STUDIES ON THE ACTION OF ANTIBACTERIAL CHEMOTHERAPEUTIC DRUGS

THESIS

For The Degree Of

DOCTOR OF PHILOSOPHY

Submitted By

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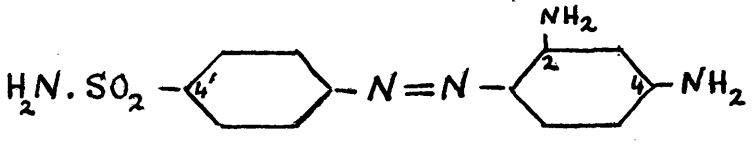
INTRODUCTION

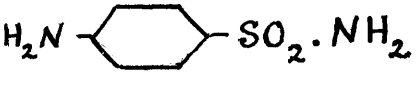
Chemotherapy, the treatment of infections by compounds of known chemical constitution, had its first successes in combating general infections due to protozoa and spirochaetes, but the discovery of drugs suitable for use in general infections due to bacterial invasion has proved less easy. Local bacterial infections may be treated successfully by means of compounds which possess powerful antiseptic properties and can be applied to readily accessible local lesions in sufficiently concentrated form to damage the vitality of the organisms without undue harm to the surrounding tissues. But whenever a bacterial infection becomes generalised it is obvious that the therapeutic agent must be a substance which can be administered either orally or by injection

to the host without toxic effects, and which will diffuse into the tissues in such a way as to meet the invading organisms in adequate concentration for their death or attenuation.

In 1935, Domagk (24) used a red dyestuff "Prontosil" (4'-sulphonamido-2:4-diaminoazobenzene)



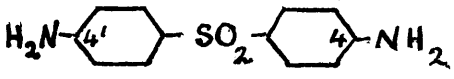
to treat experimental bacterial infections in mice and rabbits with promising results, and in the same year the Tréfouëls, Nitti and Bovet (112) confirmed these results and suggested that the activity of this azo-dye was due to its breaking down in the tissues of the infected host with the formation of a less complex compound, p-aminobenzene-sulphonamide, which they then used successfully in the treatment of haemolytic streptococcal infections in mice. This comparatively simple compound



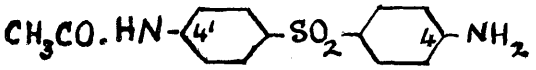
p-aminobenzene-sulphonamide or sulphanilamide may therefore

be regarded as the parent substance to which the many related compounds since suggested for therapeutic trial may be compared. It had long been known as a synthetic organic compound (Gelmo, 35) but had not been previously employed as a synthetic drug.

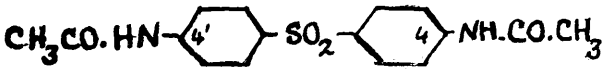
Among the new drugs obtained by substitution in or addition to the sulphanilamide molecule are several of equal or greater therapeutic activity. It has also been stated that they may vary in efficacy according to the particular type of infection against which they are used. In addition, certain drugs of "sulphone" structure have proved specially effective in treating haemolytic streptococcal infections in mice. From the large series of sulphonamide drugs tested in this laboratory for therapeutic effect, the following three sulphones have been chosen for study along with sulphanilamide:



4:4'-diamino-diphenyl-sulphone.



4:4'-monoacetyl-diamino-diphenyl-sulphone.



4:4'-diacetylamino-diphenyl-sulphone.

All four compounds were supplied by Imperial Chemical Industries Ltd.(Dyestuffs Group).

In considering the antibacterial properties of the three sulphones and sulphanilamide, it was first necessary to determine their toxicity for the particular species of animal to be employed in trials in vivo. Section I of this thesis describes investigations made on the solubility of each drug, the possibility of its use in the form of a dispersion when solubility was low, the best method of administration, assessment of the maximum dose tolerated, and the rate of absorption by and excretion from the body. Next followed the choice of a suitable experimental infection against which the action of the four drugs might be compared. Such an infection must be capable of producing a uniform course in successive series

of experiments, and the choice of suitable strains therefore takes into account many characteristics of the organisms - virulence, presence or absence of capsules, effect of low temperatures on fluid cultures, and ease of maintenance. An unexpected finding in the case of one of the strains of streptococci employed was the development of capsules during serial passage of the strain through normal animals and this altered character had an interesting effect on the response to therapy. An account of the general technique of the therapeutic experiments and of the results obtained is given in Section II. Treatment by means of the four drugs was carried out prophylactically, immediately after inoculation, or some hours after inoculation, and fractions of the maximum dosage were also tested to find out the possible therapeutic range. Combined treatment with more than one drug and the effect of p-aminobenzoic acid on the results of treatment were investigated in addition.

In studies hitherto published little or no attempt

had been made to follow the course of infection from day to day in treated mice, and a method was devised by means of which the bacterial content of the blood was examined at regular intervals. It was found that with slowly excreted drugs, such as the monoacetyl-sulphone, it was possible for a treated mouse, apparently in normal health, to carry in its blood for a considerable period of days abundant organisms which were lacking in virulence towards their present host and yet could cause fatal septicaemia when a minute drop of this blood was used to infect a fresh animal. Such observations, of which a preliminary report was given by Browning and Leckie (13), are described in detail in Section III, and their significance is discussed in considering the mode of action of the sulphonamide drugs *in vivo*.

Studies of the antibacterial action of the sulphonamide drugs should include investigations of the mode of action in the test-tube even although views based on conditions *in vitro* do not really take into consideration the constant

variation in environment provided in the tissues of the host and so may be mainly of academic interest. The very variable results obtained from *in vitro* investigations of the influence of the drugs on the growth of organisms are dealt with in Section IV.

It has been stated that "drug-resistant" strains of organisms may develop in the course of treatment with sulphonamide drugs, and it would naturally be expected that similar strains might be developed by repeated transfer of the organisms in nutrient medium containing the drug. Although success by such methods has been claimed in the case of the pneumococcus by several workers, the streptococcus has so far proved a more difficult problem. No evidence of acquired drug-resistance has been found in the course of the author's experiments *in vivo*, but the possibility of the development of such strains both *in vivo* and *in vitro* must be considered. The published results are therefore discussed also in Section IV along with certain of the present

author's experimental findings.

In Section V the various theories to account for the curative effect of the sulphonamide drugs are reviewed in the light of all the experimental results in conjunction with published data. This attempt to explain the action of the sulphonamides within the living host stresses the importance of the part played by that host in conducing to cure of the infection.

In addition to the author's own findings certain results in this thesis have been included by permission of Professor Browning under whose supervision the research has been carried out in the Department of Bacteriology. Such results are clearly indicated in both text (pp. 20, 48, 52, 53, 54, 55, 56, 63, 66) and in Tables III, VIII, IX, X, XII, XIII, XIV, XV, XVI, XXIII.

The author is indebted to Imperial Chemical Industries Limited (Dyestuffs Group) for supplies of compounds and also for support of part of the investigations.

SECTION I

TOXICITY AND ABSORPTION

SOLUBILITY.

The first step in investigating the properties of a compound of possible antibacterial activity *in vivo* is the assessment of its toxicity for the particular species of animal to be employed in the therapeutic trials. The toxicity of a drug is closely bound up with its solubility. The compound may be readily soluble in water, but the solution may be either too acid or too alkaline to be administered to the body by any route until it has been neutralised. Again, it may be insoluble in watery medium, in which case it is unlikely to be absorbed by the body at all and will either form an inert mass in the tissues at the site of subcutaneous injection or else be completely excreted in an unchanged condition after oral administration. Between these two extremes lie compounds of sparing solubility in

watery medium for some of which a suitable vehicle must be devised before they can be administered. Most frequently such compounds are finely ground and prepared as aqueous dispersions with or without the addition of a trace of a chemical dispersing agent or a small amount of gum acacia.

FORMATION OF DEPOT OF DRUG.

These dispersions are suitable for both oral and subcutaneous administration, especially the latter. A depot of drug is formed at the site of injection and is gradually absorbed by the animal over a period of days or even weeks depending on the relative solubility of the drug in the body-fluids. This has the effect of maintaining a concentration of drug in circulation without further administration and is therefore especially suitable in the case of the sparingly soluble sulphones. It is only logical that whereas a highly soluble or readily absorbed drug has to be administered sufficiently frequently to maintain an adequate concentration in the body-fluids, a less soluble drug may well be given to mice by a single

subcutaneous injection, or divided over two or three such injections when the toxic effect due to absorption following a single large dose might prove critical, and the depot of drug so formed be then gradually absorbed by the body-fluids. It is presupposed, of course, that absorption will proceed more or less continuously, and it has been verified in the case of the compounds discussed in this thesis that the depot does not become encysted and so, as it were, walled off, but that absorption takes place until there are no visible traces of deposit at the site of injection. Colebrook et al.(21) in treating a haemolytic streptococcal infection in mice gave a subcutaneous injection of a strong suspension of Prontosil and found that the depot of dye so formed became gradually absorbed by the animal for days or weeks, and that this produced very successful therapeutic results, but a survey of the literature shows that only very few other workers with the sparingly soluble drugs have administered them subcutaneously. Long and Bliss (67, p.20) favour drug

administration per os for the less soluble compounds, although, on the other hand, they consider (67, p.52) that figures for acute toxicity of the less soluble compounds arrived at on the basis of oral dosage are valueless because of the large proportion of unabsorbed drug excreted as an essentially inert substance.

SULPHANILAMIDE.

Sulphanilamide is a white powder, moderately soluble in hot water, only slightly soluble in cold water. A solution of 1:100 is obtained by heating almost to boiling-point in the requisite volume of water and allowing the solution to cool to body-temperature at which it is suitable for injection. Some workers prefer to make up a suspension of the finely powdered drug in water containing 4 to 10 per cent. gum acacia, especially when oral administration is to be employed.

A summary of the published results for the toxicity of sulphanilamide for mice by oral and subcutaneous administration is given in Table I and shows considerable

variations. With oral dosage, from 70mg. to 125mg. is given as a maximum tolerated dose, 200mg. or more causing death. Results of subcutaneous dosage are still more varied, the lethal dose being placed as low as 50mg. and as high as 120mg.. However it must be taken into account that several different preparations of the drug were used, and that, owing to slow absorption of drug from oily dispersions, presumably 120mg. in olive oil would have to be administered to give the same effect as 50mg. of the drug in aqueous solution, thus explaining the apparent disparity in these two values for the lethal dose.

Colebrook (19) gave a series of five doses of sulphanilamide intraperitoneally to mice, a total of 40mg. per 20g. mouse, over a period of 72 hours, and found this to be non-toxic and well tolerated. Yet another method of administration not referred to in the table is that of Litchfield et al.(61) who incorporated the drug in the normal feeding of mice. They first studied the normal feeding-periods and ascertained that

after the initial 24 hours, during which the animals became accustomed to the particular diet, there were six to eight feedings equally spaced over each 24 hours. They then included not more than 2 per cent. of sulphanilamide in the normal food and studied the concentration of drug in the blood. After 1 to 2 days the mice showed a steady concentration of sulphanilamide in the blood as their intake of the new diet became regular. Litchfield claimed that an effective drug level in the blood was easily maintained by this method of administration.

The estimation of the concentration of sulphanilamide in the blood or urine may be carried out by means of a colorimetric test based on the diazotisation and coupling of the amino-group in the sulphanilamide molecule. This method was first suggested by Fuller (31) and by Marshall et al.(74) and since then various modifications of the original test have been adopted. Several coupling compounds have been suggested for combination

with the diazo-compound, the criteria being that the action is independent of the presence of excess nitrous acid, and that there is rapid production of a soluble and relatively stable colour. N-β- sulphatoethyl-m-toluidine has proved a reliable reagent especially when dealing with very small amounts of blood (Rose et al.,97).

As regards sulphanilamide, all the necessary data as to estimation in the blood and urine are already available in the literature, but the sparing solubility of the sulphone drugs has presented certain difficulties. Considered purely as chemical analysis, it is apparent that the small concentrations reached in blood or urine are quite unsatisfactory for reliable colorimetric results, especially with the minute quantities employed when the experimental animal is the mouse.

The distribution of sulphanilamide throughout the body after administration and also the rate of excretion of the drug have been studied by many workers, and while much of the published work was carried out on larger

animals (dogs, rabbits, etc.,). only that dealing with drug concentrations in the mouse need be considered here. Feinstone et al.(26) with a single subcutaneous injection of 6 to 10mg. per 20g. mouse, found that the maximum blood concentration was reached in one hour, and only a trace could be found after 9 to 10 hours. With oral administration the values were lower. Marshall et al.(72) detected no difference in the rapidity of rise to a maximum blood concentration following different methods of administration. The value reached a maximum in ½ to 1 hour and had fallen to a low level in 5 to 6 hours. Hoare (46) noted toxic symptoms - incoordination of limbs, difficulty in walking, etc. - after a subcutaneous dose of 30mg., but recovery took place in about 6 hours. Long et al.(65) also observed toxic symptoms following two doses each of 9mg. given subcutaneously at an interval of 3 hours, but these disappeared within 4 hours. From consideration of the above observations on absorption and excretion it seems necessary to give regular doses of sulphanilamide

every few hours in order to maintain a sufficiently high concentration in the blood to bring about the desired therapeutic effect.

Author's Experiments.

The mice used for all the toxicity and therapeutic estimations were healthy animals weighing 18 to 25g.. They were kept separately, each in a glass jar (9" high, 5½" diameter, fitted with a wire gauze lid) containing ½" dry sawdust and changed at weekly intervals. Feeding consisted of a daily allowance of a 1" cube of stale bread soaked in a mixture of equal parts of milk and water, while a supply of about 3 to 5g. of oats was introduced with the fresh sawdust each week. All the doses of drug given in the text and in the tables are calculated for a mouse of standard weight - 20g.

Table II summarizes the results obtained experimentally with sulphanilamide. Doses of up to 100mg. were tolerated, and although 2 mice died following administration of 133mg., other mice were unaffected by 167mg. and 200mg..

these large doses, however, are necessarily given in the form of suspensions of the drug, and therefore the rate of absorption into the body-fluids is the deciding factor in determining toxicity. No marked toxic symptoms were observed apart from a temporary loss of weight in some cases.

While Litchfield's drug-diet method (p. 13) of administration of sulphanilamide appeared to have a great deal in its favour, early results with large parenteral doses of sulphanilamide in experimental haemolytic streptococcal infections gave such favourable results (see Table VIII) that subcutaneous dosage was continued, especially as this was the most suitable method of administration of the sulphone drugs which were being compared with sulphanilamide. A uniform method in comparing the two types of drug appeared desirable even although there seems to be no chemical relationship between the sulphones and sulphanilamide which would explain the chemotherapeutic action of the former in some such manner as that of

Prontosil (see p. 2).

As regards absorption of sulphanilamide, even a large dose of 167mg. injected subcutaneously in the form of a suspension was found to be completely absorbed by the tissues when the mouse was examined post mortem six days later, no trace of any undissolved drug being visible at the site of injection.

4:4'-DIAMINO-DIPHENYL-SULPHONE.

4:4'-diamino-diphenyl-sulphone was first investigated by Buttle et al.(17) for chemotherapeutic properties. It is a creamy-white powder of low solubility - 0.01g. per 100cc. in water at room-temperature, 0.05g. per 100cc. in "hot" water. Buttle stated that the diamino-sulphone by oral administration was 10 times as toxic as sulphanilamide when a single dose was given - about 5mg. tolerated by a 20g. mouse - and 25 times as toxic when repeated daily. Fourneau et al.(30) gave the tolerated oral dose as 5mg. per 20g. mouse, and Feinstone et al.(26) as 13mg. per 20g. mouse whether as a single dose or distributed over 5 days.

Author's Experiments.

Table III shows the experimental results obtained with diamino-diphenyl-sulphone, and includes some earlier results from the laboratory records (see p.8). With single doses 10mg. caused the death of 4 out of 6 mice, though larger amounts occasionally caused only toxic and not lethal effects. With divided doses, a total of 8mg. given as two doses in 5 hours was lethal for 2 out of 4 mice, but the same amount given in three doses over 24 hours was readily tolerated, while 11mg. in three doses over 24 hours caused only temporary loss of weight. The other typical toxic symptoms manifested were incoordination of movement and excitability.

As regards absorption of the drug from the subcutaneous deposit, a single dose of 7mg. was found to be completely absorbed from the site of injection within 4 days, while a slight deposit of drug from an identical dose was still visible 2 days after injection. Long and Bliss (67, p.78) observed that though absorption of the drug following

small oral doses was rapid and fairly complete, blood concentrations were maintained longer than in the case of sulphanilamide. Feinstone et al.(26), also giving oral doses, found the maximum blood level at 1 hour and a fair amount of drug still present in circulation 6 hours later, but as the dose was increased above 4mg. a limiting value was reached for the concentration in the blood.

4:4'-DIACETYLAMINO-DIPHENYL-SULPHONE.

When the diamino-diphenyl-sulphone is acetylated to give 4:4'-diacetylamino-diphenyl-sulphone the toxicity of the compound is considerable lowered (Fourneau et al., 30). Long and Bliss (67, p.52) considered this to be due to poor absorption of the drug and a low rate of conversion to the toxic non-acetylated form. The diacetyl compound is a white powder of low solubility, less than 30 parts being dissolved per million parts of water, and, as in the case of the diamino-sulphone, is generally used in the form of a suspension. A trace of a chemical dispersing agent such as Dispersol OG (an auxiliary product for the dyeing trade

prepared by Imperial Chemical Industries Limited) may be added.

Fourneau et al.(30) stated that mice tolerated 200mg. per os, Molitor et al.(79) 400mg. per os, and Buttle et al.(15) 800mg. per os, while Feinstone et al.(26) also placed the oral tolerated dose at over 400mg..

Author's Experiments.

As shown in Table IV, subcutaneous doses of 200mg. and 330mg. were very well tolerated by mice. The depot of drug at the site of injection was very slowly absorbed; and even 3½ months after a dose of 50mg., there was still a deposit in the subcutaneous tissue. The question must be left open whether, as Nitti et al.(90) believe, the diacetyl-sulphone is slowly hydrolysed and absorbed as the corresponding diamino-sulphone.

4:4'-MONOACETYL-DIAMINO-DIPHENYL-SULPHONE.

The monoacetyl derivative of diamino-diphenyl-sulphone has been found very effective in the treatment by subcutaneous injection of mice infected with haemolytic streptococci (Browning, 6). Fourneau (29) in the course of reviewing the organic sulphur derivatives including the diphenyl-sulphones, passed over the monoacetyl compound as of little interest, since it was as toxic as the corresponding non-acetylated sulphone, and he does not appear to have tested its therapeutic efficiency. On the contrary, the present experimental findings have shown the monoacetyl-sulphone to be of very great activity despite a certain degree of toxicity for mice, which, however, is much less than that of the non-acetylated substance.

Author's Experiments.

Like the other two sulphone compounds already considered, this is a white powder relatively insoluble in water and so readily administered in the form of a suspension. Although the drug has a very low solubility

- certainly less than 1.0g. per 1,000cc. in water at room-temperature - there is ample evidence of its absorption into the body-fluids, as shown by the rapid appearance of typical toxic manifestations. Mice which received a single feed of bread to which was added 1:100 or 1:200 suspension of drug at the rate of 1cc. per 20g. mouse (10 or 5mg.), all showed great excitability, incoordination of movements, and unsteadiness within 1½ hours after feeding, these symptoms gradually disappearing during the next 4 or 5 hours. The following day, after the next feed, which contained no drug but was simply a similar cube of bread soaked in diluted milk, there was a temporary recurrence of the nervous symptoms due apparently to further absorption of drug from the residue of the previous day's dose not yet fully excreted. Oral administration was not extensively tested owing to the excellent results given by subcutaneous injection with the formation of a depot of drug.

When the monoacetyl-sulphone was injected subcutaneously, evidence of absorption of the drug by mice was again shown

by marked excitability with incoordination of movements which started within 2 or 3 hours of injection; and those symptoms might continue for 3 days. A dose of 20mg. occasionally caused death within a few days although other animals showed only temporary toxic symptoms following doses of 330mg. and 200mg. given in the form of thick aqueous suspensions. Depending on the initial amount injected, the depot of drug slowly diminishes in size over a period of several weeks. A single dose of 100mg. was completely absorbed from the site of injection within 3½ months, while with three doses of 6.7mg., 4.0mg., and 4.0mg. injected at 0, 5, and 24 hours (14.7mg. in all) complete absorption took 8 to 11 weeks, and a single dose of 10mg. 6 weeks.

SUMMARY OF TOXICITY RESULTS FOR SULPHONE DRUGS.

The three sulphones varied in toxicity - the non-acetylated compound being the most toxic and the diacetylated compound the least - but it seemed advisable to give all three by the triple dosage method thereby building up the drug depot over 24 hours and thus avoiding the possibility of a single large dose having a critical toxic effect. The system of optimum or standard dosage decided on from consideration of the toxicity results is shown in Table V.

Acetylation of an amino-group has frequently been noted to reduce the toxicity of the substance for the host while leaving the chemotherapeutic effect weight for weight practically unchanged, and thus allowing a higher dosage to be administered with the possibility of a more powerful therapeutic effect (Browning et al., 7; Fourneau et al., 29). In the case of the three sulphones now under consideration, acetylation of the amino-groups may be regarded as taking place in two stages - the diamino-sulphone to the monoacetyl-sulphone and thence to the diacetylamino-

diphenyl-sulphone. The toxicity of the compounds diminishes in this same order together with the solubility, and therefore the rate of absorption from the drug depot also decreases. Since therapeutic efficiency depends greatly on the maintenance of an adequate drug-concentration in the blood, the best results would therefore be expected from the monoacetyl compound due to its intermediate rating as regards absorption. If the therapeutic effect is due to the presence of the non-acetylated compound and, as suggested by Nitti et al.(90), the diacetyl-sulphone acts by being gradually hydrolysed with formation of the diamino-sulphone, then the more rapid absorption of the monoacetyl-sulphone might be explained as due to a similar hydrolysis with, in this case, a single acetyl-group involved. When the non-acetylated sulphone is administered subcutaneously in maximum tolerated doses it is very quickly absorbed (Table V). The relation of this varying rate of absorption to the therapeutic results obtained experimentally will be consedered in Section II.

SECTION II

ESTIMATION OF THERAPEUTIC ACTIVITY

EXPERIMENTAL BACTERIAL INFECTIONS.

For assessing therapeutic activity it is essential to select both the type of infection and the host which will give consistent results in repeated trials and so allow a comparison of the efficacy of the various drugs under consideration.

The white mouse has been widely accepted as a very suitable experimental animal. It is relatively cheap and therefore large numbers may be employed in order to give statistically significant results. The individual response to infection also is supposed to vary very little from animal to animal, although experimental results to be detailed later demonstrate the importance of the "mouse factor" and its effect on the ultimate success or

failure of therapy.

Throughout the thesis, all calculations of "statistical significance" have been made by the simplified method of Loewenthal and Wilson (64). This is a modification of Fisher's factorial method of calculation of probability and was devised as a means of comparing experimental results when the total numbers are small or when there is unavoidable inequality in the sizes of the series compared. The differences between experimental results are classified as "highly significant", "significant", or "of no statistical significance".

The infective agent must be selected primarily for its power to produce in the host a course of infection which can be readily reproduced with little or no modification in successive experimental series. A great variety of organisms has been employed *in vivo* experimentally with the sulphonamide drugs - streptococci, staphylococci, gonococci, meningococci, *B. typhosus*, *B. coli*, *B. friedlander*, *B. proteus*, *B. aertrycke*, and

Pasteurella - and many of these are readily adapted by animal passage to give a course of infection suitable for testing drug therapy. The virulence of an organism, i.e. its power to invade the body of the host and produce disease, often varies from one species of animal to another, and a strain freshly isolated from a human source may not at first appear to possess a high degree of virulence for a different host. This is well illustrated in the case of the haemolytic streptococcus which was the organism employed in the present work. 86 strains of haemolytic streptococci of human origin (mainly from suppurative lesions) have been isolated in pure culture in this laboratory and a dense broth emulsion from a 24 hour agar culture in each case injected intraperitoneally into mice in considerable dose - 0.3 to 0.5cc.. 31 of the strains were sufficiently virulent at first to cause death, but on further passage lost virulence or in a few cases produced local lesions along the track of the infecting needle instead of general septicaemia. With only 28 strains virulence

was maintained or increased during succeeding animal passages and 10 of these strains were used in various therapeutic trials. The remaining 27 proved to be completely non-virulent towards mice.

Strains of haemolytic streptococci of naturally high virulence, and especially those in which enhancement of virulence has occurred as the result of passage, readily produce septicaemia when injected intraperitoneally, with death of the mouse occurring in about 24 hours. An infection of this nature has the great advantage of regularity of action over strains of a more chronic or sub-acute character, even although such a rapid course allows only a short time in which successful therapy can play its part in averting the death of the host.

Long and Bliss (67, p.23), from a study of published results with experimental streptococcal infections suggested a standard method for testing new chemotherapeutic compounds in which strains of streptococci of maximal virulence for mice and belonging to Lancefield Group A should be employed.

They stated that Lancefield Group B strains - either of bovine or human origin - were quite resistant to sulphonamide therapy while Lancefield Group A strains - which include many of those causing infections in human beings - were susceptible to such treatment. It has also been pointed out by various workers that infections due to streptococci of low mouse virulence are not affected by sulphanilamide - the usual explanation for this being the very large number of organisms inoculated.

The question of the presence of capsules can also be considered at this stage. Some strains of streptococci are readily shown by eosin relief staining to possess capsules similar to those of the pneumococci, a property which may well affect both virulence and response to therapy. For instance, as will be shown, the Kruger strain of haemolytic streptococcus was originally a non-capsulated organism, producing infections readily susceptible to sulphanilamide, but later developed a capsule. While the appearance of capsules also coincided with consistently high values in

estimations of virulence, there was no evidence of increased curability of the infection by sulphanilamide being associated with the increase in virulence. Such increased curability might have been expected according to the view that strains with high mouse-virulence are more susceptible to sulphonamide therapy than strains of low virulence. Actually, the non-capsulated phase of the strain was already highly virulent for mice, and the presence of capsules supplied an additional protection for the organisms. Schlossberger and Bär (104) in a very small number of experiments on treatment with sulphanilamide, auro-detoxin, or specific antiserum in mice inoculated with haemolytic streptococci, pointed out that the addition of mucin to the inoculum protected the invading organisms against all three forms of treatment. They deduced from this that the mode of action of sulphanilamide must be similar to that of specific antibodies in the homologous antiserum; a simpler and more likely explanation is that the mucin confers on the organisms increased protection

against all defence mechanisms whether in the form of drug or antibodies in treated animals or the normal defensive processes in untreated animals.

ESTIMATION OF VIRULENCE.

The virulence of the cultures of streptococci was assessed by making dilutions in broth from a 24 hour culture of the organisms in horse-heart infusion broth containing 1 per cent. peptone and inoculating a set of mice intraperitoneally with 0.25 to 0.3cc. of each of the various dilutions.

In making the dilutions of culture, the question arose as to whether the use of one pipette throughout might tend to introduce an appreciable error in the higher dilutions due to the carrying-over on the pipette of a slightly richer transfer than was intended. A number of tests was accordingly carried out in duplicate on several different cultures, an initial dilution of 0.02cc. of culture in 10.0cc. of broth being taken as 1:500. From this dilution ten-fold dilutions were made (1) with the same pipette

throughout, and (2) with separate sterile pipettes for each stage of dilution. For the range 1:500,000 to 1:500,000,000 it is seen from Table VI that with method (1) a total of 17 out of 33 mice died in 24 hours and with method (2) 14 out of 22, while the individual results at each level of dilution also showed practically no significant difference.

The most frequent inoculum employed in therapeutic tests is ten times the lowest concentration which can be confidently expected on the basis of recent virulence assessments to kill mice in 24 hours, i.e. the minimum certainly lethal dose (M.L.D.). With the strains used, 10 M.L.D. was generally contained in a dilution of 1:1,000 to 1:100,000. With less virulent strains or strains which give irregular results it is better to use an inoculum of at least 100 times the estimated smallest lethal dose.

MAINTENANCE OF STRAINS.

Strains of streptococci may be maintained by several methods. When not required for immediate use the organisms may be preserved for very long periods by removing aseptically and keeping in a vacuum desiccator over concentrated sulphuric acid the spleens of mice which have died from a lethal dose of culture; or by culturing the heart-blood in boiled-blood-broth and freezing the culture after 24 hours' incubation. Both of these methods have yielded growths of virulent organisms on subculture after long periods, e.g. after freezing for five years at about -16°C. When the strain is required for therapeutic experiments it should be passed two or three times by alternate mouse-inoculation and broth subculture, i.e. mice are inoculated intraperitoneally with 0.25cc. of pure culture and on their death broth cultures are made from the heart-blood and incubated for 18 to 24 hours ready for the next mouse-passage. This has the effect of ensuring that the virulence of the strain is at a high level, but prolonged maintenance of

the strain by this method may cause alterations in the morphology and behaviour of the organisms, as will be described later in the case of the Kruger strain of streptococci.

Studies on the maintenance of virulent strains of streptococci have produced some interesting facts with reference to the freezing of cultures. When the culture medium was ordinary meat-infusion broth containing 1 per cent. peptone but no blood, inoculated by transfer of one or more loopfuls of virulent culture, incubated at 37°C for 24 hours, and then frozen at about -16°C, the organisms died off fairly rapidly. Such cultures have been found to be completely innocuous to mice when injected after keeping for five days in the frozen state and to be sterile on subculture.

If the culture contained a small amount of blood, as was necessarily the case when the heart-blood of a dead mouse was used as the inoculum, the virulence of cultures was found to be unchanged even after freezing for 45 days,

but cultures which had been frozen for 9½ months - though showing typical organisms in stained films, capsulated as in the original living culture - neither gave growth on subculture nor had any pathogenic effect when injected undiluted into mice. Indeed these dead cultures conferred a certain degree of protection against the homologous strain. Thus 21 mice received 1.0cc. of such dead culture intraperitoneally in two weekly doses of 1/3cc. and 2/3cc. respectively; the second dose was followed a week later by inoculation with 0.25cc. of a 1:100,000 dilution of a virulent culture of the same strain: 10 mice remained well and the others died in two days, while the 6 unvaccinated controls all died in 24 hours. These results are statistically significant.

Cultures made in boiled-blood-broth containing 5 per cent. rabbit-blood have a long period of viability when frozen, the outstanding example being that of a culture of a non-capsulated strain in this medium which yielded virulent streptococci after being kept frozen for five

years. Before being frozen this culture caused death in 24 hours when 0.25cc. of dilutions up to 1:1,000,000 were injected intraperitoneally, and although on thawing the undiluted culture took 3 days to kill a mouse, the original virulence was manifested by the first subculture in broth made directly from the frozen culture.

The two strains of streptococci chiefly used in the therapeutic trials ("Kruger" and Thomson") both possessed high virulence for mice.

STRAINS OF STREPTOCOCCI.

Streptococcus Kruger.

This strain was received from Dr. Feldt of Berlin in March 1931. In fluid medium it yielded a flocculent growth with many long chains. Virulence for mice was stated to be "1:10,000,000" intraperitoneally, but after passage through one mouse of an emulsion of the spleen received, even 1:1,000 dilution took 48 hours to kill, and further passages and trials during the next month showed no improvement. Similar results were obtained on reviving

the strain again from spleens 3½ and 4 years later; but in April 1937, after passage at irregular intervals through altogether 13 mice since March 1931, a culture was isolated which was lethal for mice in 24 hours in dilutions up to 1:1,000, and in 48 hours for dilutions of 1:10,000 to 1:100,000; on further passage the virulence almost at once reached the level at which it remained - a dose of 1:100,000 being certainly lethal in 24 hours. Fairly constant passage of the strain now followed; more than 112 passages through mice took place during the next 20 months, in the course of which there were over 40 therapeutic experiments. In January 1939, the organisms were observed to be capsulated, and in view of this the results of therapeutic experiments carried out at different periods have been grouped, those before this date being assigned to Period I (Table VII). The strain of organisms in the capsulated state was maintained for therapeutic experiments till July 1943, the results being considered somewhat arbitrarily in three further periods. Period II extends from January to

September 1939, during which infections with the capsulated organisms responded fairly well to treatment with the more powerful drugs. Then, after an interval of two months (September to October 1939), during which maintenance of the strain took place in the usual manner by alternate mouse-passage (through about 40 mice) and broth culture, resumption of therapeutic tests showed a marked falling-off in the number of cures obtained with drugs which had hitherto been regarded as fairly effective. This is designated Period III and extended from October 1939 till February 1942. Thereafter the strain was revived - still in capsulated form - from a spleen which had been stored since early in September 1939, this marking the beginning of Period IV. In July 1943, the non-capsulated form was recovered in pure culture from a boiled-blood-broth culture which had been kept frozen since February 1938, and was used in tests grouped under Period V. In view of the experience described above, the stock culture of the non-capsulated strain was now kept frozen in boiled-blood-

broth from which a subculture could readily be made and the organisms passed once through mice prior to therapeutic experiments. In this manner maintenance of the strain by animal passage is reduced to a minimum.

During Period II virulence tests showed much more consistent results than in Period I, an aggregate of 34 out of 63 mice (54 per cent.) inoculated with a dilution of 1:10,000,000 of culture dying in 24 hours, and 8 out of 12 mice (67 per cent.) receiving 1:100,000,000 dying in the same time, results which show no statistically significant difference. In Period III virulence was at practically the same level as in Period II, 65 out of 110 mice (59 per cent.) receiving 1:10,000,000 being dead in 24 hours. In Period IV the higher dilutions were seldom tested, but 26 mice out of 44 (59 per cent.) receiving an inoculum of 1:1,000,000 of culture were dead in 24 hours. Virulence was not so high in Period V, an inoculum of 1:1,000 killing 24 out of 36 mice (67 per cent.) in 24 hours.

In Periods II, III, and IV, growth in broth culture was homogeneous, but the organisms of Period V, like those of Period I, gave extremely granular growths in fluid medium, which rendered accurate dosage of the diluted culture more difficult.

Lancefield grouping of Streptococcus Kruger carried out during Periods I and IV was positive for Group B.

Streptococcus Thomson.

This strain was obtained in 1942 from a patient with fatal septicaemia. Immediately after isolation the organisms were seen to be capsulated, but did not give a definite result when tested against Lancefield Group B serum. Virulence was at first low and rather irregular, but after 21 passages through mice during three months cultures were lethal for these in 24 hours at a dilution of 1:1,600, while the Lancefield grouping became definitely positive for Group B.

Streptococcus Aronson.

This strain was also obtained from Dr. Feldt of Berlin in January 1931. The streptococci were capsulated and possessed a high virulence for mice after one or two passages, a dilution of 1:1,000,000 being certainly fatal in 24 hours when 0.25cc. was injected intraperitoneally.

Streptococcus Cook.

A few trials have been carried out with Streptococcus Cook, a strain isolated from a carbuncle. This organism was non-capsulated and belonged to Lancefield Group A. At the time of the therapeutic trials, the virulence had reached a high level, 0.25cc. of 1:1,000,000 dilution of culture killing a mouse in 24 hours.

ROUTE OF APPLICATION OF THERAPEUTIC AGENT IN RELATION TO INOCULUM.

In early experiments on the treatment of streptococcal infections with anil-quinoline drugs and acriflavine in mice, Browning et al.(8) injected the drug intraperitoneally

one hour after intraperitoneal inoculation and obtained very good results, which will be discussed later in Section V. By this method the drug entered the body of the host by the same route as the inoculum and so followed up the latter. In the case of the sulphonamides, however, the drug is effective when injected subcutaneously or given orally after the intraperitoneal inoculation; thus the therapeutic agent and the organisms enter in opposite directions, meeting, as it were, within the tissues.

In all but a very few of the therapeutic tests carried out with sulphanilamide and the sulphones the drug has been given by the subcutaneous route and the inoculum intraperitoneally.

OBSERVATION OF TEST ANIMALS AFTER TREATMENT.

In very many of the published reports on the treatment of mice with the sulphonamide drugs, the animals were considered cured if they survived for as short a period as one or two weeks after inoculation. Long and Bliss (66)

reported the occurrence of very late deaths due to streptococcal septicaemia both in mice treated with Prontosil Soluble and with sulphanilamide - in the latter case deaths occurred 49 and 194 days after the completion of prolonged sulphanilamide treatment. Despite these results, however, few workers seem to have considered the necessity of keeping treated animals under observation for a sufficiently long period to allow for the possibility of such delayed relapses. Many results classify as "cured" animals which survived in apparent health for anything from 10 to 30 days after infection and treatment, when a period of 8 to 10 weeks would have been more advisable; and in some published work on pneumococcal infections observation for 7 days was considered adequate. With the less soluble drugs which are gradually absorbed from a depot in the body, it is possible to envisage a state of equilibrium set up in the tissues between drug and invading organisms and brought to an end by the loss of drug through excretion,

but when the drug is a soluble and readily excreted compound like sulphanilamide it is difficult to explain on these lines the occurrence of fatal relapses at 14, 26, 32, and even 68 days after inoculation. The problem of these late deaths will be referred to again in Section V.

RESULTS IN THE TREATMENT OF EXPERIMENTAL INFECTIONS.

In the following account of experimental results all the inoculated control mice died of streptococcal septicaemia, and treated animals which died from any other cause are omitted from the totals reported. In every case films, and frequently cultures, were made from the heart-blood *post mortem* and examined for the presence of bacteria.

SULPHANILAMIDE.

Streptococcus Kruger.

† Period I.

Varying inocula. It has been generally agreed (p.16) that since sulphanilamide is a fairly soluble drug which is readily absorbed and excreted, it should be administered sufficiently frequently to maintain an adequate concentration in the blood. In early therapeutic trials it was found that the standard triple dosage of 10mg., 6.7mg., 6.7mg. injected subcutaneously during the first 24 hours gave good results (Table VIII), 70 mice having been cured out of a total of 112, i.e. 63 per cent. of cures. These results, taken from 28 separate experiments, show some variation in the proportion of cures, but this does not appear to be closely related to the dilution of the inoculum. The percentage of cures with 1:100, 1:1,000 and 1:100,000 dilution of inoculum correspond closely with one another and with the average value for the whole set, showing no statistically

† The results grouped as Period I are from earlier laboratory records - see p.8.

significant difference. An unaccountably high percentage (e.g. 92 per cent.) of cures was obtained in the 8 experiments in which the inoculum was a 1:10,000 dilution, these being fairly uniformly distributed throughout the series. Accordingly, it may be concluded that over the range employed a 10,000-fold difference in the inoculum had no uniform influence on the therapeutic result with the standard treatment. Hence in what follows it has been accepted that the therapeutic results obtained within this range may be compared without reference to the exact size of the inoculum.

Varying dosage. The effect of varying the total dose of sulphanilamide is shown in Table IX. It appears that a divided dose of under 10mg. prolongs life 1 to 3 days, but seldom effects cure; actually the only mouse cured out of 10 was one which received the smallest amount of drug - 2.34mg.. Amounts from 10mg. to 23.4mg. have led to cure in 89 out of 145 animals (61 per cent.). It is not very

clear what constitutes the best system of dosage, but since a single subcutaneous administration of 10mg. at the time of inoculation cured 5 out of 13 mice, it appeared advisable to supplement this by two additional doses of 6.7mg. at 5 and 24 hours: this treatment referred to as "standard triple dosage" cured 70 out of 112 mice (63 per cent.)

Delayed treatment. When there was a delay of two hours after inoculation before beginning treatment with the standard triple dosage 7 out of 7 mice were cured, but a delay of 6 hours resulted in only 4 cures out of 7 mice - Table X A.

Prophylactic treatment. As would be expected from the rapid absorption and equally rapid excretion of the drug, treatment with sulphanilamide administered from 72 hours to 2 hours before inoculation was unsuccessful - Table X B.

Periods II, III, and IV.

In these periods similarly successful results could not

be repeated with the standard triple dosage, there being only 2 cures out of 32 mice treated (Table XI), owing apparently to some change in the behaviour of the strain of streptococcus which coincided with the appearance of capsules in a hitherto non-capsulated organism.

Period V.

With the non-capsulated organisms of Period V, results were even better than those in Period I. Of 47 mice inoculated with a 1:1,000 dilution and treated with the standard triple dosage, 31 were cured, the others dying in 2 to 7 days, except 2 late deaths at 44 and 62 days. Of 5 mice similarly treated, but with only one-third of the above quantities (i.e. 7.8mg.), all were cured.

Other Strains of Capsulated Streptococci.

With the Thomson strain of streptococci, which was capsulated when isolated, all the 24 mice inoculated with a 1:1,000 dilution and treated with the standard triple dosage of sulphanilamide succumbed to the infection in

1 to 3 days. This corresponds with unpublished results obtained in this laboratory with the Aronson strain of capsulated haemolytic streptococci and quoted here for purposes of comparison (see p.8). Of 25 mice receiving inocula varying from 1:10,000 to 1:1,000,000 dilution of a 24 hours' broth culture of the Aronson strain and treated with the standard triple dosage of sulphanilamide, only 1 was cured, the rest all dying from streptococcal septicaemia in 2 to 5 days.

From these results as well as those with the capsulated phase of the Kruger streptococcus it may be concluded that the action of sulphanilamide against haemolytic streptococci in vivo depends greatly on the character of the particular strain of streptococci involved. While the drug seems capable of controlling and, in the majority of cases, of overcoming an invasion of non-capsulated streptococci, it appears as a rule to be able only to prolong by a day or two the life of animals infected with a capsulated streptococcus.

4:4'-DIAMINO-DIPHENYL-SULPHONE.

The diamino sulphone is the first of the three sparingly soluble depot-forming sulphone compounds to be considered. It is the most readily absorbed and also the most toxic of the three (pp. 26 - 27). A summary of therapeutic experiments is shown in Table XII. The results with Streptococcus Kruger Period I† were fairly satisfactory, provided that the dilution of inoculum was 1:1,000 or higher, 11 out of 12 mice being cured by triple doses totalling from 2mg. to the maximum tolerated dosage of 8.3mg.. With Period III streptococci (capsulated), however, the infection was unaffected even by large doses of the drug; death of the animals was merely delayed for a few days.

Results in Period V were again satisfactory, the maximum triple dose of 3.3, 2.5, 2.5mg.(8.3mg.) at 0, 5, and 24 hours curing 29 out of 35 mice which had received

† The results grouped as Period I are from earlier laboratory records - see p.8.

an inoculum of 1:1,000, the rest dying in 1 to 7 days with 1 late death at 21 days, and untreated controls all dead in 24 hours.

Delayed treatment with Streptococcus Kruger Period I,† even when begun 6 hours after inoculation (Table XIII A), gave very similar results to those obtained with immediate treatment, and incidentally supplied two good examples of late deaths at 26 and 28 days after inoculation.

Administration of heavy prophylactic treatment also showed similar results to those obtained by treatment at the time of inoculation (Table XIII B).

4:4'-DIACETYLAMINO-DIPHENYL-SULPHONE.

It has been seen that just as the diamino sulphone is the most toxic and most rapidly absorbed of the three sulphones, the diacetyl sulphone is the least toxic and also the least rapidly absorbed (pp.26-27).

† The results grouped as Period I are again from earlier laboratory records - see p.8.

Streptococcus Kruger.

As shown in Table XIV, the results of treatment at Period I† were quite satisfactory even when the dosage was reduced from the standard of 10.0, 6.7, and 6.7mg. at 0, 5, and 24 hours (23.4mg. in all). Triple doses totalling 5.84 to 23.4mg. cured 46 out of 78 mice and triple doses totalling 1.16 to 3.87mg. cured 5 out of 31 mice. With Period II streptococci reduction of the size of the dose gave results which showed no significant difference, the corresponding figures being 7 out of 14 and 1 out of 12.

The influence of continued absorption of the therapeutic agent from the depot was evident from the periods of survival of treated mice which eventually died from the infection. These showed a much greater range than had been observed following sulphanilamide treatment.

† The results grouped as Period I are again from earlier laboratory records - see p.8.

Treatment of Period V infections with the standard triple dosage cured 26 out of 31 mice receiving an inoculum of 1:1,000, the others dying in 5 to 9 days, with 1 late death at 30 days and the untreated controls all dead in 24 hours.

Prophylactic treatment with a single large dose, as shown in Table XV† gave marked but irregular effects.

4:4'-MONOACETYL-DIAMINO-DIPHENYL-SULPHONE.

Streptococcus Kruger.

†Period I.

The monoacetyl sulphone yielded very satisfactory therapeutic results - Table XVI. All of 9 mice treated with the standard triple dosage - 6.7, 4.0, and 4.0mg. at 0, 5, and 24 hours - survived, and even with a tenth of this dosage there were some cures, while the survival times of the mice which died from the infection - 3 to 5 days-

† The results grouped as Period I are again from earlier laboratory records-see p.8.

were greater than those of the untreated controls, which all died in 24 hours following a similar inoculum.

Period II.

In this period results were again of much the same order as in Period I throughout the range tested, 26 out of 29 mice being cured by the standard triple dosage - Table XVII.

Period III.

The therapeutic efficiency of the monoacetyl sulphone was diminished (Table XVIII), the standard triple dosage curing only 41 out of 121 mice. The survival times of mice dying from the infection were from 4 to 18 days, and there were also several cases of late deaths due to streptococcal septicaemia, one occurring 89 days after inoculation.

Period IV.

The proportion of cures with the standard triple dosage of monoacetyl sulphone in Period III (Table XVIII) and

Period IV (Table XIX) shows no statistically significant difference, whereas a comparison of the totals of cures obtained in Periods II and IV shows a significant difference.

Period V.

Here the infection again showed the same high degree of curability as in Period I. Of 41 mice receiving an inoculum of 1:1,000 and treated with the standard triple dosage, 37 were cured, the other 4 dying in 2, 3, 13, and 30 days; and the untreated control mice in 24 hours. With half and also with a quarter of the standard dosage all of 6 mice were cured.

Effects of Varying Dosage.

Single doses equal to the total standard triple dosage were tested with Streptococcus Kruger Periods II and III (Tables XX A and B). On the whole, however, the triple dosage method seemed to produce slightly more cures over these periods than did single doses.

Delayed Treatment and Prophylactic Treatment.

Delayed treatment and prophylactic treatment with monoacetyl sulphone were both tested against Streptococcus Kruger Period II.

Delayed Treatment. With a single dose of 6.7mg. there was little or no alteration in the number of cures obtained when treatment was delayed for 1½ or 2 hours, but a delay of 3 hours or longer before administering the single dose of drug showed a decided decrease in therapeutic efficiency which was also highly significant statistically (Table XXI A). This suggests that up to 2 hours after inoculation, but not after 3 hours, the degree and rate of absorption of active drug are still sufficient to counteract the infection.

Prophylactic Treatment. A single dose of 14.3mg. given up to 5½ hours before the inoculum was effective in Period II in preventing fatal infection in nearly all the animals treated - Table XXI B. However, a slightly smaller dose (13.3mg.) had very little effect on the infection when

administered 5 days before the inoculum, as also had 11.0mg. given 4 days before. Since there is still an abundant depot of drug in the subcutaneous tissue 5 days and even later after the injection of such doses, these results suggest that absorption of drug from the subcutaneous depot proceeds at a reduced rate after the first few hours, but there is no evidence of any change in the character of the drug forming that depot. This view was supported by other experimental findings. A set of 10 mice was injected subcutaneously each with 13.3mg. of monoacetyl sulphone per 20g. body-weight. After allowing 5 days for absorption of the drug to take place to some extent, the mice were sacrificed and the subcutaneous deposits of drug removed and pooled, the whole being dispersed in water to a volume sufficient to provide 10 doses of approximately 1cc. each. This "residue" was used as treatment for 10 mice inoculated with Streptococcus Kruger Period III, while, as a control, 10 similar mice were treated with triple doses totalling

13.3mg. in 24 hours. Of the 10 mice receiving "residue", 6 were cured, the others dying in 5 to 13 days; while of the 10 mice treated with approximately equivalent triple doses 2 were cured, the remainder dying in 5 to 19 days. Although the difference between the numbers of cured mice here seems considerable it has no statistical significance, the "residue" drug retaining its curative properties even after a period of absorption lasting 5 days.

Prophylaxis of Relapses. With Streptococcus Kruger Period III, the effect was studied of an additional dose of monoacetyl sulphone given a few days after the standard triple dosage (Table XXII). This was found in general to produce an increase in the number of animals cured when the extra subcutaneous dose was given 3, 4, or 5 days after the inoculum, but the increase is not statistically significant. A beneficial effect due to an additional dose would have supported the theory of the bacteriostatic action of the sulphonamide drugs. The balance between drug and invading

organisms has been effectively weighed down on the side of the drug by further subcutaneous injection, and this allows the normal body defences to operate. Also in view of evidence stated above there might be more effective action of the recent dose than of the older depots.

Combined Treatment with Sulphanilamide and Monoacetyl Sulphone.

It seems reasonable to suppose that combined treatment with a rapidly absorbed drug along with a less rapidly absorbed one should be advantageous in combating a bacterial infection *in vivo*, since the effect of the slowly absorbed drug would come into play as that of the more soluble drug lessened due to excretion.

Various treatments were tested against Streptococcus Kruger Period III, both sulphanilamide and monoacetyl sulphone being used, but the results obtained showed no advance on those with the sulphone alone. In one experiment, the control mice were given a subcutaneous dose of 12.5mg.

monoacetyl sulphone at the time of inoculation, which cured 11 out of 21 mice. Other 15 mice each received in addition 6 doses of 10mg. sulphanilamide, the first dose one hour before the inoculum and the others at 24 hours' intervals: 6 were cured and the other 9 died 6 to 22 days after inoculation. There is no significant difference between these two sets of findings; this is scarcely unexpected, since it has already been found that sulphanilamide alone has practically no effect upon the capsulated organisms of this particular period of Streptococcus Kruger.

Treatment of Infections with Other Capsulated Strains.

Results of treatment of Streptococcus Aronson† with monoacetyl-diamino-diphenyl-sulphone are shown in Table XXIII A. Only the standard triple dosage - 6.7, 4.0, and 4.0mg. at 0, 5, and 24 hours - was found to have any therapeutic effect on this infection - 3 cures out of 8 mice - but the survival times of all the treated mice which died, were

† The results with Streptococcus Aronson are from earlier laboratory records - see p.8.

greater than for untreated mice given the same inoculum - 4 to 18 days as compared to 24 hours. In the case of this capsulated streptococcus therefore monoacetyl sulphone has to be given in maximum dosage to produce a curative effect.

Therapeutic tests with the monoacetyl sulphone were also carried out with Streptococcus Thomson - the capsulated and rather less virulent Lancefield B streptococcus of human origin. As in the case of Streptococcus Aronson and Streptococcus Kruger Periods III and IV, maximum dosage gave about 33 per cent. cures (Table XXIII B) with survival times of 2 to 20 days in treated mice dying from the infection, as compared with 24 hours for untreated control mice receiving the same inoculum.

Hence, of the five strains of capsulated streptococci, only Streptococcus Kruger Period II has shown any considerable response to treatment with monoacetyl sulphone. With all the other four strains - Aronson, Thomson, and Kruger Periods III and IV - there was equal and relatively low curability

even with maximum dosage, indicating a high degree of resistance, which may be associated with full capsular development. As regards Period II of Streptococcus Kruger, it should be noted that definite capsules were present, although the organisms were still sensitive to treatment with certain drugs; but the fact that the much less sensitive organisms of Periods III and IV developed spontaneously from those of Period II suggests that the latter represented an early stage in the development of increased natural resistance of the organism.

Treatment of Infection with a Lancefield-A Strain.

It has already been mentioned (p.32) that strains of streptococci belonging to Lancefield Group A and showing maximal virulence, are reputed to be much more susceptible to sulphonamide therapy than are Group B strains. Streptococcus Cook is a highly virulent, non-capsulated streptococcus belonging to this category, and a small number of experiments were carried out with mice infected with

this organism. No cures were obtained out of 4 mice treated with up to one-third of the standard triple dosage of sulphanilamide, but with up to one-third of the standard triple dasage of monoacetyl-diamino-diphenyl-sulphone, 8 out of 9 mice were cured, while one-sixth of the standard dosage cured 4 out of 8 mice. With diacetylamino-diphenyl-sulphone results were very similar, 6 out of 7 cures with up to one-third of the standard triple dosage and 1 out of 5 with one-sixth of the standard triple dosage. Accordingly, while this strain is not highly responsive to treatment with sulphanilamide, a marked response is shown to treatment with two of the sulphone drugs.

† The results with Streptococcus Cook are from earlier laboratory records - see p.8.

The Effect of p-Aminobenzoic Acid on the Results of Treatment.

In 1940 Woods (119) studied the reversal of the inhibitory action of sulphanilamide *in vitro* by a substance which was present in yeast extracts. He showed that p-aminobenzoic acid possessed many of the properties characteristic of this unknown yeast constituent. In continuation of this idea, Selbie (107) examined the effect of p-aminobenzoic acid on the therapeutic action of sulphanilamide *in vivo* in haemolytic streptococcal infection of mice. In this case the sodium salt of p-aminobenzoic acid was given together with sulphanilamide *per os*; and it was found that small doses of the acid greatly reduced the survival rate of mice treated with sulphanilamide.

In the experiments summarised in Table XXIV the usual technique for therapeutic experiments was employed - inoculum intraperitoneally and three subcutaneous doses of monoacetyl sulphone at 0, 5, and 24 hours - but p-aminobenzoic acid was administered to a proportion of

the mice two or three times daily as a 1:50 suspension added to the bread-ration, this being begun either a few days before or after inoculation. Feeding of the same amount of p-aminobenzoic acid to normal mice was without toxic effects. With standard triple dosage of monoacetyl sulphone, administration of p-aminobenzoic acid beginning at any time from 2 days before inoculation to 5 days afterwards, reduced the number of cures to 2 out of 33 mice as compared with 7 out of 19 mice for those which did not receive any p-aminobenzoic acid - a statistically significant difference - and in nearly every case death took place within a day or two of commencement of the special feeding. With smaller doses of monoacetyl sulphone, similar results were obtained. p-Aminobenzoic acid itself does not exert any anti-streptococcal activity *in vivo* and is quite harmless when fed to mice even in large quantities. Its effect in reversing the therapeutic activity of monoacetyl sulphone may be regarded as resembling therapeutic interference. This phenomenon was described by Browning and

Gulbransen (11,12) in the case of trypanosome infections in mice. Administration of a dose of trypaflavine normally curative for the particular strain of parafuchsine-fast trypanosomes, did not effect cure if parafuchsine was already present in the body of the host. The interfering agent - parafuchsine - must, however, be already present when the curative compound is administered, and this points to a direct action of parafuchsine upon the parasites. Parafuchsine probably combines with the protoplasm of the trypanosomes in such a way as to block the trypanocidal action of trypaflavine. In the case of p-aminobenzoic acid, however, it has been shown that interference may be produced by administration of p-aminobenzoic acid commencing as long as 5 days after inoculation and treatment with the monoacetyl sulphone. The theory has been suggested, principally by Fildes (27), that the reversal of sulphanilamide action by p-aminobenzoic acid is due to the fact that p-aminobenzoic acid (or some compound closely allied to it) is essential for the maintenance of bacterial metabolism

and that sulphanilamide blocks the related enzyme system, so that p-aminobenzoic acid must be supplied artificially to compensate for the stoppage in normal synthesis. p-Aminobenzoic acid is described as an "essential metabolite" for the organisms. This theory will be further noted in Section IV.

COMPARISON OF PUBLISHED RESULTS ON THERAPY.

It is almost impossible to draw any comparison between the various published results in the treatment of experimental infections with sulphanilamide and allied drugs. The source of infection, the method of administration of the drug, the size of the dose, and the period of observation after treatment are all variable factors and the only possible comparison is between the best results obtained by each of the various combinations of methods. The percentage of survivors is seen to depend upon the duration of treatment with sulphanilamide. Table XXV shows some of the results published for the treatment with sulphanilamide of experimental haemolytic streptococcal infections in mice.

The best results by either oral or subcutaneous administration are obtained by using a fairly large dosage divided over several days - a fact which was emphasized by Long et al.(68) who found that the survival rate and average duration of life were definitely better when the total amount was given as divided doses on each of three days instead of being given as three daily doses.

With diamino-diphenyl-sulphone quite favourable results have been obtained (Table XXVI A). Bauer and Rosenthal (2) stated that the diamino sulphone given per os was 30 times as active by weight as sulphanilamide, and Marshall et al.(75) using the drug-diet method claimed it to be three times as active on the basis of blood concentrations of the drugs. Buttle et al.(15) also found the diamino sulphone much more active than sulphanilamide, and Fourneau et al.(29) mentioned 0.05mg. as an effective oral dose against streptococcal infections in mice for both the diamino- and diacetylamino- sulphones.

Diacetylamino-diphenyl-sulphone also gave good

therapeutic results against haemolytic streptococci (Table XXVI B). Bauer and Rosenthal (2) claimed a therapeutic index six times that of sulphanilamide, and Buttle et al.(15) stated the diacetyl sulphone given per os to be ten times as active as sulphanilamide.

There is no record of published work on treatment with the monoacetyl-diamino-diphenyl-sulphone.

DISCUSSION.

Table XXVII shows the varying responses to treatment with sulphanilamide or the sulphone drugs in standard dosage as to amounts and times of administration, which were exhibited at different periods by the Kruger strain of streptococcus. It is clear that the outcome of sulphanilamide therapy was immediately affected when the organisms acquired capsules, the percentage of cures falling from 63 per cent. in Period I to under 10 per cent. in Periods II, III, and IV. Treatment with the monoacetyl sulphone was almost as successful in Period II as with the non-capsulated organisms of Period I - over 90 per cent.

of cures. During the further maintenance of the strain the least responsive phase of the organisms was reached in Period III with 34 per cent. of cures. The organisms of Period IV, derived directly from a spleen dating from Period II (Table VII), showed apparently a slightly greater sensitivity to sulphone treatment than those of Period III (49 per cent.) although the difference is not statistically significant. Excellent results were obtained with all four drugs against the non-capsulated organisms of Period V.

It would appear, therefore, that while the initial development of capsules quickly decreased the sensitivity of the organisms to treatment with sulphanilamide, cure could still be effected by treatment with monoacetyl-diamino-diphenyl-sulphone. A further, but morphologically unrecognisable, alteration occurred, however, in the course of continued passage of the strain and this caused a marked reduction in its curability by the sulphone drug.

This phenomenon can scarcely be considered as a manifestation of "drug-resistance" in the generally accepted

sense, since these alterations of the streptococci had come about without any exposure of the organisms to the specific drug. The fact that the non-capsulated phases of the strain showed rather less virulence for mice, suggests that continued animal passage had not only increased the virulence, but by doing so had increased the resistance of the organism both to the natural defences of the host's body and to the chemotherapeutic agents.

Monoacetyl-diamino-diphenyl-sulphone has proved to be the most efficient of the sulphonamide drugs in the author's experiments. It gives the highest proportion of cures with both capsulated and non-capsulated strains of streptococci. The diacetyl sulphone is next to the monoacetyl sulphone in therapeutic efficacy, but gives a lower percentage of cures, and the diamino sulphone is only slightly less active against haemolytic streptococci. Sulphanilamide is the least effective of the drugs tested.

The survival times of mice dying from the infection are related to the rates of absorption and excretion of the

drugs; sulphanilamide and the diamino sulphone, the most soluble of the three sulphones, showed the shortest survival times for treated mice in which therapy failed.

When attempts were made to assess the results of alterations in the dasage and in the time of commencing treatment, anomalous results were frequently obtained. Reduction in the amounts given by triple dosage usually resulted in a decrease in the number of cures, although this was less evident in the case of sulphanilamide and diamino-sulphone - the two more readily absorbed drugs. Administration of the monoacetyl sulphone in a single dose instead of by equivalent triple dosage had very little effect in reducing the number of cures. Prophylactic treatment with all four compounds was of practically no value as a protection against infection unless administration of the drug took place within a few hours before inoculation with the organisms. Either, as in the case of sulphanilamide, the therapeutic agent was too rapidly excreted, or else absorption from the depot of a relatively insoluble drug such as the

monoacetyl sulphone was proceeding at an insufficiently high rate by the time of inoculation. The explanation of this slowing down process remains so far obscure. Delayed treatment was of value only in the case of sulphanilamide and diamino sulphone, since both of these are readily absorbed following injection, and administration of drug was still effective in controlling an infection up to 6 hours after inoculation of streptococci. Treatment with the monoacetyl sulphone, which is less soluble and therefore less readily absorbed, had to be begun not later than 2 hours after the inoculum to obtain a high percentage of cures. Prophylaxis of relapses by administration of an additional dose of monoacetyl sulphone a day or so after the standard triple dosage did not give a statistically significant increase in the number of cures. Also, the use of two drugs in treatment which would have been expected to supply the advantages both of a readily absorbed drug and of a more slowly absorbed compound failed to improve the percentage of cures obtained with either drug alone.

Investigation of the inhibitory effect of p-aminobenzoic acid on the therapeutic action of drugs of sulphonamide type confirmed the findings of other workers but gave no further insight into the mechanisms which might be involved.

SECTION III

THE COURSE OF INFECTION IN TREATED MICE

During the experimental work already described on the treatment of streptococcal infections in mice, several instances of late deaths were recorded in which the cause of death was shown to be streptococcal septicaemia - an experience also reported by other workers, Colebrook et al.(21), Long and Bliss (65,66). This suggested an investigation of the day-to-day condition of the blood of infected animals undergoing treatment with sulphonamide drugs, a procedure which does not seem to have been employed hitherto.

TECHNIQUE.

As previously described, mice were inoculated intraperitoneally with a 24 hours' broth culture of haemolytic streptococci diluted so that the dose, 0.25cc., was ten times the inoculum sufficient to cause death from

septicaemia of all untreated control mice in about 24 hours. Subcutaneous treatment was given at 0,5, and 24 hours after the inoculum, dosage depending on the particular drug employed.

For examining the blood of treated animals a simple method was devised to minimise possible contamination during the taking of the sample. The mouse was placed in a closed box of a size just sufficient to accommodate it, with its tail protruding through a slot in the lid; the tip of the tail was dipped in a solution of crystal-violet and brilliant-green (0.5 per cent. of each in absolute alcohol) and allowed to dry for at least a minute. The surface of the tail was then wiped with cotton-wool soaked in spirit and the tip refreshed with sterile scissors. A drop of blood was expressed and one loopful (taken with a small platinum loop of standard size - 2mm. in diameter) used to make a single stroke on the surface of an agar plate - a process in which practically the whole loopful of blood was transferred to the agar surface. The plate

was incubated for 24 hours and the number of colonies counted. It was not found necessary to repeat the whole sterilising process for every plating, a single swabbing with spirit being sufficient to remove contaminants from the tail-surface on several occasions after the original painting, but the dye treatment was usually carried out at weekly intervals during the period of the observations. The difficulty previously met with in obtaining cultures of tail-blood free from surface contaminants was no longer found after the above procedure had been adopted. Daily examination of treated animals was not found to have any harmful effects on the mice and even the precaution of enclosing the animal in a box during withdrawal of the blood was unnecessary after the second or third day, as the mice very rapidly became accustomed to the process. When it was desired to inoculate fresh mice from animals under observation, one drop of tail-blood added to 0.25cc. broth was used for intraperitoneal inoculation of each new mouse.

For these investigations two different strains of haemolytic streptococci - Streptococcus Kruger Period IV and Streptococcus Thomson, both capsulated types - were used, and treatment was with sulphanilamide and 4:4'-monoacetyl-diamino-diphenyl-sulphone.

UNTREATED CONTROLS.

In mice which were inoculated with a dilution of the culture equal to 10 M.L.D. and received no treatment, there was a rapid multiplication of the invading organisms in the blood, and death from septicaemia usually within 24 hours. Examination of the blood of such mice by plating at frequent intervals during the first 9 hours after inoculation showed that even after 1½ hours a single drop of blood contained up to 100 organisms, judging by the number of colonies growing on agar. In 4 to 6 hours from the time of inoculation multiplication of the invading organisms had reached a stage at which enumeration was practically impossible, and no further difference in numbers could be distinguished by the present methods of observation.

TREATMENT WITH SULPHANILAMIDE.

As shown in Table XXVIII, out of 15 mice (taken from three separate experiments) treated with the standard triple dosage of sulphanilamide only one was cured. The blood of this mouse remained negative throughout the 31 days of examination, while the animal itself was still alive and well 5 months later. The other mice so treated died in 2 or 3 days after inoculation with no evidence of any control of the number of streptococci in the blood, except in 2 cases (E in Table XXVIII) where the blood was free from organisms on the afternoon following the third dose, but became positive the next day, and both mice died of streptococcal septicaemia on the fourth day of the experiment. This series illustrates well the general experience that in the mouse sulphanilamide is a relatively inefficient therapeutic agent against certain streptococcal infections.

TREATMENT WITH 4:4'-MONOACETYL-DIAMINO-DIPHENYL-SULPHONE.

Dosage was generally at the rate of 6.7,4.0,4.0mg. at 0, 5, and 24 hours, although three doses of 2.5mg. or three doses of 1.67mg. were used in some of the earlier experiments.

In general the types of behaviour among mice inoculated and treated as described above, may be divided into the following categories :-

Cures (33%) (A) Organisms never found in the blood (38% of cures).

(B) Few organisms present at some stage (38% of cures).

(C) Abundant organisms at some stage (24% of cures).

Death due to streptococcal septicaemia (67%) :-

(D) Blood never free from organisms (29% of deaths).

(E) Organisms gradually appeared in the blood before death (59% of deaths).

(F) Blood negative up to day of death (12% of deaths).

(See Table XXIX.)

Cures in which few or no organisms were ever found in the blood were represented by about 25 per cent. of the total mice treated, while about 8 per cent. showed cure occurring even after abundant streptococci had been observed in the blood. In the latter, the period during which streptococci were continuously present in large numbers was generally 1 to 4 days, but in one instance was 10 days (beginning 3 days after a free period of 4 days), while another mouse was eventually cured after a 23-day period of high infection lasting from the third day after inocultion. When the blood was never free from streptococci - (D) - the period of survival after inoculation usually varied from 1 to 13 days - a survival period of 36 days being exceptional.

In the most common type of behaviour - (E) - death occurred from 3 to 20 days after inoculation and the blood was in many cases completely free from organisms for a period of 1 to 10 days during that time.

In 2 animals included in class (F) the blood showed scanty streptococci only on one and three occasions within

the first week, but after free periods of 8 and 31 days, they died suddenly of streptococcal septicaemia.

Certain facts of importance have appeared from these tail-plating studies.

(1) A mouse infected with highly virulent haemolytic streptococci and then treated, may appear to be in perfect health with sleek normal coat and good appetite, and yet be found to harbour large numbers of fully virulent organisms in its blood. A single small loopful of this blood diluted in 0.25cc. of broth and at once injected intraperitoneally into a fresh mouse caused death in 24 hours, while the original mouse continued to live apparently unaffected by the infected condition of its bloodstream. This has been demonstrated in the case of 9 separate animals of which 3 were eventually cured, the above results being obtained on 17 examinations. Two animals, however, similarly inoculated with a loopful of blood each from an infected mouse survived without showing illness, although broth cultures from other drops taken at the same time and

incubated overnight caused fatal infections when 0.25cc., undiluted, was injected intraperitoneally. This result indicates that in these cases the virulence of the organisms had been considerable reduced in the body of the host.

(2) In only 2 out of 27 cases in which the organisms isolated in culture from the blood of treated mice were tested for virulence did the streptococci prove to be non-virulent. In both these instances only scanty streptococci were found on plating and while these grew freely in subculture in broth, they did not kill mice injected with 0.25cc. of the undiluted cultures. In neither case did the original mouse die from streptococcal septicaemia, although one of these had three later relapses of one or two days' duration when streptococci again appeared in the blood. The virulence of these relapse organisms was only tested by direct inoculation of blood once, and they then proved to be non-virulent. The organisms were capsulated.

(3) Blood from mice which were resistant to the large numbers of virulent streptococci in their circulation,

was on two occasions used to inoculate further mice in the manner described above, i.e. direct inoculation of blood; and these mice were then treated, but with smaller doses than the original mice. In one series, 1 survived out of 3 mice treated with 3.3,2.0,2.0mg.(7.3mg. in all), the other 2 dying in 5 and 10 days respectively; while of 3 mice receiving 2.0,1.4,1.4mg.(4.8mg. in all) all died in 6 to 8 days. The untreated controls died in 24 hours, thus indicating a highly virulent infection. In another series the untreated controls lived for between 24 and 48 hours, the virulence of the organisms being somewhat diminished, and all the treated mice were cured - the dosages of drug being the same as those just mentioned. Meanwhile the blood of the original mouse became free for 4 days, and although death occurred then, no bacteria of any kind were recovered post mortem from the heart-blood.

DISCUSSION.

An analysis of all the findings of these tail-plating experiments allows the following points to be defined.

Infected mice which were treated with the standard triple dosage of sulphanilamide only very rarely showed immediate and complete sterilisation of the blood in the case of the strains of streptococci employed. The usual result was a prolongation of life by a day or two as compared with the untreated controls; occasionally the drug produced a transient initial sterilisation of the blood-stream, but generally organisms were continually present in the blood.

With the depot-forming monoacetyl sulphone, the picture of events following treatment was rather more varied and seemed to be strongly influenced by the individual host. Permanent sterilisation of the blood-stream occurred almost at once in 25 per cent. of the treated mice, indicating a rapid response to treatment which did not permit of further analysis of the factors

involved in therapy. A more interesting finding was the demonstration of the balance set up between invading organisms on one hand and drug and defensive mechanisms of the host on the other. In the case of mice which succumbed to infection despite treatment, the same contest between opposing forces was again evident, but in these the organisms eventually gained mastery.

The importance of the part played by the host in bringing about control of the infection is emphasized by the variety of the individual responses to treatment as demonstrated by the study of the continued presence or gradual disappearance of virulent organisms in the blood. This is no simple contest between a certain number of virulent organisms and a measured amount of an active drug, for success or failure depends on the ability of the host to assist in the destruction of the organisms by means of the defensive mechanisms naturally provided. A similar inoculum of organisms injected without therapy readily overwhelms the animal's normal methods of defence,

but administration of a drug of fairly prolonged action frequently enables the body to oppose such an invasion successfully.

However, the possibility must not be overlooked that the natural response of the host to infection shows considerable variations in different mice, and even with mice of equal weight receiving identical inocula of organisms and doses of drug, the final result depends on the natural resistance of the animal to infection. That this "mouse factor" may not be quite great enough to turn the scale in favour of complete cure, is shown in the following instance. In this animal the numbers of streptococci in the blood showed alternate rises and falls until the 17th day of the experiment when a very high level was reached and maintained for 12 days. A sudden fall in numbers was observed on the 30th day, but there was not complete disappearance of the organisms; these had increased again by the following day and death occurred from streptococcal septicaemia on the 37th day.

It has been demonstrated by the above method of examination that virulent organisms may persist in the blood of a mouse for a considerable period without any apparent ill effects on the host, which may eventually recover completely and show a negative blood culture. It also appears that in some animals the circulating organisms become diminished in virulence just before they finally disappear from the blood. Both these findings agree with the theory that a balance is set up between the opposing elements in the body of an infected and treated animal, and the second illustrates the means by which the invading organisms are finally overcome.

It has already been pointed out by Browning (6), in confirmation of results reported by Mellon et al.(77), that films made from the peritoneal content of inoculated and treated mice showed no very great abnormalities as regards the types and numbers of cells present. Scanty streptococci were found to persist, however, and these yielded abundant growth on culture of a small loopful of

peritoneal exudate. The heavy dose of virulent organisms had been almost completely suppressed by the combined action of drug and body defences, but recrudescence of the infection followed rapidly on cessation of treatment. The organisms recovered on one occasion after such a failure of therapy were found to be distinctly attenuated in virulence when compared with the organisms of the original inoculum. This suggests that, as in the reaction of immunised animals towards inocula of virulent anthrax bacilli (Preisz,92), the conditions in the body of a treated mouse are often unsuited for the continued proliferation and development of pathogenic properties. A similar occurrence has already been described in the present work (p.87). Blood from a mouse which was resistant to the large numbers of streptococci present in its circulation, was used to inoculate a set of fresh mice. The untreated mice of this set lived for up to 48 hours, thereby indicating a slight diminution in the virulence of the organisms, while the treated mice all survived infection. The conditions of

organisms versus host's tissues plus drug had merely been continued in the newly infected and treated animals.

In no case has any alteration occurred in the capsulation of organisms during the course of observations.

The significance of these experimental findings is discussed further in Section V along with other theories of sulphonamide action.

SECTION IV

THE ACTION OF SULPHANILAMIDE IN VITRO

In view of the considerable antibacterial action shown by the sulphonamide drugs in experiments *in vivo* with certain organisms, it is surprising at first to find how slight an effect is produced when these drugs are tested against the same organisms *in vitro* in the ordinary types of culture medium. This is in direct contrast to the results with drugs which have powerful antiseptic properties in vitro, since the latter are of little value for the treatment of a generalised bacterial infection.

The particular type of organism used is of great importance. The parasitic nature of certain organisms is shown by their metabolic requirements; if an organism requires for its food-material a compound occurring naturally only as a result of the activity of some other living system, it becomes obligatorily a parasite.

Organisms which produce a generalised infection in the living body are by nature parasitic, and the presence of blood, serum, or some form of protein digest in the culture medium is usually, but not always, essential for their unrestricted growth *in vitro*. By differentiating between the necessary and the superfluous components of those added materials, the so-called "synthetic media" have been evolved. In the case of the streptococcus, however, the complexity of the synthetic medium required has led several workers to carry out *in vitro* experiments with the sulphonamide drugs by means of *B. coli* - an organism which grows abundantly in medium of fairly simple composition. It is doubtful in how far the results with one species of organism can be applied directly to another. Cooper *et al.*(23), using a strain of Flexner's dysentery bacillus in several types of medium, suggested that there was a direct relationship between the nutritive quality of media and the degree of bacteriostatic or bactericidal activity observed - the medium most favourable by itself to growth giving the best

inhibitory results in presence of the drug. While enrichment of a simple medium by the addition of blood or serum, etc. undoubtedly increases the nutritive value of the medium, it was found that other factors were introduced at the same time which interfered with drug activity, and the identification of these widened the field of investigation of sulphonamide action.

It is proposed in this section to deal with the published and experimental results of *in vitro* trials in so far as they are concerned with the different degrees of antibacterial activivy of the sulphonamide drugs in varying types of media and the theories of their mode of action which have been evolved. Finally the development of resistance to the drug both *in vitro* and *in vivo* will be considered.

PUBLISHED RESULTS.

Effect on Haemolytic Streptococci.

Colebrook et al.(20) found that 1:10,000 sulphanilamide in Wright's broth was sufficient to slow down the growth of a small inoculum of streptococci for 2 to 5 days, but large inocula were not inhibited by 1:100 sulphanilamide. Sulphanilamide in defibrinated human blood or serum caused slow destruction of the organisms after a period of about 6 hours' multiplication in presence of the drug; but the blood of rabbits, guinea-pigs, or mice, and deleucocyted human blood were all less satisfactory media in which to demonstrate bacteriaidal action. Finklestone-Sayliss et al.(28) emphasized that growth of the inoculum was stimulated at first by sulphanilamide and then there followed a rapid decline in population; and also that 26-hour cultures were almost immediately affected by 1:10,000 sulphanilamide as compared to young cultures. Mellon et al.(77) noted the discrepancy between reports that sulphanilamide in concentrations of 1:10,000 to

1:25,000 in broth, human serum or human blood was distinctly bacteriostatic and sometimes bactericidal to haemolytic streptococci (Lancefield Group A) and denials that there was such bacteriostasis, and emphasized the variation in results caused by small changes in technique. The effect of temperature on the antibacterial properties of sulphanilamide was noted by White et al.(116). Bactericidal action was observed at 40°C, whereas under the same conditions at 37°C only inhibition of growth of streptococci in peptone-glucose broth could be demonstrated. According to Chandler et al.(18) there is a period of normal multiplication of the organisms before the bacteriostatic effect of the drug becomes manifest, but organisms which had been grown overnight in sulphanilamide medium showed only a very brief period of multiplication when subcultured in fresh sulphanilamide medium before growth was markedly inhibited. This agrees with the general finding and with the theory of Kohn et al.(51) that certain substances essential to growth and multiplication are stored within

the bacterial cell and the rate of their synthesis is inhibited by the sulphonamides, but before the effect of the drug can be seen the reserves within the organisms of the inoculum must first be depleted. Wolff et al.(117) thought that sulphanilamide acted only when the organisms were in course of reproduction and had divided a certain number of times. They calculated that a minimum number of 2^8 divisions of the bacterium in presence of the drug was required before sulphanilamide acted on the organisms.

Sulphonamide Inhibitors.

It soon became evident that the failure to demonstrate the antibacterial action of sulphonamides in certain types of media must be due to the presence of some opposing agent. Lockwood (62) pointed out that even a small quantity of peptone - a common ingredient of nutrient media - when added to serum prevented the antibacterial action of sulphanilamide, a fact which was confirmed by many other workers. Gay et al.(34) compiled lists of factors which either aid or inhibit the growth of

streptococci, and stated that when tested under conditions favouring growth, sulphanilamide produced bacteriostasis to a degree depending inversely on the sum total of adjuvant growth factors present. Long and Bliss(67,pp.97-101) have also supplied a list of the variables which may influence the result in vitro when sulphanilamide is added to haemolytic streptococci: (1) size of inoculum; (2) age of culture; (3) virulence of culture; (4) composition of medium, both that in which the test is carried out and that in which the cultures may be diluted for use; (5) temperature at which the test is carried out; (6) duration of contact.

Stamp (111) found that in heart-broth the bacteriostatic effect of sulphanilamide was interfered with by the presence of 1 per cent. or more of peptone, similar amounts of serum or glucose, and relatively small amounts of heat-killed homologous organisms, while Green (36) isolated a growth- or "P" factor from broth cultures of Br. abortus which had an inhibiting effect on sulphonamide action, and showed that this "P" factor could also be obtained

from "marmite", yeast, casein digests, and certain peptones.

p-Aminobenzoic Acid.

Finally Woods (119) isolated from yeast extracts a sulphanilamide antagonist which was apparently identical with p-aminobenzoic acid. It was thought that the chemical similarity between sulphanilamide and p-aminobenzoic acid might account for the phenomenon of interference. This explanation of sulphenamide antagonism was followed up by Fildes (27), who considered that the antagonist, e.g. p-aminobenzoic acid, competed with sulphanilamide for an enzyme of which the former was the natural substrate. Under conditions in which p-aminobenzoic acid was present only in normal quantities or was synthesised by some earlier stage of metabolism, the structurally similar sulphanilamide - if present in sufficient concentration - might be capable of displacing p-aminobenzoic acid from its enzyme and stopping an essential line of metabolism. Addition of p-aminobenzoic acid therefore supplied a necessary growth factor of which normal utilisation had

been prevented, and so allowed continued growth of the organisms despite the presence of sulphanilamide.

Other Inhibitors of Sulphonamides.

Sulphonamide inhibitors were demonstrated in extracts of fresh normal muscle, pancreas, spleen (MacLeod, 85), rabbit-blood, mouse-blood (in red cells only), mouse-urine, but not in human blood, mouse serum or lymph (Fuller et al., 31). The presence of such inhibitors gives a possible explanation of the failure to demonstrate the bacteriostatic action of sulphanilamide in various media. The sulphonamide-antagonising action of methionine which is not structurally related to sulphanilamide and does not stimulate bacterial growth, was noted by Bliss et al.(5) and Harris et al.(42), and was thought to be involved in an enzyme reaction secondary to that involving p-aminobenzoic acid. Also McIlwain (80) noted further examples of reversal of antibacterial action even although the drug and its inhibitor were not structurally related.

To overcome the difficulty attending the use of

peptone and meat infusion MacLeod and Mirick (87) claimed that culture medium should be treated by boiling with charcoal in the course of preparation, since this removes the sulphonamide-inhibitors.

Effect on Enzyme Reactions.

Several suggestions have been made on the basis of experiments in vitro as to the nature of the enzyme reaction affected by the presence of sulphanilamide. Sevag et al.(109) studied the respiration of Streptococcus pyogenes and considered that it was the blocking by the sulphonamide drugs of the respiratory enzymes of the organism which eventually inhibited growth, while Shinn and Main (110,70) favoured the anticatalase theory which may be briefly stated as follows. When one of the by-products of cell-metabolism is hydrogen peroxide, the accumulation of this compound will tend to kill the organisms unless catalase is present to keep the hydrogen peroxide level below a critical value. It was suggested that sulphanilamide is converted by mild oxidation to a derivative which is a highly active

anticatalase. The fact of increase in hydrogen peroxide and consequent retarding of growth in the presence of sulphanilamide was demonstrated *in vitro*, but it is doubtful whether this can be accepted as an explanation of the mechanism of the action *in vivo*. It has also been shown (Mann *et al.*,71) that sulphanilamide inhibits the action of the enzyme carbonic anhydrase *in vitro*, and an *in vivo* demonstration of this inhibition was given by Benesch *et al.*(4). Carbonic anhydrase which catalyses the reaction

$$H_2CO_3 \rightleftharpoons CO_2 + H_2O$$

plays an important part in the production of calcium carbonate for egg-shell formation, and fowls which receive non-toxic doses of sulphanilamide lay eggs of which the shells are either very thin and pitted or completely absent owing to carbonate deficiency. This effect is purely temporary and disappears as the sulphanilamide is eliminated. The immediate application of this phenomenon to chemotherapeutic effects is not apparent, however.

Influence of Oxidation on Action of Sulphonamides.

Another theory of sulphonamide action which has been developed as a result of *in vitro* investigations is that of McLeod *et al.*(88). They consider that oxidation of sulphonamides is an essential step in the development of a bactericidal effect, although the relationship of these observations to the inhibitory effect of p-aminobenzoic acid on sulphonamide action remains obscure.

Effect on B.Coli.

Wood (118) has made a detailed study of the quantitative relationship between p-aminobenzoic acid and the bacteriostatic effect of various sulphonamide drugs, the test organism being B.coli, which is readily propagated in synthetic media free from sulphonamide inhibitors. The minimum amounts of p-aminobenzoic acid required to prevent bacteriostasis with varying amounts of aulphanilamide were found to be such that the ratio of p-aminobenzoic acid to drug was approximately constant.

If (PABA) = minimum molar concentration of p-amino-benzoic acid required to counteract the bacteriostatic action of (S) = molar concentration of sulphonamide drug, then (PABA)/(S) = K = "bacteriostatic constant".

K varied with the type of organism, the size of inoculum, and the conditions under which growth of the organisms occurred; but when these variables were standardised, K became an index of the relative bacteriostatic power of the drug tested - the greater the value of K the more potent the drug.

It has been estimated that one molecule of p-amino-benzoic acid is sufficient to antagonise 23,000 molecules of sulphanilamide (Rubbo et al.,102). But the striking difference between the effective concentrations of antagonist and drug does not necessarily invalidate the theory that the two compounds compete for the same enzyme system; the affinity of the antagonist - p-aminobenzoic acid - for the enzyme may be far greater than that of the drug, in which case a great excess of drug will be necessary

to block the enzyme in competition with a small amount of antagonist.

AUTHOR'S EXPERIMENTS.

In preliminary experiments to ascertain the bactericidal properties of sulphanilamide trials were made by the method regularly employed in this laboratory for testing the potency of antiseptics (Browning and Gulbransen, 10), but with some modifications in the media employed. Thus 1 per cent. Bacto-peptone meat-infusion broth and sheep serum (previously heated at 56°C for 30 minutes) were used; the organism was the capsulated Streptococcus Kruger of Period IV. The antibacterial effect of sulphanilamide demonstrated by this means was not in any way noteworthy, and a comparison with the action of a powerful antiseptic such as acriflavine is shown in Table XXX. In peptone broth some bacteriostatic activity was shown by sulphanilamide in a concentration of 1:1,000 against the highest dilution of organisms used - 1:100,000 - an effect which was more marked when sheep serum was the test medium. With

acriflavine also a relationship was demonstrated between the dilution of organisms and the concentration of drug required to produce sterilisation under the test conditions. In this method the organisms are exposed to the drug over a period of 48 hours during which the nutritive properties of the medium may have undergone marked changes, especially if there has been initially abundant proliferation. Accordingly it appeared important to renew the environment by daily subculturing into fresh medium containing the drug.

Both peptone broth and sheep serum were used for such a course of daily subculturing, at first without the addition of sulphanilamide. The organism was Streptococcus Kruger in the capsulated phase of Period IV. In peptone broth, 32 successive subcultures were made with no obvious alteration in the appearance or behaviour of the test organisms. Growth throughout was readily visible within the incubation period of 24 hours, while the organisms retained unaltered their virulence for mice and their capsulated

appearance. In sheep serum, however, growth of the organisms was never visible, although it could be demonstrated by plating out on agar medium; but the organisms, while still capsulated, rapidly lost their virulence for mice. It was therefore decided to restrict the experimental work to the study of antibacterial effects in peptone broth.

The basis of peptone broth is horse-heart from which a meat-infusion is prepared either by extracting 500g. of meat with 1000cc. of cold water for 24 hours or by heating with water gradually to boiling-point and allowing to boil for 1½ hours. The particular method of extraction used has not affected the present results to any extent. 1 per cent. Bacto-peptone and 0.5 per cent. sodium chloride are added to the infusion and the whole rendered distinctly alkaline to litmus by addition of 4 per cent. sodium hydroxide solution in order to precipitate phosphates. After filtration, the pH is adjusted to 7.8 and the broth autoclaved ready for use. While this medium is one of

fair nutritive value and capable of supporting good growth of most organisms, it seems to be impossible to ensure that the composition of the horse-heart infusion will always be the same, and the slight variations resulted in some difference in behaviour when streptococci were inoculated into broth plus sulphanilamide or acriflavine, although drug-free broth of the same batch provided apparently normal growth of the organisms.

With each batch of peptone broth the general scheme was to distribute the medium in 5.0cc. amounts with or without added drug, and to subculture daily or, when growth was slow, every 48 hours. The inoculum throughout was two loopfuls of a standard platinum loop of about 2.0mm. in diameter, the initial inoculum in each experiment being taken from a 24 hours' broth culture showing normal growth, capsulation, and mouse-virulence. When a tube showed no visible growth after 24 hours' incubation it was examined again after 48 hours and finally discarded as sterile if there was no growth after 72 hours. Frequent estimations

of mouse-virulence and examinations of capsulation were made in the course of the experiments.

Nine different batches of broth made from altogether four different horse-hearts were used in fourteen separate series of experiments. With seven of these specimens of broth (eleven series of experiments) the primary culture inoculated in presence of 1:1,000 sulphanilamide yielded full growth of streptococci with diminished virulence; but no further growth could be obtained on subculture into fresh tubes of the same drug-containing broth. Alternate passages through broth containing 1:1,000 sulphanilamide and drug-free broth gave continuous full growth, but while the virulence for mice was high in the alternate cultures in drug-free broth, the effect of growing the streptococci in presence of 1:1,000 sulphanilamide for 24 hours was to diminish the virulence slightly - 0.25cc. of a dilution of 1a10,000 taking 2 to 4 days to kill a mouse, whereas a dilution of 1:1,000,000 of a culture in drug-free broth was usually lethal in 36 hours. The other two specimens of

broth (used in three series of experiments) showed slight diminution of virulence in the primary subculture containing 1:1,000 sulphanilamide, slowing of growth in the second subculture necessitating further incubation, and regular growth in the following subcultures, but the virulence for mice was very greatly diminished or completely lost by the third subculture. Capsulation was unaltered.

Lower concentrations of sulphanilamide in the broth, 1:2,000 to 1:6,000, still produced effects similar to 1:1,000. On three occasions when continued passage was attempted in broth containing 1:6,000 the growth of streptococci was inhibited completely in the second culture. 1:7,500 to 1:100,000 sulphanilamide allowed good growth of streptococci on serial transfer, and the latter concentration had produced no alteration in the virulence of the cultures for mice after ten passages.

Discrepancies in results, however, were found even during the use of one batch of broth in parallel series of experiments. In one instance, 1:1,000 sulphanilamide

inhibited growth of streptococci slightly in the second subculture, but the third and following subcultures in the same medium showed good growth with complete loss of virulence. On the other hand, growth took place in the first subculture with 1:3,000 sulphanilamide in the same broth, but was completely inhibited thereafter.

Since some of these variable results (although not that last mentioned) are due presumably to slight differences in the composition of the culture medium already referred to, MacLeod's (87) method of removing sulphonamide-inhibitors from meat-infusion broth was tried. In the preparation of the medium the filtered broth was acidified to pH 5.0 to 5.5 and 2g. of powdered charcoal added for every 100cc.. The mixture was brought to the boil over an open flame and immediately filtered. The final pH was adjusted to 7.8 and the medium autoclaved. This medium certainly produced an increase in sulphanilamide bacteriostasis when treated and untreated broth prepared similarly from the same source of meat were compared. Broth which without charcoal

treatment had given regular growth of streptococci in presence of 1:1,000 sulphanilamide (with loss of virulence), showed after charcoal treatment, in one experiment, no growth in the second and subsequent subcultures with the same concentration of drug; in two other experiments complete absence of growth in the primary culture in concentrations of 1:1,000 to 1:8,000 was found.

The most interesting results were those obtained from growth of the organisms in presence of increasing amounts of the drug in untreated broth. The content of sulphanilamide was gradually increased from 1:9,000 to 1:1,000 in the course of 7 daily passages; at the end of this time the virulence of the organisms was high (0.25cc. of a dilution of 1:1,000,000 being lethal for mice) and remained practically unchanged after 7 further daily passages through 1:1,000 sulphanilamide broth. With the same batch of broth, however, after 6 successive subcultures in 1:3,000 sulphanilamide followed by 7 subcultures in 1:1,000 a distinct diminution in virulence was found, even

a dilution of 1:10,000 sometimes failing to kill in a dose of 0.25cc. There was again no visible alteration in capsule formation.

It was found in parallel experiments that those batches of broth which showed bacteriostasis in presence of 1:1,000 sulphanilamide, followed by complete absence of growth in subculture in the same mixture, also gave inhibition of growth of the streptococci in presence of 1:1,000,000 acriflavine. On the other hand, good growth in sulphanilamide broth with gradual loss of virulence was accompanied by corresponding growth, but slightly more rapid loss of virulence, in acriflavine broth. Accordingly, the effects of a sulphonamide compound on virulence are not unique, but are paralleled by acriflavine and depend to a great extent on factors, so far undefined, in the nutritive medium used.

Loss of virulence for mice following growth in presence of 1:1,000 sulphanilamide was not restored by further subculturing in absence of the drug in a highly nutritive

culture medium - meat-infusion broth containing 1 per cent. peptone and boiled after addition of 5 per cent. rabbit-blood. Also, while a subculture in plain broth in a control series still gave a Lancefield Group B reaction after 11 passages, the non-virulent organisms of the corresponding sulphanilamide broth subculture reacted to neither Lancefield Group A nor Group B, and remained the same even after 4 subcultures in drug-free broth.

A small number of experiments have been carried out with the non-capsulated organisms of Streptococcus Kruger Period V, but the virulence of the strain was readily diminished by a number of passages through drug-free broth. Loss of virulence observed when the non-capsulated organisms were grown in presence of gradually increased concentrations of sulphanilamide has therefore little significance.

Conclusions.

The results *in vitro* with the capsulated phase of Streptococcus Kruger are highly variable and while so far this difficulty has not been overcome, the following

statements may be made:-

(1) Full growth of streptococci in high concentrations of sulphanilamide (1:1,000) may occur occasionally along with undiminished virulence. This result was obtained by gradually increasing the amount of drug in the medium from an initial level of 1:9,000.

(2) Frequently full growth of the streptococci occurs in the initial culture containing a high concentration of sulphanilamide (1:1,000), but the virulence is diminished; on subculture there is no growth in the same medium. Alternate subculture in drug-free and drug-containing media may succeed, and in the latter the growth is diminished in virulence, whereas in the absence of drug it is fully virulent.

(3) The variability in results, apparently due to the medium, was obtained with acriflavine as well as with sulphanilamide.

(4) No changes have been observed in the state of capsulation of the organisms grown in drug-broth.

(5) Cultures which had been rendered non-virulent by sulphanilamide did not regain their virulence on further passages in drug-free medium.

ACQUIRED RESISTANCE TO SULPHONAMIDES.

When organisms are exposed continually to the action of a drug there are several possible results:-

(1) If the concentration of drug is sufficiently great, the organisms may be killed.

(2) If the concentration of drug is below the bactericidal level, the effect may be merely bacteriostatic, with renewed growth on transfer to drug-free medium. In this case a balance is set up between organisms and drug.

(3) Still smaller concentrations of drug, perhaps gradually increased in successive subcultures, may have the tendency to set up drug-resistance; the organisms become accustomed to the presence of the drug and are no longer susceptible to its antibacterial powers.

The third possibility is on the analogy of drug-resistance of trypanosomes and much work has been done on these lines.

OBSERVATIONS OF OTHER WORKERS.

Sulphonamide Resistance of Pneumococci.

In the case of the pneumococcus evidence is fairly conclusive that drug-resistance has been encountered in both clinical and experimental work. Hamburger et al.(41) found drug-resistance occurring in a clinical case of pneumococcal infection - ultimately fatal - where treatment with a sulphonamide drug was carried out intermittently over 6 months, and Ross (101) had previously reported a similar increase in tolerance of the drug by the organism. Maclean et al.(83) passed a pneumococcal infection three or four times through mice treated with sulphapyridine and found that the strain was altered in its sensitivity to the drug, although remaining unchanged in virulence, capsulation, and resistance to phagocytosis. MacLeod et al.(86) produced similar drug-fastness in an otherwise practically unchanged pneumococcus by serial transfer (33 times) in serum-broth containing increasing amounts of sulphapyridine; this drug-fastness was not affected by 30 further transfers in plain

broth or 10 passages through untreated mice. Horsfall (47) produced similar increased resistance of pneumococci to sulphathiazole by successive subculturing on solid medium, and found a marked decrease in virulence for mice although the organisms remained encapsulated and type-specific; the reduced virulence did not change through numerous subcultures in absence of the drug. Schmidt et al.(106) established resistance both in vivo and in vitro. They considered such resistant strains to be developed by selective propagation of drug-resistant variants, while Sesler et al.(108) compared various drugs of sulphanamide type and found that though the rate of production of resistance varied from drug to drug, resistance acquired to one sulphonamide drug conferred on the organism resistance to the others.

Sulphonamide Resistance of B.Coli.

Kirby et al.(50), with B.coli as the test organism, developed drug-resistance by daily transfer in vitro in culture media containing various sulphonamide drugs. It was confirmed that organisms resistant to one sulphonamide

drug were also resistant to other drugs of that group, but development of drug-resistance was thought to be due to alteration in metabolism rather than to selective propagation. Harris et al.(43) also investigated the development of resistance by B.coli to sulphonamide drugs and showed that the resistance of the organism depended not only on the medium in which the organism had been treated, but also on the type of medium in which resistance was tested, e.g. when resistance was measured in synthetic medium containing 1 per cent. proteose peptone, a strain of B.coli trained to grow in presence of sulphonamide in that same medium was more resistant than one trained in sulphonamide-medium without peptone. So a strain which was actually quite resistant in vivo might not show such resistance in vitro owing to the difference between the in vivo and in vitro environments.

Sulphonamide Resistance of Streptococci.

Hendry (44) described the isolation from the blood of an infected horse just before death of Group A haemolytic

streptococci which had developed a certain degree of resistance to sulphanilamide during treatment with that drug. These organisms showed reduced susceptibility to sulphanilamide as compared with the original strain when tested *in vitro*. McIntosh *et al.*(81) have claimed the ready development of drug-resistant strains of streptococci and also staphylococci by serial transfer in broth containing the drug, a careful balance being maintained between the nutritive value of the medium and its property of inhibiting the action of the drug. The drugs used included various aminoacridines, propamidine, and quindoline methochloride in addition to sulphonamide compounds, and the authors supported the view that the production of resistance was due to an alteration in metabolism. Serial transfer of the organisms in medium containing the drug was also studied by Rosenthal (99) who used 2 per cent. neopeptone broth containing 1:1,000 sulphanilamide and found that there was no inhibition of a fairly large inoculum of streptococci. Growth became more flocculent as a result of daily transfers

in this medium, but with no appreciable change in colony form or mouse-virulence. Chandler et al.(18) carried out repeated serial transfer of a strain of haemolytic streptococci in neopeptone-water plus horse serum containing 1:10,000 sulphanilamide and found after 18 transfers no permanent morphological change nor any evidence of attenuation. Hadley et al.(39), on the other hand, with two kinds of broth, both containing glucose and peptone but no blood or serum, found 1:10,000 to 1:40,000 sulphanilamide to be markedly bacteriostatic at 37°C. Serial passage in broth containing increasing concentrations of the drug gave temporary transformation to the smooth phase associated with sub-acute or chronic infections, and great diminution in virulence for mice. Further serial passage in broth containing still increasing concentrations of drug, however, restored completely the mucoid appearance of the streptococci, but it is not clear whether the virulence also was restored.

An example of the changes accompanying the development

of drug-resistance was given by Howie (48) who subcultured the capsulated Aronson strain of streptococcus every few days alternately on agar and broth media in the presence of gradually increasing amounts of acriflavine. The organism adapted itself to growth in presence of the antiseptic, tolerance to acriflavine increasing 40 times. A non-capsulated variant of the streptococcus appeared as a result of the treatment; this differed from the original organism not only in its lack of capsules but in producing granular growth in broth, as contrasted with even turbidity, longer chains of organisms, and rough colonies on agar instead of smooth, while the virulence for mice completely disappeared. In Howie's experiments the altered strain tended to revert quickly to the original form when subcultured on drug-free media, whereas drug-resistant trypanosomes usually retain their resistance after many passages through untreated animals.

In general, there has been little or no attempt to correlate sulphanilamide resistance acquired in vitro with

the virulence for mice of the streptococci concerned. It should be noted that loss of virulence is the rule when adaptation of pathogenic bacteria to drugs is effected *in vitro*; accordingly the drug-resistant organisms have frequently been deprived of their most important character and have become degraded to saprophytes from which no conclusions as to chemotherapeutic behaviour can be drawn.

Theories of Development of Drug-resistance.

Attempts have been made to correlate sensitivity to sulphanilamide with the yield of anti-sulphanilamide factor, and Green *et al.*(37) have suggested the most probable explanations of sulphanilamide resistance to be:- (a) more rapid synthesis of p-aminobenzoic acid; (b) more rapid release of p-aminobenzoic acid from the organisms into the surrounding medium; (c) (a) and (b) occurring only in presence of sulphonamide; or (d) reduction in p-amino-benzoic acid requirements. Landy *et al.*(52) considered sulphonamide resistance (studied in the case of *Staphylococcus aureus*) as due to increased synthesis and liberation of

p-aminobenzoic acid. The result of continued exposure to sulphonamide is the development of the ability to synthesize p-aminobenzoic acid in excess of normal metabolic requirements, and hence sulphonamide resistance appears. Diffusion of sulphonamide inhibitors from colonies of sulphonamide-resistant strains of organisms has been shown by Zimmerman et al.(120) to allow the growth of satellite colonies of a more susceptible strain within the zone of diffusion on the surface of agar containing sulphonamide, but the property of stimulation of satellite growth depended on the liberation of inhibitor rather than on sulphonamide-resistance in general.

AUTHOR'S EXPERIMENTS.

In the course of the in vitro experiments described above, a strain of streptococci with nine-fold increased resistance to sulphanilamide developed on only one occasion (p.114). The resistance achieved in this case consisted in the ability of the organisms to yield normal growth in presence of an amount of sulphanilamide which

had previously proved bacteriostatic. These organisms were still capsulated and retained their original virulence for mice. Attempts to reproduce this result all failed.

As far as experiments in vivo are concerned - Section III - there is no evidence of the development of any lasting drug-resistance by the organisms during continuous treatment by absorption from a depot of drug.

The acquisition of drug-resistant characteristics by the Kruger strain of streptococci during repeated passages through mice in absence of sulphonamides has already been described - pp.51,73. It has been seen that this result was associated with the development of capsules by the organisms.

SECTION V

THE MODE OF ACTION OF SULPHONAMIDE DRUGS *IN VIVO*

The natural defences of the body against infection are (a) phagocytosis and subsequent destruction of the organisms, (b) the bactericidal action of the body fluids (i.e. serum), and (c) the possible neutralisation by the serum of toxins produced by the invading organisms. It is when such normal defences are insufficient to arrest the progress of the infection that artificial methods such as drugs or antiserum-therapy must be employed. On the basis of the observations on drug action *in vivo* already described, it is of interest to examine the theories which have been advanced to explain the effects of chemotherapeutic agents of the sulphonamide group in living tissue either as a direct action on the infecting organisms, an enhancement of the defensive mechanism of the host, or a

combination of the two.

While quite clearly there is no possibility that the diphenyl-sulphones are acted upon in the body of the host with formation of sulphanilamide, as occurs in the case of Prontosil, it is convenient to study the sulphone drugs along with sulphanilamide, since there seems to be a distinct similarity in their mode of action. The question is not considered here as to whether the actual chemotherapeutic agent is the drug as such or a derivative formed within the body as suggested by Burton et al.(14) on the basis of in vitro experiments with p-hydroxylamino-benzene sulphonamide.

ANTICAPSULOGENIC THEORY.

It is obvious that a capsulated organism is likely to be more resistant to agents from without than a non-capsulated one, and Levaditi and Vaisman (59) advanced the view that the action of sulphanilamide might consist in rendering the medium within the host unsuitable for

the development of the protective capsule and hence exposing the organisms to phagocytosis, as the same authors had already suggested in the case of Prontosil (54). To prove this they studied the course of pneumococcal infections in treated mice; and they also suggested that the drug neutralised bacterial toxins. But Long and Bliss (65) and Colebrook and Kenny (21) were unable to confirm the anticapsulogenic property in the case of sulphanilamide and streptococcal infections. On the other hand, Schlossberger and Bär (104), as already noted (p.33) found that the addition of mucin to the inoculum of streptococci protected the organisms from sulphanilamide by formation, as it were, of an artificial capsule. The experimental results already given (p.52) for the action of sulphanilamide on the non-capsulated and capsulated phases of Streptococcus Kruger and on the other capsulated strains of streptococci show a definite lack of therapeutic efficacy for the drug against capsulated organisms. No evidence has been found in the present work of any loss of capsules occurring

in the course of successful treatment of a streptococcal infection (p.93), while the fact that such degenerative changes in the capsules have been observed in the course of experimental pneumococcal infections is usually interpreted as being merely the normal process by which the bodily defences attack and dispose of the invading pneumococci (Whitby, 113).

ANTI-TOXIC AND ANTI-ENDOTOXIC THEORY.

The view has been advanced that the sulphonamide drugs act by neutralising the toxins or endotoxins produced by the invading organisms - Levaditi *et al.*(55). However, Gross *et al.*(38) reported that sulphanilamide exerted no anti-endotoxic action in mice against formalin-killed meningococci or streptococci, or diacetylamino-diphenyl-sulphone against formalin-killed streptococci. Levaditi and Vaisman in various reports (56,60,57,58) claimed successful results *in vivo* with sulphonamide drugs against the endotoxins of gonococci, meningococci, and *B.dysenteriae*

(Shiga and Flexner), but failure against staphylococcal endotoxin. Bayliss (3) reported also that sulphanilamide did not inactivate toxins of staphylococci *in vitro*. Meyer (78) and Osgood *et al.*(91) considered that sulphanilamide did not neutralise toxins already present, but probably inhibited the formation of toxins by organisms. Nitti and Bovet (87) noted, however, in treating experimental streptococcal infections in rabbits with sulphanilamide that during a period of 3 to 6 days following treatment blood cultures frequently gave negative results, but the organisms then reappeared. Retreatment with sulphanilamide at this stage might cause sterilisation of the blood, but death occurred within 1 to 3 days, there being no apparent lesions and no reinfection of the blood. They suggested that death was due to the liberation of toxins owing to the action of the drug on the organisms. On the whole, the evidence for an anti-toxic action of the sulphonamide drugs seems exceedingly inconclusive.

THEORY OF INCREASED PHAGOCYTOSIS.

It has been suggested that the action of the sulphonamide drugs consists in stimulating phagocytic response, as has been observed with serum-therapy. Long and Bliss (66), Gay and Clark (33), Reid (96), Levaditi *et al.*(59) and others have all studied this problem both by *in vivo* and *in vitro* methods. The general conclusion is not that phagocytosis is stimulated, but rather that the organisms are in some way affected by the drug so as to render them more susceptible to phagocytosis.

THEORY OF CHANGE IN INVASIVE PROPERTIES.

It is possible that interference with the metabolic processes of the organisms modifies their invasive character (Whitby, 115; McIntosh *et al.*,82); but this behaviour is not confined to the sulphonamide drugs. Browning and Gulbransen as early as 1919 (9) published results of treatment of experimental pneumococcal infections in mice by various antiseptics. An

intraperitoneal inoculum was followed within a few minutes by an injection of the antiseptic solution given by the same route, and cures resulted with proflavine, phenol, and corrosive sublimate. It was apparent that the antiseptic cooperated in some way with the defensive mechanisms of the tissues to produce cure, and it was also shown that addition of proflavine to the organisms immediately before inoculation prevented the death of the animal, but did not necessarily sterilise the infection, as a smear from the surface of the liver one month later contained abundant pneumococci. This suggested a modification of the virulence of the organisms by the antiseptic to such a degree as to produce a mild chronic, rather than an acute, infection. Hilles et al.(45) have reported also the isolation of non-virulent pneumococci from the blood of mice treated with sulphapyridine. In 1931 Browning and Gulbransen (8) studied the therapy of experimental streptococcal

infections in mice by means of compounds of the anil-quinoline type and of acriflavine. Injection of solutions of these drugs intraperitoneally one to two hours after inoculation by the same route again led to a large number of cures; the results were as follows:-

Drug.	Cures/Number treated.
2(p-dimethylamino anil)6 n-caproylamino quinoline methochloride.	
¾ to ¼ of tolerated dose used - - - - - -	53/82
2(p-dimethylamino anil)6 methylallyl-acetylamino quinoline methochloride.	
¾ to 1/6 of tolerated dose used - - - - -	34/47
Acriflavine.	
¾ to 1/5 of tolerated dose used - - - - -	13/21

All untreated controls were dead in 24 to 48 hours. A protracted course of infection was observed in some insufficiently treated mice, which appeared well for some weeks after inoculation and then died of streptococcal septicaemia.

The experimental findings described in Section III may be similarly analysed as regards the behaviour of drug and organisms within the living host. It has been seen that following treatment of an infected animal streptococci can continue to multiply within the tissues without any apparent harmful results. In fact, the organisms have temporarily lost their virulent properties towards the individual host, although transfer of such organisms to a fresh host may result in the usual fatal septicaemia. A similar transition from a chronic non-virulent phase to an acute and virulent form may also occur within the original host, especially when treatment has been with a slowly absorbed compound. In this case the drug has been substantially eradicated from the tissues with consequent diminished influence over the organisms, which are now free to grow under normal body conditions with return of their pathogenic characteristics. This explanation, however, does not cover the fact that late deaths have been reported following treatment with sulphanilamide; and these cannot

be attributed in any way to the continued effect of the drug within the tissues, as sulphanilamide is rapidly excreted. It has been suggested by Mellon et al.(77) that the invading organisms, being reduced in virulence by the action of the drug, are ingested by the phagocytes and there lie dormant; then on liberation by lysis of the phagocytes they renew their pathogenic action in absence of the drug and cause the death of the host.

THEORY OF PRODUCTION OF IMMUNITY RESPONSE.

There is also the possibility that the initial effect of the drug in killing some of the organisms results in a stimulation of antibody production by the host similar to that induced by inoculation of dead or attenuated cultures, and that such enhancement of the defensive mechanism is then sufficient to dispose of the remaining organisms. This immunity response, of course, would vary with the particular organism concerned.

In the case of the pneumococcus, immunity to

reinfection is readily established in mice as a result of successful treatment of a primary infection. Whitby (114), McIntosh et al.(82), and Schmidt et al.(105) found that mice cured with sulphapyridine were nearly all immune to reinfection with the homologous strain of pneumococci, although according to the last authors the immunity gradually disappeared; thus 86 per cent. of the mice were immune to reinfection 7 to 14 days after the original inoculum and therapy, but only 6 per cent. were still immune 28 days after. Feinstone et al.(26) reinoculated cured mice 30 days after the original inoculum of pneumococci and treatment with 4:4'-diamino-diphenyl-sulphone and reported no immunity. The fact that immunity to pneumococcal infections can be readily established by vaccination with attenuated cultures also strengthens the theory that the therapeutic effect is due at least in part to an immunity response.

With experimental streptococcal infections on the other hand, it is frequently considered that little or no

immunity to reinfection results in mice which have been cured with sulphonamide drugs - Nitti et al(89), Levaditi et al.(53). Loewenthal (63) and Colebrook et al.(22) have both attempted to combine drug-therapy and treatment with the corresponding antiserum. Loewenthal concluded that the majority of failures in sulphanilamide therapy were due to a failure of immunity response. With a preparatory dose of antiserum 16 to 18 hours before inoculation of streptococci and therapy with sulphanilamide, 75 per cent. of mice were cured as compared with none out of 60 receiving antiserum alone and 8 out of 60 receiving drug alone. Colebrook obtained less striking results by a similar method and did not consider that any advantage was gained in treatment of experimental streptococcal infections by combining serum and drug administration. Immunisation of mice by means of streptococcal vaccines has also been rather uncertain. Long and Bliss (65) have reported repeated failures, but in the present author's experience injection of fluid cultures which had died

spontaneously through keeping in the frozen state for 9½ months was found to protect half the mice so treated against an inoculum of the homologous capsulated strain which killed all untreated controls (see p.38). Jui-Ping-Wu (49) found that a fairly heavy dosage of killed vaccine, 1.75 to 8.25cc,, over 2 to 4 weeks, generally produced immunity in mice to highly virulent strains of streptococci. It must be borne in mind that a very minute dose of living virulent pneumococci or streptococci constitutes the inoculum and that administration of the drug, begun at the time of inoculation, prevents any considerable multiplication of the organisms in a large proportion of the mice which ultimately survive. Therefore in these cases the very small amount of antigenic material available in the body may be insufficient to effect immunisation. But when control of the infection is not immediate and therefore multiplication of the invading organisms is not rapidly checked, it is quite possible that the immunity response plays a more important part in conducing to cure.

The occurrence of late deaths, especially those following sulphanilamide therapy, is readily explained by this theory. Thus recovery does not demand a continued action on the part of the drug, e.g. by absorption from a depot at the site of injection, while relapse results from failure to maintain a balance between the organisms and the host's immunity responses.

CONCLUSIONS.

Various theories have been advanced to account for the anti-infective action of the sulphonamides. Certain of these, namely the view that capsule formation is interfered with, that the drug neutralises exotoxins and endotoxins, or that it stimulates phagocytosis, obtain little support from experimental findings - the first probably substitutes an effect for a cause, while the second and third are fairly definitely disproved.

Much stress has been laid on the antiseptic or bacteriostatic properties of the drugs, but observations

in vitro show that they possess such action only to a relatively slight extent, whereas compounds which are extremely powerful in this respect *in vitro*, e.g. acriflavine, fail to act *in vivo* when the attempt is made to influence a general bacterial infection. Since a general trypanosome infection is influenced by acriflavine, it can scarcely be argued that the drug is not available. Therefore in the case of bacteria the results obtained *in vitro* do not appear to be applicable to occurrences *in vivo*. The present observations on the behaviour of streptococci in the circulation of infected and treated animals has afforded definite new information. It has been found that while in the majority of those which become cured the organisms rapidly disappear, nevertheless in a considerable proportion the infection persists for a period extending sometimes to many days. During their persistence in the blood the streptococci may be highly virulent for other animals, at least for a time. On the other hand, no prolonged prophylactic effect follows

the administration of a rapidly excreted drug such as sulphanilamide, so that the defence mechanisms of the body show no evidence of stimulation. Therefore it would seem that the metabolic processes of the organisms must be controlled under the influence of the drug so as to enable the natural defensive mechanisms of the host to come into play effectively. It is emphasized that while these observations were made with a drug which is slowly absorbed and excreted, the occurrence of late relapses in mice treated with the rapidly excreted sulphanilamide point conclusively in the same direction.

Observations *in vitro* show that the sulphonamides affect the metabolism of the organisms, although in an erratic manner, which is difficult to reproduce constantly. This, coupled with the interfering effect of p-aminobenzoic acid on the action of sulphonamides both *in vivo* and *in vitro*, strongly supports the view that the results of sulphonamide therapy are initially due to some direct effect on the organisms, and subsequently to the action exerted on the latter by the host's tissues.

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TABLES

TABLE I

Toxicity of Sulphanilamide for Mice.

Summary of Published Results.

Dosage per os.

Dose per 20g. mouse	Nature of preparation	Result.	Author.
18mg.	in acacia.	well tolerated.	Marshall (73)
50mg.	" "	innocuous.	Buttle (16)
"	aqueous.	tolerated.	Fourneau (30)
"	"	toxic.	Whitby (113)
70-80mg.	in acacia.	acutely toxic.	Feinstone (26)
76mg.	" "	50% survivors.	Marshall (73)
100mg.	" "	toxic.	Buttle (16)
120mg.	aqueous.	50% survivors.	Halpern (40)
125mg.	in acacia.	minimum lethal dose.	Barlow (1)
200mg.	" "	lethal.	Buttle (16)
>200mg.	" "	100% lethal.	Marshall (73)

Dosage subcutaneous.

Dose per 20g. mouse	Nature of preparation	Result.	Author.
10mg.	aqueous.	distinctly toxic.	Ruegsegger (103)
18mg.	"	toxic, not lethal.	Long (65)
>20mg.	"	lethal.	Ruegsegger (103)
30mg.	saline.	toxic, not lethal.	Hoare (46)
40mg.	aqueous.	tolerated.	Raiziss (93)
50mg.	"	lethal.	" (93)
55mg.	"	minimum lethal dose.	Barlow (1)
60mg.	" (Na salt)	56% survivors.	Donovick (25) Long (69)
75mg.	in acacia.	minimum lethal dose.	Barlow (1)
120mg.	in olive oil.	lethal.	Rosenthal (98)

TABLE II

Toxicity of Sulphanilamide for Mice.

Summary of Author's Results.

Dosage subcutaneous.

Dose per 20g. mouse.	Nature of preparation.	No. of mice.	Effect.
50mg.	in acacia.	2	tolerated, practically no loss of weight.
67mg.	" "	2	tolerated, no loss of weight.
100mg.	" "	2	" , practically no loss of weight.
133mg.	" "	2	lethal, (both mice died within 24 hours.)
167mg.	" "	1	tolerated without loss of weight.
200mg.	" "	1	tolerated without loss of weight.

TABLE III

Toxicity of Diamino-diphenyl-sulphone for Mice.

Summary of Author's Results.

Dosage subsutaneous.

	Dosage in mg. per 20g. mouse.	Total	Nature of preparation.	No. of mice.	Effect.
*	3, 2.5, 2.5 (0, 5, 24 hours)	8	aqueous.	2	Readily tolerated, practically no loss of weight.
*	5, 3. (0, 5 hours)	8	"	4	2 dead within 24 hours.
*	5, 3, 3 (0, 5, 24 hours)	11	"	2	Tolerated with temporary loss of weight.
	10	10	"	6	4 died in 1-3 days, others recovered, all showed toxic symptoms.
	12.5	12.5	"	6	1 died in 24 hours, 5 others recovered completely.
	14	14	"	3	All recovered completely.
	17	17	"	3	All dead in 24 hours.
	20	20	"	3	All dead in 24 hours.

* Results marked are from earlier laboratory records-see p.8.

TABLE IV

Toxicity of Diacetylamino-diphenyl-sulphone for Mice.

Summary of Author's Results.

Dosage subcutaneous.

Dose per 20g. mouse.	Nature of preparation	No. of mice.	Effect.
5mg.	aqueous.	1	tolerated without loss of weight.
10mg.	"	4	tolerated without loss of weight.
20mg.	"	3	tolerated without loss of weight.
50mg.	"	1	very well tolerated.
100mg.	"	4	tolerated without loss of weight.
200mg.	"	1	very well tolerated.
330mg.	"	1	very well tolerated.

TABLE V

Standard Dosage of Sulphone Drugs for Mice.

Aqueous dispersion injected subcutaneously.

Drug.	Dosage in mg. at 0,5,and 24 hours.	Total.	Duration of depot.
Diamino-diphenyl-sulphone.	3.3,2.5,2.5	8.3	4 days.
Monoacetyl derivative.	6.7,4.0,4.0	14.7	8-11 weeks.
Diacetyl derivative.	10,6.7,6.7	23.4	- ※

※ Single dose of 50mg. - deposit still present 3½ months later.

TABLE VI

Virulence of Streptococcus Kruger as Assessed by Two Methods of Dilution.

Initial dilution: 0.02cc. of pure culture in 10cc. of broth (=1:500).
Ten-fold dilutions made in broth thereafter.

Results from 4 experiments.

No. of experiment.	Dilution.	Method 1. (Single pipette throughout.)				Method 2. (Separate pipette for each dilution.)			
		Total.	Deaths up to 24 hours.	Deaths at 24–48 hours.	Deaths after 48 hours.	Total.	Deaths up to 24 hours.	Deaths at 24–48 hours.	Deaths after 48 hours.
1.	1:500,000	–	–	–	–	3	3	–	–
	1:5,000,000	3	3	–	–	3	3	–	–
	1:50,000,000	3	2	1	–	3	3	–	–
	1:500,000,000	3	2	1	–	3	0	2	1
2.	1:500,000	2	2	–	–	2	1	1	–
	1:5,000,000	2	0	2	–	2	1	1	–
	1:50,000,000	2	1	1	–	2	0	2	–
	1:500,000,000	2	0	2	–	–	–	–	–
3.	1:500,000	2	1	1	–	2	1	0	1
	1:5,000,000	2	2	–	–	–	–	–	–
	1:50,000,000	2	1	1	–	–	–	–	–
	1:500,000,000	2	0	1	1	–	–	–	–
4.	1:500,000	2	2	–	–	2	2	–	–
	1:5,000,000	2	1	1	–	–	–	–	–
	1:50,000,000	2	0	2	–	–	–	–	–
	1:500,000,000	2	0	2	–	–	–	–	–
Total.	1:500,000	6	5	1	–	9	7	1	1
	1:5,000,000	9	6	3	–	5	4	1	–
	1:50,000,000	9	4	5	–	5	3	2	–
	1:500,000 000	9	2	6	1	3	0	2	1
		33	17	15	1	22	14	6	2

TABLE VII

Alteration in Character of Streptococcus Kruger.

Period.	Duration of period.	Source.	Capsulation.	Virulence.
I	Jan.1931 - Jan.1939 (Lancefield Group B)	Spleen. (From Dr. Feldt.)	Non-capsulated.	1:100,000 112/154 (73%) dead in 24 hours.
II	Jan.1939 - Sept.1939.	Heart-blood of mouse.	Capsulated.	1:10,000,000 34/63 (54%) dead in 24 hours.
III	Oct.1939 - Feb.1942	Heart-blood of mouse.	Capsulated.	1:10,000,000 65/110 (59%) dead in 24 hours.
IV	Feb.1942 - July 1943. (Lancefield Group B)	Spleen. (Stored since Sept.1939)	Capsulated.	1:1,000,000 26/44 (59%) dead in 24 hours.
V	July 1943 - - - -	Boiled-blood-broth culture frozen since Feb.1938.	Non-capsulated.	1:1,000 24/36 (67%) dead in 24 hours.

TABLE VIII

Treatment with Sulphanilamide - Varying Inocula.

† Streptococcus Kruger Period I.

Inoculum intraperitoneal: untreated controls with the same inoculum all died in 24 hours.

Treatment subcutaneous: 10, 6.7, and 6.7mg. (23.4mg. in all) at 0, 5, and 24 hours respectively.

Inoculum.	Total No. of mice.	Cures. No.	Cures. %	Survival time of mice dying from infection.
1:10	5	3	-	3 days.
1:100	24	16	67	3-5 "
1:1,000	38	18	47	3-5 "
1:5,000	3	1	-	4 "
1:10,000	25	23	92	6 "
1:25,000	2	0	-	2-6 "
1:50,000	2	1	-	8 "
1:100,000	13	8	62	3-6 "
	112	70	63	

† All results are from earlier laboratory records - see p.8.

TABLE IX

Treatment with Sulphanilamide - Varying Dosage.

†Streptococcus Kruger Period I.

Inoculum intraperitoneal: untreated controls with the same inoculum (1:10 to 1:100,000) all died in 24 hours.

Treatment subcutaneous.

Dosage in mg.	Total	Timing of dosage in hours.	Total No. of mice.	Cures. No.	Cures. %	Survival time of mice dying from infection.
1,0.67,0.67	2.34	0,5,24	2	1	-	2 days.
1.67,1.1,1.1,	3.87	0,5,24	2 (10)	0 (1)	-	2 "
1.67,1.25,1.25	4.17	0,5,24	4	0	-	3 "
3.3,2.2,2.2	7.7	0,5,24	2	0	-	2-4 "
10	10	0	13	5	38	2-3 "
2.5,2.5,2.5, 2.5	10	0,5,24, 29	5 (145)	5 (89)	100 (61)	-
10,6.7	16.7	0,5	9	4	45	3-9 "
5,5,5,5.	20	0,5,24 29	6	5	83	4 "
10,6.7,6.7	23.4	0,5,24	112	70	63	3-4 "

Results grouped independently of the size of the inoculum.

† All results are from earlier laboratory records - see p.8.

TABLE X

(A) Delayed Treatment with Sulphanilamide.

† Streptococcus Kruger Period I.

Inoculum intraperitoneal (1:10 to 1:100,000): untreated controls with the same inoculum all died in 24 hours.

Treatment subcutaneous in 3 doses of 10,6.7,6.7mg.(23.4mg. in all).

Timing of dosage in hours.	Total No. of mice.	Cures.	Survival time of mice dying from infection.
6,11,24	7	4	4–5 days.
2,7,24	7	7	-
0,5,24	112	70	3–4 "

(B) Prophylactic Treatment with Sulphanilamide.

† Streptococcus Kruger Period I.

Inoculum intraperitoneal (1:100 to 1:10,000): untreated controls with the same inoculum died in 24 hours.

Treatment subcutaneous.

Dosage in mg.	Total.	Timing of dosage in hours before inoculum.	Total No. of mice.	Cures.	Survival time of mice dying from infection.
10,6,7	16.7	24,19	3	0	2–3 days.
10,6.7,6.7	23.4	24,5,0	6	1	3–4 "
10.10.10.	30	50,26,2	3	0	2 "
10,10,10	30	53½,29½,5½	3	0	2 "
10,10,10	30	72,48,24	3	0	2 "
10,10,10,10,10	50	72,48,24,3,0	4	3	2 "

† All results are from earlier laboratory records - see p.8.

TABLE XI

Variation in Curability of Streptococcus Kruger at Different Periods - Treatment by Sulphanilamide.

Inoculum intraperitoneal (1:10 to 1:100,000): untreated controls with the same inoculum all died in 24 hours.

Treatment subcutaneous: 10,6.7,6.7mg.(23.4mg. in all) at 0,5,and 24 hours respectively.

Period.	Condition of strain.	Total No. of mice.	Cures. No.	Cures. %	Survival time of mice dying from infection.
I	Non-capsulated.	112	70	63	3-4 days.
II	Capsulated.	12	1	.	2-3 "
III	Capsulated.	12	0	6.3	2-4 "
IV	Capsulated.	8	1	.	2-3 "
V	Non-capsulated.	47	31	66	2-7 " ※

※ 2 mice also died at 44 and 62 days respectively.

Results grouped independently of the inoculum.

TABLE XII

Treatment with Diamino-diphenyl-sulphone.

(A) †Streptococcus Kruger Period I.

Inoculum intraperitoneal (1:10 to 1:100,000): untreated controls with the same inoculum all died in 24 hours.

Treatment subcutaneous.

Inoculum.	Dosage in mg.	Total.	Timing of dosage in hours.	Total No. of mice.	Cures.	Time of survival of mice dying from infection.
1:10	3.3,2.5,2.5	8.3	0,5,24	2	0	5 days.
1:100	1.67,1.25,1.25	4.17	0,5,24	2	1	5 "
1:100	3.3,2.5,2.5	8.3	0,5,24	2	0	3 "
1:1,000	1.67,1.25,1.25	4.17	0,5,24	2	1	5
1:1,000	3.3,2.5,2,5	8.3	0,5,24	4	4	-
1:10,000	2.5	2.5	0	1 } 12	1 } 11	-
1:100,000	1.0,0.5,0.5	2.0	0,5,24	2	2	-
1:100,000	2.0,1.0,1.0	4.0	0,5,24	2	2	-
1:100,000	2.5,2.0,2.0	6.5	0,5,24	1	1	-

†These results are from earlier laboratory records - see p.8.

(B) Streptococcus Kruger Period III.

Inoculum intraperitoneal (1:100,000): untreated controls with the same inoculum all died in 24 hours.

Treatment subcutaneous.

Dosage in mg.	Total.	Timing of dosage in hours.	Total No. of mice.	Cures.	Survival time of mice dying from infection.
0.5,0.41,0.41	1.32	0,5,24	3	0	2-3 days.
1.0,0.5,0.5	2.0	0,5,24	3	0	3 "
2.08	2.08	0	4	0	3-5 "
0.83,0.625,0.625	2.08	0,5,24	4	0	1-3 "
4.16	4.16	0	4	0	3-4 "
1.67,1.25,1.25	4.17	0,5,24	4	0	3-4 "
8.3	8.3	0	4	1	4-6 "
3.3,2.5,2.5	8.3	0,5,24	3	0	4-5 "

TABLE XIII

Delayed and Prophylactic Treatment with Diamino-diphenyl-sulphone.

(A) Delayed treatment.

†Streptococcus Kruger Period I.

Inoculum intraperitoneal (1:1,000 to 1:10,000): untreated controls with the same inoculum all died in 24 hours.

Treatment subcutaneous.

Dosage in mg.	Total	Timing of dosage in hours.	Total No. of mice.	Cures	Survival time of mice dying from infection.
2.5	2.5	2	1	0	6 days.
1.25,0.83,0.83	2.91	6,11,24	4	4	-
2.5,1.67,1.67	5.84	6,11,24	4	3	6 days.
2.5,2.0,2.0	6.5	2,5,24	2	1	28 "
2.5,2.0,2.0	6.5	6,11,24	3	1	2-26 "

(B) Prophylactic treatment.

†Streptococcus Kruger Period I.

Inoculum intraperitoneal (1:2,000 to 1:10,000): untreated controls with the same inoculum all died in 24 hours.

Treatment subcutaneous.

Dosage in mg.	Total	Timing of dosage in hours.	Total No. of mice.	Cures	Survival time of mice dying from infection.
2.5,2.5,2.5,2.5, 2.5	12.5	72,48,24, 3,0	4	3	10 days.

†All results are from earlier laboratory records - see p.8.

TABLE XIV

Treatment with Diacetylamino-diphenyl-sulphone.

(A)† Streptococcus Kruger Period I.

Inoculum intraperitoneal (1:1,000 to 1:300,000): untreated controls with the same inoculum all died in 24 hours.

Treatment subcutaneous at 0, 5, and 24 hours.

Dosage in mg.	Total.	Total No. of mice.	Cures.	Survival time of mice dying from infection.
0.5, 0.33, 0.33	1.16	3	0	2–4 days.
0.67, 0.4, 0.4	1.47	3	0	3–4 "
1.0, 0.67, 0.67	2.34	11	1	1–8 "
1.25, 0.83, 0.83	2.91	6	3	3–7 "
1.67, 1.1, 1.1	3.87	8	1	1–13 "
2.5, 1.67, 1.67	5.84	15	6	2–8 "
3.3, 2.2, 2.2	7.7	11	4	2–9 "
5.0, 3.3, 3.3	11.6	6	3	3–8 "
10, 6.7, 6.7	23.4	46	33	2–16 "

†These results are from earlier laboratory records - see p.8.

(B) Streptococcus Kruger Period II;

Inoculum intraperitoneal (1:1,000 to 1:300,000): untreated controls with the same inoculum all died in 24 hours.

Treatment subcutaneous at 0, 5, and 24 hours.

Dosage in mg.	Total.	Total No. of mice.	Cures.	Survival time of mice dying from infection.
0.41, 0.33, 0.33	1.07	4	0	2–3 days.
0.625, 0.5, 0.5	1.625	4	0	2–3 "
1.25, 0.83, 0.83	2.91	1	0	2–4 "
1.67, 1.1, 1.1	3.87	3	1	2–3 "
2.5, 1.67, 1.67	5.84	1	1	–
3.3, 2.2, 2.2	7.7	3	1	3–4 "
5.0, 3.3, 3.3	11.6	1	1	–
10, 6.7, 6.7	23.4	9	4	9–19 "

TABLE XV

Prophylactic Treatment with Diacetylamino-diphenyl-sulphone.

†Streptococcus Kruger Period I.

Inoculum intraperitoneal (1:2,000 to 1:100,000): untreated controls with the same inoculum all died in 24 hours.

Treatment subcutaneous: a single dose of 14.3mg. per 20g.mouse.

Period elapsing between treatment and inoculation.	Total No. of mice.	Cures.	Survival time of mice dying from infection.
5½ hours.	3	2	5 days.
4½ "	4	1	3-5 "
3½ "	3	0	3-5 "
2 "	3	0	2-5 "
1¼ "	4	4	-
1 hour.	3	2	4 "
0 hours.	6	4	4-5 "

†All results are from earlier laboratory records - see p.8.

TABLE XVI

Treatment with Monoacetyl-diamino-diphenyl-sulphone.

†Streptococcus Kruger Period I.

Inoculum intraperitoneal (1:10,000 to 1:300,000): untreated controls with the same inoculum all died in 24 hours.

Treatment subcutaneous at 0, 5and 24 hours.

Dosage in mg.	Total.	Total No. of mice.	Cures.	Survival time of mice dying from infection.
0.5,0.33,0.33	1.16	3	0	3-4 days.
0.67,0.4,0.4	1.47	5	2	3-5 "
1.0,0.67,0.67	2.34	3	0	4-6 "
1.1,0.67,0,67	2.44	2	2	-
1.25,0.83,0.83	2.91	6	4	5 "
1.67,1.0,1.0	3.67	3	0	4 "
2.2,1.3,1.3	4.8	2	2	-
2.5,1.67,1.67	5.84	3	1	5-6 "
5.0,3.3,3.3	11.6	6	5	8 "
6.7,4.0,4.0	14.7	9	9	-

†These results are from earlier laboratory records - see p.8.

TABLE XVII

Treatment with Monoacetyl-diamino-diphenyl-sulphone.

Streptococcus Kruger Period II.

Inoculum intraperitoneal (1:100,000): untreated controls with the same inoculum all died in 24 hours.

Treatment subcutaneous at 0, 5, and 24 hours.

Dosage in mg.	Total.	Total No. of mice.	Cures.	Survival time of mice dying from infection.
0.42,0.33,0.33	1.08	4	2	2-3 days.
0.67,0.4,0.4	1.47	3	0	3 "
0.625,0.5,0.5	1.625	4	2	3-4 "
1.1,0.67,0.67	2.44	9	3	4-10 "
1.25,1.0,1.0	3.25	4	3	5 "
1.67,1.0,1.0	3.67	4	3	3 "
2.2,1.3,1.3	4.8	9	5	4-7 "
6.7,4.0,4.0	14.7	29	26	3-11 "

TABLE XVIII

Treatment with Monoacetyl-diamino-diphenyl-sulphone.

Streptococcus Kruger Period III.

Inoculum intraperitoneal (1:100,000 to 1:3,000,000): untreated controls with the same inoculum all died in 24 hours.

Treatment subcutaneous at 0, 5, and 24 hours.

Dosage in mg.	Total.	Total No. of mice.	Cures.	Survival time of mice dying from infection.
0.83,0.5,0.5	1.83	5	0	2-4 days.
0.83,0.63,0.63	2.09	4	0	4-5 "
1.0,1.0,1.0	3.0	5	0	4 "
1.67,1.25,1.25	4.17	4	0	5-8 "
2.0,1.4,1.4	4.8	6	1	3-12 "⊗
2.2,1.33,1.33	4.86	12	0	4-9 "
1.67,1.67,1.67	5.01	10	0	3-10 "
2.0,2.0,2.0	6.0	12	6	5-6 "
2.2,2.2,2.2	6.6	29	9	4-10 "
3.3,2.0,2.0	7.3	26	2	5-22 "
2.5,2.5,2.5	7.5	8	1	3-15 "
3.3,2.2,2.2	7.7	15	1	2-11 "
3.3,2.5,2.5	8.3	4	1	5-10 "
2.9,2.9,2.9	8.7	8	3	6-13 "
3.3,3.3,3.3	9.9	10	4	5-14 "
5.0,2.9,2.9	10.8	13	2	5-15 "
4.7,3.2,3.2	11.1	9	2	5-10 "
5.0,3.3,3.3	11.6	18	9	5-16 "
7.1,2.5,2.5	12.1	10	2	5-19 "
5.7,3.3,3.3	12.3	6	0	10-18 "
6.7,4.0,4.0	14.7	121	41	4-18 "※

⊗Also one late death at 48 days.

※Also late deaths at 21, 23, 26, 26, 35, 89 days.

TABLE XIX

Treatment with Monoacetyl-diamino-diphenyl-sulphone.

Streptococcus Kruger Period IV.

Inoculum intraperitoneal (1:100,000): untreated controls with the same inoculum all died in 24 hours.

Treatment subcutaneous at 0, 5, and 24 hours.

Dosage in mg.	Total.	Total No. of mice.	Cures.	Survival time of mice dying from infection.
1.67, 1.67, 1.67	5.01	3	1	5-6 days.
2.2, 2.2, 2.2	6.6	9	4	1-6 "
2.5, 2.5, 2.5	7.5	5	1	5-14 "
6.7, 4.0, 4.0	14.7	37	18	1-16 "※
5.0, 5.0, 5.0	15.0	20	9	1-12 "⊗

※Also one late death at 29 days.

⊗Also one late death at 21 days.

TABLE XX

Treatment with a Single Dose of Monoacetyl-diamino-diphenyl-sulphone.

(A) Streptococcus Kruger Period II.

Inoculum intraperitoneal (1:50,000 to 1:3,000,000): untreated controls with the same inoculum all died in 24 hours.

Treatment subcutaneous at time of inoculation.

Dose in mg.	Total No. of mice.	Cures.	Survival time of mice dying from infection.
2.5	4	1	3-4 days.
5.0	4	2	7-17 "
6.7	19	16	8-13 "
10.0	4	2	5-11 "
14.3	6	6	-

(B) Streptococcus Kruger Period III.

Inoculum intraperitoneal (1:50,000 to 1:3,000,000): untreated controls with the same inoculum all died in 24 hours.

Treatment subcutaneous at time of inoculation.

Dose in mg.	Total No. of mice.	Cures.	Survival time of mice dying from infection.
1.67	4	0	2-4 days.
1.8	5	0	2-3 "
2.0	4	0	3-4 "
2.2	4	0	3-5 "
3.3	4	0	4-7 "
3.6	5	0	3-4 "
4.15	4	0	2-7 "
6.7	4	0	3-10 "
7.1	5	0	3-5 "
8.3	4	0	2-12 "
11.0	6	1	5-8 "
12.5	21	11	8-15 "
14.3	35	7	3-15 "

TABLE XXI

Delayed and Prophylactic Treatment with a Single Dose of Monoacetyl-diamino-diphenyl-sulphone.

Streptococcus Kruger Period II.

Inoculum intraperitoneal (1:50,000 to 1:1,000,000): untreated controls with the same inoculum all died in 24 hours.

Treatment subcutaneous.

(A) Delayed treatment with a single dose of 6.7mg.

No. of experiment.	Timing of treatment.	Total No. of mice.	Cures.	Survival time of mice dying from infection.
1	0 hours.	16	14	8-13 days.
	1½ "	16	12	9-12 "
	6 "	16	2	5-20 "
2	2 "	12	11	12 "
	6 "	10	4	8-17 "
3	3 "	15	2	3-11 "
4	2 "	12	11	8 "
	6 "	12	6	7-14 "
Totals.	0 hours.	16	14	8-13 "
	1½ "	16	12	9-12 "
	2 "	24	22	8-12 "
	3 "	15	2	3-11 "
	6 "	38	12	5-20 "

(B) Prophylactic treatment with a single dose.

No. of experiment.	Timing of treatment before inoculation	Total No. of mice.	Cures.	Survival time of mice dying from infection.
1	14.3mg. at 5½ hrs.	3	2	1 day.
	" 3½ "	3	3	-
	" 2 "	3	3	-
	" 1 "	3	2	1 day.
	" 0 "	3	3	-
2	14.3mg. at 4½ hrs.	4	4	-
	" 1¼ "	4	4	-
	" 0 "	3	3	-
3	6.7mg. at 10 days.	4	0	2-3 days.
	" 5 "	4	0	3-5 "
	" 2 "	4	1	5-6 "
	" 0 hrs.	4	3	7 "

TABLE XXII

Treatment with Monoacetyl-diamino-diphenyl-sulphone.

Prophylaxis of relapses - the effect of an additional dose after 3, 4 or 5 days.

Streptococcus Kruger Period III.

Inoculum intraperitoneal (1:100,000): untreated controls with the same inoculum all died in 24 hours.

Treatment subcutaneous at 0, 5, and 24 hours, and then at 3, 4, or 5 days.

Primary dosage in mg.	Total	Additional dose.	Total No. of mice.	Cures.	Time of survival of mice dying from infection.
2.2,2.2,2.2	6.6	0	33	13	4–10 days.
		2.2mg. at 3 days.	6	1	2–8 " †
		2.5mg. " 3 "	12	7	6–11 "
		2.5mg. " 4 "	12	5	11–20 "
		2.5mg. " 5 "	12	5	6–14 " ⊗
		5.0mg. " 5 "	6	5	11 "
		6.7mg. " 5 "	6	6	–
2.9,2.9,2.9	8.7	0	6	2	6–13 "
		5.0mg. at 5 days.	6	5	11 "
3.3,3.3,3.3	9.9	0	6	4	5–14 "
		6.7mg. at 5 days.	4	3	5 "
5.0,3.3,3.3	11.6	0	6	5	10 "
		5.0mg. at 3 days.	9	9	–
		5.0mg. " 5 "	6	1	6–18 " ⁂
6.7,4.0,4.0	14.7	0	6	4	11–12 "
		6.7mg. at 5 days.	3	3	–

† Inoculum - Streptococcus Kruger Period IV.

⊗ Also one late death at 38 days.

⁂ Also one late death at 40 days.

TABLE XXIII

Treatment with Monoacetyl-diamino-diphenyl-sulphone.

(A) † Streptococcus Aronson.

Inoculum intraperitoneal (1:100,000 to 1:1,000,000): untreated controls with the same inoculum all died in 24 hours.

Treatment subcutaneous.

Dosage in mg.	Total.	Timing of dosage in hours.	Total No. of mice.	Cures.	Survival time of mice dying from infection.
1.17,1.0,1.0	3.17	0,5,24	6	0	4–6 days.
7.15	7.15	0	3	0	5–8 "
3.3,2.0,2.0	7.3	0,5,24	5	0	6–15 "
14.3	14.3	0	2	0	10–13 "
6.7,4.0,4.0	14.7	0,5,24	8	3	6–18 "

† These results are from earlier laboratory records - see p.8.

† These

(B) Streptococcus Thomson.

Inoculum intraperitoneal (1:100, to 1:1,000): untreated controls with the same inoculum all died in 24 hours.

Treatment subcutaneous.

Dosage in mg.	Total.	Timing of dosage in hours.	Total No. of mice.	Cures.	Survival time of mice dying from infection.
6.7,4.0,4.0	14.7	0,5,24	77	26	2–20 days.

TABLE XXIV

Treatment with Monoacetyl-diamino-diphenyl-sulphone.

The effect of p-aminobenzoic acid.

Streptococcus Kruger Period III.

Inoculum intraperitoneal (1:100,000): untreated controls with the same inoculum all died in 24 hours.

Treatment subcutaneous at 0, 5, and 24 hours.

p-Aminobenzoic acid:- 1:50 suspension, 1cc. per 20g. body-weight 2 or 3 times daily.

Combined results of 4 experiments.

Dosage in mg.	Total.	Duration of feeding with relation to inoculation.	Total No. of mice.	Cures.	Time of survival of mice dying from infection.
6.7,4.0,4.0	14.7	From 1 day before.	10	0	1-4 days.
		" 2 days after.	12	2	3-6 "
		" 3 " "	6 (33)	0 (2)	4-7 "
		" 5 " "	5	0	5-7 "
		Controls.	19	7	5-16 "
5.0,3.3,3.3	11.6	From 1 day before.	11	0	1-3 "
		Controls.	18	9	5-16 "
3.3,2.0,2.0	7.3	From 1 day before.	4	0	1-2 "
		Controls.	6	1	3-5 "
2.2,2.2,2.2	6.6	From 4 days after.	5	1	5 "
		" 6 " "	5	3	7 "
		Controls.	6	4	4-10 "

TABLE XXV

Published Results for the Treatment of Haemolytic Streptococcal Infections with Sulphanilamide.

Mice treated per os.

Author.	Total drug in mg.	Duration of treatment. (in days)	Survivors. %	Duration of observation.
Tréfouël (112)	3.75	2	50	10 days.
Buttle (16)	450	13	100	3 weeks.
Nitti (89)	5	2	0	-
Whitby (113)	200	10	58	10 days.
Raiziss (93)	50	5	67	5 weeks.
Mellon (76)	250	10	50	25 days.
	81.25	10	100	30 days.
Raiziss (95)	50	5	60	28 days.
" (94)	50	5	67	28 days.
Feinstone (26)	180	7	94	30 days.

Mice treated subcutaneously.

Author.	Total drug in mg.	Duration of treatment. (in days)	Survivors. %	Duration of observations.
Colebrook (21)	63	6	100	5 weeks.
Raiziss (93)	50	5	86	3-10 weeks.
	30	3	100	10 weeks.
Rosenthal (100)	10 (oil)	4	80	-
Raiziss (94)	50	5	64	28 days.
Feinstone (26)	18	3	29	14 days.

TABLE XXVI

Published Results for the Treatment of Haemolytic Streptococcal Infections with the Sulphones.

4:4'-diamino-diphenyl-sulphone.

Treatment per os.

Author.	Total drug in mg.	Duration of treatment in days.	Survivors. %	Duration of Observation.
Raiziss (95)	2.5	5	$66\frac{2}{3}$	28 days.
	5.0	5	$66\frac{2}{3}$	28 "
	10.0	5	80	28 "
Feinstone (26)	3.75	3	94	14 "
	13.5	7	96	30 "

4:4'-diacetylamino-diphenyl-sulphone.

Treatment per os.

Author.	Total drug in mg.	Duration of treatment in days.	Survivors. %	Duration of Observation.
Raiziss (95)	5.0	5	55	28 days.
	10.0	5	60	28 "
	25.0	5	80	28 "
Feinstone (26)	15.0	3	57.3	14 "
	90.0	7	72	30 "

TABLE XXVII

Comparison of the Results of Treatment of Streptococcus Kruger at Different Periods.

Inoculum intraperitoneal (1:10 to 1:100,000): untreated controls with the same inoculum all died in 24 hours.
Treatment subcutaneous at 0, 5, and 24 hours.

Drug and dosage.	Period I Non-capsulated. cures/total	Period II (Derived from I) Capsulated. cures/total	Period III (Derived from II) Capsulated. cures/total	Period IV. (Derived from II) Capsulated. cures/total	Period V (Derived from I) Non-capsulated. cures/total
Sulphanilamide. 10,6.7,6.7mg. (23.4mg.)	70/112 (63%) (3-4 days)	1/12 (2-3 days)	0/12 2/32 (6%) (2-4 days)	1/8 (2-3 days)	31/47 (66%) (2-7 days)†
Diamino-diphenyl-sulphone. 3.3,2.5,2.5mg. (8.3mg.)	4/8 (50%) (3-5 days)	-	0/3 (4-5 days)	-	29/35 (83%) (1-7 days)
Diacetylamino-diphenyl-sulphone. 10,6.7,6.7mg. (23.4mg.)	33/46 (72%) (2-16 days)	4/9 (9-19 days)	-	-	26/31 (84%) (5-9 days)
Monoacetyl-diamino-diphenyl-sulphone. 6.7,4.0,4.0mg. (14.7mg.)	9/9 (100%) -	26/29 (90%) (3-11 days)	41/121 (34%) 59/158 (37%) (4-18 days)※	18/37 (49%) (2-16 days)	37/41 (90%) (2-13 days)⊗

The number of days given in brackets indicates the time of survival of treated animals dying from the infection.

† Also late deaths at 44 and 62 days.

※ Also late deaths at 21, 23, 26, 26, 35, 89 days.

⊗ Also one late death at 30 days.

TABLE XXVIII

Course of Infection following Treatment with Sulphanilamide.

Inoculum intraperitoneal with dilutions of culture shown: untreated controls with the same inoculum all died in 24 hours.

Treatment subcutaneous - 10,6.7,6.7mg. at 0,5, and 24 hours.

Classification.	Streptococcus Kruger. 1:100,000	Streptococcus Thomson. 1:100	Streptococcus Thomson. 1:1,000	Total.
Cures. A	1	-	-	1
Failures D	-	4	8	12
Failures E	2	-	-	2
				15

A = Organisms never found in the blood.

D = Blood never free from organisms.

E = Organisms appeared and gradually increased in the blood before death.

TABLE XXIX

Course of Infection following Treatment with Monoacetyl sulphone.

Inoculum intraperitoneal with dilutions of culture as shown: untreated controls with the same inoculum all died in 24 hours.

※ Treatment subcutaneous at 0, 5, and 24 hours.

Classification.		Streptococcus Kruger. 1:100,000	Streptococcus Thomson. 1:100	Streptococcus Thomson. 1:1,000	Total.
Cures.	A	1	-	10	11
	B	2	-	9	11
	C	-	-	7	7
Failures.	D	3	4	10	17
	E	15	-	20	35
	F	5	-	2	7
15 further animals died from causes other than streptococcal septicaemia.					88

A = Organisms never found in the blood.

B = Few organisms present at some stage.

C = Abundant organisms at some stage.

D = Blood never free from organisms.

E = Organisms appeared and gradually increased in the blood before death.

F = Blood negative up to day of death.

※ In treatment of Streptococcus Kruger: 2.5,2.5,2.5mg.(7.5mg.) or 1.67,1.67,1.67mg.(5.01mg.)

In treatment of Streptococcus Thomson: 6.7,4.0,4.0mg.(14.7mg.)

TABLE XXX

Antibacterial Action of Sulphanilamide and Acriflavine.

Inoculum: Streptococcus Kruger Period IV, 24-hour culture in broth, 0.1cc. to 1.0cc, of medium.

(1) In broth.

Dilution of culture for inoculum.	Sulphanilamide.			Acriflavine.			Controls.
	$1:10^5$	$1:10^4$	$1:10^3$	$1:5\times10^6$	$1:10^6$	$1:5\times10^5$	
1:1	+	+	+	+	+	inh.	+
1:100	+	+	+	+	+ or inh.	−	+
1:10,000	+	+	+ or inh.	+ or inh.	−	−	+

(2) In sheep serum (previously heated at 56°C for 30 minutes and stored frozen.)

Dilution of culture for inoculum.	Sulphanilamide.			Acriflavine.			Controls.
	$1:10^5$	$1:10^4$	$1:10^3$	$1:5\times10^6$	$1:10^6$	$1:5\times10^5$	
1:1	+	+	+ or inh.	+	+	+	+
1:100	+	+ or inh.	inh.	+	+	+ or inh.	+
1:10,000	+	inh.	−	+	+	−	+

Bactericidal effects were read at the end of 48 hours at 37°C.

In broth absence of growth in subculture corresponded with the medium remaining clear; in serum sterility was judged by subculture only, as the medium never became turbid from growth of the organisms.

\+ = growth of organisms on subculture equal to that from control

inh. (inhibition) = " " " " " much less than that from control.

− = no growth of organisms on subculture.

Streptococcus Finger

March 1931 - isolated from ulcerated finger $\frac{1}{1000}$ = MLD

Apr (1937) lost of to $\frac{1}{100,000}$ after passage

Jan 1939 found capsulated

Period I : precapsulation - virulence variable over [illegible], culture granular

II = capsulated Jan 1939 to Apr 1939 = high virulence, [illegible] finger

III = from October 1939 - Feb 1942 still capsulated but not so susceptible to drugs = high virulent culture finger

IV = ~~July 1943 NON CAPSULATED~~ Recovered from [illegible] culture ~~finger [illegible]~~ stored since 1939 still capsulated = high virulence culture finger

V July 1943 recovered from NON CAPSULATED from blood broth culture finger since Feb 1938 : 5 years = virulence low culture ~~[illegible]~~ granular

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I & IV Typing = Lancefield B !

Strept Human

1942 ex [illegible] low virulence, after 3 months $\frac{1}{1000}$ and became Lancefield B capsulated

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Aronson - capsulated & highly virulent $\frac{1}{1,000,000}$

Cork

Lancefield Group A non capsulated virulent $\frac{1}{1,000,000}$

Velocity vector of aircraft