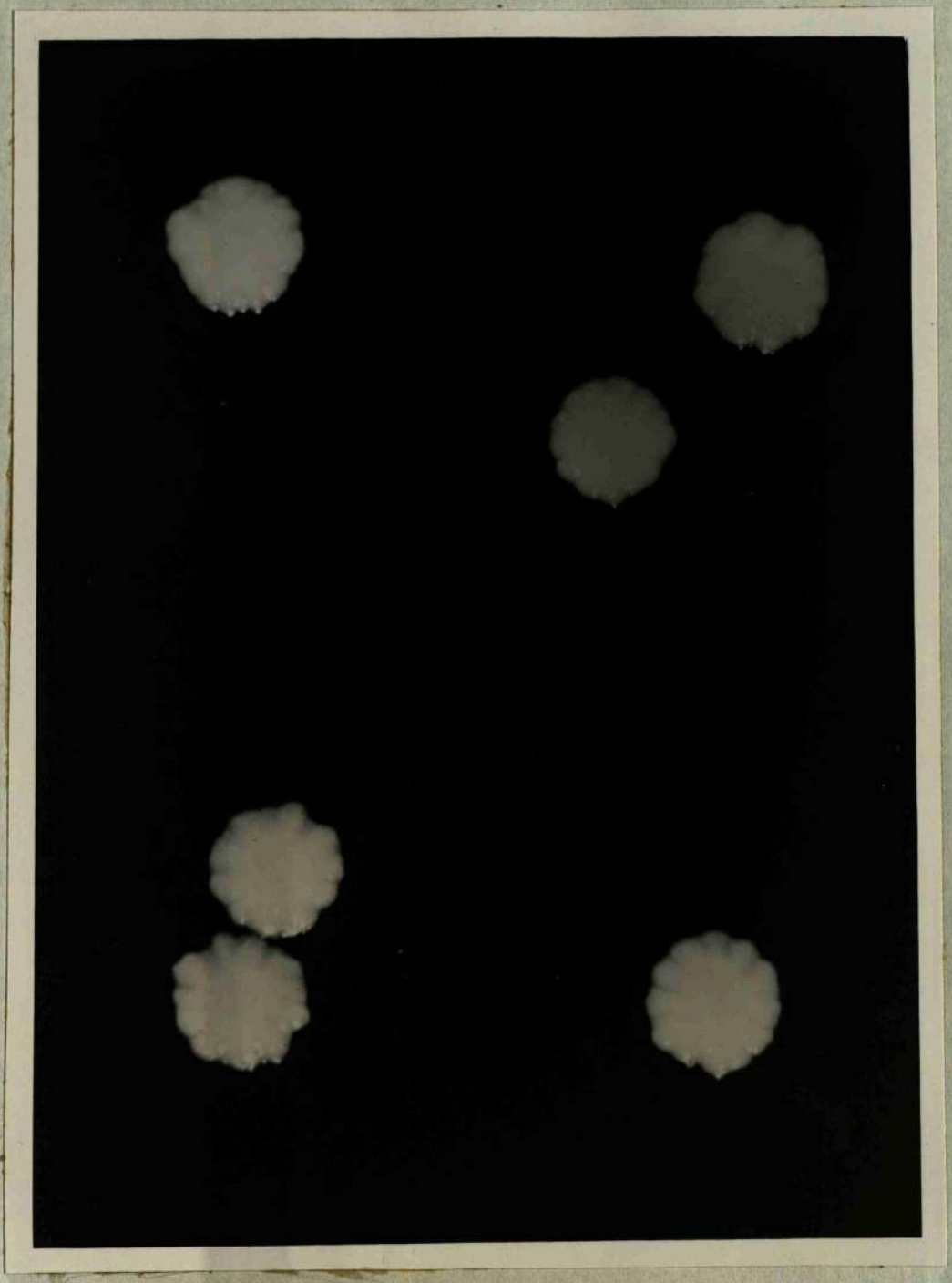
Broth cultures of, on left, R fast-clotting variant; on right, S slow-clotting variant x ca. 2.

Figure 2.



Colonies of R, fast-clotting variants on heated blood agar. x ca. 10.

Figure 3.

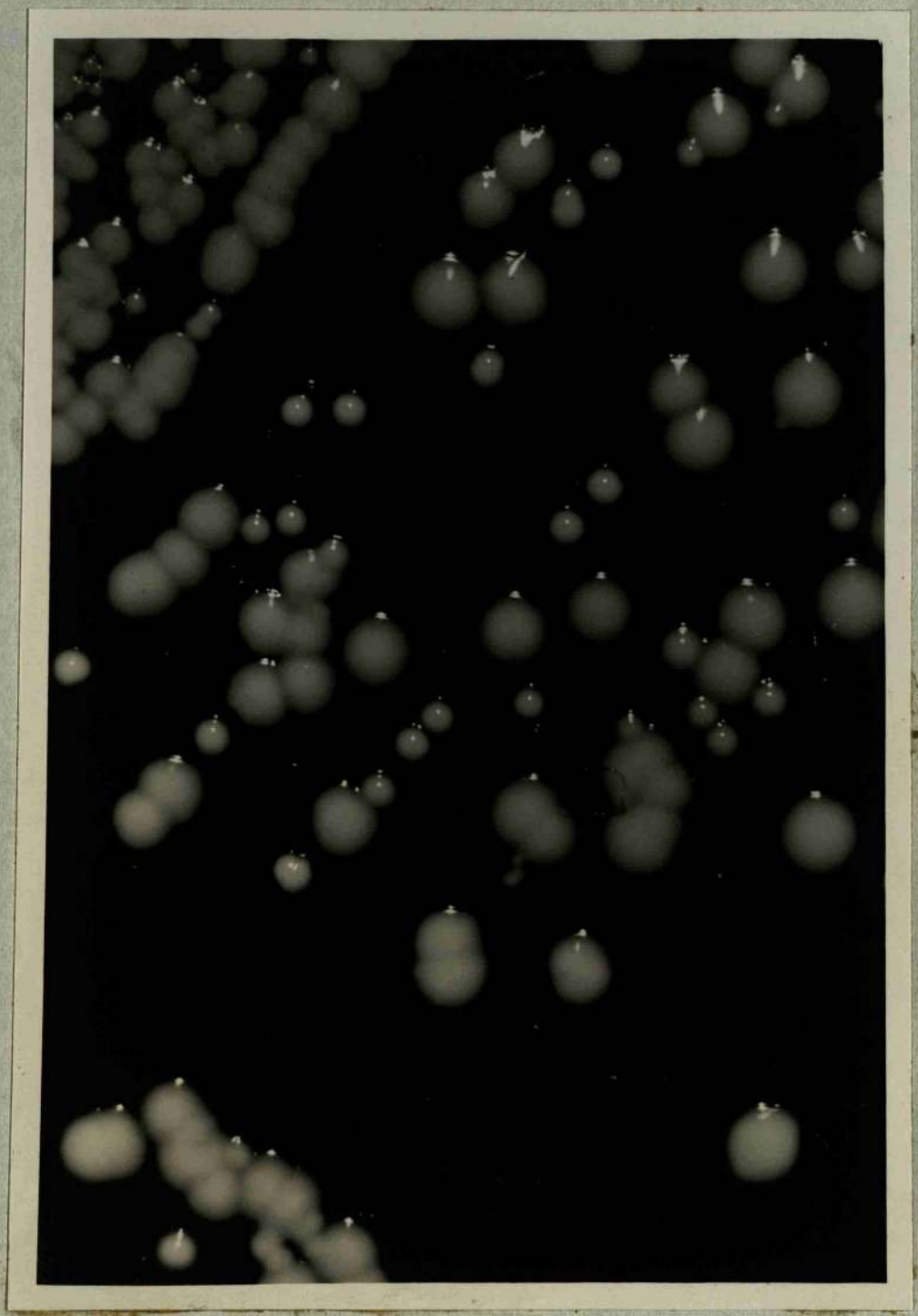


Colonies of R fast-clotting variants on heated blood agar. x ca. 20.

Figure 4.

Colonies of R, fast-clotting variants (small and irregular) and S, slow-clotting variants (large and circular) on blood agar. x ca.10.

Figure 5.



Colonies of fast-clotting variants (small and opaque) and slow-clotting variants (large) x ca. 10.

## Table 1.

Growth characters of variants of Staph. aureus.

| Medium | R fast-clotting variant | S slow-clotting variant |
|---|---|---|
| Broth | Granular with pellicle formation (Fig.1) | Uniform turbidity; no pellicle (Fig.1) |
| Heated-blood agar | Rough or irregular (Figs.2-4); or small, dull and opaque (small colonies of Fig.5); little or no clearing or discolouration of medium. | Large, circular, glistening and smooth (large colonies of Figs.4-5); often discolouration and clearing of medium. |

## Table 2.

Coagulase production of variants of Staph. aureus

| Strain | Clotting time of original strain (minutes)* | R or fast-clotting variant | | S or slow-clotting variant | |
|---|---|---|---|---|---|
| | | Clotting-time (minutes)* | Coagulase titre+ | Clotting-time (minutes)* | Coagulase titre+ |
| "5" | 5 | 1 | 32,000 | >25 | 4 |
| $M^cC$ | 15 | 1 | 32,000 | >25 | 256 |
| $M^cD$ | >20 | 2 | 16,000 | >25 | 32 |
| K | >20 | 1 | 16,000 | >25 | 2 |
| C | >20 | 1 | 32,000 | 20 | 1000++ |
| $M^cA$ | >20 | 1 | 16,000 | >25 | 2 |
| W | >20 | 1 | 32,000 | 16 | 2000++ |
| M | >20 | 1 | 16,000 | 16 | 1000++ |
| Wils | >20 | 1 | 8,000 | >25 | 2 |
| Ken | >20 | 2 | 16,000 | >25 | 32 |

* The relation between clotting time and titre of coagulase varies with the strain.

\+ The titre of coagulase was described in the section on "Materials and methods". The figures are the reciprocals of the highest culture dilutions to show coagulase activity under the condition described.

++ In these three cultures inoculated from smooth colonies the relatively high production of coagulase was accompanied by the appearance of R variants.

Table 3.

Relation of coagulase production to dry cell weight in Strain 5*

| Variant tested | Coagulase titre+ | Dry weight of bacteria (gm. per 100 ml.) |
|---|---|---|
| Rough | 8,000 | 0.0627 |
| Smooth | 64 | 0.0382 |
| Ratio Rough:Smooth | 125:1 | 1.64:1 |

* The figures in the table refer to 48-hour cultures grown in 40 ml. volumes, with a large surface area.

+ The titre of coagulase was ascertained as described in the section on "Materials and methods". The figures are the reciprocals of the highest culture dilution to show coagulase activity under the condition described.

often showed granular growth and gave high yields of coagulase. Plates spread from such broths revealed the presence of R colonies.

Changes in coagulase production within strains

As already mentioned, strains kept in the laboratory over a number of years often showed sudden changes in coagulase production. Experiments showed that at the peak of coagulase production strains yielded a high proportion of R fast-clotting variants and few or none during the phase when coagulase production was at its lowest level. Observations with strain 5 illustrate this point. An agar slope made from a typically granular broth culture was shown by plating to contain mainly rough variants. Subcultures made a few days later still gave granular growth in broth and a high yield of coagulase. Two months later, subcultures from the same slope gave smooth colonies on plates and uniform turbidity in broth with little coagulase production. The same change from R to S happened also with freeze-dried cultures of strain 5. Fourteen days after drying subcultures gave rough colonies, granular growth in broth and a high yield of coagulase; but three months later subcultures gave smooth colonies, uniform turbidity in broth and poor production of coagulase. The instability of these particular freeze-dried cultures may have been due to imperfect drying. For some strains which had considerably

declined in coagulase production it was possible to restore this property; but only after repeated platings from old broth cultures were we able to isolate R fast-clotting colonies which on subculture gave high yields of coagulase. To sum up, high yields of coagulase were always associated with the presence of R fast-clotting variants and low yields of coagulase with their absence.

## Phage-typing

The instability of the variants strongly suggested that the starting material did not consist of a mixture of strains, but in order to check this point the variants of three strains were submitted for phage-typing. The results (Table 4, p.70) confirm the view that, for each of the strains tested, the starting material indeed consisted of only a single strain.

## Preservation of variants

Despite the instability of the variants it was possible to maintain them by daily platings on blood or heated-blood agar from colonies showing the characteristic morphology. Thus over a period of four months the R fast-clotting variant of strain 5 was kept through 100 subcultures and the S slow-clotting variant through 50. It is important that subcultures should be made from plates not more than 24 hours old because, as already mentioned, after this time the colonies tend to lose their characteristic appearance. Also on plating from old R colonies an increased proportion of S colonies was obtained.

Table 4.

Phage-typing of the variants of 3 strains of Staph. aureus.

| Strain | Variant | Phage-type* |
|---|---|---|
| '5' | R (fast-clotting) | 6/7/47/53/54 |
| " | S (slow-clotting) | 7/47/54 |
| " | S (coagulase negative) | 6/7/47/54 |
| M$^{c}$C | R (fast-clotting) | 29/29A |
| " | S (slow-clotting) | 29/29A |
| M$^{c}$D | R (fast-clotting) | 53/76/77 |
| " | S (slow-clotting) | 53/75/76/77 |

*Those phage patterns are regarded as establishing close relationship between the variants within each particular strain.

## Summary

1. The existence of variants differing considerably in coagulase production and growth character was demonstrated in each of ten strains studied.

2. High production of coagulase was found to be associated with the R (rough) form of growth and low coagulase production with the S (smooth).

3. R variants were convertible to S and *vice versa*, the ease of conversion varying from strain to strain.

This work was carried out in collaboration, Smith, Morrison & Lominski (1952).

## SECTION II

## The production of coagulase in chemically defined media.

(Experimental ..... p.79 et seq.)

# A. Review of work on growth and metabolism of *Staph. aureus* in chemically defined media.

A. Growth of Staph. aureus in chemically defined media

Introduction

The first attempt to grow staphylococci in a simple medium of known composition was made by Hughes (1932) in the course of an investigation into the chemistry of bacterial metabolism. He found that an acid hydrolysate of casein would not support the growth of Staph. albus but that the addition of 0.01% of meat extract gave heavy growth; the meat extract alone had to be at a concentration of more than 0.5% to give equivalent growth. An acetone-precipitated concentrate of the growth factor was effective at a concentration of one part in 50 million parts of casein hydrolysate. It was found to be heat stable in neutral or slightly acid solutions and was dialysable. Hughes was unable to identify this factor beyond saying that its behaviour placed it in the class of chemical compounds known collectively as "Vitamine B".

Knight (1935), using a basal medium of acid-hydrolised gelatin plus tryptophan, tyrosine, cystine and glucose, found that a growth factor, which he obtained from yeast preparations (marmite), was required by Staph. aureus. An even more powerful concentrate than Hughes's preparation was obtained by solvent extraction of marmite, precipitation of impurities and vacuum distillation of the active material.

Tests showed that this "staphylococcus factor" enabled most strains of pathological origin (whether aureus or albus) to grow in the basal medium but that saprophytic staphylococci required in addition some other nutrient factor. The general properties of Knight's and Hughes's preparations agreed fairly well, which is not surprising since it is likely that they were both impure preparations of the same growth factor.

A further step towards the production of a medium of known chemical composition was made by Fildes, Richardson, Knight & Gladstone (1936). They obtained aerobic growth in a medium containing only amino acids, glucose, salts and "staphylococcus factor". The medium used was a slightly altered version of one which had been found to support the growth of Cl. sporogenes (Fildes & Richardson, 1935) and no detailed attempt was made to find out which amino acids were essential. Anaerobic growth required the addition of pyruvic acid, the presence of 5% carbon dioxide and an additional component extracted from yeast which they called "Factor III". When Factor III was found to be uracil (Richardson, 1936) only the so-called "staphylococcus factor" remained to be identified for the growth requirements of Staph. aureus to be known.

This last step in devising a medium containing only known chemical compounds was made by Knight (1937a). He

investigated the "staphylococcus factor" of marmite using two media, an amino-acid medium and acid-hydrolysed gelatin. From the high vacuum distillate of marmite (Knight, 1935) a chloroplatinate was isolated; this had growth-promoting properties in gelatin but not in the amino-acid medium. Thus "staphylococcus factor" appeared to consist of at least two fractions, one of which was already present in gelatin hydrolysate. Chemical examination of the active principle suggested the presence of cyclic nitrogen compounds (e.g., pyridine derivatives). For this reason, and since other micro-organisms had been shown to need these substances, Knight decided to try the effect of adding cozymase and vitamin $B_1$ (aneurin). Cozymase and nicotinic acid and amide, which are possible degradation products of cozymase, were indeed found to be active in the basal gelatin medium but not in the amino-acid medium. The second factor, required in the amino-acid medium only, was subsequently found to be identical with or replaceable by aneurin. Koser, Finkle, Dorfman & Saunders (1938) later confirmed the need of Staph. aureus for the two vitamins aneurin and nicotinamide.

Knight (1937b) also determined the amounts of nicotinic acid and aneurin needed for full growth of Staph. aureus and the degree of specificity of aneurin. He found that the two components of the aneurin molecule, the pyrimidine and the thiazole residues, could be utilised in

place of the complete molecule when added together but only when the spatial configurations were exactly as in aneurin. Aneurin itself with one substituent group lacking was found to be inactive, which illustrates the highly specific nature of the growth requirements of Staph. aureus. A similar high degree of specificity was found for nicotinic acid or nicotinamide by Knight & McIlwain (1938). This was later confirmed by Landy (1938a, 1938b) who found that derivatives and even isomers of nicotinic acid were, with few exceptions, inactive as growth factors for Staph. aureus.

The vitamin requirements of Staph. aureus have been investigated by other workers and it now seems that the matter is not so simple as at first appeared. Kogl & Wagtendonk (1938), for instance, were able to obtain an increase in growth of over 800% in a simple gelatin hydrolysate medium by the addition of the methyl ester of biotin, and Porter & Pelczar (1941) found that 2 of 17 strains of Staph. aureus which failed to grow on continued subculture in a simple medium containing aneurin and nicotinamide grew well in the same medium after biotin had been added. The latter authors also found that with some strains growth in the basal medium was stimulated by the addition of biotin, but that a number of other bacterial vitamins had no effect on any of the strains tested. On the basis of these findings Porter & Pelczar suggested that Staph. aureus

could be divided into three groups in respect of vitamin requirements: (1) strains which can grow on continued subculture in the presence of nicotinamide and aneurin and are not influenced by biotin, (2) strains which can grow in the presence of nicotinamide and aneurin and are stimulated by biotin, and (3) strains which will not grow without biotin.

Kligler, Grossowicz & Bergner (1943) found that in a series of strains examined all needed nicotinic acid for growth and most needed aneurin, but a few were able to grow without aneurin.

Thus not only are there strains with more complex vitamin requirements than the work of Knight (1937a, 1937b) indicated, but there are some with simpler requirements.

Despite these qualifications, the medium devised by Fildes et al. (1936) and modified by Fildes & Richardson (1937) and Gladstone (1937) satisfies the growth requirements of the majority of strains and has been a tool of prime importance in the investigation of the metabolism of Staph. aureus. In some later work casein hydrolysate supplemented by vitamins was used as a matter of convenience rather than the more complex amino-acid medium, but even so it was based on the nutritional requirements of Staph. aureus worked out on the amino acid media. Media of known chemical composition have been used for a variety of purposes some of which are outlined in the following pages.

These included (1) the function of aneurin and nicotinamide in the metabolism of Staph. aureus, (2) the amino-acid requirements of Staph. aureus, (3) the assimilation of amino acids and penicillin resistance, and (4) the influence of nutrients on the formation of bacterial products such as toxins and coagulase.

The function of aneurin and nicotinamide

Hills (1938) studied the effect of aneurin on the metabolism of lactic and pyruvic acids by Staph. aureus. He found that aneurin was active at such a low concentration that its effect could be observed only by using organisms grown on synthetic media deficient in aneurin. With such organisms he found that aneurin was necessary for the dismutation of pyruvic acid into lactic acid, acetic acid and carbon dioxide (see below) and that in the absence of aneurin pyruvic acid accumulated and inhibited the oxidation of lactic acid.

$$\underset{\text{pyruvic acid}}{2\ CH_3CO\ COOH} + H_2O = \underset{\text{lactic acid}}{CH_3CHOH\ COOH} + \underset{\text{acetic acid}}{CH_3COOH} + CO_2$$

Smyth (1940) using washed cells grown in an aneurin-deficient medium found that oxaloacetic acid or fumaric acid could replace aneurin in the dismutation of pyruvic acid and suggested that aneurin acted by catalyzing the formation of oxaloacetic acid which acted as a hydrogen carrier in the pyruvic acid dismutation.

Bovarnick (1943) claimed that a mixture of asparagin and glutamic acid heated at 100°C. for several days could replace nicotinamide as a growth factor for Staph. aureus, but did not use this finding to throw light on the function of nicotinamide. The most detailed study of the rôles of both aneurin and nicotinamide was that carried out by Kligler et al. (1943). They found that the rôle of nicotinamide was to produce a primary glycolysis in which the oxidation of the pyruvic acid was catalysed by aneurin. Strains capable of growing without aneurin used only about 40% of the glucose utilised in the presence of both aneurin and nicotinamide; without aneurin pyruvic and lactic acids accumulated. With both vitamins present the end product consisted, mainly, of acetic and lactic acids with only small amounts of pyruvic acid.

Sevag & Swart (1947) and Sevag, Shelburne & Madd (1951) made the curious finding that cells which had been grown in the presence of glucose were unable to metabolise pyruvate; cells which had been grown in a glucose-free medium could metabolise pyruvate completely in 2 - 8 hours. This effect was not modified by the presence of a large excess of aneurin. The mechanism of the breakdown of pyruvate is therefore more complex than was suggested by Kligler et al. (1943).

The amino-acid requirements of Staph. aureus

The amino-acid requirements of 26 strains of Staph. aureus were very thoroughly investigated by Gladstone (1937) using a modified form of the medium of Fildes & Richardson (1937). He found that 25 of 26 strains grew well in this 16-amino-acid medium, but that withdrawal of each amino acid in turn revealed widely differing growth requirements; thus for one strain phenyl alanine was essential, for another strain leucine, for another valine and so on. Such differences disappeared when strains were trained to grow in deficient media. When cells were left in a deficient medium growth eventually occurred; transfers in such media ultimately resulted in good early growth. By successive withdrawals Gladstone was able to train his strains to grow in a medium from which all amino acids had been excluded and in which the main source of nitrogen was ammonia. The ease of withdrawal of individual amino acids varied, however, with the complexity of the medium. Thus leucine could be withdrawn from a complex medium (16 amino acids) but as amino acids were progressively omitted a stage was reached where no more withdrawals could be made unless leucine was restored. Staphylococci could be trained to grow without leucine, however, but during training required the presence of other amino acids which in a complex medium did not appear to be essential. Both

leucine and these amino acids could be withdrawn once training had occurred. Cystine was the most difficult amino acid to dispense with, partly because it was the only organic form of sulphur present and partly because it was the last source of amino nitrogen in the medium. When finally the strain had been trained to dispense with amino nitrogen it was found possible to replace cystine with thioglycollate and thus grow Staph. aureus in a medium containing no amino acids.

No permanent alteration in the morphological, cultural and biochemical characteristics resulted from adaptation to growth in simple media and when the strains were transferred to the usual laboratory media such properties as gelatin liquefaction, coagulase production and haemolysin production were unimpaired. Very little haemolysin and no coagulase were produced in any of the amino acid media.

Gladstone's results suggest that differences in the nutritional requirements of strains of Staph. aureus are largely dependent on their previous nutritional history and that the application of the term "indispensable amino acids" is a relative term as far as growth is concerned. This does not apply, however, to the amino acid requirements for coagulase and haemolysin production. The fact that haemolysin production was slight and coagulase was not produced in the chemically defined media suggests that although all

amino acids are dispensable for growth some may be indispensable for coagulase and haemolysin production. It was on the basis of this assumption, in fact, that the work to be described in a later section was carried out (see p.88).

An investigation of the sulphur requirements of Staph. aureus was undertaken by Fildes & Richardson (1937). They took great care to ensure that no sulphur was present as impurities in the constituents of their medium, using as far as possible synthetic amino acids and reducing to the lowest possible level any natural amino acids present. Their results showed that cystine was the most effective source of sulphur but that it could be replaced, though less effectively, by methionine, any mercapto (-SH) compound, dithio (-S-S-) compounds or finally any compound such as a thionic acid (RCSOH) or amide ($RCSNH_2$) containing a potential mercapto group. The outstanding effectiveness of cystine as a source of sulphur ( $5 \times 10^{-8}$ M.) in comparison with methionine ($10^{-3}$M.) and various other mercapto compounds led the authors to suggest that the effect on growth of a mercapto compound is not exerted by virtue of its sulphur content but by the ease with which it can be used in the synthesis of cystine. Apart from its intrinsic interest in regard to the metabolism of Staph. aureus this paper contains an excellent discussion of the criteria of purity to be satisfied in order to obtain valid results in the study of bacterial nutrition.

The amino-acid metabolism of Staph. aureus and penicillin resistance

An interesting application of the synthetic medium of Gladstone (1937) was the demonstration by Gale & Rodwell (1948) that strains of Staph. aureus which had been trained to grow in very high concentrations of penicillin had become almost non-exacting in their amino acid requirements and were even able to dispense with nicotinamide. But, as was first noted by Bellamy & Klimek (1948), not only were these highly resistant organisms Gram-negative, but they were bacillary in shape. They bear so little resemblance to Staph. aureus in biochemical, morphological and staining characters that it is difficult to be sure that the parent and the resistant strains belong to the same bacterial species.

This work of Gale & Rodwell formed part of a very extensive investigation by Gale and his associates of the assimilation of amino acids by Gram-positive bacteria (Gale, 1947a, b & c; Gale & Taylor, 1946, 1947; Taylor, 1947; Gale & Mitchell, 1947). A summary of their main conclusions is given below.

Staph. aureus assimilates and accumulates certain amino acids within the cell. Some amino acids, particularly lysine, pass into the cell by diffusion; others, particularly

glutamic acid, by an active process. In either case an equilibrium is reached with the concentration inside the cell markedly higher than in the external environment. With glutamic acid (the amino acid mainly studied) this accumulation within the cell acts as a metabolic pool for a direct condensation to protein and for another metabolic process inhibited by the triphenylmethane dyes. According to Gale, penicillin prevents the assimilation of glutamic acid by Staph. aureus so that the glutamic-acid content of the growing cell is rapidly exhausted and lysis follows. The acquisition of penicillin resistance seems to depend on the ability of staphylococci to change from an assimilative to a synthetic type of metabolism. Thus strains which have acquired a high penicillin resistance are also non-exacting and, conversely, strains adapted to simple nutritional requirements show increased resistance to penicillin.

The above summary gives some indication of the wide field covered by Gale and his colleagues and of the originality of their approach to the study of the nitrogen metabolism of Staph. aureus. Owing to the limitations of the analytical methods used, their work has been mainly restricted to glutamic acid. It is possible, however, that an extention of Gale's methods will eventually throw light on the part played by different amino acids in specific

aspects of the metabolism of Staph. aureus, such as the synthesis of coagulase.

The influence of nutrients on the formation of bacterial products

The first attempt to study the influence of individual amino acids on the formation of bacterial products by Staph. aureus appears to have been carried out by Gengou (1935). His investigation was prompted by the observation that some of his strains did not produce α-haemolysin when grown in meat-extract broth. He studied the effect of adding individual amino acids to meat-extract broth and found that some stimulated the production of α-haemolysin while others had no effect. The addition of arginine to the medium gave a much quicker and greater production of α-haemolysin than any of the other amino acids with the exception of ornithine, which gave almost as marked a response. Arginine, ornithine and citrulline have part of the molecule in common, but unfortunately Gengou was unable to obtain any citrulline which is even more closely related to arginine than ornithine.

The next attempt was made by Gladstone (1938) in a very extensive investigation of factors influencing α-haemolysin production. He used a chemically defined medium (Gladstone, 1937) modified on the basis of the amino-acid analysis of hydrolised edestin which was found to give good

$\alpha$-haemolysin production. His Staph. aureus was adapted to grow on an amino-acid-free medium but produced $\alpha$-haemolysin only when transferred to a richer medium. Much adjustment of the proportions of the various amino acids was needed for good yields of $\alpha$-haemolysin because some amino acids were stimulatory to haemolysin production at one concentration and inhibitory at a higher concentration. Gladstone confirmed Gengou's finding that arginine was of primary importance in $\alpha$-haemolysin production and was replaceable by ornithine; his order of importance of the other amino acids was roughly in agreement with Gengou's findings.

It was not until twelve years later that the formation of coagulase in a chemically defined medium was reported (Lominski, O'Hea, Goudie & Porter, 1950). Their medium consisted of 17 amino acids, 4 vitamins and inorganic salts and gave coagulase after 3 - 5 days' incubation of the cultures but only in low yields. This medium provided the starting point for the experiments now to be described.

## SECTION II

### B Production of coagulase in a chemically defined medium

## B Production of coagulase in a chemically defined medium

### Introduction

It was thought that it might be possible, by varying the components of a medium and observing the effect on coagulase production, to gather information about the synthesis of coagulase. The first need was for a chemically defined medium which would give yields comparable to those given by the usual laboratory media such as meat-extract broth. The medium described by Lominski et al. (1950) gave such small amounts of coagulase that it was not suitable for this type of study. Further unpublished work by Lominski, O'Hea, Morrison & Porter, however, led to the devising of a medium in which a suitable strain of Staph. aureus produced coagulase of a titre* of 8000. It contained a higher concentration of most of the amino acids than the earlier medium (with some omissions and some additions) and was arrived at by observing the effect of withdrawal of each constituent in turn. The composition of the medium is shown on p. 94 (Medium I).

This medium gave high yields of coagulase on occasion, but at other times under apparently identical conditions, and with the same strain of organism gave poor yields. It was at this point that the experiments now to be described

---

* See definition on p. 92.

were begun with the aim of finding out the cause of the erratic results obtained with Medium I. The work comprised a study of the effect of varying the concentration of amino acids and vitamins. A section is devoted to the parts played by arginine, ornithine and citrulline on coagulase production. In the course of this work the finding was made that citrulline is formed from arginine by Staph. aureus and an account of this is also given. Finally an account is given of the effect of bacterial variation on coagulase production.

## Materials and Methods

Glassware. All glassware used in these experiments was cleaned in chromic acid or sodium hypochlorite cleaning mixture, thoroughly washed in tap water, washed in distilled water, steeped in distilled water and finally rinsed in distilled water.

Preparation of media. The media were prepared by one of the following methods:-

### Method A

Stock solutions of each amino acid in Sörensen's buffer ($K_2HPO_4$ 0.2 M; citric acid 0.1 M.) of pH 7.3 were sterilised by autoclaving, except for tryptophan which was filtered through a Seitz pad to avoid heat destruction. The inorganic salts and vitamins were dissolved in Sören-

sen's buffer and sterilised by Seitz filtration. With sterile precautions throughout, appropriate amounts of the different amino acids were added to the vitamin/salts solution and the mixture dispensed in 10 ml. lots into universal containers.

Method B

The amino acids and salts were dissolved in Sörensen's buffer and sterilised by Seitz filtration. Stock solutions of the individual vitamins were made in distilled water and sterilised by Seitz filtration. 10 ml. lots of media in universal containers were made by the addition of the amino acid base and the individual vitamin solutions, with all precautions to maintain sterility.

Cultures. The inocula were obtained by centrifuging 24-hour meat-extract broth cultures of Staph. aureus, washing the cells three times with physiological saline and transferring a small loopful of the washed cells into synthetic medium. After three successive subcultures in synthetic medium at 12 - 24-hour intervals the various experimental media were inoculated with a small loopful from the third subculture. The different media were incubated at 37.5°C. and samples withdrawn at one or two day intervals and tested for coagulase.

Estimation of coagulase. Serial doubling dilutions of the cultures were made in 0.85% saline containing 0.1% merthiol-

ate (sodium ethyl mercurithio-salicylate); to each 0.5 ml. of diluted culture was added 0.5 ml. of human plasma (diluted 1 in 5 with 0.85% saline). The merthiolate, present in a concentration of 0.05% (W/V), was found to prevent bacterial growth. The tubes were incubated at 37.5°C. for 24 hours and examined for the greatest dilution to give a clot. Frequently a second reading was made after the tubes had been left for another 24 hours at room temperature. The amount of coagulase present in a culture was expressed as the titre, which was the reciprocal of the greatest dilution of the culture to give a definite plasma clot.

The effect of altering the concentrations of amino acids

In attempting to discover why Medium I should have given erratic results possible explanations were considered. Gross errors in weighing seemed to be ruled out by the fact that in a series of careful repetitions erratic results were still obtained. It was then thought that these might be due to very small variations in the composition of the media. This thought was prompted by the findings of Gladstone (1938) that some amino acids which stimulated -haemolysin production at one concentration became inhibitory at a higher concentration, and that individual amino acids toxic to bacterial growth might in combination show

no toxic activity (Gladstone, 1939). It seemed possible that coagulase production might be inhibited without growth being affected. The experiments which follow were designed to test this possibility.

The effect of increased concentration of single amino acids

Since the possible variations in the composition of a complex medium are limitless it was decided in the first place to see whether any single amino acid was present at an inhibitory concentration and whether coagulase production depended on neutralisation of the inhibitory effect of one amino acid by some other.

Different media were prepared in each of which one amino acid was kept at the concentration shown in Medium I and the remaining amino acids were reduced to one fifth of the concentration shown. Vitamins and salts were at the concentrations shown for Medium I. In a control medium all the amino acids were kept at one fifth of the concentration given for Medium I. The inoculations were made from the third subculture in the control medium of a good coagulase producing strain of Staph. aureus. Samples were tested for coagulase after two and four days' incubation of the cultures.

The results given in Table 5 (p.96) show that even a five-fold increase in the concentration of each amino

## Medium I

Composition (per 100 ml. buffer* at pH 7.3) of a medium which gave unpredictably variable yields of coagulase

| | | | |
|---|---|---|---|
| L-arginine base | 0.20 gm. | DL-alanine | 0.089 gm. |
| L-glutamic acid | 0.16 " | L-leucine | 0.06 " |
| DL-aspartic acid | 0.16 " | DL-phenylalanine | 0.06 " |
| DL-methionine | 0.16 " | L-tryptophan | 0.06 " |
| DL-valine | 0.12 " | L-histidine HCl | 0.06 " |
| DL-threonine | 0.12 " | L-proline | 0.06 " |
| DL-isoleucine | 0.12 " | DL-serine | 0.06 " |
| L-lysine HCl | 0.10 " | | |

$MgSO_4 \cdot 7H_2O$ = 0.25 gm. $K_2HPO_4$ = 0.25 gm.

Aneurin 0.5 ugm.; pyridoxin 120 ugm.; nicotinamide 20 ugm.

---

* The buffer was 0.2 M $K_2HPO_4$/0.1 M citric acid Sorensen's buffer.

acid made little difference to coagulase production. This appeared to rule out the possibility that the erratic results obtained with medium I (p.94) had been due to slight variations in the concentration of amino acids. If any one amino acid had been present at a concentration on the borderline between inhibition and stimulation of coagulase production one would have expected that its use at a concentration five times greater than in the control would have had a marked effect. Also, if coagulase production had depended on the exact neutralising of the inhibitory effect of one amino acid by another, the large changes in the relative proportions of the amino acids in this experiment should have been reflected in results differing from each other by more than a single tube of the doubling dilution series.

Effect of varying the concentration of serine and isoleucine

In addition to the neutralising of the toxicity of one amino acid by another it is known that the toxicity of iso-leucine and serine for B. anthracis can be neutralised by a combination of valine and leucine but not by either alone (Gladstone, 1939). It was thought interesting to see whether the same phenomenon could be shown in coagulase production since if the concentrations of each of these four amino acids were critical, small variations (within experimental error) in the composition of media might have a marked

## Table 5.

Growth and coagulase production with one amino acid at full-strength and remainder at one-fifth normal concentration

| | 2-day cultures | | 4-day cultures | |
|---|---|---|---|---|
| Amino acid at full strength | Cell count per ml. x $10^8$ + | Coagulase titre* | Cell count per ml. x $10^8$ + | Coagulase titre* |
| None | 9.5 | 8 | 11.4 | 8 |
| Arginine | 7.6 | 8 | 11.4 | 8 |
| Glutamic acid | 9.5 | 16 | 11.4 | 16 |
| Aspartic acid | 9.5 | 16 | 11.4 | 8 |
| Methionine | 9.5 | 8 | 11.4 | 8 |
| Valine | 7.6 | 8 | 11.4 | 4 |
| Threonine | 9.5 | 16 | 11.4 | 8 |
| Isoleucine | 9.5 | 8 | 11.4 | 8 |
| Lysine | 7.6 | 8 | 11.4 | 8 |
| Alanine | 7.6 | 8 | 11.4 | 8 |
| Leucine | 9.5 | 8 | 11.4 | 16 |
| Phenyl alanine | 5.7 | 16 | 11.4 | 8 |
| Tryptophan | 7.6 | 8 | 11.4 | 8 |
| Histidine | 9.5 | 8 | 11.4 | 8 |
| Proline | 5.7 | 8 | 11.4 | 8 |
| Serine | 9.5 | 16 | 11.4 | 16 |

*The titrations were read after 48 hrs. (24 hours incubation at 37.5°C. followed by 24 hours at room temperature). The titre is the reciprocal of the highest dilution to cause clot. Range of dilutions 1 in 2, 4, 8, 16, etc.

+ Cell counts were measured by opacity tube methods.

effect on the yield of coagulase.

Accordingly, media were prepared in which all the amino acids were at the concentrations shown in Medium I with the exception of serine and iso-leucine. These two were reduced singly and jointly to three-quarters of their normal level. Cultures inoculated with a good coagulase-producing Staph. aureus, strain 5, were tested after two and four days' incubation.

The results given in Table 6 (p.98) show that alteration in the concentrations of serine and iso-leucine far greater than could be found between different batches of the same medium had little effect on coagulase production.

The effect of varying the proportions of arginine and alanine

Unpublished work by Lominski, Morrison, O'Hea & Porter indicated that the relative and absolute concentrations of alanine and arginine affected coagulase production. An experiment was therefore carried out in which all the amino acids except arginine and alanine were at the concentrations shown in Medium I; arginine was varied between M/20 and M/80 (0.87% - 0.22%) and alanine between M/10 and M/1000 (0.89% - 0.0089%). Strain 5 was again used and the cultures were tested for coagulase after four, six and eight days. The results are given in Table 7 (p.99).

The highest titre reached was low in comparison with

## Table 6.

The effect on coagulase production by Staph. aureus of varying the concentrations of serine and isoleucine

| Serine* concentration | Isoleucine* concentration | 2-day cultures titre + | 4-day cultures titre + |
|---|---|---|---|
| Normal | Normal | 4 | 32 |
| $\frac{1}{2}$ Normal | Normal | 16 | 32 |
| Normal | $\frac{1}{2}$ Normal | 8 | 32 |
| $\frac{1}{2}$ Normal | $\frac{1}{2}$ Normal | 8 | 32 |

* Normal concentration of serine = 0.06 gm. per 100 ml.
" " " isoleucine = 0.12 " " " "

+ See footnote to Table 5.

## Table 7.

Effect of varying concentrations of arginine and alanine

| Arginine concentrn. | Alanine concentrn. | 4-day cultures | | 6-day cultures | | 8-day culture | |
|---|---|---|---|---|---|---|---|
| | | Cell count per ml. x $10^{8}$+ | Coagulase titre* | Cell count per ml x $10^{8}$+ | Coagulase titre* | Cell count per ml x$10^{8}$ + | Coagulase titre |
| M/80 | M/10 | 13.3 | 32 | 17.2 | 16 | 17.2 | 16 |
| M/80 | M/100 | 15.2 | 32 | 19.0 | 64 | 22.7 | 64 |
| M/80 | M/1000 | 15.2 | 32 | 19.0 | 32 | 22.7 | 64 |
| M/40 | M/100 | No growth | | 9.5 | 4 | 13.3 | 8 |
| M/40 | M/1000 | No growth | | 11.4 | 8 | 15.2 | 32 |
| M/20 | M/100 | No growth | | No growth | | No growth | |
| M/20 | M/1000 | No growth | | 5.7 | 4 | 11.4 | 4 |

* Titration read after 48 hours (24 hours' incubation at 37.5°C followed by 24 hours at room temperature). See footnote to Table 5.

+ See footnote to Table 5.

the titres which this strain gave in meat-extract broth (2000 - 4000), but clear differences can be seen between the various experimental media. Arginine and alanine both became inhibitory as their concentrations were raised so that M/20 arginine + M/1000 alanine gave delayed growth and M/20 arginine + M/100 alanine no growth at all.

Thus the same effect is apparent with coagulase production as Gladstone (1938) noticed in regard to -haemolysin; arginine which, as later experiments show, is of primary importance for coagulase production, becomes inhibitory to growth above a concentration of M/80. Alanine does not appear to have much influence on coagulase production at low concentrations but is inhibitory to both growth and coagulase production at the highest concentration tested.

The influence of arginine, ornithine and citrulline on coagulase production

In view of Gengou's finding that arginine greatly influenced the production of -haemolysin (Gengou, 1935), and that it was replaceable by ornithine, experiments along similar lines were carried out. A series of media were prepared in which arginine was replaced by ornithine or citrulline; in controls the arginine was replaced by norleucine or simply omitted. The vitamin content of the media was high and was dictated by the results of experiments on

the influence of vitamin concentration on coagulase production described on p. 118 *et seq*. The media were prepared in the following way:

A basal medium of the same composition as Medium II (see p.102) but without arginine, was prepared by dissolving the components in 80 ml. of buffer, the addition of stock solutions of arginine or its replacements bringing the concentration of ingredients to the desired level (Series A). The same basal medium was also used four times diluted (Series B). In both series arginine was at the concentration shown in Medium II or was replaced in turn by buffer or equimolar amounts of ornithine, citrulline and norleucine. In each case 8 ml. of concentrated or diluted basal medium plus 2 ml. of arginine or replacement was inoculated with a rough variant of strain 5, known to give a titre of 8000 - 16,000 in meat-extract broth, and passed previously through three successive subcultures in the norleucine medium. Each culture was tested for coagulase after two, four and seven days, and the results are shown in Table 8 (p.103).

The question arose whether the differences in coagulase production merely reflected differences in the number of cells present. Inspection of Series A in Table 8 will show that this is not so. The cell counts of the 4-day arginine and ornithine cultures are the same, but the arginine medium contains 16 times more coagulase than the

## Medium II

Composition (per 100 ml. buffer* at pH 7.3) of a medium used in arginine, ornithine, citrulline experiments

| | | | |
|---|---|---|---|
| L-arginine base | 0.20 gm.+ | DL-alanine | 0.089 gm. |
| L-glutamic acid | 0.32 " | L-leucine | 0.06 " |
| DL-aspartic acid | 0.16 " | DL-phenylalanine | 0.06 " |
| DL-methionine | 0.16 " | L-tryptophan | 0.06 " |
| DL-valine | 0.12 " | L-histidine HCl | 0.06 " |
| DL-threonine | 0.12 " | L-proline | 0.06 " |
| DL-isoleucine | 0.12 " | DL-serine | 0.06 " |
| L-lysine HCl | 0.10 " | | |

$MgSO_4 \cdot 7H_2O$ 0.25 gm. $K_2HPO_4$ 0.25 gm.

Aneurin 60 ugm. ; nicotinamide 4000 ugm.

---

* The buffer was 0.2M.$K_2HPO_4$/0.1 M. citric acid. Sörensen's buffer.

\+ In the other media the arginine was replaced by one of the following:- norleucine 0.15 gm.; ornithine dihydrochloride 0.257 gm; citrulline 0.20 gm.

Table 8.

Replacement of arginine by ornithine, citrulline and norleucine

| Basal medium used | Amino acid added | 2-day cultures | | 4-day cultures | | 6-day cultures | |
|---|---|---|---|---|---|---|---|
| | | Cell count per ml. x $10^6$+ | Coagulase titre* | Cell count per ml. x $10^6$+ | Coagulase titre* | Cell count per ml. x $10^6$+ | Coagulase titre* |
| Series A | none | 3.8 | <4 | 11.4 | 64 | 13.3 | 16 |
| As for Medium II but without arginine | norleucine | 3.8 | <4 | 7.6 | 16 | 13.3 | 16 |
| | ornithine | 5.7 | <4 | 11.4 | 64 | 38.0 | 2000 |
| | citrulline | 5.7 | <4 | 13.3 | 1000 | 38.0 | 8000 |
| | arginine | 3.8 | <4 | 11.4 | 1000 | 38.0 | 8000 |
| Series B | none | <3.8 | 4 | 3.8 | 16 | 3.8 | 16 |
| As for Series A but diluted 1 in 4 | norleucine | <3.8 | <4 | 3.8 | 16 | 5.7 | 8 |
| | ornithine | 3.8 | 8 | 9.5 | 32 | 9.5 | 16 |
| | citrulline | 7.6 | 16 | 11.4 | 256 | 11.4 | 256 |
| | arginine | 7.6 | 16 | 7.6 | 128 | 11.4 | 256 |

+ See footnote to Table 5.

* Titrations read after 24 hours' incubation at 37.5°C.

See footnote to Table 5.

ornithine medium. The same disparity may be seen with the coagulase production in the 4-day arginine culture and the culture of basal medium in Series A, where the growth is identical. In the 6-day cultures growth in Series A media containing arginine, ornithine and citrulline proceeded much further than in the controls. But the differences in cell counts are much less than the differences in the amounts of coagulase produced. If the 6-day arginine and norleucine cultures of Series A are considered, the ratio of cell counts is seen to be less than 4 to 1, but the ratio of coagulase produced is 500 to 1. This dissociation of growth and coagulase production was observed in many other experiments.

It is clear, therefore, that arginine plays an important part in the production of coagulase just as in the production of haemolysin. It also seems that it is fully replaceable by citrulline and less effectively by ornithine. It would seem then that in order to obtain high yields of coagulase either arginine or the amino acids ornithine and citrulline, which have common structural features, must be present in the medium. With arginine simply omitted or replaced by norleucine the yield of coagulase was poor.

These results were obtained with a strain of Staph. aureus capable of giving high yields of coagulase in meat-extract broth. It was therefore decided to try the effect of arginine on another strain producing less coagulase

(Strain Kelly). In this experiment meat-extract broth, basal medium, basal medium + arginine and basal medium + ornithine were compared. The results are given in Table 9 (p.106). It is surprising to note that this organism, which is a poor coagulase producer, gives faster initial growth than strain 5 though it never attains as high a final cell count. Apart from this point it is obvious that the conclusions drawn from the previous experiment are not fully applicable to this strain. The influence of arginine is slight, indicating that for this type of strain conditions for good coagulase production were not attained in the synthetic media and that another factor or factors were needed.

(INSERT P.105a HERE)

Formation of citrulline from arginine by Staph. aureus

The experiments described above showed that at least for certain strains arginine was of great importance for coagulase production. It could be replaced by citrulline or ornithine, but one of these three amino acids had to be present for good yields of coagulase. It was thought that they might be interconvertible or that they might be able to act as precursors of some factor essential for the synthesis of coagulase.

The formation of ornithine from arginine had already been demonstrated by Hills (1940) and Gale (1945) who postulated an enzyme, arginine dihydrolase, catalysing the

## Summary

These experiments indicated that strains of Staph. aureus differ among each other in their nutritional requirements for coagulase production. Similar differences were later found even between variants of a single strain (see p.131). It was decided to investigate the behaviour of different strains and their variants in chemically defined media since the important rôle of arginine might be obscured by variability in regard to other nutritional requirements. The work described on p.52 et seq. on the effect of R —— S variation on the production of coagulase in ordinary media emphasised the need for this aspect to be considered.

Before passing to these experiments, however, an account is given of the formation of citrulline from arginine by Staph. aureus; this is followed by a description of the experiments in which vitamin concentrations were varied.

Table 9.

Effect of replacing arginine with ornithine on a poor coagulase producing strain of Staph. aureus.

| Medium | 2-day cultures | | 4-day cultures | | 7-day cultures | |
|---|---|---|---|---|---|---|
| | Cell counts per ml. x $10^8$ + | Coagulase titre* | Cell counts per ml. x $10^8$ + | Coagulase titre* | Cell counts per ml. x $10^8$ + | Coagulase titre* |
| Basal medium | 7.6 | 8 | 13.3 | 8 | 11.4 | 4 |
| Added ornithine | 11.4 | 16 | 15.2 | 16 | 15.2 | 16 |
| Added arginine | 11.4 | 32 | 15.2 | 32 | 15.2 | 16 |
| Meat-extract broth | 13.3 | 128 | 15.2 | 256 | 15.2 | 256 |

* Titrations read after 48 hours (24 hours' incubation at 37.5°C. followed by 24 hours at room temperature). See footnote to Table 5.

+ See footnote to Table 5.

following reaction (reaction (1)):-

Arginine + 2 $H_2O$ ⟶ ornithine + 2 $NH_3$ + $CO_2$ ..... (1)

Since reaction (1) involves the simultaneous reaction of two molecules of water with one molecule of arginine it almost certainly proceeds in two stages. In a personal communication Professor H.A. Krebs suggested that these might be as follows, citrulline being formed in the first stage and ornithine in the second:

Arginine + $H_2O$ ⟶ citrulline + $NH_3$ ..... (2)

Citrulline + $H_2O$ ⟶ ornithine + $NH_3$ + $CO_2$ ..... (3)

The reactions (2) and (3) had not been shown with Staph. aureus but had been demonstrated in Streptococcus faecalis by Tsutomu Sekine (1947) and Knivett (1951).

The work described in the following pages was not undertaken with the object of finding out whether there was a similar sequence of reactions with Staph. aureus, but mainly to acquire some information about the materials used and products formed in the synthesis of coagulase. For this reason a culture medium containing a high concentration of amino acids and giving a good yield of coagulase was used.

## Materials and methods

Strain. The strain of Staph. aureus used was coagulase positive, of human origin and with typical fermentation reactions.

Media and cultures. The medium used was that shown in Medium III (p.109) or with the arginine replaced by equimolar amounts of citrulline, ornithine or norleucine. These four media are referred to as the arginine, citrulline, ornithine and norleucine media. Each contained 16 amino acids, 4 vitamins and inorganic salts; glucose was omitted, as in all other experiments with chemically defined media, because of its inhibitory effect on coagulase production (Weter, 1937). The inocula were prepared by centrifuging 18-hour old broth cultures of Staph. aureus, washing the cells well with saline, and subculturing successively through three lots of arginine-free norleucine medium. From the third subculture 10 ml. lots of the various media were inoculated with a small loopful. In each experiment five media were used - a norleucine medium, an ornithine medium, a citrulline medium, an arginine medium, and a blank consisting of an uninoculated arginine medium. The norleucine medium was used as a control, giving similar growth to the arginine medium, since simple omission of arginine caused much poorer growth with the strain used. Cultures were incubated for 9 days and samples withdrawn at intervals for examination by paper chromatography.

Chromatographic methods. The samples from the different cultures were de-salted electrolytically by the method of Consden, Gordon & Martin (1947). After de-salting, the

## Medium III

Composition (per 100 ml. buffer* at pH 7.3) of medium used in experiments on formation of citrulline from arginine.

| | | | |
|---|---|---|---|
| L-arginine base | 0.16 gm. | DL-alanine | 0.16 gm. |
| DL-glutamic acid | 0.16 " | DL-leucine | 0.06 " |
| DL-aspartic acid | 0.16 " | DL-phenylalanine | 0.06 " |
| DL-methionine | 0.16 " | DL-tryptophan | 0.06 " |
| DL-valine | 0.12 " | DL-histidine HCl | 0.06 " |
| DL-threonine | 0.12 " | DL-proline | 0.06 " |
| DL-isoleucine | 0.12 " | DL-serine | 0.06 " |
| DL-lysine HCl | 0.06 " | glycine | 0.06 " |

$MgSO_4 \cdot 7H_2O$ = 0.5 gm.; $K_2HPO_4$ = 0.5 gm.

Biotin 1.0 µgm.; aneurin 0.3 µgm.; pyridoxin 120 µgm.: nicotinamide 20 µgm.

---

* The buffer was 0.2 M. $K_2HPO_4$/0.1 M. citric acid Sörensen's buffer.

samples were evaporated to dryness and taken up to the original volume in 50% ethyl alcohol. 30 micro-litre spots were used in two dimensional chromatograms consisting of a 24-hour ascending run using phenol saturated with water as the solvent, followed by a 48-hour descending run with a water-saturated solution of one part glacial acetic acid in four parts n-butyl alcohol. The spots were neutralised with ammonia and the butyl alcohol/acetic acid run was carried out in the presence of ammonia vapour. After removal of the solvent the amino acid spots were developed by spraying with a solution of ninhydrin.

## Results

The chromatograms of the 3-day and 5-day cultures showed only the amino acid spots to be expected from the known composition of the media in the case of the uninoculated arginine blank medium and the ornithine and norleucine media. The 3-day and 5-day arginine cultures showed an additional ornithine spot and the 3-day and 5-day citrulline cultures showed an ornithine spot and an unidentified spot.

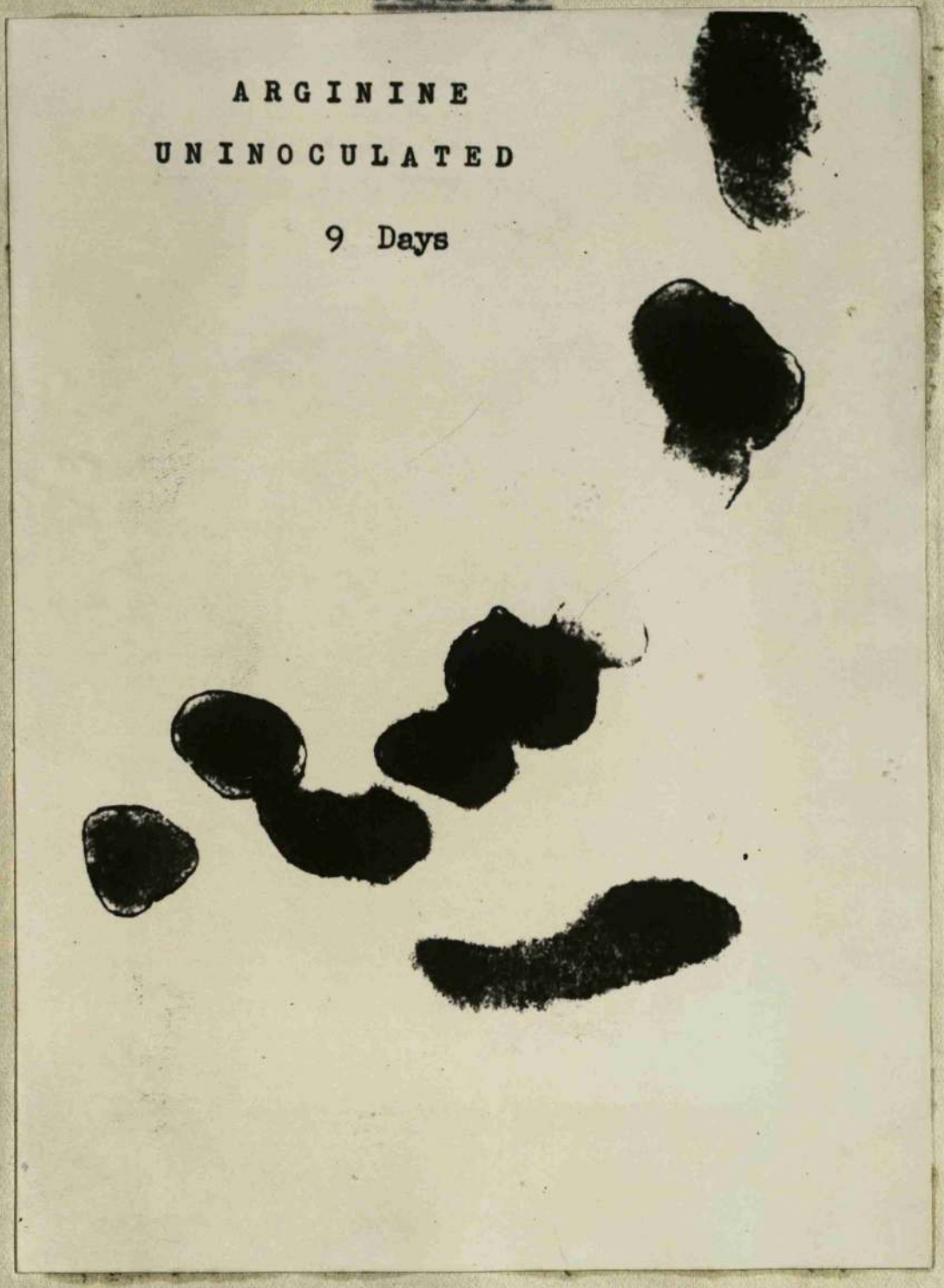
The chromatogram of the 7-day old arginine culture showed the appearance of a citrulline spot which was missing from the 7-day norleucine and ornithine chromatograms and from the blank (uninoculated arginine) medium. This citrulline spot was even more marked on the chromatogram of

the 9-day arginine culture but again was absent from the 9-day norleucine and ornithine cultures and from the blank medium. Figs. 6 - 10 (pp.112-116) show the chromatograms of the 9-day uninoculated arginine medium and norleucine, ornithine, citrulline and arginine cultures. The separation of the spots is poor with the high concentration of amino acids used, but with lower concentrations the small amount of citrulline formed would not have been detectable.

Since the completion of these experiments Stein & Moore (1951) reported the break-down of arginine during electrolytic de-salting and their results suggested that citrulline might be one of the products of this break-down. The possibility of the citrulline formed in the arginine cultures having arisen as an artefact of de-salting was small, however, since all the specimens were subjected to the same de-salting procedure; yet no citrulline was found in the younger arginine cultures or in the uninoculated arginine medium. To clinch the matter, further experiments with resting cells in which de-salting was omitted were carried out; these ruled out the possibility of citrulline having arisen as a result of the electrolytic break-down of arginine.

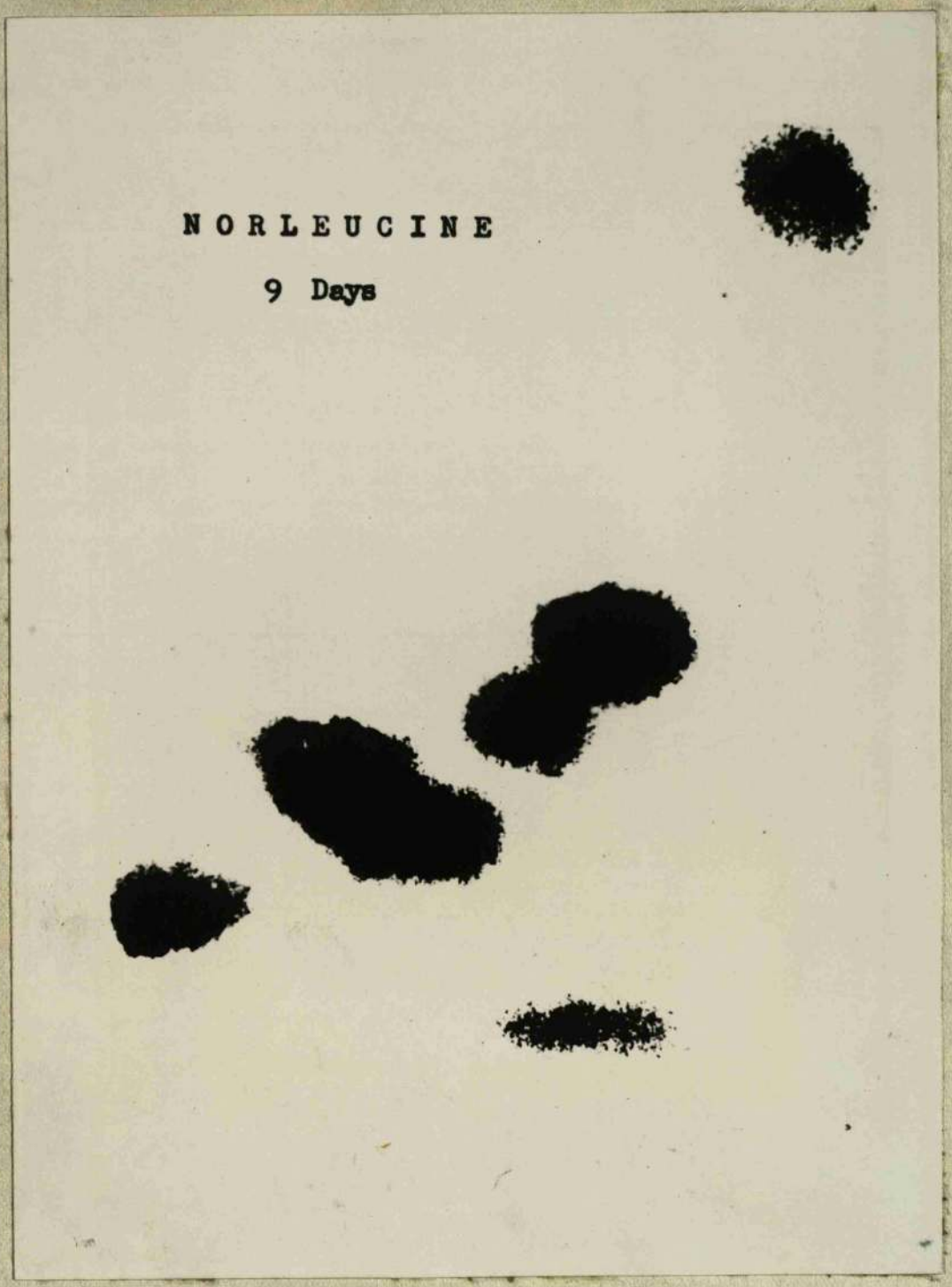
Method. A suspension of well-washed cells of Staph. aureus in distilled water was incubated for 24 hours at 37.5°C. with an equal volume of a 0.4% solution of arginine in distilled

Figure 6.



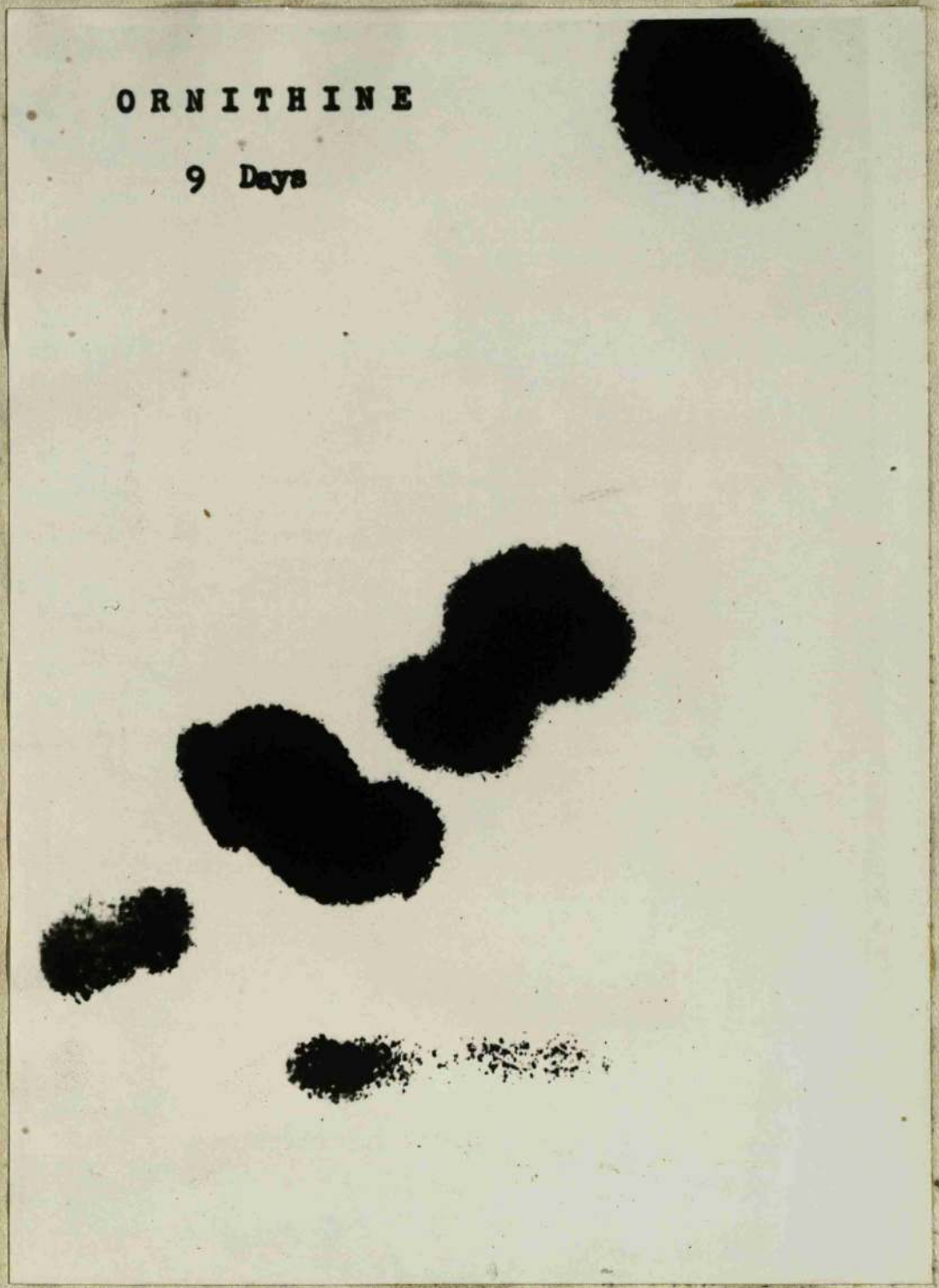

Chromatogram of uninoculated arginine medium after 9-days' incubation at 37.5°C.

Figure 7.



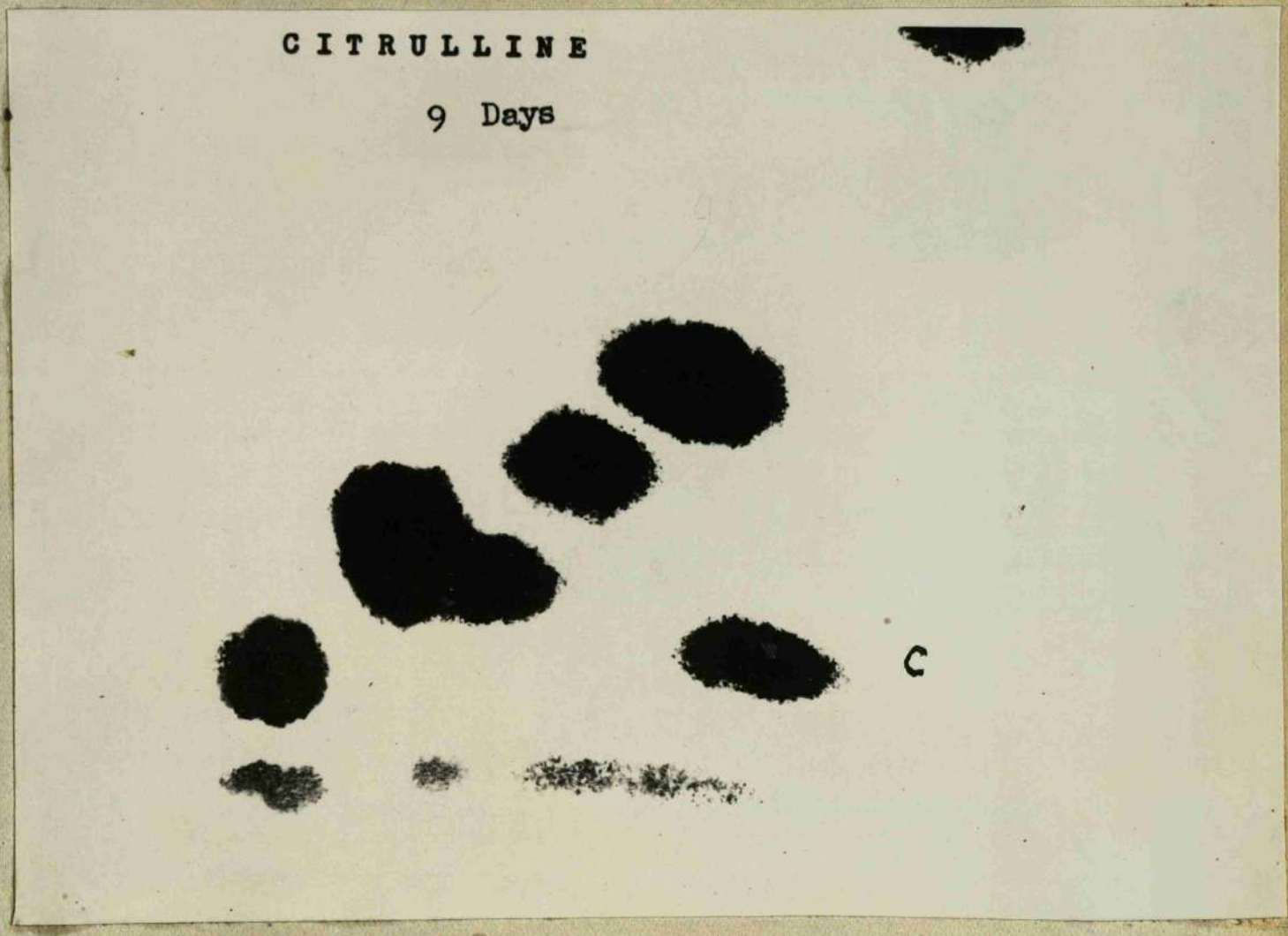

Chromatogram of norleucine culture after 9-days' incubation at 37.5°C.

Figure 8.




Chromatogram of ornithine culture after 9-days' incubation at 37.5°C.

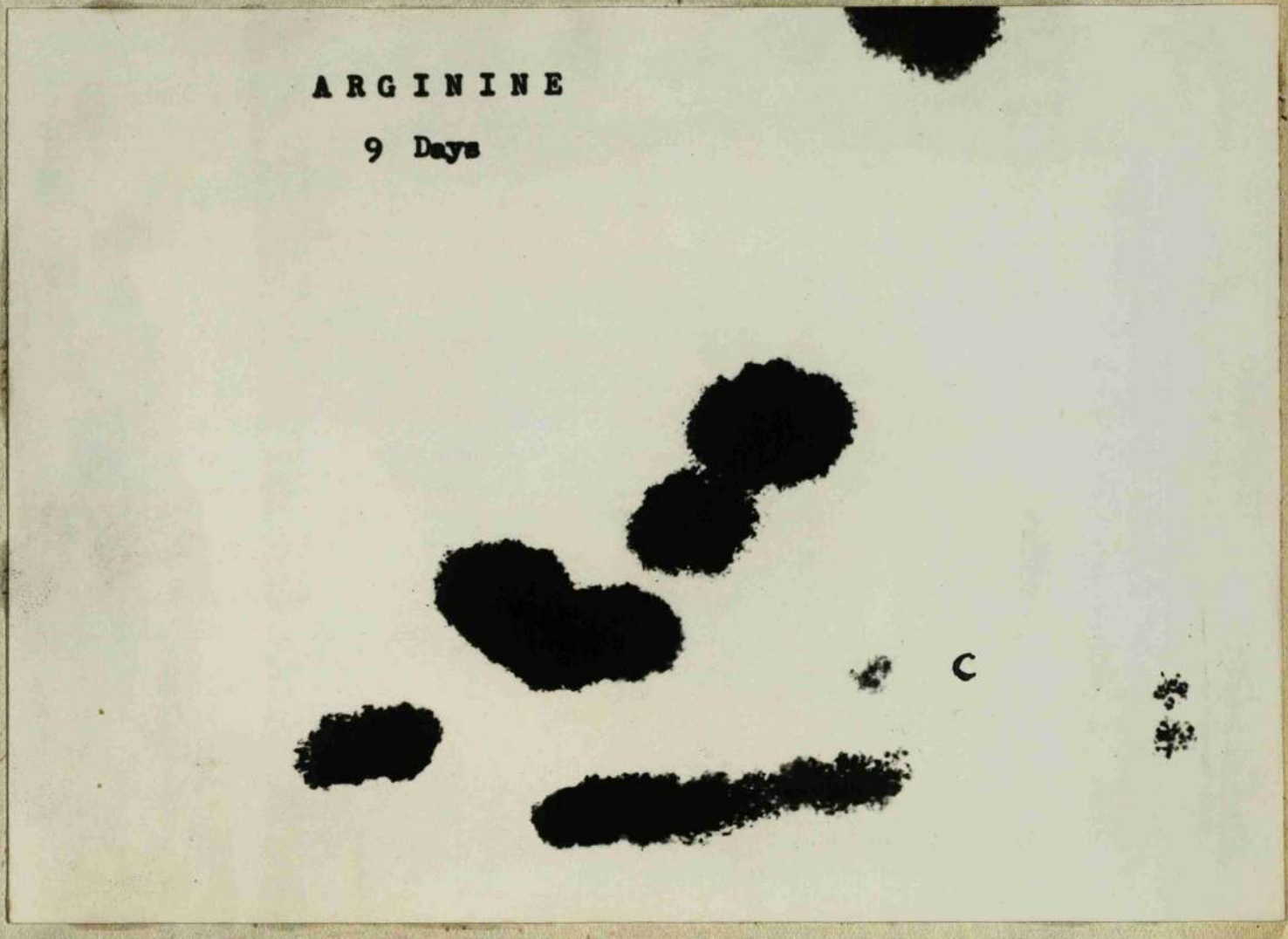
Figure 9.




Chromatogram of citrulline culture after 9-days' incubation at 37.5°C.

C shows the position of the citrulline spot.

Figure 10.




Chromatogram of the arginine culture after 9-days' incubation at 37.5°C.

C shows the position of the citrulline spot.

water. Descending chromatograms using butyl alcohol/acetic acid as solvent were carried out on 10 micro-litre spots of the following:- (1) suspension of cells in distilled water, (2) solution of arginine in distilled water, and (3) mixture of cells and arginine.

Results. The cell suspension showed no amino-acid spots at all, the arginine solution a single amino-acid spot, but the mixture of cells and arginine showed a marked formation of both citrulline and ornithine accompanied by a decrease in arginine. Figs. 11 and 12 (pp.119, 120) show the chromatograms of the arginine + cell suspension alone and with marker spots of ornithine and citrulline added.

Thus the formation of citrulline from arginine has again been demonstrated, this time in circumstances which eliminate the possibility of the citrulline having arisen as an artefact.

When this finding is considered together with the known formation of ornithine from arginine and the formation of ornithine from citrulline (shown in the citrulline 9-day chromatogram) the breakdown of arginine to ornithine in the two stages suggested by Krebs is demonstrated. It appears from Table 8 (p.103) that arginine and citrulline are about equally effective in stimulating coagulase production but that ornithine is less effective than either. From this it follows that the rôle of these three amino

acids can hardly be explained by postulating that arginine and citrulline are converted to ornithine and that ornithine is the key amino acid for coagulase production.

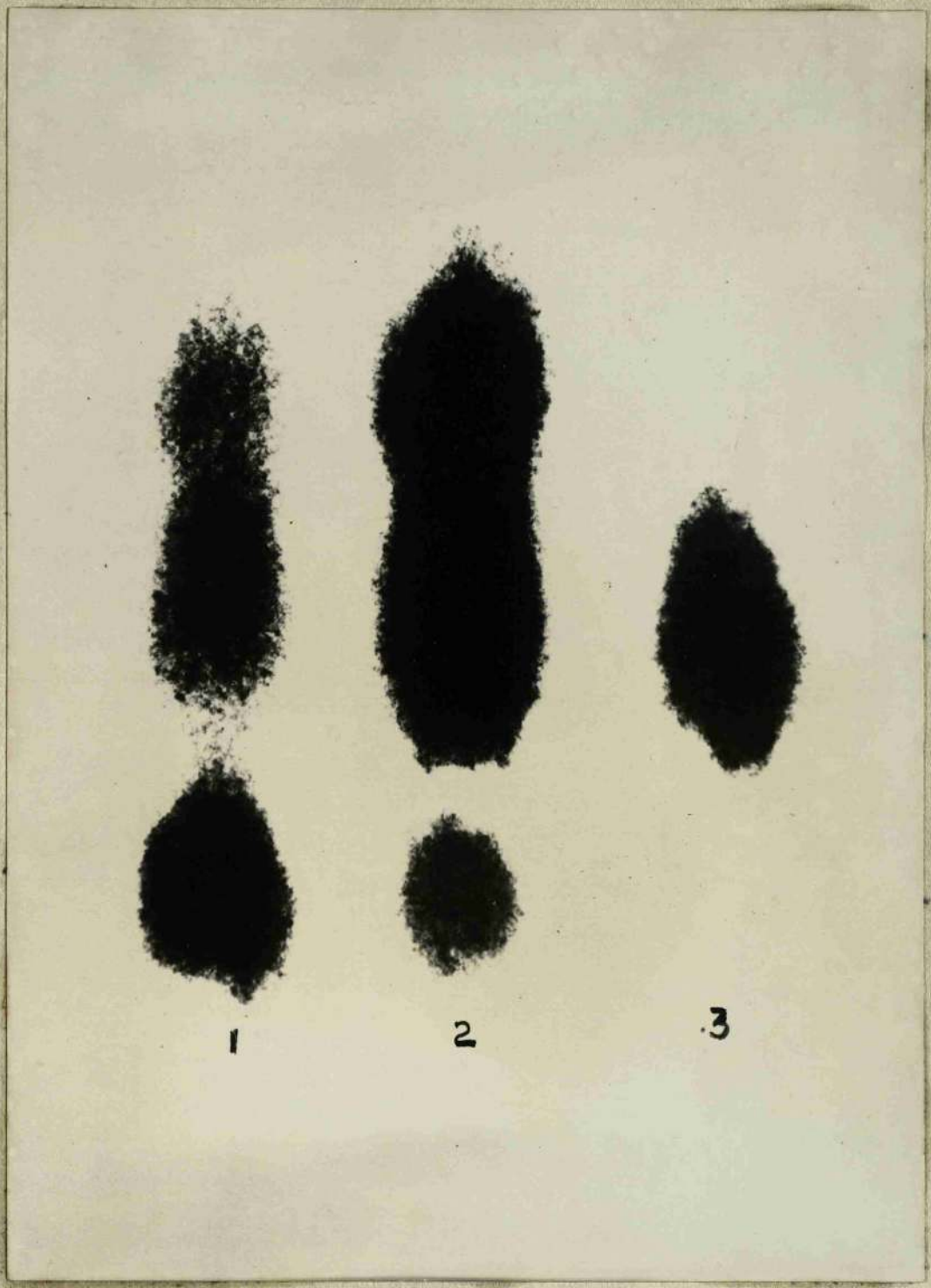
Though it is not clear what part this arginine-citrulline-ornithine conversion plays in the production of coagulase, the close relationship between the structural formulae of these three amino acids and the shared ability to stimulate coagulase production strongly suggests a common function.

Influence of vitamins on coagulase production

The experiments described earlier in which the amino-acid composition of media was varied failed to provide an explanation of the erratic results obtained. The possibility that changes in the vitamin content might have been responsible was then considered. Dilute solutions of vitamins lose their activity in the course of time and since in some of the repeat experiments (with Medium I) stock solutions of vitamins several weeks old were used, it was thought worth while to investigate the influence of vitamins.

In an initial experiment three vitamins, aneurin, pyridoxin and nicotinamide were used at the usual concentration (Medium I, p. 94) at 10 times and at 50 times greater;

Figure 11.



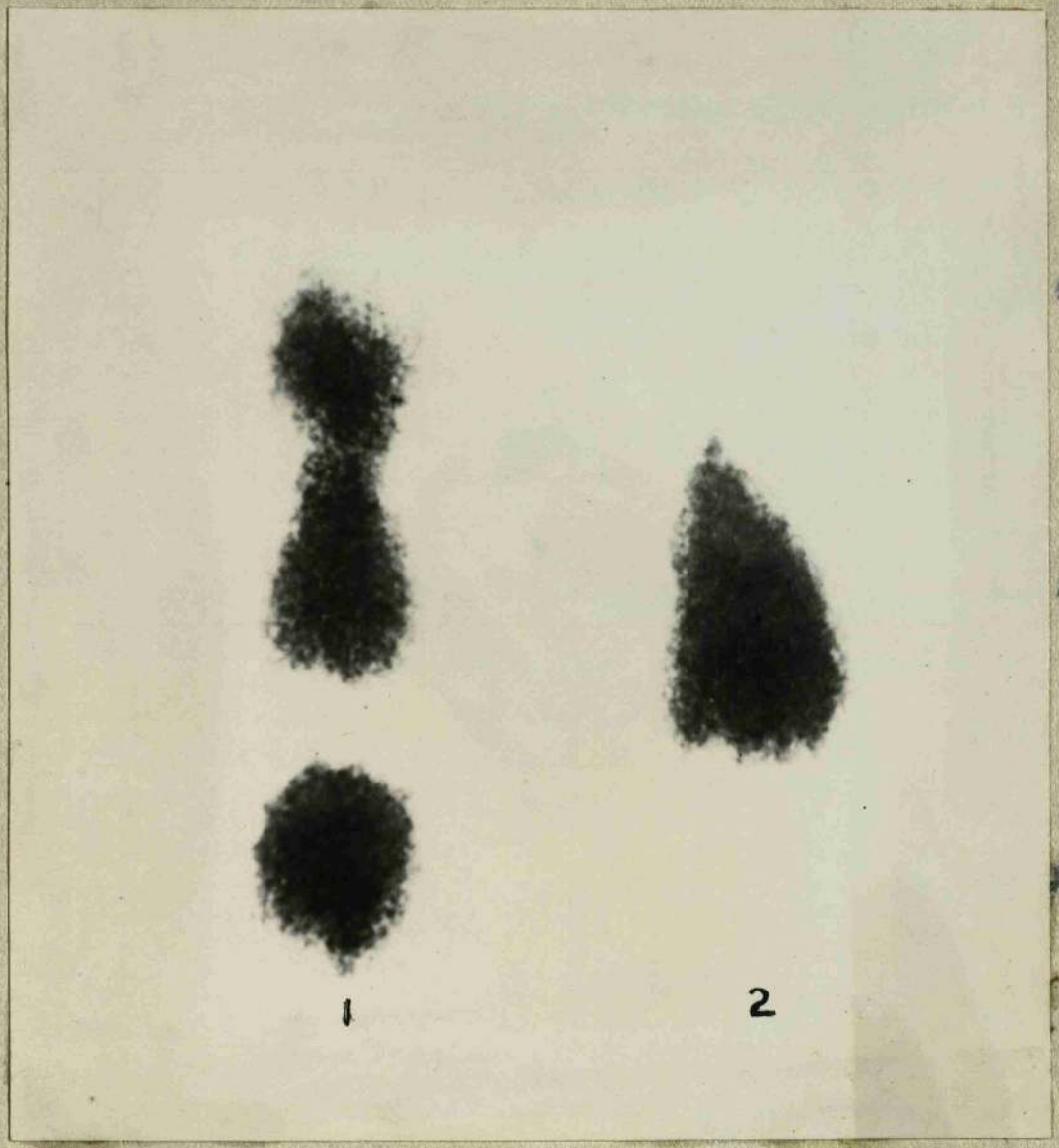

One dimensional chromatograms of the following:-

(1) Cells + arginine incubated for 24 hours at 37.5°C.

(2) Marker spots of arginine, ornithine and citrulline.

(3) Marker spot of arginine.

Figure 12.




One dimensional chromatogram of:

(1) Cells + arginine incubated for 24 hours at 37.5°C.

(2) Marker spot of arginine.

the rest of the components of the medium were at half the concentration shown in Medium I. The organism used was strain McCracken. The results given in Table 10 (p.125) show that although the amount of coagulase produced in each case is small, there is a slight but definite increase with increasing concentration of vitamins.

Subsequent tests showed that strain McCracken had lost its ability to give high yields of coagulase even in broth cultures and it was decided to repeat the experiment with a different strain (strain 5). At the same time the possibility that one or more of the vitamins might be inhibitory at higher concentrations was investigated; the individual vitamins were used at the concentrations given in Medium I or at 100 times these concentrations. Amino acids and salts were at three-quarters of the concentrations given in Medium I. The media were inoculated from the third successive subculture of strain 5 in low-vitamin medium. Table 11 (p.126) gives the results. Again only a low final titre was reached but the results suggested that pyridoxin might be inhibitory to coagulase production. A further experiment was therefore carried out with the same strain and with one or more vitamins omitted. The remaining constituents were at half strength of Medium I. The inocula were from the third successive subculture in a pyridoxin deficient medium. Table 12 (p.127) gives the results.

In this third experiment in which the concentration of the vitamins was varied the omission of pyridoxin caused no change in coagulase production. There was a very marked increase, however, in coagulase when aneurin was increased to 100 times the usual concentration. A similar increase in nicotinamide concentration did not affect the amount of coagulase produced.

The most striking feature of the results of this last experiment was the appearance of a high titre of coagulase in two of the media and it was decided, therefore, to investigate the influence of vitamins on coagulase production more fully. Experiments were carried out in which vitamins were omitted or increased and the amino acids and salts were at half, three-quarters or the full strength of Medium I. The staphylococcus used was the undissociated strain 5 or its rough variant (strain 5R), see p. 66. The highest titre of coagulase reached in the course of 6 to 7 days' culture is given. Tables 13 - 17 (pp. 128-132) give the results of different experiments which are summarised as follows:

## Summary

(1). Pyridoxin had no effect on coagulase production in three experiments and was inhibitory in one when aneurin and nicotinamide were at the normal concentrations. When aneurin and nicotinamide were at a high concentration pyridoxin was once inhibitory and once stimulatory.

(2). High concentrations of aneurin were sometimes stimulatory and sometimes without effect, but in the presence of high concentrations of nicotinamide always led to a marked increase in coagulase production.

(3). High concentrations of nicotinamide were effective in increasing coagulase production only in the presence of high concentrations of aneurin.

(4). A combination of aneurin and nicotinamide at 100 times their normal concentrations caused a marked increase in coagulase production (8 to 16 times) in every experiment.

It is obvious from these results that the response of Staph. aureus to changes in the vitamin content of a chemically defined medium is not consistent. The strain used behaved as though its vitamin requirements differed from experiment to experiment. It seemed, however, that a medium containing no pyridoxin and with aneurin and nicotinamide at 100 times normal concentration could be relied on

to give a fairly consistent yield of coagulase and some experiments on arginine replacement were carried out with a slightly modified version of this medium (see Table 8, p.103).

It must be emphasised, however, that variation in coagulase production was encountered throughout this work and could not be wholly attributed to changes in either amino acid or vitamin concentration. It assumed the aspect of a biological phenomenon and strongly recalled the findings in regard to R —— S variation of staphylococci in ordinary media, see p.53. It was therefore decided to investigate the vitamin requirements of different strains and of their variants in order to test the hypothesis that variable coagulase production might be the result of strain variation. It was thought that not only might the intrinsic powers of synthesising coagulase vary but also the nutritional requirements for the synthesis.

Table 10.

Effect of increased vitamin concentrations on coagulase production of Staph. aureus.

| Vitamin+ concentrations | 2-day cultures | | 4-day cultures | | 6-day cultures | | 8-day cultures | |
|---|---|---|---|---|---|---|---|---|
| | Cell counts per ml. x10$^8$++ | Coagulase titre* | Cell counts per ml. x10$^8$++ | Coagulase titre* | Cell counts per ml. x10$^8$++ | Coagulase titre* | Cell counts per ml. x10$^8$++ | Coagulase titre* |
| Normal | 5.7 | 4 | 11.4 | 8 | 13.3 | 4 | 13.3 | 4 |
| 10 x Normal | 7.6 | 8 | 15.2 | 16 | 15.2 | 8 | 20.8 | 8 |
| 50 x Normal | 11.4 | 8 | 19.0 | 32 | 20.8 | 32 | 22.7 | 16 |

* Titrations read after 24 hours at 37.5°C. See footnote to Table 5

\+ Normal vitamin concentration as in Medium I.

++ See footnote to Table 5.

## Table 11.

**Effect of varying the concentration of vitamins on coagulase production by Staph. aureus.**

| Vitamin concentration+ | | | 3-day cultures | | 5-day cultures | | 7 day cultures | | 9-day cultures | |
|---|---|---|---|---|---|---|---|---|---|---|
| Aneurin | Pyridoxin | Nicotinamide | Cell counts per ml. $x10^8$++ | Coagulase titre* | Cell counts per ml. $x10^8$++ | Coagulase titre* | Cell counts per ml. $x10^8$++ | Coagulase titre* | Cell counts per ml. $x10^8$++ | Coagulase titre* |
| 1 | 1 | 1 | 3.8 | 4 | 9.5 | 64 | 11.4 | 64 | 11.4 | 32 |
| 100 | 1 | 1 | 7.6 | 8 | 13.3 | 64 | 19.0 | 64 | 11.4 | 64 |
| 1 | 100 | 1 | 3.8 | <4 | 9.5 | 16 | 11.4 | 32 | 13.3 | 16 |
| 1 | 1 | 100 | 7.6 | 8 | 11.4 | 64 | 11.4 | 128 | 13.3 | 64 |

* Titrations read after 24 hours at 37.5°C. See footnote to Table 5.

\+ Vitamin concentration: 1 = concentration given in Medium 1; 100 = 100 times the concentration given in Medium 1.

++ See footnote to Table 5.

## Table 12.

Effect on coagulase production by Staph. aureus of varying the vitamin concentration

| Vitamin concentration+ | | | 2-day cultures | | 4-day cultures | | 6-day cultures | |
|---|---|---|---|---|---|---|---|---|
| Aneurin | Pyridoxin | Nicotinamide | Cell counts per ml. x $10^8$ ++ | Coagulase titre* | Cell counts per ml. x $10^8$ ++ | Coagulase titre* | Cell Counts per ml. x $10^8$ ++ | Coagulase titre* |
| 1 | 1 | 1 | 3.8 | 32 | 7.6 | 128 | 9.5 | 64 |
| 1 | Nil | 1 | 3.8 | 32 | 7.6 | 128 | 7.6 | 64 |
| 100 | Nil | 1 | 5.7 | 32 | 13.3 | 1000 | 13.3 | 1000 |
| 1 | Nil | 100 | < 3.8 | 32 | 7.6 | 128 | 7.6 | 64 |
| - | Nil | 100 | < 3.8 | 32 | 7.6 | 128 | 7.6 | 128 |
| 100 | Nil | - | No growth | | No growth | | No growth | |
| 100 | Nil | 100 | 5.7 | 64 | 13.3 | 512 | 13.3 | 1000 |
| Meat Extract | | | Not done | 4000 | Not done | 8000 | Not done | 16000 |

* Titration read after 24 hours at 37.5°C. See footnote to Table 5.

\+ Vitamin concentration: 1 = concentration given in Medium 1; 100 = 100 times the concentration given in Medium 1.

++ See footnote to Table 5.

## Table 13.

Effect of omitting pyridoxin on coagulase production

| Vitamin concentration+ | | | Highest coagulase titre reached* | | | |
|---|---|---|---|---|---|---|
| Aneurin | Nicotinamide | Pyridoxin | Expt.1 | Expt.2 | Expt.3 | Expt.5 |
| 1 | 1 | 1 | 128 | 16 | 256 | 128 |
| 1 | 1 | Nil | 128 | 16 | 512 | 128 |

## Table 14.

Effect of increasing pyridoxin concentration on coagulase production

| Vitamin concentration+ | | | Highest coagulase titre reached* | |
|---|---|---|---|---|
| Aneurin | Nicotinamide | Pyridoxin | Expt.3 | Expt.5 |
| 100 | 100 | Nil | 128 | 2000 |
| 100 | 100 | 1 | 32 | 4000 |
| 100 | 100 | 10 | Not done | 4000 |
| 100 | 100 | 100 | Not done | 4000 |

* Titration read after 24 hours at 37.5°C. See footnote to Table 5.

+ Vitamin concentrations: 1 = concentration given in Medium 1; 10 = 10 times concentration given in Medium 1; 100 = 100 times concentration given in Medium 1.

Table 15.

Effect of increasing aneurin concentration on coagulase production

| Vitamin concentration+ | | | Highest titre of coagulase reached* | | | |
|---|---|---|---|---|---|---|
| Aneurin | Nicotinamide | Pyridoxin | Expt.2 | Expt.3 | Expt.4 | Expt.5 |
| 1 | 1 | Nil | 128 | 16 | 512 | 128 |
| 100 | 1 | Nil | 1000 | 16 | 512 | 1000 |
| 1 | 100 | Nil | | - | 512 | 128 |
| 100 | 100 | Nil | | - | 2000 | 2000 |

Table 16.

Effect of increasing nicotinamide concentration on coagulase production

| Vitamin concentration+ | | | Highest titre of coagulase reached* | | | |
|---|---|---|---|---|---|---|
| Aneurin | Nicotinamide | Pyridoxin | Expt.2 | Expt.3 | Expt.4 | Expt.5 |
| 1 | 1 | Nil | 128 | -- | 512 | 128 |
| 1 | 100 | Nil | 128 | -- | 512 | 128 |
| 100 | 1 | Nil | -- | 16 | 512 | 1000 |
| 100 | 100 | Nil | -- | 128 | 1000 | 2000 |

* Titrations read after 24 hours at 37.5°C. See footnote to Table 5.

+ Vitamin concentrations; 1, 10 and 100 = 1, 10 and 100 times concentration given in Medium 1.

Table 17.

Effect of increasing both aneurin and nicotinamide concentrations on coagulase production

| Vitamin concentration+ | | | Highest coagulase titre reached* | | | | |
|---|---|---|---|---|---|---|---|
| Aneurin | Nicotinamide | Pyridoxin | Expt.2 | Expt.3 | Expt.4 | Expt.5 | Expt.6 |
| 1 | 1 | Nil | 128 | 16 | 256 | 128 | 64 |
| 100 | 100 | Nil | 1000 | 128 | 2000 | 2000 | 1000 |

* Titrations read after 24 hours at 37.5°C.

See footnote to Table 5.

+ Vitamin concentrations: 1, and 100 = 1 and 100 times concentration given in Medium 1.

The behaviour of different strains and their variants in a chemically defined medium

The investigations of the influence of amino acids and vitamins on coagulase production strongly suggested that nutritional requirements differed from strain to strain. The work on R ——— S variation (see Section I, p.53) showed that even variants of a single strain differed in their ability to produce coagulase on ordinary media.

In the light of these findings it seemed desirable to investigate more extensively the behaviour of different strains and strain variants in a chemically defined medium. Vitamin requirements were particularly selected for study.

The formula of all the media used was basically that of Medium I (p.94) but without pyridoxin. It was used (a) as such and (b) with a hundredfold increase in the concentrations of aneurin and nicotinamide. Control cultures in meat-extract broth were made in each case. All media were inoculated with washed organisms which had been sub-cultured through three successive lots of low-vitamin media.

The organisms selected for testing included the rough, high-coagulase-producing variants of three strains (5R, $M^{C}$D.R. and $M^{C}$C.R), the smooth, low-coagulase-producing variant of one of these strains (McD.S) and an undissociated strain (Kelly). In Experiment 3 a newly isolated rough variant (D.R) was substituted for strain Kelly, which had been found

to produce very small quantities of coagulase even in meat-extract broth.

During preparation of the inocula for these experiments, it became evident that growth requirements differed among the several strains, and even between the variants of a single strain. Thus the rough variant of strain ($M^{C}D.R$) gave much poorer initial growth than any of the other strains tested. In two experiments $M^{C}D.R$ became adapted but in the third it stopped growing altogether. The smooth variant of the same strain ($M^{C}D.S$) grew freely throughout the experiments. Because of the failure of the rough variant to grow in the third passage, strain $M^{C}D$ ($M^{C}D.R$ and $M^{C}D.S$) was omitted from Experiment 3. Table 18 (p.133) summarises the results and gives the highest cell count and coagulase titre reached by each culture.

A striking finding is the failure of strain 5R to give high yields of coagulase although in the past, in the same medium, high titres had been obtained; high titres were, in fact, achieved in the present experiments in meat-extract broth. The possibility of error in the preparation of the media was ruled out by making new media with new constituents. Again strain 5R failed to give much coagulase. The fact that strain $M^{C}C.R$ gave good coagulase production in Experiment 2 also argued against the medium being defective.

## Table 18.

Coagulase production of strains and their variants in high- and low-vitamin media

| Strain | Medium+ | Expt.1 Cell Counts per ml. x10⁸++ | Expt.1 Highest Coagulase titre* | Expt.2 Cell counts per ml x10⁸++ | Expt.2 Highest coagulase titre* | Expt.3 Cell counts per ml. x10⁸++ | Expt.3 Highest coagulase titre* |
|---|---|---|---|---|---|---|---|
| 5 R | 1 | 9.5 | 8 | 3.8 | 16 | 5.7 | 32 |
| " | 100 | 11.4 | 16 | 5.7 | 32 | 7.6 | 64 |
| " | M.E. | 22.7 | 16,000 | 26.5 | 32000 | 38.0 | 64000 |
| $M^{c}D$ R | 1 | 9.5 | Nil | 3.8 | Nil | Not enough growth for inoculation | |
| " | 100 | 9.5 | Nil | 5.7 | Nil | | |
| " | M.E. | 24.6 | 2,000 | 22.7 | 1000 | | |
| $M^{c}D$ S | 1 | 9.5 | Nil | 15.2 | Nil | Not done | |
| " | 100 | 11.4 | Nil | 15.2 | Nil | | |
| " | M.E. | 19.0 | 1,000 | 15.2 | 64 | | |
| $M^{c}C$ R | 1 | 9.5 | 8 | 7.6 | 500 | 15.2 | 16 |
| " | 100 | 13.3 | 16 | 7.6 | 1000 | 11.4 | 16 |
| " | M.E. | 9.5 | 28 | 28.4 | 32000 | 17.2 | 500 |
| Kelly | 1 | 9.5 | Nil | 7.6 | Nil | Not done | |
| | 100 | 9.5 | Nil | 9.5 | 16 | | |
| | M.E. | 13.3 | 256 | 11.4 | 16 | | |
| D R | 1 | Not done | | not done | | 5.7 | 128 |
| | 100 | | | | | 7.6 | 256 |
| | M.E. | | | | | 22.7 | 1000 |

* Titrations read after 24 hours at 37.5°C. See footnote to Table 5.

\+ Medium: 1 = Medium 1 with vitamins at concentration shown on p. 94.
100 = Medium 1 with vitamins at 100 times concentration shown
M.E. = Meat-extract broth.

++ See footnote to Table 5.

During these experiments evidence was beginning to accumulate that the R, high-coagulase-producing variants and the S, low-coagulase-producing variants were convertible one into the other with varying degrees of ease (see p.68). And, in fact the R —— S variation was found, by plating, to be the cause of the variations in production of coagulase with strain M$^{c}$C.R both in the chemically defined medium and in meat-extract broth. For strain 5 R, however, this does not hold since plating of cultures in chemically defined media of low coagulase titre gave R colonies.

This investigation of the behaviour of different strains and their variants in chemically defined media concludes the study of the relationship of metabolic requirements to coagulase production. As the result of this work, the conception of Staph. aureus as a species with complex but relatively fixed nutritional requirements had to undergo a radical change. The results of the last set of experiments considered jointly with those of the earlier experiments on amino acids, vitamins and R —— S variation led to a new picture of the species with the extreme variability of its metabolic needs as the dominant feature.

## Summary

1. The causes of irregular production of coagulase in chemically defined media were investigated by studying the effect of varying the concentration of amino acids and vitamins.

2. The importance of arginine in the formation of coagulase and the ability of ornithine and citrulline to act as replacements were demonstrated.

3. The formation of citrulline from arginine was demonstrated both in culture and with resting cells.

4. The vitamins aneurin and nicotinamide were found to cause a marked increase in coagulase production when present at concentrations much greater than were needed to ensure full growth of Staph. aureus.

5. Staph. aureus was found to show a wide variation in nutritional requirements which was not obvious in ordinary media but revealed itself in chemically defined media.

6. Irregular production of coagulase in chemically defined media could be partly attributed to R —— S variation. In addition it was found that strains and variants of a single strain could vary in their nutritional requirements both for growth and coagulase production while remaining in the R form.

The work on formation of citrulline from arginine was carried out in collaboration (Lominski, Morrison & Porter, 1952).

## SECTION XXI

### The mode of action of coagulase



(Experimental ... p.145 et seq.)

## A. Introduction. The components involved in staphylococcal plasma clotting

General introduction

The starting point for a discussion of the mode of action of coagulase must of necessity be the work of Smith & Hale (1944) which was described in an earlier section (p.46). They showed, it will be recalled that coagulase would not clot purified fibrinogen and required the presence of a component of human and other plasmas called activator with which it reacted to give a thrombin-like substance, active coagulase. Their findings raised a number of problems which after eight years are still unsolved.

The first of these problems is whether coagulase or activator is the quantitative precursor of active coagulase. Smith & Hale left the matter open and suggested that either explanation would fit the known facts. A further possibility is that active coagulase is the product of combination of activator with coagulase.

Another problem arising from Smith & Hale's discovery is concerned with the nature of the activator. Is it identical with any of the factors involved in the normal blood-clotting mechanism? An account of the different attempts which have been made to answer these questions is given below.

Relation of activator to the components of blood-clotting

Thrombokinase. Smith & Hale considered the possibility of activator being identical with thrombokinase but dismissed it for a number of reasons. Dialysed plasma, from which the calcium had doubtless been removed, showed no kinase activity but retained its original content of coagulase activator. They found also that guinea-pig testis although a rich source of thrombokinase was deficient in activator, and that thrombokinase survived heating which destroyed activator. Tager & Hales (1943b) in discussing this latter result regarded heat differentiation as invalid because they were able to show some survival of activator in plasma heated for 15 minutes at 56°C. They admitted, however, that in acetone-treated rabbit brain activator is much less heat stable than thrombokinase. Guinea-pig plasma (according to Tager & Hales) contains quite a high concentration of "masked" activator which can only be revealed by acid precipitation; it is possible that similar conditions apply to guinea-pig testis.

All the same, the facts that calcium is not involved in the activation of coagulase (see p.6) and that rich sources of thrombokinase are poor sources of activator have caused Tager & Hales and other workers to accept Smith & Hale's view, and no subsequent experimental evidence has been offered to controvert it.

Prothrombin. Although no satisfactory proof of the identity of activator and prothrombin has been produced, several workers have been struck by similarities between the two substances. These similarities are summarised below. Tager & Hales (1948a) found that the activator and prothrombin contents of ethanol-precipitated plasma fractions ran parallel; others have confirmed this finding (Kaplan & Spink, 1948; Miale, 1949). They (Tager & Hales) also found that both substances have a similar zone of heat inactivation in plasma and that both are adsorbed by barium sulphate, aluminium hydroxide and magnesium hydroxide. Other similarities were found by Duthie & Lorenz. Incubation of activator or prothrombin with thrombokinase + calcium caused a parallel loss of both, rabbits and humans receiving dicumarol showed a parallel decrease in both, and finally, a specimen of dried purified prothrombin prepared from human plasma functioned as activator at a dilution of 1 in $10^9$ (Duthie & Lorenz, 1950, 1952).

The chief points of difference between prothrombin and activator are detailed below. In the first place, five successive Seitz filtrations of plasma cause a complete loss of prothrombin activity but leave the activator level almost unchanged (Tager & Hales, 1948b; Duthie & Lorenz, 1950). This argument is not quite so strong as it seems since acid precipitates from such filtrates have a slight prothrombin

activity (Tager & Hales, 1948b). Secondly, activator can be destroyed by freezing or lyophilizing plasma without impairing the prothrombin level (Kaplan & Spink, 1948). Thirdly, thrombin, which can be assumed to contain little unconverted prothrombin, shows a high activator content (Tager & Hales, 1948b). Lastly, plasma clotted by coagulase shows no loss of prothrombin as occurs in a prothrombin-thrombin conversion (Duthie & Lorenz, 1950).

If activator and prothrombin are identical one must assume a different method of conversion to the active clotting agent since calcium ions are not required by coagulase. One must also assume a different end product since coagulase is not inhibited by heparin whereas thrombin is. Finally, activator does not appear to be the quantitative precursor of active coagulase (see p. 145 et seq) as prothrombin is of thrombin.

There is thus a considerable body of evidence both for and against the view that activator and prothrombin are identical. It seems that the most reasonable attitude at present is to regard them as closely associated fractions of plasma which are separable only with difficulty.

Other components of plasma. Cerbeim, Ferguson & Travis (1947) and Miale (1949) found that although activator came down with albumin in ammonium sulphate fractionation of

plasma, finer methods of fractionation showed it to be associated with the plasma globulins. The authors were unable, however, to identify it with any of the components of the blood clotting process such as Accelerator Globulin or V factor. Hiale, in fact, regarded it as a specialised globulin characterised by its reaction with coagulase and renamed it "Coagulase Globulin". In a later paper Hiale (1952) found that the activator content of blood was reduced in haemophilia and suggested that activator might be identical with the precursor of thrombokinase. His results may, however, have been due to the difficulty of separating activator from other components of plasma.

Coagulase, activator and active coagulase

Ever since Gratia (1920a) gave the name staphylocoagulase to the clotting agent produced by Staph. aureus it has generally been accepted that coagulase is an enzyme. The basis of this belief has no doubt been the fact that if the time factor was ignored a large or small quantity of coagulase produced the same effect, the clotting of plasma, but the matter has never been thoroughly investigated. The discovery of Smith & Hale (1944) that the bacterial product was inactive per se made it clear that the enzymic properties belong to active coagulase, but even here the evidence is not very strong. About the most that can be said is that

active coagulase is heat-labile, non dialysable (Hale, 1949), and can be recovered without loss after four successive clottings of plasma (Tager, 1948b).

If we accept the enzymic nature of active coagulase there remains the problem of the parts played by activator and coagulase in the formation of this enzyme. Little may be concluded from the fact that coagulase can be prepared with a potency of one part in several million (Tager, 1948b) and that plasma is effective as a source of activator at a dilution of 1 in 120,000 (Tager, 1948a) since the quantitative aspect of the reaction between them is unknown. The problem of elucidating this reaction is extremely difficult because it can be followed only by titrating the amount of active coagulase formed which in turn can be estimated only by its ability to clot plasma. No method has yet been found of interrupting the reaction at any particular stage and separating the three substances, coagulase, activator and active coagulase.

As a result of this difficulty diverse views are held on the rôles of activator and coagulase in the formation of active coagulase. Smith & Hale, by the use of the term activator perhaps implied a slight preference for coagulase as the precursor of active coagulase, but they stated that activator might equally well fulfil this function. Tager (1948a) considered the rôle of the two components to be so

doubtful that he introduced the term Coagulase Reacting Factor (C.R.F.) in place of activator. Miale (1949) without any supporting evidence preferred to regard activator rather than coagulase as the precursor of active coagulase. Kaplan & Spink (1948) suggested the possibility that instead of a simple conversion of coagulase to active coagulase there might be a more complicated reaction between coagulase, activator and fibrinogen.

The only attempt, so far, to establish the rôles of activator and coagulase in the formation of active coagulase by a study of the kinetics of the reaction is that of Walker & Berow (1949). They came to the conclusion that the reaction was stoichiometric and that the amount of active coagulase formed in mixtures of activator and coagulase is dependent on the component not in excess. Their results are not entirely convincing because, under certain conditions of enzyme and substrate concentration, the amount of end product formed may depend on the concentration of both enzyme and substrate, and the reaction only sham a stoichiometric reaction.

Their method involved incubation of coagulase with activator for 21 hours; the amount of active coagulase formed in this period was estimated by determining the reaction-velocity constant of the conversion of fibrinogen to fibrin by the mixture. They had previously found that the con-

version of fibrinogen to fibrin in the presence of a fixed amount of active coagulase follows a first order course, so that changes in the value of the reaction-velocity constant would give a measure of the amount of active coagulase formed in mixtures of activator and coagulase. Unfortunately no indication is given of the potency of their activator preparation which was the ethanol-precipitated Fraction I of human plasma, and Tager (1943a) has shown that in the purified state this fraction contains very little activator. Thus it is more than possible that the concentration of activator was so low in many of their mixtures that the reaction had not gone to completion in 21 hours. Since their reasoning is based on the assumption that in all their mixtures the coagulase-activator reaction had gone to completion, their conclusions are open to doubt. Their results do not, therefore, rule out the possibility that active coagulase is formed by an enzyme-catalysed reaction.

The experimental work described in the following pages was undertaken with a view to solving the problem which Walker & Derow had tried to answer.

## B. The formation of active coagulase from activator and coagulase

## The formation of active coagulase from activator and coagulase

The following experiments were designed to ascertain how far either coagulase or activator conformed to the ideal conception of an enzyme as a biological catalyst. In other words, would the amount of active coagulase formed in a mixture of activator and coagulase during a long enough reaction period be dependent only on the concentration of one of these two components?

The first method used was to leave mixtures of activator and coagulase until no further formation of active coagulase was detectable and then estimate the amount of active coagulase in the mixtures by titration. The same difficulty of interpretation arose as in the experiments of Walker & Berow; the amount of active coagulase formed in the mixtures appeared to depend on the amount of coagulase present, but was this due to the enzymic nature of activator or was the reaction stoichiometric with activator in large excess?

The second method determined the amount of active coagulase formed by the speed with which mixtures of activator and coagulase clotted fibrinogen. Again the results did not give a decisive answer to the question, but in conjunction with the results of the titration experiments

appeared to rule out Walker & Berow's view that the reaction was bi-molecular and suggested rather that it was enzymic in nature with coagulase as substrate and activator as enzyme.

Finally, some experiments were carried out in which the degree of completion of the activator/coagulase reaction was measured by observing the effect of adding excess of each component in turn to a mixture in which the reaction appeared to have gone to completion. Both activator and coagulase could be demonstrated in mixtures in which the formation of active coagulase had ceased. Such results could be obtained with an enzymic reaction in which the enzyme is inhibited by the product of its activity or in a stoichiometric reaction which does not go to completion.

## Materials

### Coagulase

(1). Horse-heart digest broth or meat-extract broth cultures of a Staph. aureus known to be a good coagulase producer were incubated at 37.5°C. until they had reached a coagulase titre of 2000 to 3000. They were then heat-killed (7 minutes in a Koch's steriliser) and centrifuged and the clear supernatant was used as a source of coagulase.

(2). A partly purified coagulase was made from cultures of the same strain in 2% peptone water or meat-extract broth.

Heat-killed 5-day cultures were brought to pH 4.0 with sodium acetate/acetic acid buffer and the precipitate was allowed to sediment at 4°C. for 12 hours. After decanting, the sediment was packed by centrifuging and re-suspended in digest broth at pH 7.2. The suspension was kept at 4°C. for 12 hours and the cells and insoluble materials were then centrifuged out and discarded. This procedure, which is a partial application of the method of Tager (1948b), was used to give coagulase preparations with titres ranging from 256 to 16,000 which were free of inhibitory substances present in the original cultures.

Activator

An ammonium sulphate precipitation method was used in the preparation of activator from human plasma. 40 ml. of plasma were diluted to 200 ml. with 0.85% saline and 56 gm. ammonium sulphate added, giving 40% saturation. After 30 minutes' precipitation at room temperature the material was filtered through filter paper coated with a thin layer of kieselguhr. The filtrate, which contained most of the activator of the plasma and none of the inhibitory substances, was brought to 80% saturation with ammonium sulphate and left to precipitate for 30 minutes at room temperature before being filtered once more through coated filter paper. Activator was eluted from the kieselguhr by shaking with

0.85% saline (pH 7.5 - 8.0) and centrifuging out the insoluble material. Three elutions were carried out to give a total volume of 20 ml. eluate. This eluate was dialysed for 12 hours in tap water to remove ammonium sulphate. The dialysate was brought up to the original volume of the plasma (40 ml.) and was used as a source of activator. It was found to be free of inhibitor as tested by the method of Lominski & Roberts (1946); for sterile experiments it was filtered through sintered glass.

Fibrinogen solution

0.5% and 0.25% solutions of purified bovine fibrinogen (Armour) were used in titration and clotting-time experiments respectively. No clot formed when these solutions and equal amounts of coagulase were mixed and incubated at 37.5°C. for 24 hours; the solutions were also found to be free of coagulase-inhibitory substances. Since it was found that fibrinogen tended to precipitate out on keeping, solutions were prepared each evening for use throughout the next day.

Active coagulase

Active coagulase was made by leaving a mixture of equal parts of coagulase and activator for 48 hours at room temperature. No further formation of active coagulase was observed on longer interaction.

## Methods

### Titration of active coagulase, coagulase and activator

The titre of active coagulase was determined by making doubling dilutions in 0.85% saline or in 0.85% saline containing 0.1% merthiolate. To 0.5 ml. of each dilution of active coagulase 0.5 ml. of fibrinogen solution was added and the tubes kept at 37.5°C. for 24 hours and sometimes for a further 24 - 72 hours at room temperature.

The titre of coagulase was determined by a titration in which the test-tubes contained: 0.5 ml. of serial doubling dilutions of coagulase + 0.2 ml. of activator + 0.3 ml. of fibrinogen solution.

The titre of activator was determined by a titration in which the test-tubes contained: 0.5 ml. of serial doubling dilutions of activator + 0.2 ml. of coagulase + 0.3 ml. of fibrinogen solution.

The reciprocal of the greatest dilution to give a clot was taken in all cases as the titre.

### Estimation of active coagulase by clotting times

To 0.5 ml. of active coagulase or a mixture of activator and coagulase containing an unknown amount of active coagulase, 0.5 ml. of fibrinogen solution were added. The 4 x 1/2 in. test-tube containing the mixture was tilted gently through 45° from the vertical at 15-second intervals until one minute from clotting (determined by preliminary experiments)

and thereafter continuously. The time was noted at which a clot formed in the more concentrated solutions of active coagulase or macroscopic granules appeared in the more dilute solutions. By replicate readings it was found that the error was 5 to 10 seconds in clotting times up to 5 minutes and 5 to 10 minutes in a clotting time of 60 minutes, when the clot was forming slowly. The amount of active coagulase present was found by interpolation of the clotting time in a curve prepared from known dilutions of active coagulase.

Stability of reagents

The stability of active coagulase was determined by measuring the clotting times immediately after making dilutions in saline and after the dilutions had stood for 24 hours at 37.5°C. or at room temperature.

The stability of activator was determined by adding a fixed amount of coagulase (a) to freshly prepared and (b) to 24-hours-old dilutions of activator and measuring the clotting times immediately after making the addition.

The stability of coagulase was determined by adding a fixed amount of activator to (a) freshly prepared and (b) 24-hours-old dilutions of coagulase and measuring the clotting times immediately after making the additions.

When the clotting times of the freshly prepared and

24-hours-old dilutions were approximately the same the reagents were accepted as stable.

## Results

### Experiments in which coagulase was estimated by titration

A series of mixtures of different quantities of activator and coagulase were prepared. The dilute solutions of activator and coagulase used were made by ten-fold dilution in saline. The composition of the mixtures is shown in Table 19 (p.152). Each mixture was left for 2 hours at room temperature to allow formation of active coagulase, and the amount formed was determined by titrating doubling dilutions against fibrinogen solution. The composition of the mixture and the results of a typical experiment in which the doubling dilutions were made in merthiolated saline and the titrations carried out at 37.5°C. are shown in Table 19 (p.152). It will be seen that when the concentration of coagulase was decreased by 100 times (Mixtures 3 to 1) the amount of active coagulase formed decreased by 128 times, but even when the concentration of activator was decreased by 10,000 times the amount of active coagulase formed again dropped by 128 times (Mixtures 5 to 9).

Further experiments were performed in which the preparations of coagulase and activator, the temperature, the

## Table 19.

**Titration of active coagulase formed in mixtures containing varying proportions of coagulase and activator**

| Mixture No. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|
| Coagulase | 0.1 ml. 1/100 | 0.1 ml. 1/10 | 0.1 ml. | 1.0 ml. | 1.0 ml. | 1.0 ml. | 1.0 ml. | 1.0 ml. | 1.0 ml. |
| Activator | 1.0 ml. | 1.0 ml. | 1.0 ml. | 1.0 ml. | 0.1 ml. | 0.1 ml. 1/10 | 0.1 ml. 1/100 | 0.1 ml. 1/1000 | 0.1 ml. 1/10,000 |
| Titre of mixture | 2 | 16 | 256 | 512 | 512 | 256 | 64 | 16 | 4 |
| Titre of coagulase | 2000* | 1760* | 2320* | 1024 | 564 | 282 | 70 | 18 | 4 |
| Titre of activator | 2 | 18 | 282 | 1024 | 5640 | 28,200 | 70,400 | 176,000 | 440,000 |

Coagulase prepared by 1st method; temperature, 37.5°C.; merthiolated saline; titrations read after 24 hours.

* With doubling dilutions these three figures may represent the same amount of active coagulase: for example, if the coagulase had been potent to a titre of 3000 the above results could have been obtained.

diluent, the reaction period; and the time of reading the titrations were varied. With some exceptions, traced to the use of unsuitable materials, the results showed a similar pattern to those quoted in the typical experiment (Table 19, p.152). Changes in the concentration of coagulase in the mixtures usually led to a proportional change in the amount of active coagulase formed, but even very large changes in the concentration of activator caused relatively small changes in the amount of active coagulase produced.

Most of the experiments were carried out with mixtures which had been allowed to react for 1 - 2 hours before titrating the active coagulase formed. The justification for this procedure was the finding that varying the length of reaction time apparently made little difference to the amount of active coagulase produced (see p.154, Table 19a). On carrying out some clotting-time measurements, however, it became evident that the reaction was by no means complete within 2 hours and was in fact proceeding during the subsequent 24 hours when the diluted mixtures were in contact with fibrinogen. As a consequence of this finding and because of the sources of error introduced by the large number of pipettings which titration techniques require, it was decided to seek confirmation of the findings by another method of active coagulase estimation. Before passing to a description of the results of this other method - clotting-time measure-

## Table 19(a).

Effect of varying the length of reaction time on the formation of active coagulase in mixtures of activator and coagulase

| Coagulase | 2.7 ml. | 2.7 ml. |
|---|---|---|
| Activator | 0.3 ml. | 0.3 ml. $\frac{1}{1000}$ |
| Reaction period | Titre of active coagulase formed | |
| 1 hour | 576 | 72 |
| 6 hours | 576 | 72 |
| 24 hours | 576 | 72 |

ment - two interesting features of the titration experiments are worth mentioning.

It was found by reading titrations after 24, 48 and 72 hours that the mixtures containing low concentrations of activator showed a continued increase in titre of active coagulase, whereas mixtures containing small amounts of coagulase did not. Another point of interest was the observation that with mixtures containing a small amount of activator the titre of active coagulase took a much longer time to reach a maximum when the titration was done at room temperature rather than at 37.5°C.; this difference was not found with mixtures containing little coagulase.

Estimation of active coagulase formation by clotting time measurements

As mentioned previously, it was thought that it might be possible to confirm the results of the titration experiments by using a different method for the estimation of active coagulase. Since active coagulase is a thrombin-like substance it was decided to try to estimate it by a similar method to that used for thrombin (Quick, 1942). This proved feasible. When the clotting times of known dilutions of active coagulase were plotted against concentration the points fell on a hyperbolic curve (Fig.13). Provided that the reagents remained stable the amount of

active coagulase that had been formed in any mixture of activator and coagulase could be estimated by interpolating the clotting time of the mixture in Fig.13. In practice stable preparations of active coagulase proved difficult to obtain, the stability varying with the source of the coagulase. The only preparation of coagulase which gave a stable active coagulase had a very low titre. This was used in the first experiment. In the second experiment coagulase and activator of equal potency which gave a less stable active coagulase were used.

Experiment 1. Coagulase titre, 256: activator titre 256,000

A mixture of equal volumes of coagulase and activator was left at room temperature until the clotting time had reached a constant value. A series of dilutions of this mixture as shown in Table 20 (p.159) was prepared and the clotting time of each dilution measured. The clotting time of the undiluted mixture was taken as the clotting time of 100% active coagulase and the times of the different dilutions were used to prepare fig.13. To determine the amount of active coagulase present in any mixture of activator and coagulase the clotting time of the mixture was taken and interpolated in fig.13. Table 21 (p. 159) shows the clotting times of mixtures of 2 ml. of activator with 2 ml. of different dilutions of coagulase at intervals during the 24

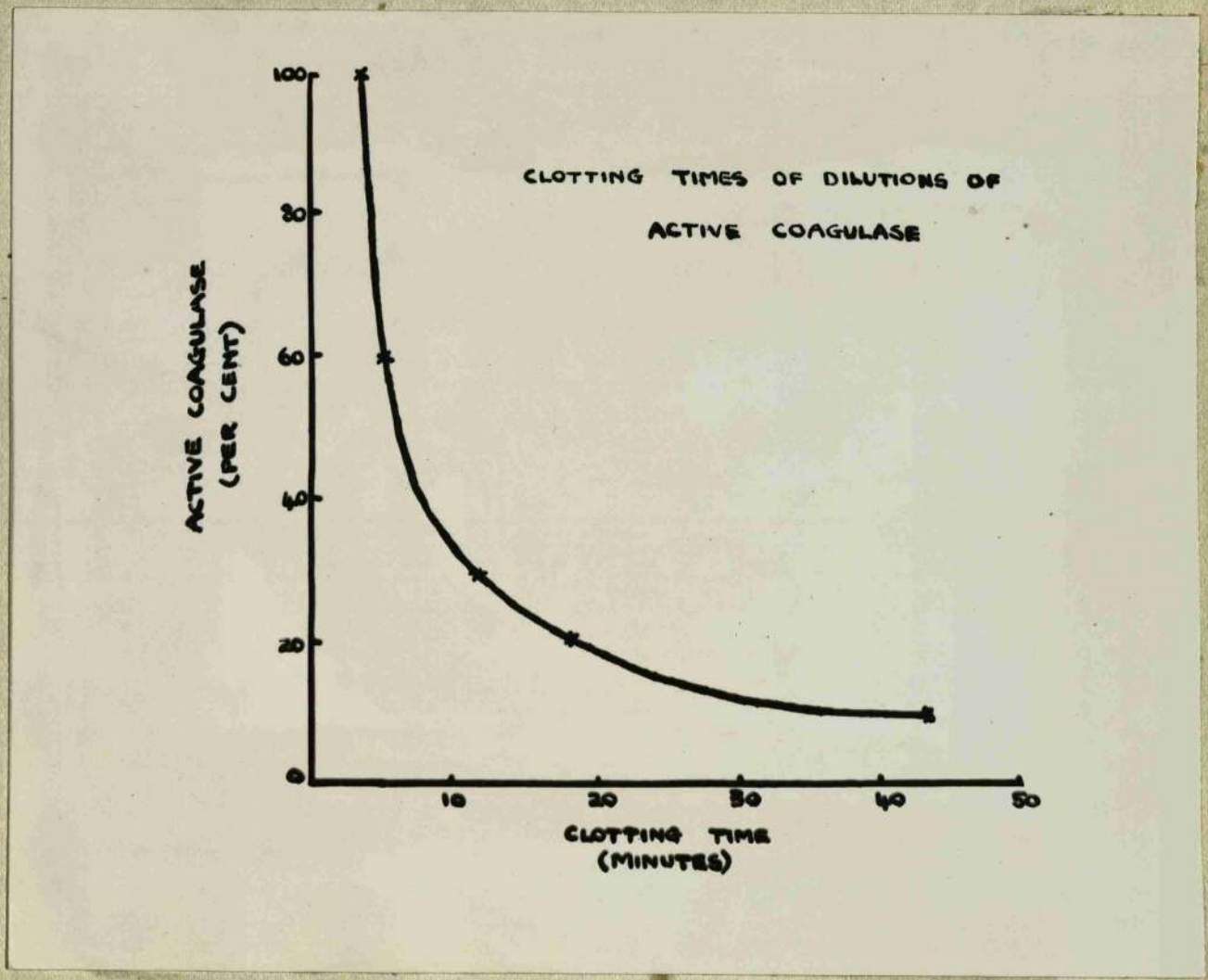
hours after mixing. Table 22 (p. 160) shows the clotting times of mixtures of 2 ml. of coagulase with 2 ml. of different dilutions of activator. Tables 25 - 27 (p. 162) give the results of the stability tests.

In Tables 23 and 24 (p. 161) are given the amounts of active coagulase formed in the various mixtures immediately after mixing and by the end of 24 hours, the figures being obtained by interpolation in fig. 13. It will be noticed that the amount of active coagulase formed in mixtures containing diminishing amounts of coagulase decreases in proportion to the decrease in coagulase. But in mixtures containing diminishing amounts of activator the amount of coagulase formed remains constant until the mixture containing 20% activator is reached and even in the case of 2.5% activator the amount of active coagulase formed has dropped only to 74%. This would be a very convincing demonstration of the enzymic nature of activator were it not for the great disparity in titres of the two reagents. A bi-molecular reaction with activator in great excess might give a similar result. Because of this the experiment described below was carried out.

Experiment 2. Coagulase titre, 8000: activator titre, 8000

In this experiment it was decided to use activator and coagulase of equal potency as measured by titration of each

Figure 13




Curve in which clotting times of different dilutions of active coagulase are plotted against amount of active coagulase.

Table 20

Clotting times of different dilutions of active coagulase

| Concentration of active coagulase | 100% | 80% | 60% | 40% | 30% | 20% | 10% |
|---|---|---|---|---|---|---|---|
| Clotting time in min.' and sec" | 3'25" | 4'20" | 5'05" | 7'30" | 11'20" | 17'30" | 43' |

Table 21.

Clotting times of mixtures of diminishing amounts of coagulase with a fixed amount of activator.

| Coagulase 2.0 ml. | 100% | 80% | 40% | 30% | 20% | 10% |
|---|---|---|---|---|---|---|
| Activator 2.0 ml. | 100% | 100% | 100% | 100% | 100% | 100% |
| Period of reaction | Clotting time in min.' and sec." | | | | | |
| 0 hours | 6'40" | 7'45" | 14'30" | 20'30" | 33' | 78' |
| 1 " | 5'15" | 6'0" | 12'20" | 18'45" | 30' | 60' |
| 2 " | 4'40" | 5'30" | 10'30" | 16'15" | 30' | 60' |
| 4 " | 4'35" | 5'20" | 10'15" | 15'30" | 27' | 60' |
| 8 " | 4'30" | 5'00" | 10'25" | 16'30" | 28' | 63' |
| 24 " | 3'35" | 4'15" | 8'0" | 12'30" | 25' | 65' |

Table 22.

Clotting times of mixtures of diminishing amounts of activator with a fixed amount of congulase

| Activator 20 ml. Congulase 2.0 ml. | 100% 100% | 50% 100% | 40% 100% | 30% 100% | 20% 100% | 10% 100% | 5% 100% | 2½% 100% |
|---|---|---|---|---|---|---|---|---|
| Period of reaction | Clotting time in min.' and sec." | | | | | | | |
| 0 hours | 6'40" | 8'00" | 8'55" | 9'10" | 10'25" | 12'25" | 18' | 22' |
| 1 " | 5'15" | 5'30" | 5'45" | 5'50" | 6'05" | 6'15" | 7'30" | 8'25" |
| 2 " | 4'45" | 5'25" | 5'20" | 5'20" | 5'20" | 5'45" | 6'35" | 7'25" |
| 4 " | 4'35" | 4'35" | 4'35" | 5'00" | 5'00" | 5'25" | 5'35" | 6'30" |
| 8 " | 4'30" | 4'25" | 4'30" | 4'30" | 4'40" | 4'50" | 5'10" | 5'50" |
| 24 " | 3'35" | 3'40" | 3'40" | 3'35" | 3'30" | 3'45" | 4'00" | 4'35" |

Table 23.

Amount of active coagulase formed in mixtures of diminishing amounts of coagulase with a fixed amount of activator

| Coagulase 2.0 ml. | 100% | 80% | 40% | 30% | 20% | 10% |
|---|---|---|---|---|---|---|
| Activator 2.0 ml. | 100% | 100% | 100% | 100% | 100% | 100% |
| Reaction period | Amount of active coagulase formed | | | | | |
| 6 hours | 45% | 40% | 24% | 13% | 14% | 4% |
| 24 hours | 100% | 82% | 39% | 28% | 10% | 6% |

Table 24.

Amount of active coagulase formed in mixtures of diminishing amounts of activator with a fixed amount of coagulase

| Activator 2.0 ml. | 100% | 50% | 40% | 30% | 20% | 10% | 5% | 2½% |
|---|---|---|---|---|---|---|---|---|
| Coagulase 2.0 ml. | 100% | 100% | 100% | 100% | 100% | 100% | 100% | 100% |
| Reaction period | Amount of active coagulase formed | | | | | | | |
| 0 hours | 45% | 39% | 36% | 35% | 32% | 23% | 20% | 16% |
| 24 hours | 100% | 100% | 100% | 100% | 100% | 96% | 90% | 74% |

## Table 25.

### Stability of active coagulase

| Concentration of active coagulase | 100% | 80% | 60% | 40% | 30% | 20% | 10% |
|---|---|---|---|---|---|---|---|
| Age of dilution | Clotting time in min' sec" | | | | | | |
| 0 hours | 3'25" | 4'20" | 5'05" | 7'30" | 11'30" | 17'30" | 43' |
| 24 hours | 3'40" | 4'15" | 5'00" | 7'15" | 10'30" | 15'20" | 39' |

## Table 26.

### Stability of coagulase

| Concentration of coagulase | 100% | 80% | 60% | 40% | 30% | 20% | 10% |
|---|---|---|---|---|---|---|---|
| Age of dilution | Clotting time in min' sec" | | | | | | |
| 0 hours | 7'00" | 8'15" | 10'40" | 15'30" | 21'30" | 36' | 93' |
| 24 hours | 7'00" | 8'45" | 11'00" | 18' | 22'30" | 38' | 96' |

## Table 27.

### Stability of activator

| Concentration of activator | 100% | 50% | 40% | 30% | 20% | 10% | 5% | 2½% |
|---|---|---|---|---|---|---|---|---|
| Age of dilution | Clotting time in min' sec" | | | | | | | |
| 0 hours | 7'45" | 8'55" | 9'50" | 10'35" | 12'30" | 15' | 20' | 22' |
| 24 hours | 8'25" | 9'55" | 10'25" | 11'10" | 12'15" | 15' | 19' | 23' |

in the presence of excess of the other. Under these conditions if the reaction were bi-molecular a mixture of equal volumes of activator and coagulase would contain the same molecular concentration of both activator and coagulase. The general equation giving the initial reaction velocity for a bi-molecular reaction between activator and coagulase is given by equation (1):

$$V = K A C \quad \ldots\ldots\ldots\ldots (1)$$

where V = the reaction velocity, A = the molecular concentration of activator, C = the molecular concentration of coagulase, K = the reaction-velocity constant.

With a mixture of equal parts of equipotent activator and coagulase the initial reaction velocity would be given by equation (2):

$$V = K a^2 \quad \ldots\ldots\ldots\ldots (2)$$

where $a = A = C$.

After any time, t, when x molecules of activator have combined with x molecules of coagulase to give x molecules of active coagulase the reaction velocity is given by equation (3):

$$V_t = K(a-x)^2 \quad \ldots\ldots\ldots\ldots (3)$$

where $V_t$ = the reaction velocity at time, t, and x = the molecular concentration of active coagulase formed.

If, however, the initial molecular concentrations of activator and coagulase were not identical, then the re-

action velocity after time, t, is given by equation (4):

$$V_t = K(p-x)(q-x) \quad \ldots\ldots\ldots\ldots\ldots\ldots (4)$$

where p = the initial molecular concentration of activator, q = the initial molecular concentration of coagulase, and x = the amount of active coagulase formed in time, t.

Let us consider two mixtures used in this experiment, one of which consists of 2 ml. of 100% activator + 2 ml. 40% coagulase, and the other of 2 ml. of 100% coagulase + 2 ml. of 40% activator. The reaction velocity of the concentrated activator, diluted coagulase mixture is given by equation (5):

$$V_1 = K(a-x)(\tfrac{4}{10}a-x) \quad \ldots\ldots\ldots\ldots\ldots\ldots (5)$$

where p, the initial concentration of activator = a, and q, the initial concentration of coagulase = a, and x = the amount of active coagulase formed.

The reaction velocity of the concentrated coagulase plus dilute activator mixture, when the same amount of active coagulase, x, has been formed is given by equation (6):

$$V_2 = K(\tfrac{4}{10}a-x)(a-x) \quad \ldots\ldots\ldots\ldots\ldots\ldots (6).$$

Thus in these two mixtures the rate of formation of active coagulase will be the same when any specified amount of active coagulase has been formed, and this should hold for all values of x.

In other words, if the reaction is bi-molecular then for any two mixtures of the equipotent activator and coagulase preparations used in this experiment, when the ratio of

coagulase to activator in one is equal to the ratio of activator to coagulase in the other the initial and subsequent rates of formation of active coagulase will be identical.

The main object of this second experiment was to discover whether, in fact, the reaction between activator and coagulase did satisfy these criteria of a bi-molecular reaction.

A preparation of active coagulase was again made by leaving a mixture of activator and coagulase at room temperature until there was no further reduction in clotting time. A series of dilutions of this preparation were tested for clotting times and fig.14 (p.171) drawn from the results shown in Table 28 (p.167). Tables 29 and 30 (pp.168,169) give the clotting times of mixtures of different concentrations of activator and coagulase throughout 24 hours (after mixing) and the corresponding amounts of active coagulase formed. Tables 31-33 (p.170) give the result of stability tests on the reagents. Figs.15 and 16 (pp.172,173) illustrate the different rates of formation of active coagulase in mixtures in which the ratio of activator to coagulase was the same as the ratio of coagulase to activator.

These two graphs illustrate very clearly that the reaction between activator and coagulase does not follow a bi-molecular course. With a mixture of dilute coagulase and

concentrated activator there is a rapid initial formation of active coagulase and very little more is formed during the remainder of 24 hours. The corresponding mixture of dilute activator with concentrated coagulase, however, gives a slower initial formation of active coagulase which continues to be produced at a fairly steady rate throughout the next 24 hours.

An unsatisfactory feature of this experiment is the instability of the active coagulase. Table 31 (p.170) shows a marked loss in the higher dilutions after standing for 24 hours at room temperature. This loss may have been smaller in the mixtures than appears from the stability tests since the loss of active coagulase was found to be greater in saline than in the presence of the proteins of plasma or culture media. Even assuming that the figures for active coagulase formation are lower than they should be owing to loss during the time of the experiment, the comparison between rates of formation remains valid. The reaction does not follow a bi-molecular course.

Another feature of this experiment was the finding that the amounts of active coagulase formed in mixtures of decreasing amounts of coagulase with a fixed amount of activator were greater than in corresponding dilutions of the active coagulase preparation. This raised the possibility that a mixture of equal parts of activator and coagulase the

Table 23.

Clotting times of different dilutions of active coagulase

| Concentration of active coagulase | 100% | 80% | 40% | 20% | 10% | 5% |
|---|---|---|---|---|---|---|
| Clotting time (min' sec") | 2'50" | 3'05" | 4'14" | 7'50" | 14'55" | 30' |

Table 29.

Clotting times and amounts of active coagulase formed for mixtures of diminishing amounts of coagulase with a fixed amount of activator. Activator and coagulase were equipotent.

| Coagulase 2.0 ml. Activator 2.0 ml. | 40% 100% | | 20% 100% | | 10% 100% | | 5% 100% | |
|---|---|---|---|---|---|---|---|---|
| Reaction period | Clotting time min' sec" | Active coagulase % | Clotting time min' sec" | Active coagulase % | Clotting time min' sec" | Active coagulase % | Clotting time min' sec" | Active coagulase % |
| 0 hours | 7'55" | 20 | 9'40" | 16 | 14'30" | 10 | 20'10" | 6 |
| 1 " | 5'30" | 27 | 8'30" | 19 | 12'10" | 12 | 18'30" | 8 |
| 2 " | 5'10" | 29 | 7'40" | 20 | 12'20" | 12 | 21'15" | 6 |
| 6 " | 4'10" | 42 | 7'00" | 22 | 11'15" | 14 | 19'10" | 8 |
| 24 " | 4'00" | 46 | 6'00" | 25 | 10'05" | 16 | 15'10" | 10 |

Table 30.

Clotting times and amounts of active coagulase for mixtures of diminishing amounts of activator with a fixed amount of coagulase. Activator and coagulase were equipotent.

| Activator 2.0 ml. Coagulase 2.0 ml. | 40% 100% | | 20% 100% | | 10% 100% | | 5% 100% | |
|---|---|---|---|---|---|---|---|---|
| Period of reaction | Clotting time min' sec" | Active coagulase % | Clotting time min' sec" | Active coagulase % | Clotting time min' sec" | Active coagulase % | Clotting time min' sec" | Active coagulase % |
| 0 hours | 9'55" | 16 | 14'30" | 10 | 19'45" | 7 | 31'00" | 4 |
| 1 " | 7'05" | 22 | 9'25" | 17 | 12'15" | 12 | 22'50" | 6 |
| 2 " | 5'55" | 26 | 7'15" | 21 | 10'20" | 15 | 16'10" | 9 |
| 6 " | 4'30" | 32 | 5'00" | 30 | 7'27" | 21 | 12'45" | 11 |
| 24 " | 3'40" | 64 | 3'55" | 58 | 5'15" | 28 | 8'50" | 18 |

Table 31.

Stability of active coagulase

| Concentration of active coagulase | 100% | 80% | 40% | 20% | 10% | 5% |
|---|---|---|---|---|---|---|
| Age of dilution | Clotting time in min' sec" | | | | | |
| 0 hours | 2'50" | 3'05" | 4'14" | 7'50" | 14'55" | 30' |
| 24 hours | 2'55" | 4'00" | 6'25" | 14'00" | 26'30" | 40' |

Table 32.

Stability of coagulase

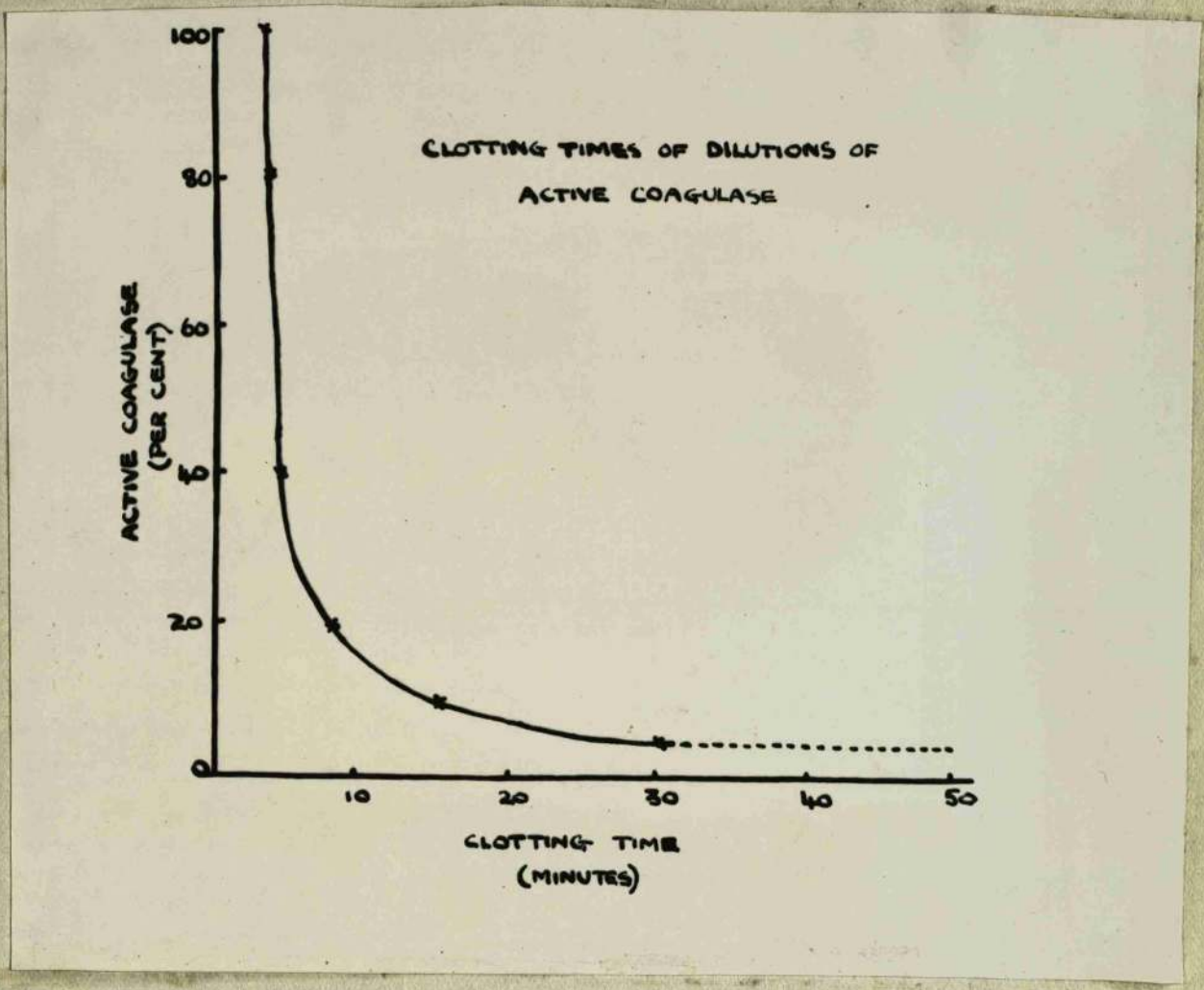
| Concentration of coagulase | 100% | 80% | 40% | 20% | 10% | 5% |
|---|---|---|---|---|---|---|
| Age of dilution | Clotting time in min' sec" | | | | | |
| 0 hours | 5'45" | 6'00" | 7'55" | 9'40" | 14'30" | 20' |
| 24 hours | 5'55" | 6'10" | 7'45" | 8'55" | 15'00" | 17'30" |

Table 33.

Stability of activator

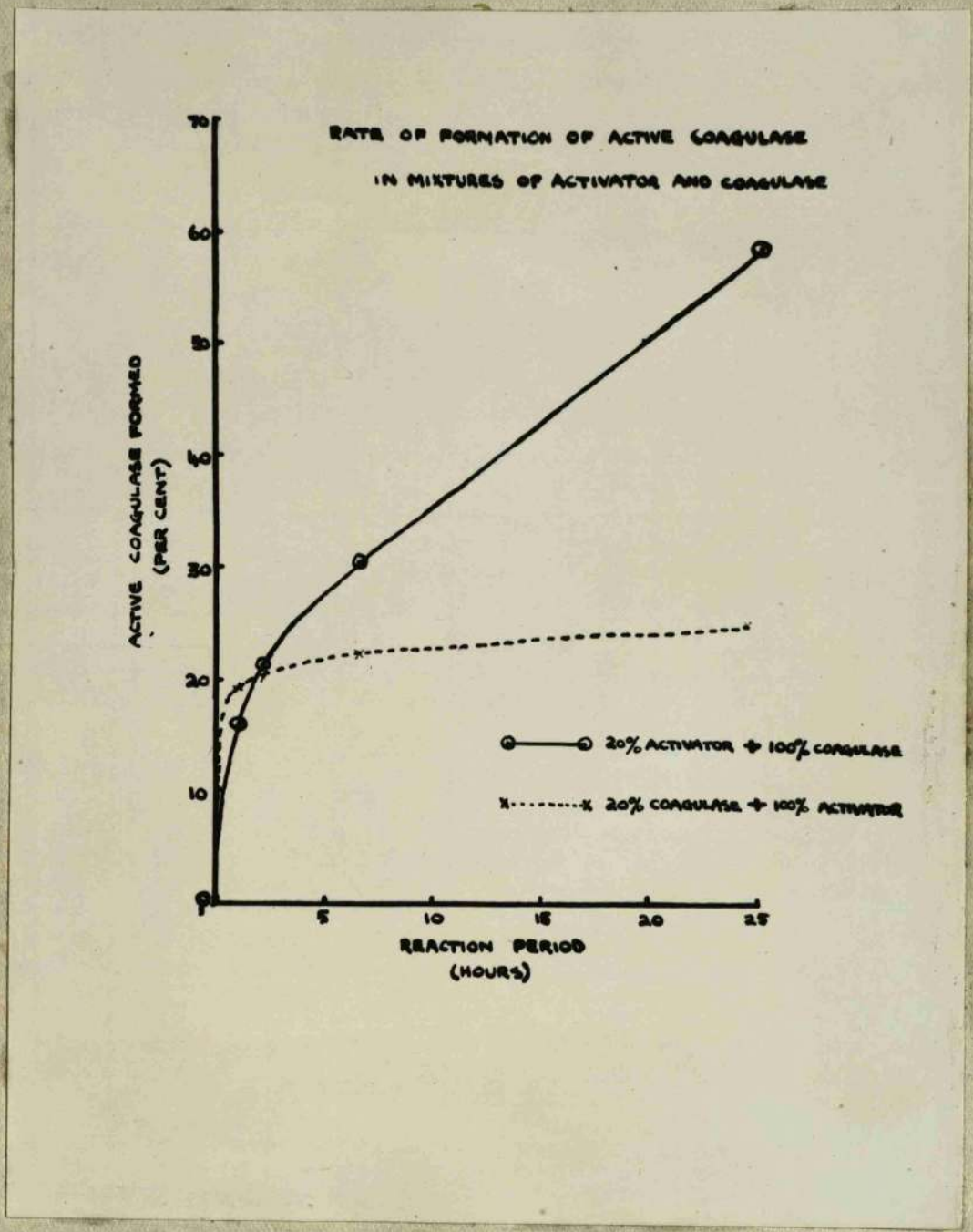
| Concentration of activator | 100% | 80% | 40% | 20% | 10% | 5% |
|---|---|---|---|---|---|---|
| Age of dilution | Clotting time in min' sec" | | | | | |
| 0 hours | 5'45" | 6'50" | 9'55" | 14'30" | 20' | 31' |
| 24 hours | 6'05" | 7'45" | 11'45" | 15'05" | 15' | 31' |

## Figure 14.



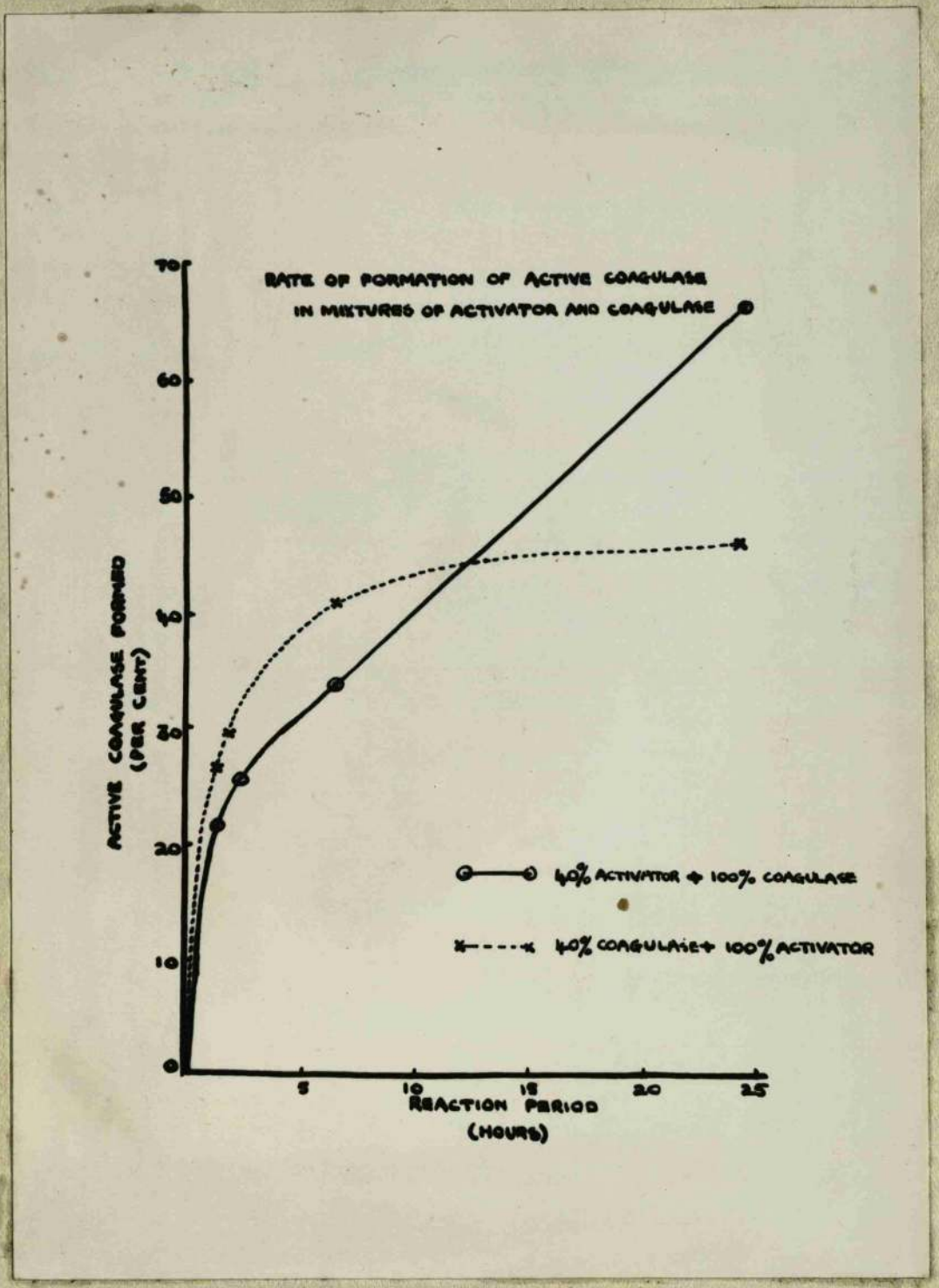

Curve in which clotting times of different dilutions of active coagulase are plotted against amount of active coagulase.

Figure 15.




Curves showing rates of formation of active coagulase in two corresponding mixtures of activator and coagulase.

Figure 16.




Curves showing the rates of formation of active coagulase in corresponding mixtures of activator and coagulase.

reaction might not have gone to completion. The next two experiments were designed to find out if this were so.

Effect of adding activator and coagulase to active coagulase

Experiment 1. Active coagulase, prepared by leaving a mixture of activator and coagulase for 48 hours, was diluted to a concentration of 20% in saline. Mixtures of equal parts of this 20% active coagulase were made with saline, activator or coagulase and the clotting time of each mixture was observed over 24 hours. The results are given in Table 34 (p.175).

Experiment 2. Two preparations of active coagulase were made. In the first the activator used was four times more potent than the coagulase, and in the second activator was first diluted in saline to the same potency as the coagulase. Mixtures of 20% dilutions of these two active coagulase preparations with equal amounts of saline, activator or coagulase were made and the clotting times measured during the following 24 hours. The results are given in Tables 35 and 36 (p.176).

Both experiments show that the active coagulase preparations contained both free activator and free coagulase since addition of either component caused a shortening of the clotting time. The suspicion that the reaction might not have gone to completion was therefore confirmed. In the

Table 34.

The effect of adding activator and coagulase to a 20% dilution of active coagulase

| Mixture | 20% active coagulase 0.75 ml. + saline 0.75 ml. | 20% active coagulase 0.75 ml. + activator 0.75 ml. | 20% active coagulase 0.75 ml. + coagulase 0.75 ml. |
|---|---|---|---|
| Age of mixture | Clotting time min' sec" | | |
| 0 hours | 13'25" | 7'35" | 14'50" |
| 5 " | 13'10" | 8'15" | 8'15" |
| 24 " | 11'30" | 8'20" | 6'35" |

Table 35.

Effect on clotting time of adding activator and coagulase to a 20% dilution of active coagulase prepared from activator (titre 16,000) and coagulase (titre 4,000).

| Mixture | 20% active coag. 2.0 ml. + saline 2.0 ml. | 20% active coag. 2.0 ml. + activator 2.0 ml. | 20% active coag. 2.0 ml. + coagulase 2.0 ml. |
|---|---|---|---|
| Age of mixture | | Clotting time | |
| 0 hours | 35' | 17' | 12' |
| 1 " | 38' | 11' | 12' |
| 24 " | 55' | 8' | 16' |

Table 36.

Effect on clotting time of adding activator and coagulase to a 20% dilution of active coagulase prepared from activator (titre 4,000) and coagulase (titre 4,000).

| Mixture | 20% active coag. 2.0 ml. + saline 2.0 ml. | 20% active coag. 2.0 ml. + activator 2.0 ml. | 20% active coag. 2.0 ml. + coagulase 2.0 ml. |
|---|---|---|---|
| Age of mixture | | Clotting time | |
| 0 hours | 57' | 32' | 11' |
| 1 " | 53' | 23' | 12' |
| 24 " | 29' | 16' | 14' |

results of the second experiment a curious fact emerges. More dilution of the mixture of equipotent coagulase and activator caused a marked shortening of the clotting time, but this did not happen with the mixture of coagulase with more potent activator.

## Summary

1. An investigation of the formation of active coagulase from activator and coagulase by titration and clotting-time measurements suggested that the reaction was enzymic with activator as enzyme and coagulase as substrate.

2. The results from both titration and clotting-time experiments were incompatible with a bi-molecular reaction between activator and coagulase.

3. When active coagulase formation had ceased, activator and coagulase were still present in the reaction mixture.

This work was carried out in collaboration (Lominski, Goulie & Morrison).

## DISCUSSION

When the investigation of conditions affecting the production of coagulase was begun the main object was to study its formation in chemically defined media. *Staph. aureus* is primarily a non-synthetic organism and it seemed possible that the use of media of known composition might enable the parts played by different amino acids in the synthesis of coagulase to be determined. In the course of this work, however, it became clear that uncontrolled variation in the coagulase-producing power of strains of *Staph. aureus* were responsible for inconsistencies.

### Production of coagulase in ordinary media and R —— S variation

It became necessary to acquire some knowledge of the conditions giving rise to fluctuations in coagulase production even in ordinary media and this prompted the investigation of a number of laboratory cultures of *Staph. aureus*. The finding that strains may consist of variants differing considerably in their ability to produce coagulase not only helps to explain the inconsistent results with chemically defined media, but also throws some light on the relationship of coagulase production to pathogenicity.

The coagulase test used in the identification of pathogenic staphylococci is performed either on colonies

from solid media or on broth cultures. In both instances a large population of organisms is tested. The problem therefore arises of whether it is permissible to look on a positive result in this test as representative of the whole population; a few R fast-clotting variants might be present in a strain which consisted largely of coagulase-negative variants. On the other hand a negative result need not exclude the possibility that the strain is capable of producing coagulase-positive variants; some basis for this exists in the finding of a coagulase-negative variant of strain 5. Certainly the results do not suggest that there is an unbridgeable gap between coagulase-positive and coagulase-negative staphylococci.

Apart from their bearing on the coagulase test the findings of this work raise interesting speculations in regard to the relationship between the amount of coagulase produced and the pathogenicity of a strain. Despite the general acceptance of coagulase production as an index of pathogenicity, very little has been done in the way of comparing the amount of coagulase produced with the virulence of strains. The only work of this nature appears to be that of Yager & Hales (1947) who tried to relate the clinical source of a number of strains to their capacity to produce coagulase. They found that staphylococcal sepsis and other severe systemic diseases might be associated

with strains of high, intermediate or low coagulase activity; it seemed to be sufficient that some coagulase and some toxin should be produced for an organism to be a potential pathogen.

The method which Tager & Hales used was to pick colonies at random from primary cultures of staphylococci isolated from lesions. After ensuring that they were coagulase-positive, the amounts of coagulase which they produced were compared. There is a possibility that any single colony selected was only one of many variants of the strain causing the infection. The wide variation which they found in coagulase production in strains causing severe infection may reflect only the wide differences in coagulase production found in the variants of one strain. Indeed they refer to other unpublished work which indicated that a given strain may give rise to daughter strains differing significantly in their capacity to elaborate coagulase. They also mention the possibility that the substrate provided by the host at one particular area favours the selection of strains of different coagulase-producing powers, and that factors may come into play within the host which modify the capacity of the organism to produce coagulase.

These speculations were no doubt prompted by the intellectual difficulty of accepting a phenomenon of such qualitative importance as quantitatively insignificant.

While the work on R - S variation bears only indirectly on this problem it suggests a way in which high coagulase production might develop within the host even with strains showing low in-vitro activity. Since the majority of the ten strains studied showed a greater tendency to give S variants from R than R from S it might well be that even if in-vivo conditions favour the R variants (high coagulase producers), the transference to laboratory media causes a change to the S form with a much lower coagulase activity. This suggestion is, of course, purely speculative, but the finding of variants differing greatly in coagulase activity raises the possibility that the lack of correlation between the amount of coagulase produced and the severity of infection may be an in-vitro phenomenon.

The findings of the work on R —— S variation help to explain why laboratory cultures of Staph. aureus sometimes show sudden changes in coagulase-producing powers and in particular why such strains often deteriorate as producers of coagulase. With most strains the tendency is greater for the R variants to change to the S form than vice versa so that a strain which was in the R form when first isolated and therefore giving good yields of coagulase may revert to the more stable S form with poorer coagulase producing powers. In a few strains, however, the tendency is in the reverse direction.

This R —— S variation also provides a partial explanation of why the same strain in the same chemically defined medium under identical conditions of culture shows wide fluctuations in coagulase production. It does not provide a complete explanation since strains have been found which, while growing in the R form vary in their nutritional requirements for coagulase production.

Production of coagulase in chemically defined media

As mentioned earlier, the main object of the investigation of the nutritional requirements of Staph. aureus in chemically defined media was to try and gain some information about the part played by different nutrients in the synthesis of coagulase. Some information was obtained, viz., that arginine, ornithine and citrulline play an important role in the production of coagulase and that without the presence of one of these three amino acids in the medium coagulase is formed only in very small amounts. Sometimes, however, even with adequate amounts of arginine present, poor coagulase yields were obtained. It was also found that citrulline is formed from arginine; this finding taken in conjunction with the known formation of ornithine from arginine and the close structural similarity of the three amino acids suggests that they share some common function in the synthesis of coagulase. The nature of this function, however, is still unknown.

Another finding was that aneurin and nicotinamide, when present at concentrations much greater than needed for full growth, have a markedly stimulatory effect on coagulase production. Again, however, the effect of high vitamin concentrations was not consistent and sometimes a strain failed to produce much coagulase in a high-vitamin medium which had previously given good results.

This inconsistency in the behaviour of Staph. aureus as regards coagulase production led to the investigation of the nutritional requirements of several strains and strain variants. As a result of this work and of the earlier work with amino acids, vitamins and R —— S variation, the conception of Staph. aureus as a species with complex and relatively fixed metabolic requirements underwent a radical change. The new picture of the species which emerged had as a dominant feature the extreme variability of its metabolic needs. A tentative description of the variable behaviour of Staph. aureus which led to this new conception is given below.

1. In a chemically defined medium, though complex and believed to contain all essential nutrients some, but not all, strains and strain variants of Staph. aureus may have their nutritional requirements satisfied.

2. In such a medium some strains or variants of a

single strain may produce coagulase and others, though growing satisfactorily, produce none.

3. A strain may grow and produce coagulase but the amount of coagulase formed may fluctuate as a result of R —— S variation, this dissociation being sometimes at a high and sometimes at a low level.

4. A strain may grow as an R (high coagulase producing) variant which always gives high coagulase production in meat-extract broth and yet may sometimes fail, although remaining R, to produce coagulase in a chemically defined medium. At other times the same R variant in the same medium may give high yields of coagulase.

In this study only two features were considered - growth and coagulase production. When growth alone was taken as an index of the metabolic requirements of the organism the amount of variability was less than when growth and coagulase production were considered together. An investigation of a greater number of strains and a wider range of characters would almost certainly have increased the extent of variability found.

Staph. aureus is generally regarded as an assimilative rather than a synthetic organism; it now appears that the fluidity in its metabolic requirements is another characteristic of the species. Though only a few strains were used and the changes in the media were slight, a whole

gamut of variation in nutritional requirements was encountered. It is tempting, therefore, to regard this variability of Staph. aureus in its metabolic needs as a predominant feature of the species; indeed this is the most striking finding in this study of the production of coagulase.

The mode of action of coagulase

A great difficulty in attempting to determine the rôles of activator and coagulase in the formation of active coagulase has already been mentioned (p.142); activator and coagulase can be demonstrated only by their ability to form active coagulase which in turn can be estimated only in terms of its ability to produce a fibrin clot. A similar problem arises in connection with the interaction of prothrombin with thrombokinase to give thrombin. Despite an enormous amount of work on this subject the question of whether thrombokinase is an enzyme or not still remains in doubt.

The approach to the problem was to attempt to find out how far the reaction of activator with coagulase conformed to a feature of enzyme/substrate reactions, namely, that the amount of end-product is directly related to the amount of substrate and tends to be independent of the amount of enzyme present if the reaction time is sufficiently long. To some extent the titration experiments in fact showed this feature;

with excess of activator the active coagulase formed was directly proportional to the amount of coagulase present. On the other hand, with excess of coagulase and diminishing amounts of activator the amount of active coagulase formed did not decrease proportionately with the activator. As a matter of fact small quantities of activator gave much greater yields of active coagulase than could have been possible if activator had been the substrate in an enzyme-catalysed reaction. Thus the results of the titration experiments suggest that coagulase is the quantitative precursor of active coagulase and that the conversion is brought about by an enzyme, activator. This view is supported by the observation that active coagulase continues to be formed over long periods in mixtures containing small amounts of activator. A stoichiometric reaction between activator and coagulase would appear to be ruled out by the finding that the amount of active coagulase formed is not directly proportional to the amount of activator present in the reaction mixture. The results of the titration experiments, however, are weakened by the disparity in potencies when activator and coagulase are titrated against each other.

In the first of the clotting-time experiments the amount of active coagulase produced in various mixtures of activator and coagulase was almost completely independent of activator and directly proportional to the concentration

of coagulase. This is in almost complete agreement with the ideal results which could be obtained if coagulase were the substrate and activator an enzyme. Unfortunately, a bi-molecular reaction in which activator was in large excess would lead to a similar finding. And in this experiment activator was in large excess as judged by the relative titres of 256 and 256,000. It was then decided to use equipotent activator and coagulase. This could not be done by concentrating coagulase since concentrated preparations were very unstable. Activator had to be diluted to achieve equipotency.

This raises a fundamental difficulty of this method of investigation. The strengths of the preparations of activator and coagulase are measured by the extent to which each can be diluted and still react with excess of the other to produce enough active coagulase in 24 hours to clot fibrinogen. This is quite a valid method of judging the relative strengths of the preparations if the reaction between them is stoichiometric, but is less valid if one of them is an enzyme. In the above experiment, if we assume that activator is an enzyme, it would be in accordance with the known behaviour of enzymes that it should produce a demonstrable amount of active coagulase in 24 hours at a dilution of 1 in 256,000. But it does not follow that a mixture of undiluted coagulase with highly diluted activator must give

complete conversion of coagulase to active coagulase however long the reaction is allowed to proceed. Enzymes commonly lose much of their activity during the progress of the reaction which they catalyse, and the weaker the enzyme preparation the more marked is this loss of activity. Thus the enzymic nature of activator might not reveal itself under conditions of equipotency of the components of the reaction.

Nevertheless it was thought that the use of equipotent preparations of activator and coagulase might give information not so much about the amount but rather about the rate of active coagulase formation which would indicate whether the reaction was stoichiometric or enzymic. This proved to be the case.

Tables 29 and 30 and figs. 15 and 16 show that in mixtures containing dilute coagulase the reaction proceeds quickly at first and quickly falls off. This is what one would expect if the coagulase is being used up as active coagulase is formed. The tables and graphs also show that in corresponding mixtures with dilute activator there is a much less marked falling off in the reaction rate and for most of the 24 hours active coagulase is formed at a steady rate. The conversion of coagulase to active coagulase is not nearly so complete in this as in the clotting-time experiment using concentrated activator. But the use of

equipotent reagents made it extremely unlikely that what is, after all, an ideal pattern of enzymic reaction would be manifest under such adverse conditions. Although the formation of active coagulase from coagulase is not complete in the 24 hours of the experiment the steady rate of formation with dilute activator and the rapid falling off with corresponding concentrations of coagulase are not in accordance with the results to be expected in the bi-molecular reaction postulated by Walker & Derow (1949). Despite the adverse conditions of the experiment the fact that reduction of activator has less effect on the active coagulase produced than a corresponding reduction in coagulase also argues against a bio-molecular reaction.

There remain the results of the last two experiments to be discussed. It is obvious from Tables 34-36 (pp.175, 176) that when active coagulase formation has stopped there is still available activator and coagulase in the reaction mixture since addition of either component causes further active coagulase to be produced. Any explanation of this phenomenon must be tentative but it is a common feature of enzyme-catalysed reactions for the enzymes to be inhibited by the products of their own activity, and this inhibition may be of the competitive type (Sumner & Myrbäck, 1950). In this case if activator is competitively inhibited by active coagulase it is not surprising that the addition of

more coagulase or more activator should cause more active coagulase to be formed. The incompleteness of the reaction could also occur in a stoichiometric reaction, but for the reasons given above this interpretation is ruled out. For the reasons discussed earlier (p.142) (the impossibility of separating activator, coagulase and active coagulase) it proved technically impossible to carry out inhibition experiments. It is possible that Walker & Derow's finding that the amount of activator, when not in excess, determined the amount of active coagulase produced was due to inhibition of activator by active coagulase. As mentioned earlier, Walker & Derow used a weak source of activator, human plasma Fraction I, and the concentration of activator in some of their reaction mixtures may have been quite low.

A curious feature of the last experiment is the effect of dilution on a preparation of active coagulase. Dilution of a preparation of active coagulase made from equipotent activator and coagulase caused more active coagulase to be formed during the next 24 hours. No explanation is offered of this unexpected finding, but it is possibly related to the phenomenon observed by Mensies (1952) who found that dilute preparations of activator survived heating much better than concentrated solutions.

To sum up, the explanation by Walker & Derow (1949) that the reaction between activator and coagulase is bi-

molecular is not supported by the results of either titration or clotting-time experiments. The results of both titration and clotting-time experiments suggest that coagulase is the quantitative precursor of active coagulase and that the conversion of coagulase to active coagulase is brought about by the enzymic action of activator.

## SUMMARY

1. The discovery, nature, properties, and mode of action of coagulase are described in an introductory survey.

2. In a number of strains of Staph. aureus the experimental work described in this thesis demonstrated the existence of variants differing considerably in coagulase production and growth characters. A description of their distinguishing features is given.

3. Previous work in which chemically defined media were used for investigating the growth requirements and metabolism of Staph. aureus is described.

4. Experimentally, the present work shows the importance of arginine in the formation of coagulase and the ability of ornithine and citrulline to act as its replacements in chemically defined media.

5. This work also shows that the vitamins aneurin and nicotinamide cause a marked increase in coagulase production when present at a concentration much greater than is needed to ensure full growth of Staph. aureus in amino-acid media.

6. The formation of citrulline from arginine by Staph. aureus has been demonstrated both in culture and with resting cells.

7. The growth requirements of variants of a strain have been shown to differ, and it has been found that the nutritional requirements of Staph. aureus are even more variable for coagulase production than for growth.

8. The reaction between activator and coagulase has been investigated and it appears that coagulase is the quantitative precursor of active coagulase, and that the conversion from the inactive to the active form is brought about by the enzymic action of activator.

## BIBLIOGRAPHY


AGNEW, S., Kaplan, H. & Spink, W.W. (1947) Proc. Soc. Exptl. Biol. Med., 65, 33.

BARRON, E.S.G., De Meio, P.H. & Klemperer, P. (1936) J. Biol. Chem., 112, 625.

BELLAMY, W.D. & Klimek, J.W. (1948) J. Bact., 55, 183.

BIGGER, J.W., Boland, C.R. & O'Meara, R.A.Q. (1927) J. Path. Bact., 30, 861.

BOVARNICK, M.R. (1941) J. Biol. Chem., 140, 151.

BURKY, E.L. (1933) J. Immunol., 25, 419.

CHAPMAN, G.H. (1942) J. Bact., 43, 313.

CHAPMAN, G.H. & Berens, C. (1935) J. Bact., 29, 487.

CHAPMAN, G.H., Berens, C., Wilson, E.L. & Curcio, L.C. (1938) J. Bact., 35, 311.

CHAPMAN, G.H., Berens, C., Peters, A. & Curcio, L.C. (1934) J. Bact., 28, 345.

CHAPMAN, G.H., Lieb, C.W. & Curcio, L.C. (1938) Am. J. Clin. Path., 8, Tech. Supp., 2, 5.

CHRISTIE, R. & Wilson, H. (1941) Australian J. Exptl. Biol. Med. Sci., 19, 329.

COHN, E.J., Strong, L.E., Hughes, Jr., W.L., Mulford, D.J., Ashworth, J.N., Melin, M. & Taylor, H.L. (1946) J. Am. Chem.Soc., 68, 459.

COHNHEIM, R., Gordon, A.H. & Martin, A.J.P. (1947) Biochem. J., 41, 500.

COWAN, S.T. (1938) J. Path. Bact., 46, 31.

CRUICKSHANK, R. (1937) J. Path. Bact., 45, 295.

DARANYI, J.v. (1926) Zentr. Bakt. Parasitenk., I. Orig., 99, 74.

DIENST, R.B. (1942) J. Lab. Clin. Med., 27, 663.

DUDGEON, L.S. (1908) J. Path. Bact., 12, 242.

DUDGEON, L.S. & Simpson, J.W.H. (1928) J. Hyg., 27, 160.

DUTHIE, E.S. & Lorenz, L.L. (1950) Nature, 165, 729.

DUTHIE, E.S. & Lorenz, L.L. (1952) J. Gen. Microbiol., 6, 95.

FAINEROTHER, R.W. (1940) J. Path. Bact., 50, 65.

FANKAS, H. (1947) J. Bact., 53, 401.

FILDES, P. & Richardson, G.M. (1936) Brit. J. Exptl. Path., 16, 326.

FILDES, P. & Richardson, G.M. (1937) Brit. J. Exptl. Path., 18, 292.

FILDES, P., Richardson, G.M., Knight, B.C.J.G. & Gladstone, G.P. (1936) Brit.J.Exptl.Path., 17, 461.

FISHER, A.M. (1936a) Bull. Johns Hopkins Hosp., 59, 305.

FISHER, A.M. (1936b) Bull. Johns Hopkins Hosp., 59, 415.

FLAUM, A. (1938) Acta Path. Microbiol. Scand., Supp.35, 1.

GALE, E.F. (1945) Brit. J. Exptl. Path., 26, 265.

GALE, E.F. (1947a) J. Gen. Microbiol., 1, 55.

GALE, E.F. (1947b) J. Gen. Microbiol., 1, 327.

GALE, E.F. (1947c) Nature, 160, 407.

GALE, E.F. & Mitchell, P.D. (1947) J. Gen. Microbiol., 1, 299

GALE, E.F. & Rodwell, A.W. (1948) J. Bact., 55, 161.

GALE, E.F. & Taylor, E.S. (1946) Nature, 158, 676.

GALE, E.F. & Taylor, E.S. (1947) J. Gen. Microbiol., 1, 314.

GEISSE, A. (1914) Zeit. f. Hyg., 77, 465.

GENGOU, O. (1933) Ann. Inst. Pasteur, 51, 14.

GENGOU, O. (1935) Ann. Inst. Pasteur, 55, 129.

GERMEIM, E.B., Ferguson, J.H. & Travis, B.L. (1947) Proc. Soc. Exptl. Biol. Med., 66, 525.

GERMEIM, E.B., Ferguson, J.H., Travis, B.L., Johnston, C.L., & Boyles, P.S. (1948) Proc. Soc. Exptl. Biol. Med., 68, 246.

GLADSTONE, G.P. (1937) Brit. J. Exptl. Path., 18, 322.

GLADSTONE, G.P. (1938) Brit. J. Exptl. Path., 19, 208.

GLADSTONE, G.P. (1939) Brit. J. Exptl. Path., 20, 199.

GONZENBACH, W.v. & Uemura, H. (1916) Zentr. Bakt. Parasitenk. I. Orig., 79, 97.

GORDON, M.H. (1905/04) Rept. Loc. Govt. Board, Rept. Med. Officer, 35, 398.

GRATIA, A. (1919a) Compt. rend. soc. biol., 82, 1245.

GRATIA, A. (1919b) Compt. rend. soc. biol., 82, 1392.

GRATIA, A. (1920a) Compt. rend. soc. biol., 83, 584.

GRATIA, A. (1920b) Compt. rend. soc. biol., 83, 585.

GRATIA, A. (1920c) Compt. rend. soc. biol., 83, 649.

GRATIA, A. (1921) Compt. rend. soc. biol., 85, 25.

GROSS, H. (1927) Klin. Wochschr., 6, 2281.

GROSS, H. (1928) Zentr. Bakt. Parasitenk., I, Orig., 109, 241.

GROSS, H. (1931) Zentr. Bakt. Parasitenk., I, Orig., 122, 354.

GROSS, H. (1933) Klin. Wochschr., 12, 304.

HARPER, E.N. & Conway, N.S. (1948) J. Path. Bact., 60, 247.

HALE, J.H. & Smith, W. (1945) Brit. J. Exptl. Path., 26, 209.

HILLS, G.M. (1938) Biochem. J., 32, 385.

HILLS, G.M. (1940) Biochem. J., 34, 1057.

HINE, T.G.M. (1922) Lancet, 2, 1380.

HUGHES, T.P. (1932) J. Bact., 25, 457.

JULIANELLE, L.A. (1922) J. Infectious Diseases, 31, 256.

KAPLAN, M. & Spink, W.W. (1948) Blood, 3, 573.

KELLAWAY, C.H., Burnet, F.M. & Williams, P.E. (1930) J. Path. Bact., 33, 899.

KENNES, B. (1928) Zentr. Bakt. Parasitenk., I. Orig. 109, 11.

KLEINSCHMIDT, H. (1909) Z. Immunitats., 3, 516. Quoted from Zentr. Bakt. Parasitenk. Ref. 46, 165.

KLIGLER, I.J., Grossowicz, N. & Bergner, S. (1945) J. Bact., 46, 399.

KLOPSTOCK & Bockenheimer (1904) Arch. klin. Chir., 72. Quoted from Zentr.Bakt.Parasitenk. Ref. 36,191.

KNIGHT, B.C.J.G. (1935) Brit. J. Exptl. Path., 16, 315.

KNIGHT, B.C.J.G. (1937a) Biochem. J., 31, 731.

KNIGHT, B.C.J.G. (1937b) Biochem. J., 31, 966.

KNIGHT, B.C.J.G. & McIlwain, H. (1938) Biochem. J., 32, 1241.

KNIVETT, V.A. (1952) Biochem. J., 50, xxx.

KOGL, F. & Wagtendonk, W.J.v. (1938) Rec. trav. chim., 57, 747.

KOSER, S.A., Pinkle, R.D., Dorfmann, A. & Saunders, F. (1938) J. Infectious Diseases, 62, 209.

KUTSCHER & Konrich (1904) Z. Hyg. Infektionskrankh. 2, 46, 249. Quoted from Zentr. Bakt. Parasitenk., Ref. 36, 191.

LANLY, M. (1938a) Nature, 142, 618.

LANLY, M. (1938b) Proc. Soc. Exptl. Biol. Med., 38, 504.

LOEB, L. (1903) J. Med Res., 10, 407.

LOMINSKI, I. (1944) Nature, 154, 640.

LOMINSKI, I. (1948) J. Path. and Bact., 60, 147.

LOMINSKI, I. (1949) J. Gen. Microbiol., 3, IX.

LOMINSKI, I., Conway, H.S., Harper, E.H. & Rennie, J.B. (1947) Nature, 160, 575.

LOMINSKI, I., & Milne, J. (1947) J. Path. Bact., 59, 516.

LOMINSKI, I., Morrison, R.B. & Porter, I.A. (1952) Biochem. J., 51, xvii.

LOMINSKI, I., O'Hea, A.J., Goudie, J.G. & Porter, I.A. (1950) Nature, 166, 309.

LOMINSKI, I. & Roberts, G.B.S. (1946) J. Path. Bact., 58, 187.

LOMINSKI, I., Smith, D.D. & Morrison, R.B. (1952) Nature (in press).

MARKS, J. (1952) J. Path. Bact., 54, 175.

MASON, H.C. (1945) J. Immunol., 51, 307.

MENKIN, V. (1933) J. Exp. Med., 57, 977.

MENKIN, V. (1934) Am. J. Med. Sci., 190, 585.

MENKIN, V. & Walston, H.D. (1934/35) Proc. Soc. Exptl. Biol. Med., 32, 1259.

MENZIES, D. (1952) J. Path. Bact., 54, 169.

MIALE, J.B. (1949) Blood, 4, 1039.

MIALE, J.B. (1952) Am. J. Clin. Path., 22, 218.

MOSS, E.S., Squires, G.V. & Pitts, A.C. (1941) Am. J. Clin. Path., 11, 657.

MUCH, H. (1908) Biochem. Z., 14, 143.

NEISSER, M. & Wechsberg, F. (1901) Z. Hyg. Infektionskrankh., 36, 299. Quoted from Zentr. Bakt. Parasitenk., 29, 956.

METER, E. (1937) J. Bact., 41, 243.

NOLF, A. (1919a) Compt. rend. soc. biol., 82, 567.

NOLF, A. (1919b) Compt. rend. soc. biol., 82, 1222.

OTTO, R. (1903) Zentr. Bakt. Parasitenk., I, Orig., 34, 46.

PILLEMER, L. (1946) J. Immunol., 53, 237.

PINNER, M. & Veldrich, M. (1932) J. Infectious Diseases, 50, 105.

PORTER, J.R. & Pelczar, Jr., M.J. (1941) J. Bact., 41, 173.

RAMMELKAMP, Jr., C.H., Badger, G.F., Dingle, J.H., Feller, A.E. & Hodges, R.G. (1950) J. Infectious Diseases, 86, 159.

RAMMELKAMP, Jr., C.H., Hezebicks, M.H. & Dingle, J.H. (1950) J. Exp. Med., 91, 295.

RICHARDSON, G.M. (1936) Biochem. J., 30, 2184.

RIDDON, R.H. (1942) Proc. Soc. Exptl. Biol. Med., 50, 324.

ROSENBACH, F.J. (1884) Mikroorganismen bei den Wundinfektionkrankheiten des Menschen. Wiesbaden.

SEVAG, M.G., Sholburne, M. & Mudd, S. (1945) J.Bact., 49, 65.

SEVAG, M.G. & Swart, E.A. (1947) Arch. Biochem., 13, 401.

SMITH, D.D., Morrison, R.B. & Lominski, I. (1952) J. Path. Bact., 64, 567.

SMITH, W. & Hale, J.H. (1944) Brit. J. Exptl. Path., 25, 101.

SMITH, W., Hale, J.H. & Smith, M.M. (1947) Brit. J. Exptl. Path., 28, 57.

SMITH, D.H. (1940) Biochem. J., 34, 1598.

STEIN, W.H. & Moore, S. (1951) J. Biol. Chem., 190, 103.

SUHRUS, M. & Schimrigk, R. (1933) Z. Immunitats., 80, 42.

SUMNER, J.B. & Myrback, K. (1950) The Enzymes. Academic Press, New York.

SUMNER, J.B. & Somers, G.F. (1937) Chemistry and Methods of Enzymes. Academic Press, New York.

TAGER, M. (1948a) Yale J. Biol. Med., 20, 369.

TAGER, M. (1948b) Yale J. Biol. Med., 20, 487.

TAGER, M. & Hales, H.B. (1947) Yale J. Biol. Med., 20, 41.

TAGER, M. & Hales, H.B. (1948a) Yale J. Biol. Med., 21, 91.

TAGER, M. & Hales, H.B. (1948b) J. Immunol., 60, 1.

TAGER, M. & Hales, H.B. (1949c) J. Immunol., 60, 475.

TAYLOR, E.S. (1947) J. Gen. Microbiol., 1, 86.

TOPLEY, W.W.C. & Wilson, G.S. (1929) Principles of Bacteriology and Immunity (Edward Arnold & Co.) London.

TSUTOMU SEKINE (1947) J. Japan. Biochem. Soc., 19, 79. Quoted from Chem. Abstr., 44, 10709g.

VANBREUSEGHEM, R. (1932) Compt.rend.soc.biol., 111, 159.

VANBREUSEGHEM, R. (1934a) Compt.rend.soc.biol., 115, 1235.

VANBREUSEGHEM, R. (1934b) Compt.rend.soc.biol., 116, 344.

VANBREUSEGHEM, R. (1934c) Compt.rend.soc.biol., 116, 650.

VEIEL, F. (1904) Muench. Med. Wochschr. I, 51, 15.

WALKER, B.S. & Derow, M.A. (1949) Abstr. 1st International Congress Biochem., p.121 and Personal Communication.

WALKER, B.S., Derow, M.A. & Schaffer, N.K. (1948) Science, 108, 347.

WALKER, B.S., Schaffer, N.K. & Derow, M.A. (1947) J.Bact., 56, 191.

WALSTON, H.G. (1936) J. Hyg., 35, 549.

WINSLOW, C.-E.A., Rothberg, W. & Parsons, E.I. (1930) J.Bact., 5, 145.

ACKNOWLEDGMENTS

It is difficult to single out individuals for thanks since every member of the Department of Bacteriology has contributed suggestions, criticisms and encouragement throughout the course of this work. To the colleagues with whom I collaborated, Dr. D.D. Smith on variation in coagulase production, Dr. I.A. Porter on citrulline formation, and Dr. J.G. Goudie on the mode of action of coagulase, my particular thanks are due.

I am grateful, too, for the help given by Mr. F. Lonsdale of the technical staff in selecting and subculturing strains, and by Mr. D. Colvin with the photographs.

I must also acknowledge my debt to Professor C.H. Browning, F.R.S. for finding a place for me in an already overcrowded department and for providing much subsequent encouragement and advice.

Professor J.N. Davidson was kind enough to place the facilities of the Department of Biochemistry at my disposal, and I am most grateful to Dr. G. Leaf of that department for the unfailing kindness, help, and instruction I enjoyed while working there.

My thanks are due to Miss J. Edgar of the Western Infirmary Blood Transfusion Service for her assistance in securing specimens of plasma and to the Central Staphylo-

coccal Reference Laboratory for carrying out phage-typing.

I have great pleasure in thanking Dr. I. Lominski who so willingly devoted time and thought to teach me bacteriology, provided me with some of the subjects of the research, and encouraged and advised me throughout the course of this work. His help and stimulation indeed extended far beyond the subjects with which his name is associated.

I should also like to express my gratitude to the Faculty of Medicine of Glasgow University for a grant made in 1950 in aid of my research, and to the Rankin Fund for grants towards the expenses.

The research was done during the tenure of a Carnegie Senior Research Scholarship and but for the generosity of the Carnegie Trust it would not have been possible for me to carry out this work.