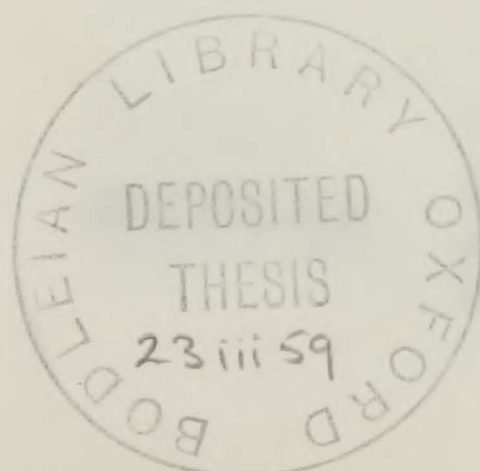


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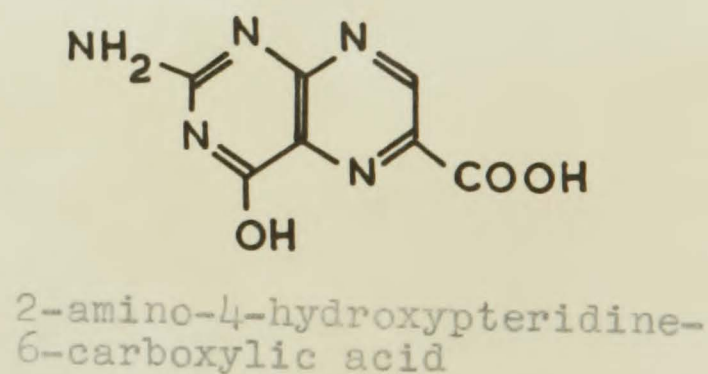
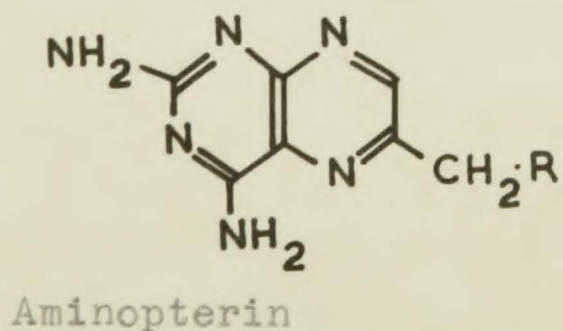
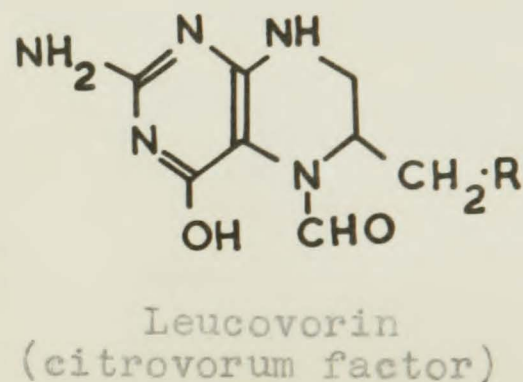
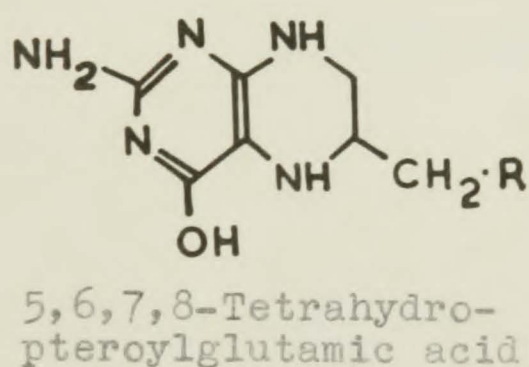
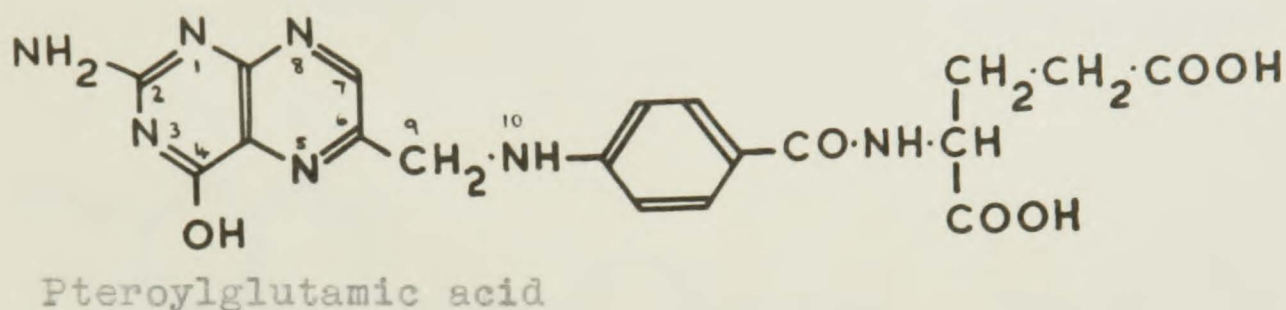
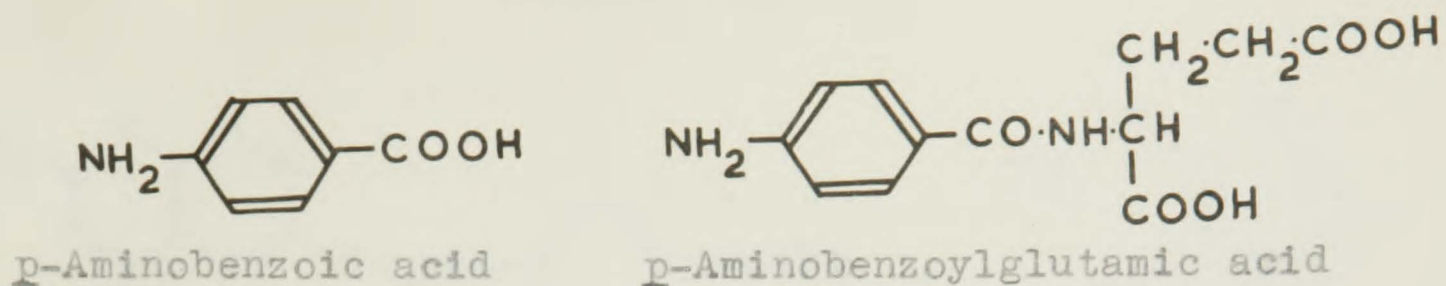
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Nomenclature and Abbreviations

For convenience the following abbreviations and conventions are used throughout this thesis:

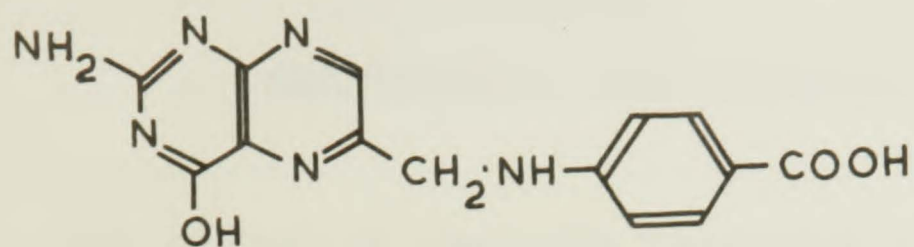
- PAB = p-Aminobenzoic acid
- Folic acid = The natural factor(s) which is required for growth of Lactobacillus casei and other organisms
- LF = The natural factor(s) which supports growth of Lb.casei in media free from folic acid
- CF = Leuconostoc citrovorum factor, either the natural factor(s) or the synthetic compound ("leucovorin") which supports growth of Lc.citrovorum in media free from folic acid
- CoF = Coenzyme F, the coenzyme form of folic acid, which participates in the transfer of one carbon units.
- PABG = p-Aminobenzoylglutamic acid
- ATP = Adenosine triphosphate; AMP = Adenosine monophosphate; PP = Pyrophosphate

The structures of compounds to which frequent reference is made are shown in Figure 1.

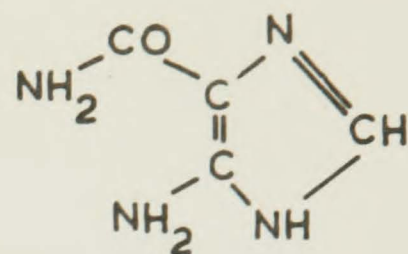


R is the p-aminobenzoylglutamyl residue.

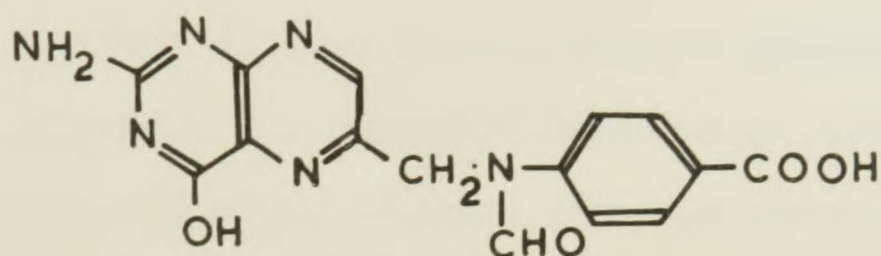
Structural formulae



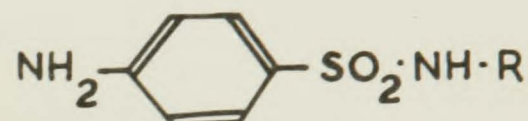
Pterioic acid



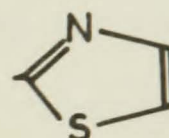
4-amino-5-imidazole-carboxamide



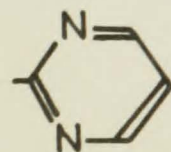
Rhizopterin



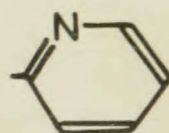
R = -H Sulphanilamide



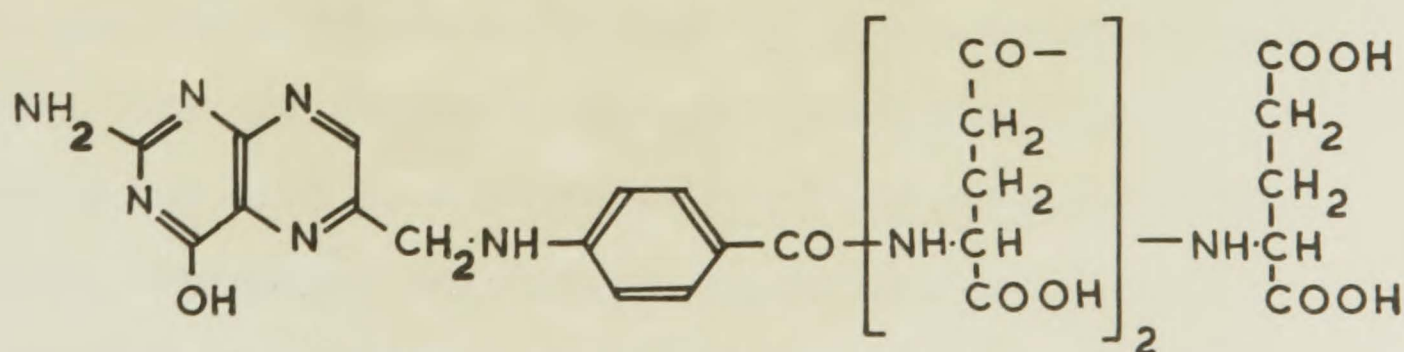
Sulphathiazole



Sulphadiazine



Sulphapyridine



Pteroyltriglutamic acid

PREFACE

The Present Investigation

The object of the work described in this thesis was to investigate the biochemical mechanisms of resistance to sulphonamide drugs.

A study of the attributes of drug-resistant strains of staphylococci is valuable because of the particular frequency with which such strains occur in clinical infections (Jawetz, 1956; Harington, 1957). Resistant strains of initially sensitive organisms may be obtained by several laboratory procedures (Chapter I). The biochemical mechanisms of resistance that have either been demonstrated or postulated are discussed in Chapter II with special reference to sulphonamide resistance. Resistance to sulphonamides was chosen for investigation because more is known about the mode of action of these drugs than of most antibacterial substances (Chapter III). Such information directs attention to the metabolism of p-aminobenzoic acid and folic acid and suggests that the development of resistance may lead to changes in the metabolism of these compounds. Present knowledge of the biosynthesis and function of folic acid is, therefore, briefly reviewed. (Chapter III).

Plan of the investigation.

The general plan for this study was first to obtain from sensitive strains of staphylococci, substrains resistant to sulphonamides. The growth, nutrition and general biochemical properties of these strains were then to be examined (Section 1). Closest attention was to be given to the metabolism of folic acid and PAB, where changes which bring about resistance seemed most likely to occur (Section 2). In particular, the investigations of Lascelles and Woods (1952), on the synthesis of folic acid by a sensitive and a resistant strain of Staph.aureus were to be extended to other strains. The metabolism of sulphathiazole, and the permeability of the organisms to this drug were also studied (Section 3).

Owing to the number of properties investigated, it was not practical to use more than three or four pairs of sensitive and resistant strains. With more strains, time would have been insufficient for each pair to be examined in any detail.

Only those aspects of drug resistance that are relevant to the present study are considered in the Introduction. More extensive information may be found in the review by Abraham (1953) and in the comprehensive treatise of Schnitzer and Grunberg (1957).

Introduction

CHAPTER I

The development and attributes of drug-resistant strains of micro-organisms

Drug-resistant strains of micro-organisms for comparative studies are usually derived in the laboratory from known sensitive cultures. Drug-resistant strains that are isolated from clinical infections do sometimes produce sensitive back-mutants, but otherwise it is very difficult to identify related sensitive strains that might be used for comparison with the resistant strains.

The majority of drug-resistant strains of micro-organisms developed in the laboratory have been obtained by exposure to a chemical agent either in vitro or in vivo. For bacteria and fungi, the former is the more convenient method; with many protozoa only the in vivo approach is practicable.

Development of resistance by serial passage in vitro.

This is the most often used method for the development of drug-resistant strains of bacteria. Organisms are incubated in a medium which contains a concentration of drug which is insufficient to suppress growth completely. When growth appears under these conditions, organisms are transferred to medium containing the same or a higher concentration of drug. Such transfers are continued either for a fixed number of times or, more frequently, until transfers cease to produce further increase in resistance.

Although the operations involved in this method are very simple, it is not always easy to obtain resistant strains. Detailed descriptions in the literature of the progress of the development of resistance are not frequent, but it appears that there is seldom a consistent decrease of sensitivity at every transfer. Usually there is a gradual but not uniform increase of resistance (e.g. McIntosh and Selbie, 1943; Grunberg and Eldridge, 1950). The degree of resistance is not dependent only on the number of passages; different strains may show considerable variation in fastness after the same number of passages in the presence of a drug (Williston and Youmans, 1947; Wright, Purcell, Wilcox, Broderick and Finland, 1953) and in other cases resistance may rise precipitously after only a single exposure to a drug (Demerec, 1948).

There may be differences in the development of resistance depending on the composition of the medium used and whether it is liquid or solid. Thus, Ramsey and Padron (1954) obtained resistance to chloramphenicol in Staph.aureus with a complex medium, but failed to do so in a chemically defined medium. Haight, Wilcox and Finland (1952) obtained a two-fold increase of streptomycin-resistance with a streptococcus in blood broth, but an almost one hundred and thirty-fold increase if the organism was subcultured on blood agar.

While a certain degree of resistance can be obtained against practically all antibacterial agents, increase of

used extensively to select resistant

resistance greater than one hundred-fold seems to be limited to a few drugs, such as the sulphonamides, penicillin and streptomycin. Chloramphenicol and the tetracyclines show much less tendency to give rise to high resistance (Schnitzer and Grunberg, 1957).

The method of serial passage was the only procedure by which resistant strains were developed successfully in the present work. Certain practical difficulties were found and these will be considered later (Chapter V).

Development of resistance by single exposure *in vitro*.

Resistance to drugs of the streptomycin type may occur on a single exposure of a large inoculum to the drug (Demerec, 1948). The gradient plate of Bryson and Szybalski (1952) is a useful device in performing experiments of this kind. In bacterial cultures which have not been in contact with the drug there are individual organisms of very different degrees of sensitivity (Silver and Kempe, 1947) so that when large numbers of cells are exposed to the drug there is a reasonable chance that some highly resistant organisms will be present.

In recent years methods have been devised by which resistant organisms may be identified without exposure to a drug. While these methods are of great theoretical interest, and provide strong support for the view that resistant organisms arise by spontaneous mutation, they have not been used extensively to select resistant strains of micro-organisms.

Development of resistance by exposure to transforming principles.

Fractions containing deoxyribonucleic acid from penicillin-fast or streptomycin-fast pneumococci have been used by Hotchkiss (1951) to transfer resistance to sensitive pneumococci. The transformation of Haemophilus influenzae to streptomycin resistance has been demonstrated (Alexander and Leidy, 1953; Schaeffer, 1956), and Hotchkiss and Evans (1957) have described transformation of pneumococci to sulphonamide-resistance.

Identification of resistant organisms by the replica plate method.

This method will be described in detail because a modification was used in attempts to obtain a sensitive back-mutant from a resistant strain which was used in the present work.

The technique of Lederberg and Lederberg (1952) consists of the transfer of bacterial growth from a plate containing no drug to another which contains drug. During the transfer the original spatial location of the colonies on the first plate is maintained by using for the transplantation a piece of sterile velveteen on which an imprint is made of the first plate. On the second plate only resistant colonies will develop, and since this plate is a replica of the first, the sites on the original plate of the resistant colonies can be found. Cultures prepared from these locations contain a high number of resistant organisms, which have not been in contact

with the drug before their sensitivity is tested.

Attributes of drug-resistant micro-organisms.

Cross-resistance and specificity.

When resistance has been acquired there is usually a considerable degree of specificity of resistance towards the drug to which the organism had been exposed. Bacteria may develop resistance to a chemotherapeutic agent without becoming less sensitive to others (e.g. Collier and Phillips, 1953; Alexander and Leidy, 1947). Sometimes, however, resistance occurs to agents other than the individual drug against which resistance originally developed. For example, it is generally found that resistance developed to one sulphonamide confers resistance towards other members of the group (Kirby and Rantz, 1943; Spink and Vivino, 1943). In this case the drugs are similar in their chemical structures, but cross-resistance to chemically unrelated agents is also known. Examples are the sulphonamide-fast Streptococcus mitis described by Clapper and Heatherman (1950) which was also resistant to bacitracin, and the strain of Plasmodium gallinaceum which acquired resistance to proguanil at the same time as it became resistant to sulphonamides (Bishop and McConnachie, 1950). The explanation of such cases of cross-resistance is not often evident, although a similarity in the mechanism of action of the drugs is sometimes indicated.

Duration of drug-resistance in the absence of the drug.

In considering the duration of drug-resistance the most useful criterion would be the number of generations in drug-free medium for which resistance is stable. Such information is not available, and failing this, the number of transfers in drug-free medium for which resistance is maintained, is a good criterion. Resistance is generally maintained for several years when little growth occurs, as in lyophilized cultures.

The stability of resistant organisms subcultured in absence of drug seems to vary from one drug and one organism to another. Resistance to penicillin and chloramphenicol tends to be of relatively short duration; sulphonamides, streptomycin and isoniazid give resistant strains of greater stability (Schnitzer and Grunberg, 1957). One factor that appears generally to be involved in the stability of resistance is the degree of fastness of the organism; thus a higher degree of resistance usually confers a higher degree of stability (Monnier and Schoenbach, 1951; Lam and Sevag, 1955). When cultures of bacteria are acquiring resistance during serial passage it is sometimes found that the resistance which first develops is readily lost but that later the resistant strain becomes stable (Schmidt, Sesler and Dettwiler, 1942; Davies and Hinshelwood, 1943).

Changes in morphology, virulence and other biological properties are often observed in resistant bacteria. This is

in contrast to the position among the protozoa, where generally there are few such changes accompanying the development of resistance.

The significance of such changes is not understood. They may be caused by the altered metabolism which enables a resistant strain to grow in the presence of a drug. On the other hand many of these changes can be produced or lost without change in the sensitivity of an organism to a drug. Sulphonamide-resistant micro-organisms generally do not show marked changes in biological properties. (Schnitzer and Grunberg, 1957).

It is possible to obtain information about the cellular changes that are responsible for resistance, without being aware of the way in which these changes are brought about. In theory at least, a number of possibilities of resistance appear possible, and these have been listed by Weiss and Weiss (1952).

1. Alternative metabolic pathways bypassing the inhibited reaction.
2. Increased formation of a metabolite that antagonizes the inhibitor.
3. Decreased requirement for a product of the inhibited reaction.
4. Increased destruction of the inhibitor, or decreased conversion of an administered substance into a more active inhibitor.
5. Increased amount of the enzyme that the drug inhibits.

CHAPTER II

Biochemical mechanisms of drug-resistance

The mechanism of drug-resistance has two aspects. One is the process by which a hereditary change in resistance to an inhibitor arises in an organism and becomes transmitted to succeeding generations. The other is the nature of the biochemical changes, regardless of origin, that are directly responsible for the decreased sensitivity of the organism to the effect of the drug. The work that is described in this thesis has only been concerned with this second aspect.

It is possible to obtain information about the cellular changes that are responsible for resistance, without being certain of the way in which these changes are brought about. In theory at least, a number of mechanisms of resistance appear possible, and these have been listed by Davis and Maas (1952).

1. Alternative metabolic pathways by-passing the inhibited reaction.
2. Increased formation of a metabolite that antagonizes the inhibitor.
3. Decreased requirement for a product of the inhibited reaction.
4. Increased destruction of the inhibitor, or decreased conversion of an administered compound into a more active inhibitor.
5. Increased amount of the enzyme that the drug inhibits.

6. Formation of an altered enzyme with decreased affinity for the inhibitor, or with increased relative affinity for the substrate compared to a competitive inhibitor.
7. Decreased permeability of the organism, or of subcellular units, to the inhibitor.

Since mutations can give rise presumably to an increment of resistance by various mechanisms, all of which might be operating in a mutant, there is no reason to suppose that any of the above mechanisms will be uniquely important. For the operation of some of these possibilities there is experimental support, while others (3, 5 and 6) have been very seldom demonstrated. Although mechanisms 5 and 6 appear formally distinct it would be very difficult experimentally to distinguish between them.

Altered metabolic pathways in resistant organisms.

Changes in metabolism are much more common in drug-resistant strains of bacteria than in protozoa. This subject is reviewed extensively by Sevag (1955) and by Schnitzer and Grunberg (1957), so only a few examples bearing on sulphonamide-resistance will be quoted.

Changes in amino acid metabolism are frequently observed in drug-resistant micro-organisms. Steers and Sevag (1949) found that two sulphonamide-resistant strains of Staph.aureus suffered partial loss of the ability to synthesize amino acids, which was more pronounced in the absence of glucose. On the other hand, Gale and Rodwell (1949) found that penicillin-resistant strains of Staph.aureus produced in the laboratory

became increasingly less exacting in their amino acid requirements as sensitivity fell.

Staph.aureus made resistant to sulphonamides in the presence of glucose was not resistant in a medium where glucose was replaced by pyruvate (Sevag and Green, 1944). The authors suggested that some step in the metabolism of glucose via pyruvate was blocked by sulphonamides, and that the resistant organism developed an alternative metabolic process which by-passed pyruvate. Repeated subculture of the resistant organisms in a glucose-free medium resulted in a strain resistant to sulphonamides even in the absence of glucose.

The nature of the medium has an important influence on the biochemical characteristics developed during selection of a resistant strain. Sevag (1946) summarized this influence by postulating that an organism can more readily adapt itself to the presence of substances which interfere with its usual metabolic functions if its environment contains other substances which can be utilized for the development of alternative mechanisms of synthesis. A strain of Esch.coli resistant to sulphanilamide was developed by Kohn and Harris (1942) in a defined medium containing methionine. Methionine became an essential growth factor for the strain, which originally had been able to grow without it. A similar result was found by Harrison and

Clapper (1950). Strains of Streptococcus mitis when made resistant to sulphonamides in a medium which contained pteroylglutamic acid, developed a requirement for this factor. Both these findings may be explained by supposing that when the synthesis of a metabolite is blocked by an inhibitor, the organisms have developed the ability to utilize a new source of the product of the inhibited reaction.

It is sometimes very difficult to assess the significance of these changes in metabolism, and to decide whether they are responsible for the development of resistance or are consequences thereof. Growth requirements and metabolic functions vary so much from organism to organism with the same or different drugs that it is difficult to find common features in the various changes that have been observed in resistant organisms. The evidence for the utilization of alternative metabolic pathways as a mechanism of resistance is generally inferential rather than direct owing to the lack of knowledge of the processes inhibited by most drugs. The most direct demonstration of such a mechanism for sulphonamide-resistance would be to show that the reaction of PAB which is sensitive to sulphathiazole in the synthesis of folic acid may be replaced by a reaction or reactions that are insensitive to the drug.

An objection put forward by Davis (1957) to this type of mechanism of resistance is that in a biosynthetic sequence

proceeding from compound A to C, via B, the appearance of a new route by-passing B would probably require the insertion of more than one new enzyme in the sequence. This is unlikely to occur by a single mutation, although it is possible that one of the enzymes new in the sequence might already be present for other purposes.

Increased synthesis of a metabolite that antagonizes the inhibitor.

The best known example of this mechanism is the antagonism of PAB with the sulphonamides. Woods (1940) suggested that resistance to these drugs might be due to increased production of PAB. This view has been confirmed experimentally in several micro-organisms (MacLeod, 1940; Mirick, 1943; Landy, Larkum, Oswald and Streightoff, 1943). However, the increase in PAB formation does not always parallel the increase in resistance, and there are many cases of sulphonamide-resistance where no increase in PAB formation has been found (Lemberg, Callaghan, Tandy and Goldsworthy, 1948; Yaniv and Davis, 1953). It may be that a failure to detect increased PAB synthesis, at least in some cases, is due to all the PAB being converted into a higher product which might not be detected by an assay for PAB.

Apart from this instance of sulphonamide-fastness there seem to be very few cases where increased synthesis of a metabolite antagonistic to a drug is responsible for

resistance. In spite of much effort, none of the antibiotics has been clearly shown to act by competing with a metabolic intermediate.

Decreased requirement for a product of an inhibited metabolic system.

Before this mechanism can be investigated, it is necessary to have considerable knowledge of the mode of action of the drug to which resistance is developed. As this is the case with few drugs, the occurrence of this mechanism has not been explored, but it seems unlikely that it could cause more than a moderate increase of resistance. In the case of sulphonamide-resistance a decreased requirement for folic acid or for products of the function of folic acid would result.

Increased destruction of an inhibitor or decreased conversion of an administered compound into a more active inhibitor.

The acquisition of an ability to render a drug inactive is well known to be involved in some cases of drug-resistance, but it is uncommon. The best known example is the enzyme penicillinase, which is found in most penicillin-resistant organisms that are isolated clinically (Abraham and Chain, 1940; Eagle, 1954a; Barber, 1957). The enzyme converts penicillin to penicilloic acid, which is not bactericidal. The enzyme is constitutive in some organisms, for example in many staphylococci, and inducible in others, such as Bacillus cereus (Pollock, 1950).

A few other cases of drug inactivation by resistant organisms are known. Woolley (1944) found that a strain of Endomyces vernalis which had developed resistance to pyriithiamine was able to cleave the drug between its pyrimidine and pyridine rings. Merkel and Steers (1952; 1953) described the increased "chloramphenicol reductase" activity of resistant strains of Esch.coli. This enzyme reduces the nitro group of chloramphenicol to the corresponding amino derivative, which is subsequently degraded further (Smith and Worrel, 1949). The destruction of sulphonamides by resistant organisms has been very little investigated.

At one time it was thought that the sulphonamide drugs might be converted in micro-organisms to more active compounds (see review by Henry, 1943). The reason for this view was that p-hydroxylaminobenzene sulphonamide is in vitro a more active inhibitor of the growth of some bacteria than is sulphanilamide. The hydroxylamino compound was held to inhibit catalase activity so that toxic amounts of hydrogen peroxide accumulated. This "anti-catalase" theory of sulphonamide action is no longer maintained (Chapter III). p-Hydroxylaminobenzene sulphonamide is no more active in vivo than sulphanilamide, probably because of its instability, and its in vitro effects are not antagonized by PAB, as are the effects of sulphonamides.

There are very few reports of drugs that are metabolically converted to the actual inhibitory substance. Examples are

shown in Figure 2. These reactions are usually brought about by the tissues of the host, and there is no evidence that resistant organisms can decrease the extent of these changes.

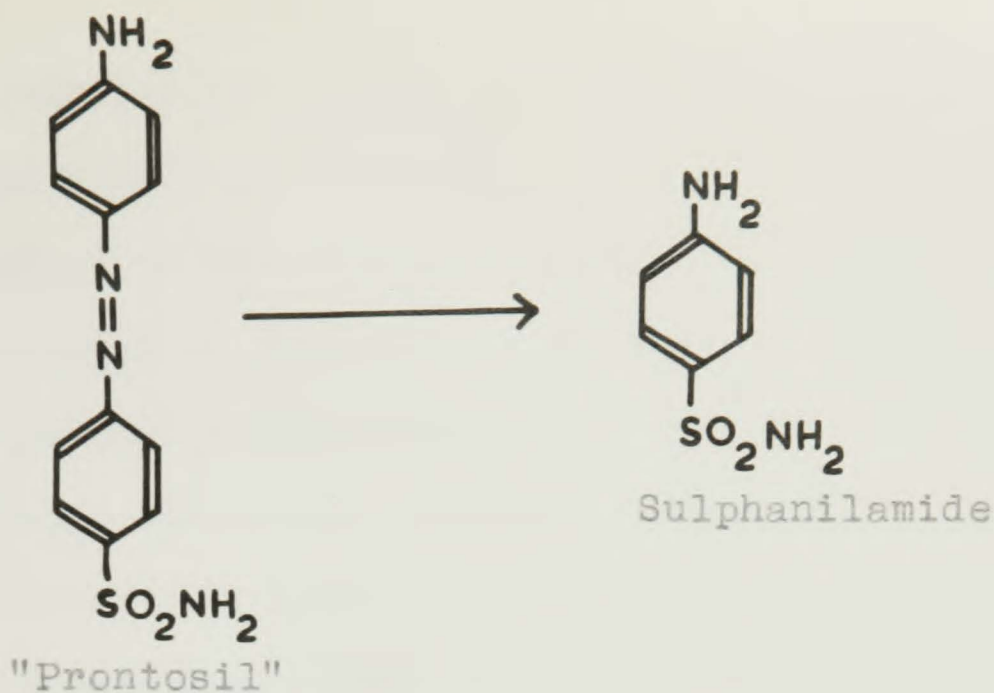
Increased concentration of the enzyme that the drug inhibits.

This possibility has never been demonstrated, perhaps because of the practical difficulty of any such attempt, especially without knowledge of the nature of the enzyme to be investigated. With the sulphonamides, only very recently has any evidence been obtained about the actual reaction inhibited by the drugs (Chapter III).

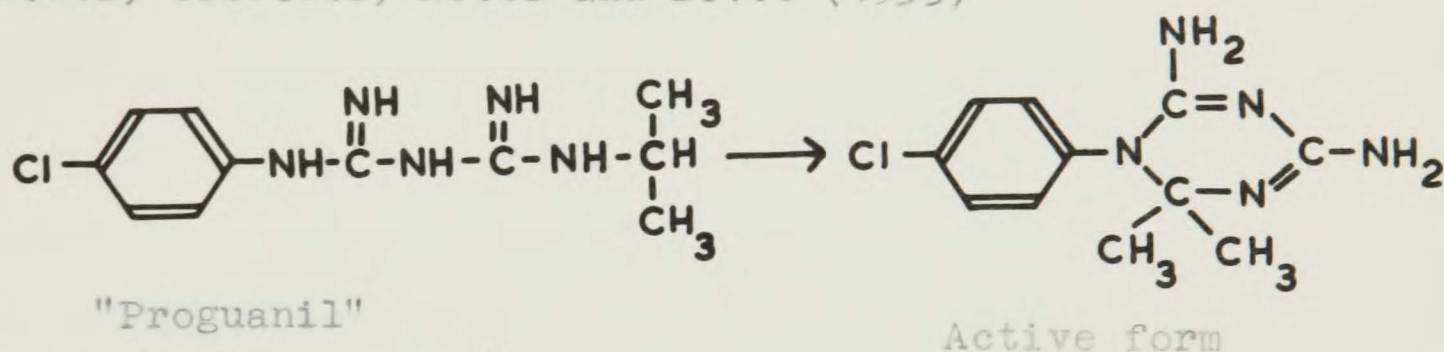
To demonstrate this mechanism it would be necessary to purify the enzyme and show its presence in increased amount in resistant strains. It might be possible to do this if the purified enzyme had distinct serological properties. Cohn and Torriani (1953) showed that the β -galactoside activity developed after addition of methyl- β -galactoside to a culture of Esch.coli corresponds quantitatively to the production of a new protein with distinct and specific immunological properties.

Formation of qualitatively altered enzyme.

Difficulties similar to those discussed for the previous mechanism of resistance arise in this case. Owing to the lack of knowledge about the enzymes with which drugs interfere, direct demonstrations of changed properties of these enzymes have not been possible.

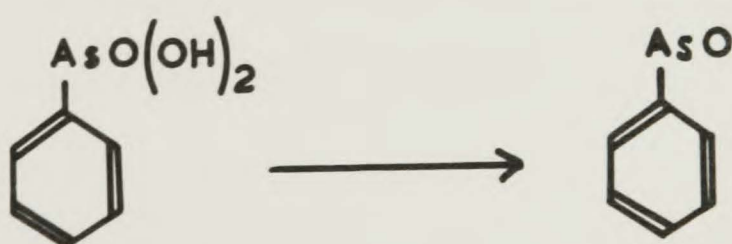


Tréfouël, Tréfouël, Nitti and Bovet (1935)



Hawking and Perry (1948)

Carrington, Crowther, Davey, Levi and Rose (1951)



Pentavalent arsenicals

Trivalent arsenicals

See review by Albert (1951)

Examples of drugs that are converted *in vivo* to therapeutically active compounds

In recent years it has been shown in animals and in micro-organisms that mutation may give rise to subtle changes in the nature of the enzyme performing a given reaction. Those resulting in increased thermal sensitivity appear to be frequent perhaps because they are easy to select (Davis, 1957). Such findings encourage the search for other qualitative changes that would lead to drug-resistance.

Davis and Maas (1952) showed that p-nitrobenzoic acid inhibits the growth of Esch.coli by competing with both PAB and p-hydroxybenzoic acid (POB). By growing the organism in the presence of p-nitrobenzoic acid and each metabolite separately, two types of resistant cells were obtained which showed no cross-resistance. It could therefore be assumed that resistance was not due to destruction of the inhibitor nor to changes in the permeability of the organisms to the inhibitor. Moreover, it was found that the two kinds of resistant organisms were distinct from strains resistant to p-aminosulphonamides and p-hydroxysulphonamides, which also compete with PAB and POB. This indicated that resistance was not due to increased production, nor more efficient utilization of the metabolites, nor to the development of new metabolic pathways which enabled the metabolites to be dispensed with. The exclusion of these mechanisms led to the conclusion that resistance was due to a qualitative change in the enzyme using PAB or POB, as a result of which the enzyme

showed a decreased affinity for the drug compared with the metabolite.

Wacker, Trebst and Simon (1957) found that in resistant strains of Esch.coli and Enterococcus Stei, ^{14}C -labelled PAB was less completely displaced from the organisms by sulphathiazole than in sensitive strains. This could be interpreted to mean that the affinity of the PAB-utilizing enzyme for its substrate had increased in the resistant strains, since experiments with ^{35}S -labelled sulphanilamide had shown that the permeability to this drug of sensitive and resistant strains was about the same.

Decreased permeability to an inhibitor.

Changes which prevent a drug reaching its site of action may be important in the development of drug-resistance. When a resistant strain is found to take up a decreased amount of a drug, this may mean that the drug is unable to penetrate the cell to its site of action, or it may mean that the sensitive component itself is changed and no longer reacts with the drug. The experimental distinction between these two possibilities may be very difficult.

Correlation between the uptake of a drug and sensitivity occurs more frequently and more convincingly in drug-resistant protozoa than in bacteria. Normal trypanosomes, for example, absorb acriflavin, while resistant organisms do not (Gonder, 1912; von Jancso, 1932). Again, normal

trypanosomes differ from those that are resistant to drugs such as atoxyl in absorbing appreciable quantities of the arsenicals concerned (Yorke, Murgatroyd and Hawking, 1934; Hawking, 1937).

Reduced uptake of the drug is not always characteristic of drug-resistance in bacteria. Eagle (1954b,c) used ^{35}S -labelled penicillin to study the binding of penicillin in different organisms. Some of the resistant variants bound less penicillin, others bound the same amounts as the parent strains, and some even more. Such discrepancies were found among strains of the same species. Barclay (1955) found that a strain of Mycobact.tuberculosis resistant to p-aminosalicylic acid took up much more of the drug than did the sensitive strain; on the other hand, isoniazid-resistant organisms took up markedly less of the drug than did the sensitive strain (Barclay, 1953). Yegian and Budd (1945) showed that sulphonamide-resistant mycobacteria did not differ from the parent strains in permeability to sulphonamides. The synthesis of citrovorum factor from pteroylglutamic acid was studied by Nichol (1954) in whole organisms and in extracts of sensitive and resistant strains of Strep.faecalis A. Large amounts of aminopterin were required to inhibit the synthesis by intact resistant organisms, in contrast to the very much smaller amounts of inhibitor that were needed to inhibit the synthesis in extracts of the same organism or in whole cells and extracts of the sensitive strain.

A possibility which has not been investigated is that the drug which is taken up by resistant strains may be inactivated by binding into some compound. This mechanism is suggested by the example of biotin, which is inactivated when it reacts with a protein, avidin, in egg white (Eakin, Snell and Williams, 1940). Evidence has been found that a penicillin-binding component is present in penicillin-sensitive organisms (see review by Cooper, 1956) but there has been no suggestion that penicillin is inactivated when it combines with this substance.

In the past few years views have altered very much on the permeability properties of bacteria. The presence has been demonstrated of a variety of permeation systems, each stereospecific for a structurally related group of substrates (see review by Cohen and Monod, 1957). Certain of these systems are adaptive, while others seem to be constitutive. Permeation systems, like enzymes, can be gained or lost by mutation.

By applying these facts to the problem of drug-resistance, it can be imagined that mutations as well as physiological adaptations could alter the transportation of a drug into the organism. Compounds that overcome an inhibition are not necessarily metabolites; they can also be analogues that interfere with the penetration of an inhibitor. Such applications of these new theories are very attractive, but work in these directions has not yet proceeded very far.

Summary of mechanisms of sulphonamide-resistance in micro-organisms.

The mechanisms that have been demonstrated for sulphonamide-resistance are gathered together for convenience.

1. Some, but not all, resistant strains have an increased ability to synthesize PAB. The increase in PAB synthesis is not always as great as the increase in resistance.
 2. Metabolic changes sometimes accompany sulphonamide resistance. In a few cases the changes can be interpreted on the basis of the anti-PAB theory of sulphonamide action, as being a means of avoiding the inhibited reaction. In other instances it is less clear whether or not the change is directly responsible for resistance.
 3. A few observations could be interpreted as suggesting a change in the amount or properties of a PAB-utilizing enzyme, but there is no unequivocal evidence for this mechanism.
- Changes in permeability to sulphonamides or inactivation of sulphonamides have not been demonstrated in resistant strains.

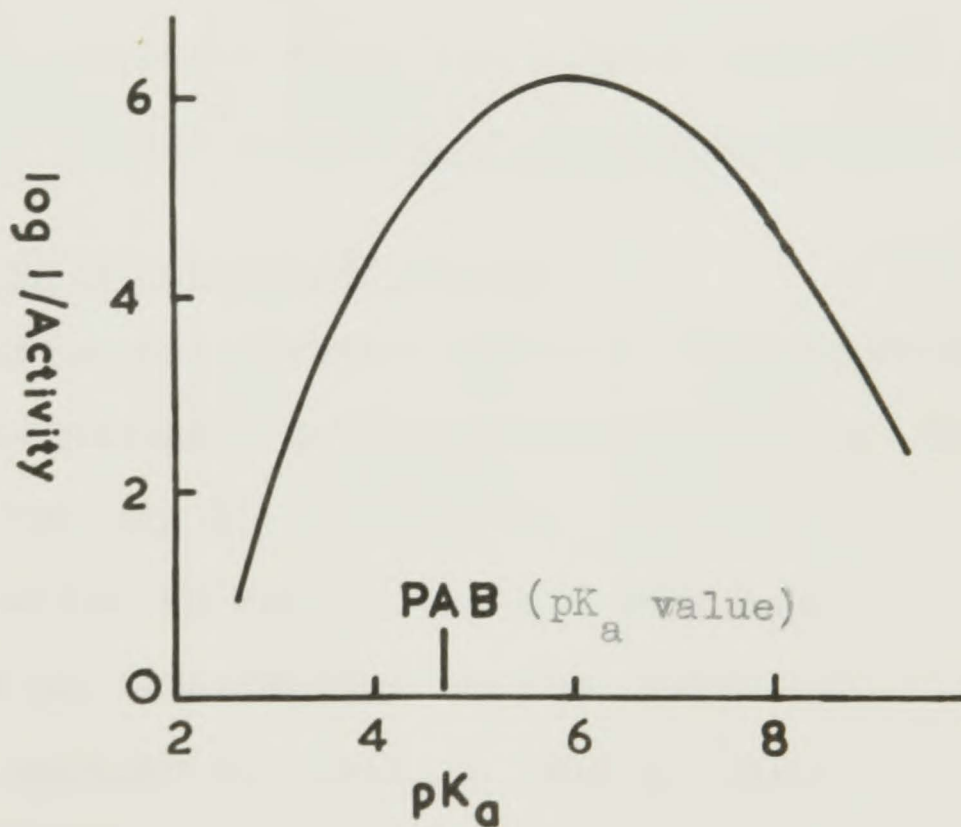
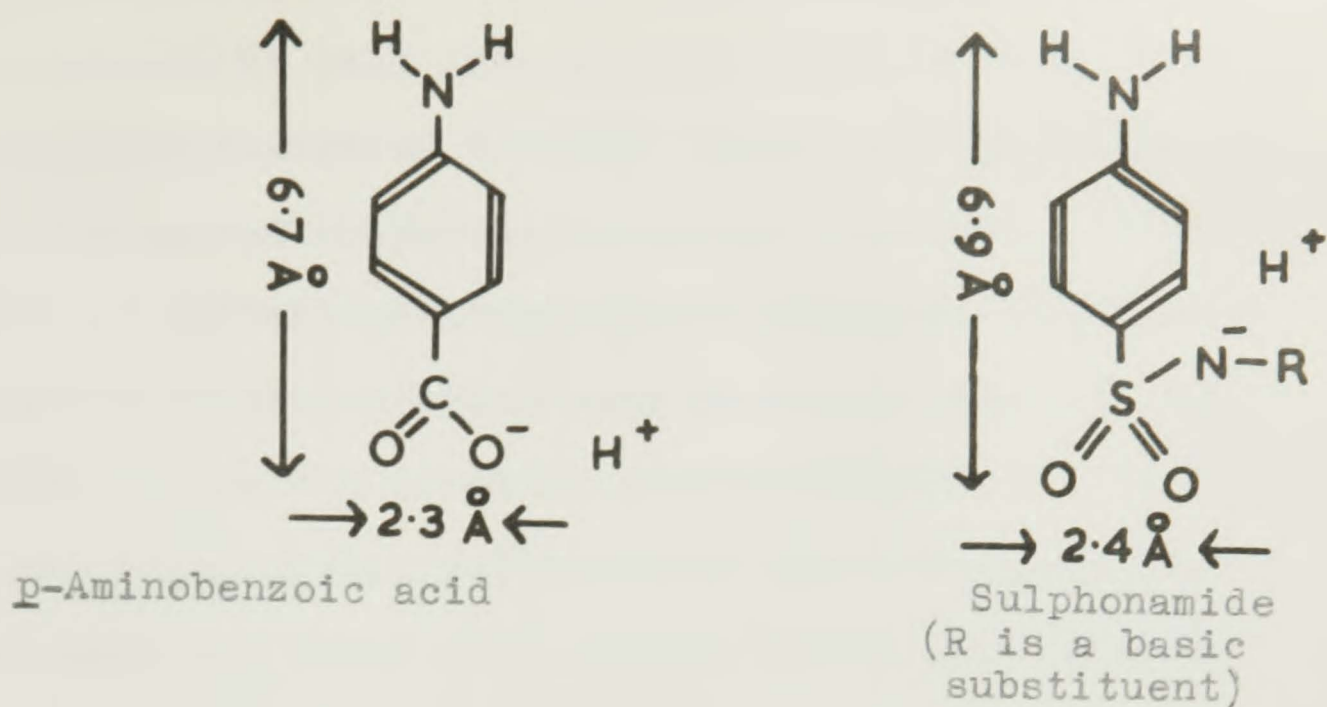
CHAPTER III

The mode of action of the sulphonamide drugs, and the biosynthesis and functions of folic acid

Competitive relation between PAB and sulphonamides.

It is now generally accepted that the primary action of the sulphonamides is to interfere with the metabolism of PAB or a closely related substance in micro-organisms. This interference seems to be a result of the structural similarity between PAB and the *p*-aminobenzenesulphonamido-residue which is common to all sulphonamides.

Bell and Roblin (1942) showed that the distance between the *p*-amino- and the *N*-substituted sulphonamido-groups in sulphonamides is very similar to the distance between the *p*-amino- and the carboxylic acid groups in PAB (Figure 3). These workers also found that when the antibacterial activities of various sulphonamides were plotted against their pK_a values a peak of activity occurred with those drugs which had a pK_a in the region of that of PAB. At neutral pH, the carboxylic acid group of PAB is almost completely ionized, so it was suggested that the activity of a sulphonamide is increased the more negatively charged, at neutral pH, the oxygen atoms of the sulphonamido-group become. Consequently ionic forms should be more active inhibitors than molecular forms. The more electrophilic the group R, the more ionization will be favoured, and the pK_a



Structures and bacteriostatic activities of sulphonamide drugs (after Bell and Roblin (1942)).

value will rise. However, as R becomes increasingly electrophilic it will draw towards itself a large part of the negative charge of the ion, which will no longer be available to the sulphonamido-group. Consequently there will be an optimal position, where the group R produces ionization without drawing away to itself much of the negative charge conferred by the ionization.

The antagonistic relationship has been shown for practically all drugs that contain the p-aminobenzene-sulphonamido-moiety (see review by Henry, 1943) and has been demonstrated in protozoa, fungi and higher organisms, and for all bacteria tested except Bact.tularensis (Tamura, 1944).

Other theories of sulphonamide action.

A series of papers between 1938 and 1940 presented evidence that sulphonamides acted by destroying or inhibiting catalase, thus causing an accumulation of toxic hydrogen peroxide (see review by Henry, 1943). This view is no longer regarded as satisfactory because sulphonamides act on organisms that contain no catalase, and on others that form no hydrogen peroxide. In addition, Mann and Keilin (1940) showed that sulphanilamide had no effect on purified catalase.

Another theory, which is summarized in reviews by Henry (1943) and Sevag (1946) is that the primary action of sulphonamides is on the respiratory enzymes of bacteria. The experimental basis of this theory is not very convincing and

it is probably of little value. Wyss, Strandkov and Schmelkes (1942) found that in Esch.coli, Staph.aureus and Strep. pyogenes therapeutically inactive isomers of sulphanilamide affected respiration as much, or more than sulphanilamide itself; sulphathiazole had no effect on respiratory enzymes. "Training" Esch.coli to resist sulphonamides had no effect on the respiratory enzymes. Since sulphonamides interfere with the synthesis of purines, which form part of the respiratory coenzymes, it is understandable that sulphonamides may affect respiration indirectly.

Secondary effects of the sulphonamides.

Some sulphonamide drugs have inhibitory effects in addition to that which is overcome by PAB. In such cases it might be necessary for a resistant organism to develop fastness towards the several modes of action of the drug. Dorfman and Koser (1942) showed that the stimulation of respiration which occurs when nicotinamide is added to suspensions of shigella harvested after growth under conditions of nicotinamide deficiency, was antagonized by sulphapyridine and by sulphathiazole, but not by sulphanilamide. Mann and Keilin (1940) found that sulphanilamide affected the enzyme carbonic anhydrase, though other sulphonamides were inactive. This effect was not due to the complete sulphanilamide molecule, as removal of the p-amino-group gave compounds which still inhibited the enzyme.

Relationship of PAB and folic acid.

In view of the presence of a PAB residue in the folic acid molecule (Figure 1) it seemed likely that one function of PAB in micro-organisms might be its use in the synthesis of folic acid. This is possibly the only function of PAB in some organisms (see review by Woods, 1952), and in others there is little evidence of other functions.

Davis (1950) found that PAB, at concentrations higher than are needed for growth of PAB-requiring organisms, can act as a source of *p*-hydroxybenzoic acid for mutants which need this factor. Sloane (1953) isolated a free base from cultures of *Mycobact. smegmatis* which had grown in the presence of large quantities of PAB. McCullough (1957) has obtained apparently the same compound from cultures of *Esch.coli* grown similarly. The base has cofactor activity in the hydroxylation of aniline and anthranilic acid by enzyme preparations and it can overcome non-competitively the action of the sulphonamides. The possibility that this base is in fact related to folic acid is suggested by the observation of Kaufman (1958) that tetrahydropteroylglutamic acid can act as a cofactor in the hydroxylation of phenylalanine by a rat liver enzyme.

The role of PAB in the synthesis of folic acid is well established. Nimmo-Smith, Lascelles and Woods (1948) and Lascelles and Woods (1952) demonstrated the synthesis of

Lactobacillus casei factor by washed suspensions of various organisms; PAB was the only essential requirement for this synthesis, though glucose and glutamic acid were stimulatory. Mitbander and Sreenivasan (1954,a,b) found that Lb.arabinosus could synthesize folic acid from PAB and glutamate. The synthesis was stimulated by Tween 80 or biotin, and by xanthine. The incorporation of carboxyl-¹⁴C labelled PAB has been demonstrated by Wacker, Grisebach, Trebst and Weygand (1954) into folic acid in Esch.coli and Enterococcus Stei.

The synthesis of folic acid and its inhibition by sulphonamides.

Although it is believed that sulphonamides inhibit the conversion of PAB into folic acid, the exact locus of this effect has been obscure, owing to the lack of knowledge about the reactions involved. Pteroylglutamic acid contains residues of PAB, glutamic acid and a pteridine. It is not necessarily the case that these three residues are synthesized separately; for example, PAB might combine with a precursor of the pteridine ring. Also, because the ultimate coenzyme form of folic acid contains a reduced pteridine ring, pteridine intermediates in the biosynthesis of the coenzyme may also be in a reduced state. Little experimental work on the synthesis of folic acid seems yet to have been done with these possibilities in mind.

A possible point of interference by sulphonamides would seem to be in the synthesis of pteric acid (Figure 1). Pteric acid can replace pteroylglutamic acid for growth of Strep. faecalis R, and utilization of pteric acid is not inhibited by sulphonamides. In other organisms, however, pteric acid is less active than PAB in reversing sulphonamide inhibition, and the reversal is competitive, indicating that pteric acid is not a product of the reaction inhibited by sulphonamides. (Lampen and Jones, 1946a,b).

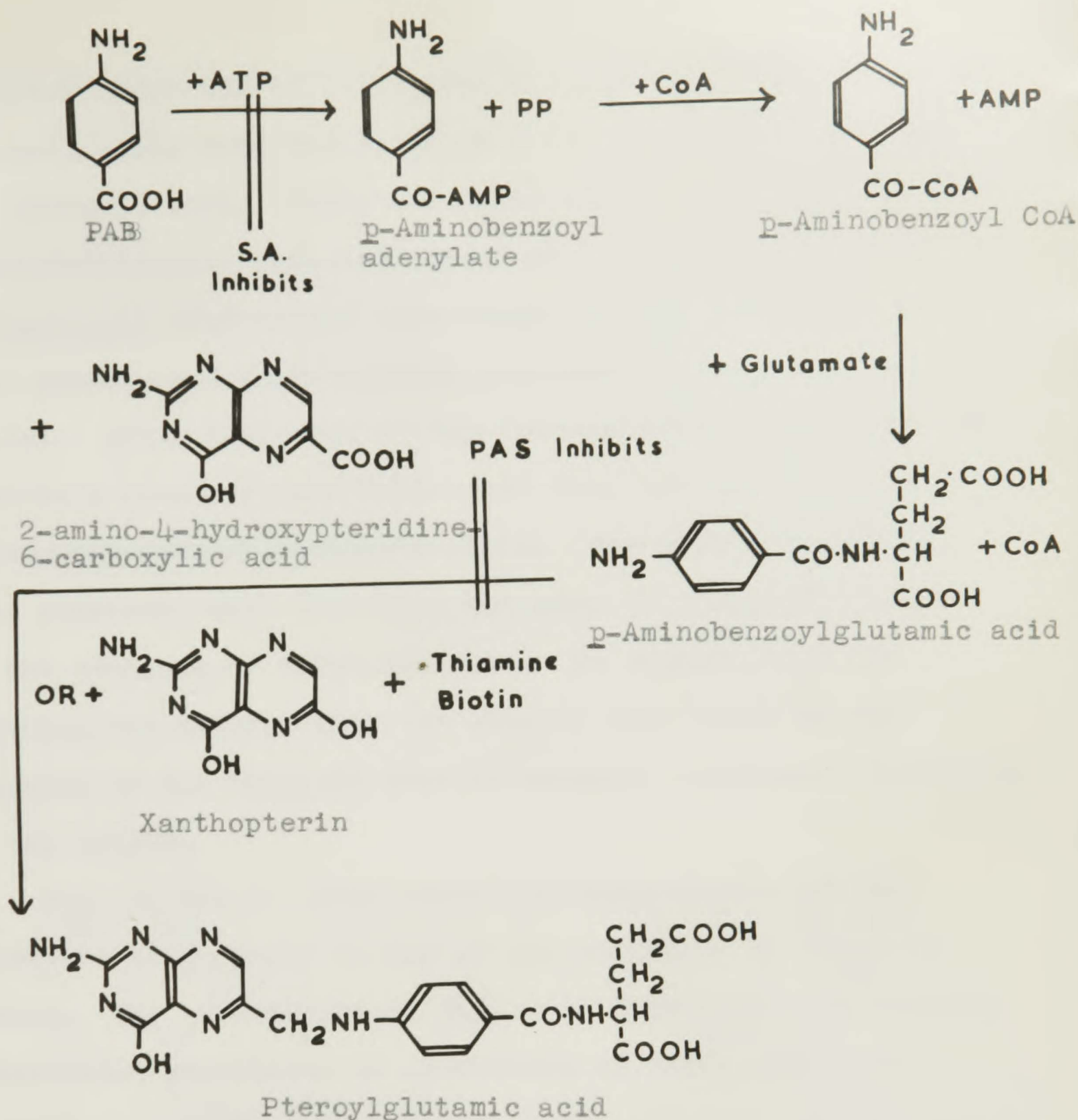
Another possible site for inhibition by sulphonamides might be the conjugation of PAB with glutamic acid to give p-aminobenzoylglutamic acid (PABG). If this reaction is suppressed by sulphonamides, then in the presence of PABG sulphonamides should be ineffective. Auhagen (1943) found PABG was eight to ten times as active as PAB in reversing sulphonamide-inhibition of Streptobacterium plantarum, though the reversal was competitive. The competition is suggestive of an initial breakdown to PAB, unless the utilization of PABG, as well as its formation, is inhibited by sulphonamide. With a number of other organisms, however, PABG is effective only at concentrations explicable by contamination with PAB, and the reversal obtained is competitive (Lampen and Jones, 1946a,b; 1947).

Recently, Japanese workers (Katunuma, Shoda and Noda, 1957; Katunuma and Shoda, 1957) have published an entire scheme of

folic acid biosynthesis in cell-free enzyme preparations from Mycobact. avium (Figure 4). Extracts, after treatment with ion exchange resin, produced PABG in a reaction mixture which contained PAB, glutamate, ATP and Mg^{++} ; the synthesis was increased four-fold by coenzyme A. In the absence of glutamate an active intermediate accumulates, as suggested by the formation of a compound giving a hydroxamic reaction with hydroxylamine and ferric chloride. Evidence was obtained for the formation of an acyl adenylate of PAB as a primary product of utilization of PAB. This first step, as well as the overall formation of PABG, was inhibited by sulphonamides (Katunuma N., private communication). It would be interesting to know whether PABG overcomes sulphonamide-inhibition of the growth of this organism; and if so, whether competitively or non-competitively. Also it is desirable that these observations should be extended to other organisms.

Pteroylglutamic acid was synthesized from 2-amino-4-hydroxy-pteridine-6-carboxylic acid and PABG in the presence of ATP. Xanthopterin could also be used as a substrate if thiamine and biotin were added as cofactors.

Comparisons of the synthesis of folic acid by sulphonamide-resistant and sulphonamide-sensitive strains of bacteria have not been numerous. Lascelles and Woods (1952) found that a sulphathiazole-resistant strain of Staph. aureus formed about ten times as much folic acid as its parent strain when



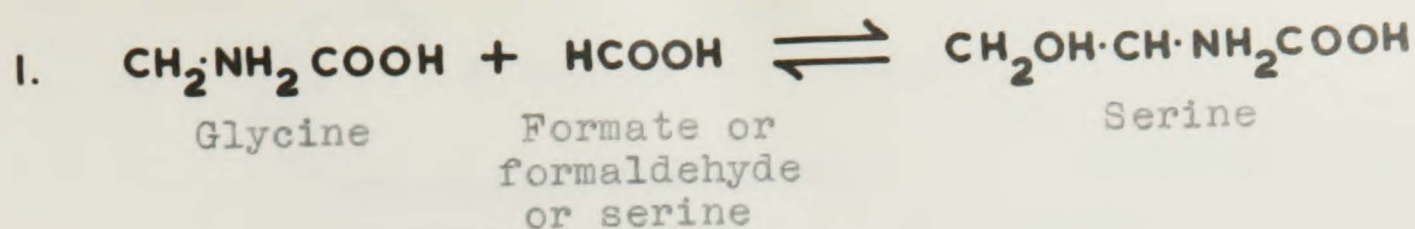
Scheme of biosynthesis of pteroylglutamic acid by cell-free enzymes from *Mycobact. avium*. (Katunuma et al. 1956; 1957).

organisms depleted of folic acid by growth in the presence of sulphathiazole were incubated in a mixture of PAB, glucose and glutamic acid. Sevag and Ishii (1958) have reported that a sulphathiazole-resistant strain of a PAB-requiring mutant of Esch.coli synthesized four times as much folic acid from PAB, glucose and intracellular precursors as the sensitive parent. After depletion of the intracellular precursors the resistant strain formed folic acid from PAB and 2-amino-4-hydroxypteridine-6-carboxylic acid. The sensitive strain also performed this reaction, but only if originally grown in the presence of sulphathiazole. It appears that the organism can acquire this new ability when grown in the presence of the drug and that it becomes a permanent character of the mutant.

Very little is known about the biosynthesis of the pteridine ring itself in any of the compounds in which it occurs. The investigations that have been made with various substituted pteridines as precursors of folic acid have recently been reviewed by Woods (1958) and will not be further discussed here as this work does not bear very directly on the problem of sulphonamide-resistance.

Functions of folic acid.

The metabolic reactions in which folic acid participates as a cofactor are shown in Figure 5. It might be that in

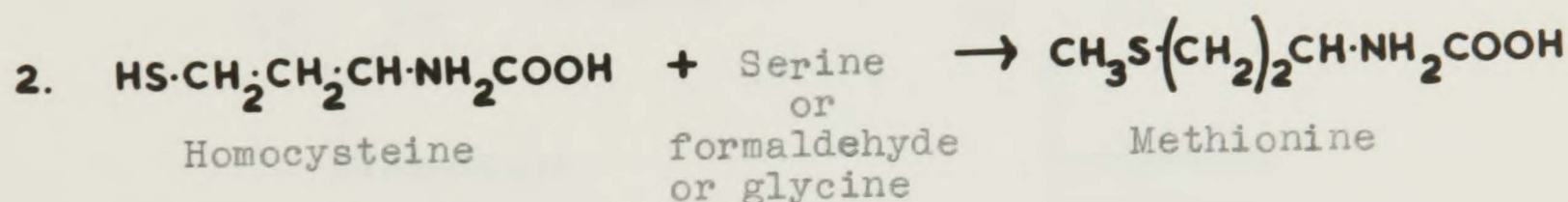


Stokstad (1954)

Lascelles and Woods (1954)

Blakley (1957 a,b)

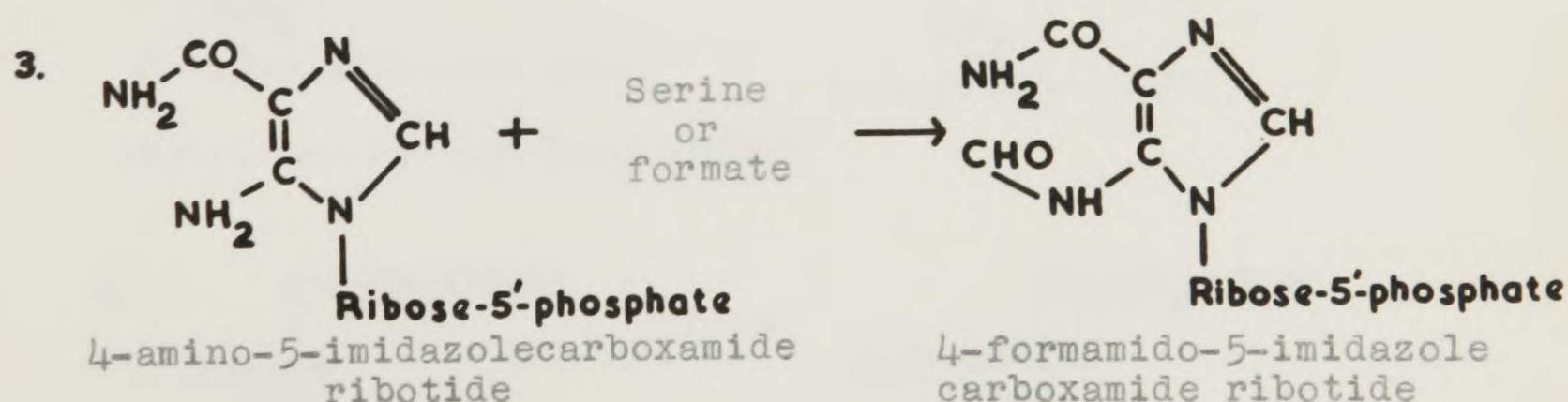
Kisliuk and Sakami (1955)



Woods (1952)

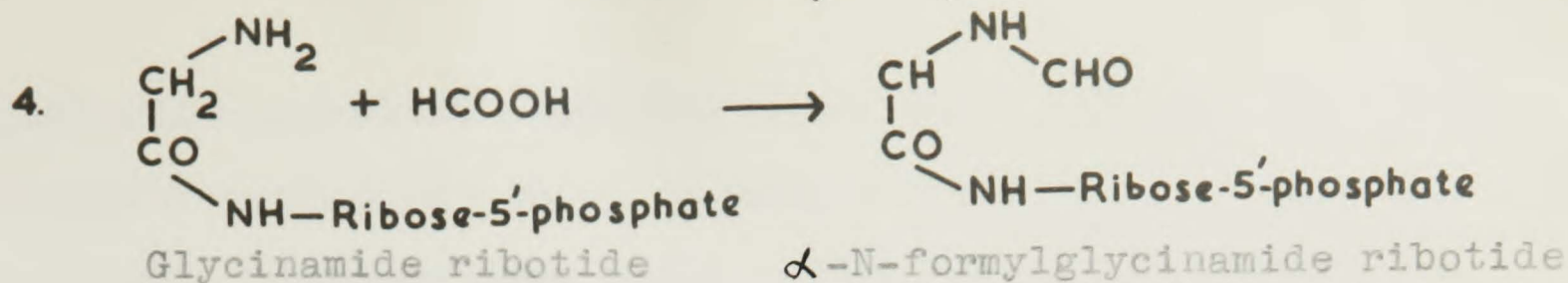
Cross and Woods (1954)

Kisliuk and Woods (1958)



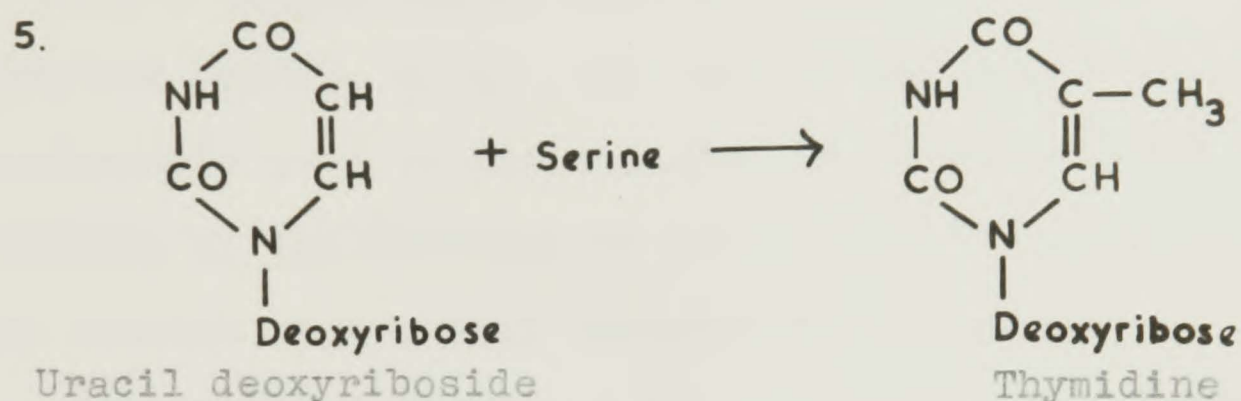
See review by Buchanan, Flaks, Hartman, Levenberg, Lukens and Warren. (1957).

Functions of folic acid coenzymes



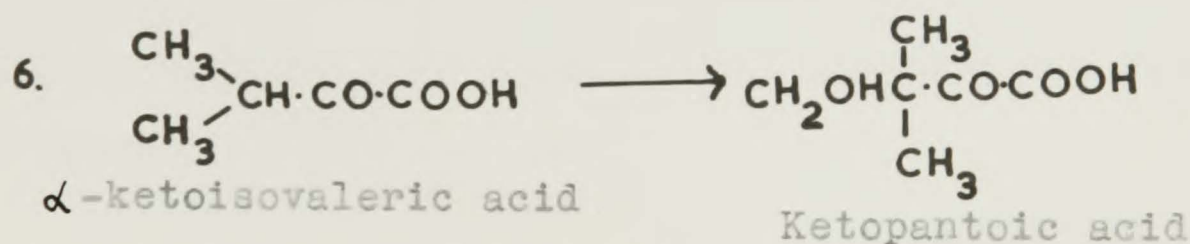
Buchanan et al. (1957)

Katunuma, Ishikawa and Watarai (1956)

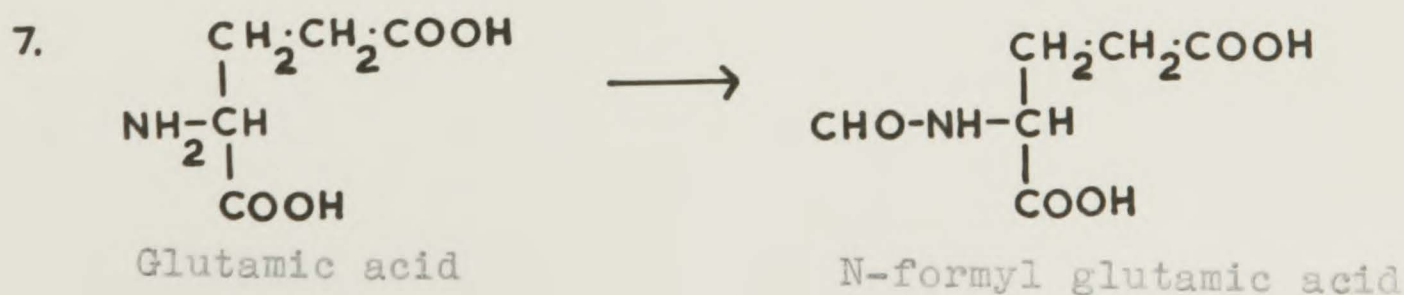


Friedkin and Roberts (1956)

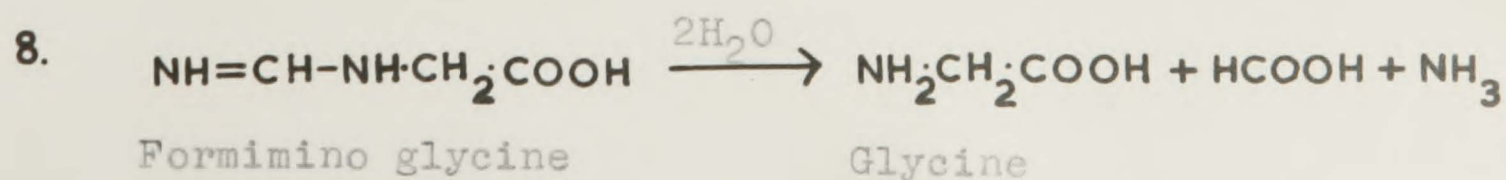
Blakley (1957 c)



Purko, Nelson and Wood (1954)



See review by Wright (1958)



See review by Wright (1958)

sulphonamide-resistant organisms these reactions take place by alternative mechanisms which no longer require utilization of folic acid. In every case where evidence has been obtained of the ultimate function of PAB or folic acid at some specific step in a biosynthetic pathway the reaction is one in which a single carbon unit is transferred (see review by Welch and Nichol, 1952). It must be emphasized that a considerable part of the experimental work on which our knowledge of the function of folic acid is based has come from studies with animal tissues and enzymes.

The chemical nature of the ultimate coenzyme form of folic acid is not known. It is certainly, however, a derivative of tetrahydropteroylglutamic acid or some closely related compound (see reviews by Greenberg and Jaenicke, 1957; and Wright, 1958).

CHAPTER IV

Materials and Methods

The usual precautions that are necessary in microbiological studies were observed, especially with regard to the purity of the chemicals and the cleanliness of the glassware. Chemicals were of A.R. quality when available. No special details about individual chemicals are given in Appendix I. Glassware was cleaned and sterilized as follows: bottles and beakers were washed with a solution of 1% sodium hydroxide followed by sterile distilled water.

Experimental Results

Section 1

Growth.

The growth, nutrition and general biochemical properties of sulphonamide-sensitive and resistant strains of staphylococci.

Sensitive and resistant strains of staphylococci, and of other strains that are resistant when isolated (Table I). A number of other organisms were used for control of pH and other tests. Details of the strains and methods of culture of the various organisms are given in Appendix II. From cultures were subcultured aseptically on agar at 37°C. For making suspensions a well known amount of a pure culture was added to a known volume of sterile distilled water and the mixture was shaken thoroughly.

Results are that strains of staphylococci are sensitive to sulphonamides (1943). The organisms were grown on agar and in broth. It is suggested that the results are not yet complete. In some cases the results are not yet complete.

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Materials and Methods

The usual precautions that are necessary in microbiological studies were observed, especially with regard to the purity of the chemicals and the cleanliness of the glassware. Chemicals were of A.R. quality when available; any special details about individual chemicals are given in Appendix I. Glassware was cleaned with chromic acid and was then washed very thoroughly with resin deionized water followed by glass distilled water.

Organisms.

The properties were investigated of three pairs of sensitive and resistant strains of staphylococci, and of one strain that was resistant when isolated (Table 1). A number of other organisms were used for assays of PAB and folic acid. Details of the origins and methods of culture of the various organisms are given in Appendix II. Stock cultures were subcultivated monthly and stored at 2°. Four working subcultures were made every month from a stock culture, and each was used for one week to provide daily cultures.

Staph.lactis 2102 is the strain that was called Staph.aureus 2102 by Lascelles and Woods (1952). The organism forms golden coloured colonies, but it is coagulase negative and does not ferment mannitol. In modern classifications

Table 1Strains of staphylococci used in present work

Parent strain	Resistant strain	Degree of resistance	Origin of resistant strain
<u>Staph.lactis 2102</u>	* <u>2102R</u>	x50	Serial passage of 2102 in medium B + sulphathiazole
"	<u>2102R2</u>	x50	"
<u>Staph.aureus JHM</u>	<u>JHMR</u>	x20	Serial passage of JHM in medium B + sulphathiazole
<u>Staph.aureus H</u>	* <u>HR2</u>	x200	Serial passage of H in medium B + sulphathiazole
	<u>Staph.aureus R122</u>		Sulphonamide-resistant when isolated

* Provided by Dr J.Lascelles.

Full details of the composition of all media used are

(Breed, Murray and Smith, 1957; Shaw, Stitt and Cowan, 1951) the distinctive property of Staph.aureus is the possession of the coagulase enzyme, while pigment formation is not regarded as of taxonomic value since variants with altered pigmentation arise frequently. Staph.aureus is also able to ferment mannitol.

In the seventh edition of Bergey's manual (Breed et al., (1957)) the coagulase negative staphylococci are all assigned to one species, Staph.epidermidis. However, the description of this species differs at several points from the properties of Staph.2102. Thus, 2102 grew very poorly anaerobically, and did not grow in presence of 10% sodium chloride; a clot as well as acid was formed in litmus milk medium and gelatin was not liquified. None of the descriptions of other species of the Micrococcaceae fitted 2102 more closely.

The classification used by the N.C.T.C. places all aerobic, catalase positive, Gram positive cocci in the genus Staphylococcus. Again, Staph.aureus is distinguished by the coagulase reaction. The coagulase negative strains are divided on the basis of the production of acid and acetoin from glucose. Staph.2102 resembles closely the description of Staph.lactis n.sp. in that it is coagulase negative, produces acid but not acetoin from glucose, and its colonies are not pink. It was decided to re-name Staph.aureus 2102 as Staph.lactis 2102.

Media.

Full details of the composition of all media and the

methods of preparation are given in Appendix III. Most growth experiments with staphylococci were done in medium B, which was a semi-defined medium based on vitamin-free acid-hydrolyzed casein. Medium B was modified from the staphylococcal medium described by Lascelles and Woods (1952) by the replacement of DL-cysteine by L-cysteine hydrochloride, and by doubling the amounts of disodium hydrogen phosphate and potassium dihydrogen phosphate. Medium B₂ was a completely defined medium suitable for the growth of the strains of Staph. aureus, in which the casein hydrolyzate was replaced by amino acids.

Procedure for growth experiments.

Except where stated otherwise, growth tests were done in 6"x $\frac{3}{4}$ " rimmed "Pyrex" test tubes. Double strength medium was added and other additions including inoculum were made to a final volume after inoculation of 5 ml. The tubes were autoclaved at 10lb per sq.in. for 7 min., cooled, and sterile additions and inocula added. The tubes were incubated at 37° in a wire basket sloped at about 10° to the horizontal to expose a large surface of the culture liquid ("semi-aerobic" conditions). Each experiment was always set up in duplicate.

Inocula were prepared from fresh 16-18 hr cultures on agar slopes by making a suspension of organisms in water and diluting this serially in water to a desired colorimeter reading. Such dilutions were made from suspensions of cell density about 10 as read in quarter inch diameter tubes, with neutral density filter (1.0) in an EEL photoelectric

colorimeter (Evans Electroselenium Co.Ltd., Harlow, Essex). Small inocula were used routinely to minimize the danger of contamination by nutrients carried over with the inoculum; normally, 5 ml medium was inoculated with 0.1 ml of a suspension of organisms diluted to $\frac{1}{50}$ EEL 1 (equivalent to about 4×10^3 organisms per ml of inoculated medium). In some cases the organisms were washed once in water before the serial dilution. Whether or not a washed inoculum was used made very little difference in growth tests.

Growth was estimated routinely by visual examination at intervals of about 12 hr during incubation. When quantitative results were required, cell density was assessed in the EEL colorimeter, with uninoculated medium to give the zero setting. The relation between EEL reading and dry weight of suspensions of organisms in water was linear for each of the strains of staphylococci, up to a reading of 30-35. The dry weights corresponding to an EEL reading of 10 were:

<u>Staph.lactis 2102</u>	0.15 mg/ml
<u>Staph.aureus JHM</u>	0.21 "
<u>Staph.aureus H</u>	0.23 "
<u>Staph.aureus R122</u>	0.18 "

The resistant substrains gave relations similar to their parent strains.

Estimations of viable organisms were made by plating on peptone agar measured volumes of suspensions which had been serially diluted to give about 50 colonies on a plate. The significance of viable counts on cultures of staphylococci is

not easy to assess, since the tendency of the organisms to grow in groups means that a single colony may have arisen either from one organism or a group. For this reason, the results of viable counts on different suspensions of the same organism were very variable. Very roughly, 1 ml of an EEL 10 suspension corresponded to 10^8 colonies.

Procedure for measurement of growth curves.

Cultures were incubated in $6 \times 6\frac{1}{2} \times \frac{1}{2}$ T-tubes which were rocked in a water bath at 37° , 35 times per min. by a mechanical shaker with an excursion of 4 in. The tubes were optically matched and shaped to fit directly into an EEL colorimeter so that growth could be assessed at intervals without the need to withdraw samples from the tubes. EEL readings measured in T-tubes and in quarter inch diameter tubes on the same culture, with neutral density filter, agreed well. The volume of culture used in T-tubes was 10 ml, with 0.2 ml of standard inoculum.

Conditions for anaerobic growth.

The usual conditions for growth experiments were followed, but the tubes were incubated upright in a McIntosh and Fildes jar containing an atmosphere of hydrogen and 5% (v/v) carbon dioxide. The jar was three times emptied and filled with this mixture before the platinized asbestos capsule was heated for 45 min. while the jar was in the incubator. The early stages

of growth were observed through the sides of the jar, which was not opened at all until the end of the experiment. With a facultative anaerobe, it is essential to remove all traces of oxygen to ensure that growth is strictly anaerobic. In most experiments, two similar series of tubes were prepared; one was incubated anaerobically, and the other aerobically.

Biochemical tests.

The strains were examined by various biochemical tests (coagulase, fermentations, etc) using methods described in standard works on bacteriology (Mackie and McCartney, 1931; Kolmer and Boerner, 1945).

Organisms were grown in a series of tubes containing saline B and sulphathiazole. Usually the concentration of inhibitor was doubled between each tube of the series, but sometimes the levels were closer. Organisms from the highest level of sulphathiazole at which growth occurred after 2-3 days were used to inoculate a fresh series of tubes. Continuation of this procedure led to a gradual loss of sensitivity, which, however, was not consistent at every step (Tables 3 and 4). The resistant strains that were produced were called *Staph. lactis* 2102R2 and *Staph. aureus* 2102R. The

CHAPTER V

Isolation of sulphonamide-resistant strains

Two pairs of sulphonamide-sensitive and resistant strains of staphylococci were available at the start of this work, Staph.lactis 2102 and 2102R and Staph.aureus H and HR2. The resistant strains were developed by Dr J. Lascelles by serial passage in the semi-defined medium B.

Further substrains resistant to sulphathiazole were developed from Staph.lactis 2102 and Staph.aureus JHM. Sulphathiazole was used because the organisms were initially more sensitive to this drug in medium B than to other sulphonamides tested (Table 11). Medium B was chosen for the development of resistant strains because of the greater sensitivity of the parent strains in this medium (Table 2).

Organisms were grown in a series of tubes containing medium B and sulphathiazole. Usually the concentration of inhibitor was doubled between each tube of the series, but sometimes the levels were closer. Organisms from the highest level of sulphathiazole at which growth occurred after 2-3 days were used to inoculate a fresh series of tubes. Repetition of this procedure led to a gradual loss of sensitivity, which, however, was not consistent at every step (Tables 3 and 4). The resistant strains that were produced were called Staph.lactis 2102R2 and Staph.aureus JHMR. The

Table 2

Sensitivity of Staph.lactis 2102 and
Staph.aureus JHM to sulphathiazole
in different media

Medium	Maximum toler- ated conc. of sulphathiazole (M)	Incubation time (hr) for growth at max.conc. of sulphathiazole	Organism
Peptone water	10^{-4}	70	<u>S.aureus JHM</u>
Tryptic meat broth	$> 10^{-3}$	16	"
Medium B	5×10^{-6}	80	"
Peptone water	10^{-3}	60	<u>S.lactis 2102</u>
Tryptic meat broth	4×10^{-3}	60	"
Medium B	10^{-5}	90	"

Inocula. Passage 1

Passages 2-15

Table 3

Development of resistance to sulphathiazole
in Staph. lactis 2102 grown in medium B.

Passage No.	Interval (days) between passages	Maximum tolerated concentration of sulphathiazole (M)	Time of incubation (days) for growth at maximum conc. of sulphathiazole	Relative inoculum size
1	2½	2x10 ⁻⁵	2½	1.0
2	3	4x10 ⁻⁵	3	1.0
3	2	6x10 ⁻⁵	2	1.0
4	2½	6x10 ⁻⁵	2	1.0
5	2½	10 ⁻⁴	2	1.0
6	4	10 ⁻⁴	3	1.0
7	3	10 ⁻⁴	3	1.0
8	3	10 ⁻⁴	2½	1.0
9	4	2x10 ⁻⁴	3½	1.0
10	3½	2x10 ⁻⁴	2½	1.0
11	3	2x10 ⁻⁴	3	1.0
12	4	4x10 ⁻⁴	4	1.0
13	4	4x10 ⁻⁴	1	1.0
14	4	2x10 ⁻⁴	3½	0.1
15	3	2x10 ⁻⁴	2	0.1
16	4	4x10 ⁻⁴	4	0.1

Inocula. Passage 1 0.1 ml of a suspension of cells from an agar slope diluted in water to $\frac{1}{5}$ EEL 1.

Inocula. Passages 2-16 0.1 ml of each preceding culture diluted in water to $\frac{1}{5}$ EEL 1 (passages 2-13) or $\frac{1}{50}$ EEL 1 (passages 14-16)

Table 4

Development of resistance to sulphathiazole
in Staph. aureus JHM grown in medium B

Passage No.	Interval (days)	Maximum tolerated conc. of S-T (μ)	Time of incubation (days) for growth	Relative inoculum size
1	2½	2×10^{-5}	2½	1.0
2	3	10^{-5}	3	1.0
3	2	4×10^{-5}	2	1.0
4	2½	6×10^{-5}	2	1.0
5	3	10^{-4}	4½	1.0
6	4	2×10^{-4}	3	1.0
7	3	2×10^{-4}	3	1.0
8	3	10^{-4}	2½	1.0
9	4	10^{-4}	2	1.0
10	3½	2×10^{-4}	3½	1.0
11	3	2×10^{-4}	3	1.0
12	4	2×10^{-4}	< 4	1.0
13	4	2×10^{-4}	3	1.0
14	4	4×10^{-4}	4	0.1
15	2½	4×10^{-4}	2½	0.1
16	2	8×10^{-4}	2	0.1
17	2	10^{-3}	2	0.1
18	2	10^{-3}	2	0.1

Inocula. Passage 1 0.1 ml of a suspension of cells from an agar slope diluted to $\frac{1}{5}$ EEL 1.

Passages 2-18 0.1 ml of each preceding culture diluted in water to $\frac{1}{5}$ EEL 1 (passages 2-13) or $\frac{1}{50}$ EEL 1 (passages 14-18).

sensitivities of the parent and resistant strains in medium B are compared in Table 5.

The first attempts to develop resistant substrains were unsuccessful (Table 6) probably owing to the use of too small an inoculum in the early passages, and to the period between subcultivations being too long.

If resistance is developed by the selection of spontaneous mutants, it is desirable to use a large inoculum to increase the probability of a mutant being present. However, the size of the inoculum must not be too large, for then the drug fails to inhibit growth so that it is impossible to select resistant organisms (Table 7). The insensitivity is not due to the selection in one step of highly resistant mutants, since cultures which have grown from a large inoculum in the presence of drug show an increased, not decreased, sensitivity on subcultivation (Table 8). The probable reason is that the organisms in the inoculum initially contain sufficient sulphonamide antagonists to permit a limited number of cell divisions, even in the presence of a high concentration of drug (Hotchkiss and Evans, 1957). If a large inoculum is used, these divisions may be enough to produce visible growth.

For the earlier passages, therefore, the size of the inoculum ($0.1 \text{ ml } \frac{1}{5}$ EEL 1 per 5 ml) was ten times that normally used in growth experiments. The inoculum was reduced to the usual size in the final passages to assess the degree of

Table 5

Sensitivities of strains of staphylococci
to sulphathiazole in medium B.

Organism (days)	Max. tolerated conc. of sulpha- thiazole(M) after various times of incubation (hr)				Approximate increase in resistance
	25	40	50	80	
<u>Staph.lactis 2102</u>	5×10^{-6}	5×10^{-6}	5×10^{-6}	10^{-5}	
" <u>2102R</u>	$< 2.5 \times 10^{-4}$	2.5×10^{-4}	5×10^{-4}	5×10^{-4}	50
" <u>2102R2</u>	2.5×10^{-5}	2.5×10^{-4}	5×10^{-4}		50
<u>Staph.aureus JHM</u>	$< 2.5 \times 10^{-6}$	2.5×10^{-6}	5×10^{-6}	10^{-5}	
" <u>JHMR</u>	2.5×10^{-5}	5×10^{-5}	10^{-4}	2×10^{-4}	20
<u>Staph.aureus H</u>	$< 2.5 \times 10^{-6}$	2.5×10^{-6}	5×10^{-6}	10^{-5}	
" <u>HR2</u>	5×10^{-4}	5×10^{-4}	10^{-3}		200
<u>Staph.aureus R122</u>	$< 2.5 \times 10^{-4}$	4×10^{-4}		7.5×10^{-4}	100 *

* Compared with Staph.aureus JHM or H.

Table 6

Failure of *Staph. lactis* 2402 to develop sulphonamide-resistance when a small inoculum was used, and intervals between subcultivations were prolonged.

Passage No.	Interval (days)	Maximum tolerated conc. of sulphathiazole (M)	Time of incubation (days) for growth at maximum conc. of sulphathiazole.
1	2	10^{-5}	2
2	$4\frac{1}{2}$	4×10^{-5}	3
3	$4\frac{1}{2}$	6×10^{-5}	$4\frac{1}{2}$
4	$7\frac{1}{2}$	4×10^{-5}	$5\frac{1}{2}$
5	7	6×10^{-5}	$6\frac{1}{2}$
6	$6\frac{1}{2}$	$< 4 \times 10^{-5}$	$6\frac{1}{2}$

Inocula. Passage 1 0.1 ml of a suspension of cells from an agar slope diluted in water to $\frac{1}{50}$ EEL 1.

Passages 2-6 0.1 ml of each preceding culture diluted in water to $\frac{1}{50}$ EEL 1.

Table 7

Relation between sensitivity to sulphathiazole
and size of inoculum

S.aureus JHM in medium B

Maximum toler- ated conc. of sulphathiazole (M)	Incubation time (hr) for growth at max. conc. of sulphathiazole	Inoculum per 5 ml	Relative inoculum size
5×10^{-6}	80	0.1 ml A	0.02
5×10^{-5}	60	0.1 ml B	0.2
2×10^{-4}	25	0.1 ml C	1.0
5×10^{-4}	13	0.2 ml C	2.0
2×10^{-3}	13	0.4 ml C	4.0
2×10^{-3}	13	0.8 ml C	8.0

Inocula Cells from an agar slope suspended in water

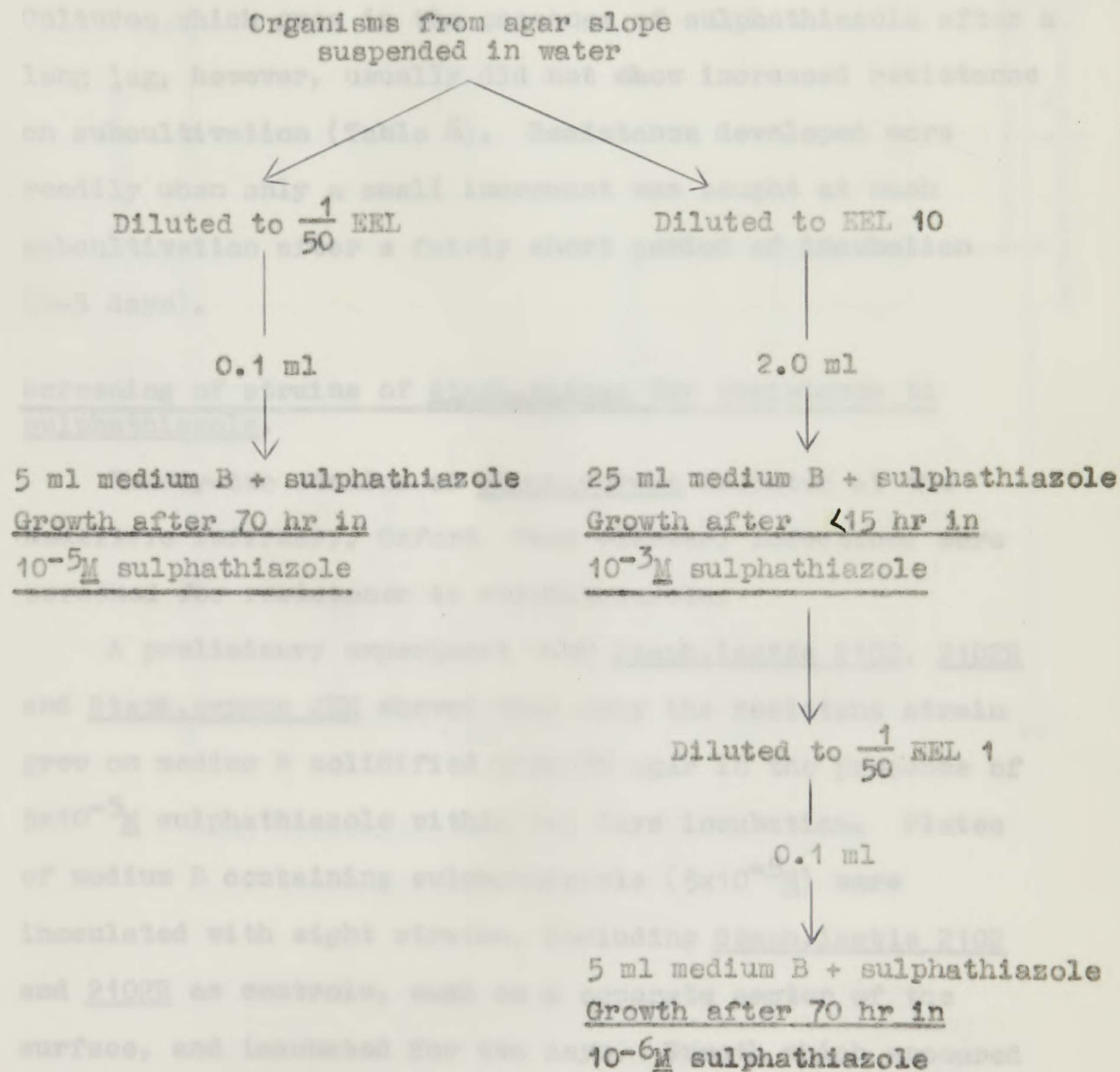
A Diluted to $\frac{1}{50}$ EEL 1

B Diluted to $\frac{1}{5}$ EEL 1

C Diluted to EEL 1.

Table 8

Increased sensitivity on subcultivation of organisms grown in presence of high concentrations of sulphathiazole.



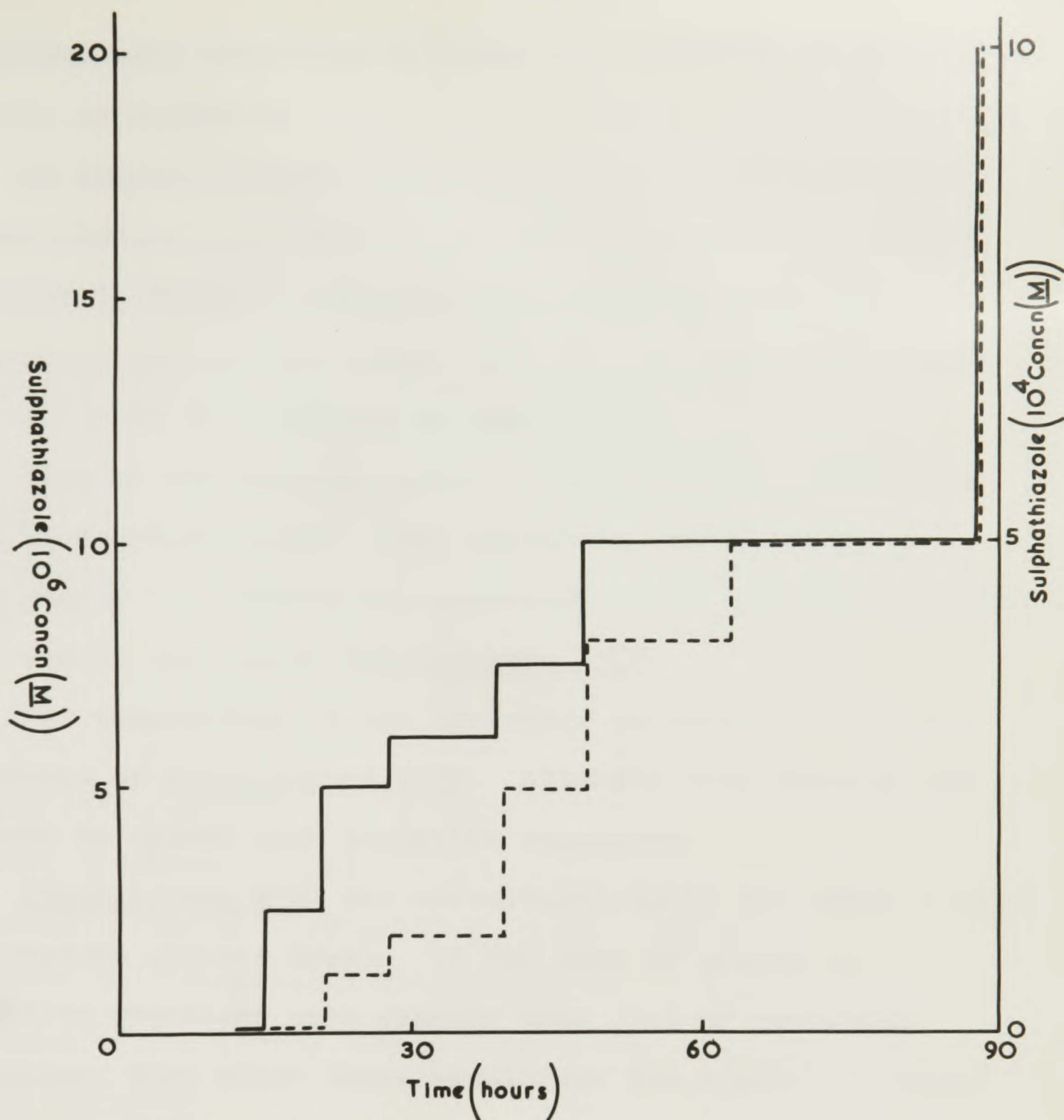
resistance when indefinite propagation was required from a small number of organisms.

Growth occurs in progressively higher concentrations of sulphathiazole as the time of incubation is increased (Figure 6). Cultures which grew in the presence of sulphathiazole after a long lag, however, usually did not show increased resistance on subcultivation (Table 6). Resistance developed more readily when only a small increment was sought at each subcultivation after a fairly short period of incubation (2-3 days).

Screening of strains of *Staph.aureus* for resistance to sulphathiazole.

Thirty-two strains of *Staph.aureus* isolated at the Radcliffe Infirmary, Oxford, from clinical infections were screened for resistance to sulphathiazole.

A preliminary experiment with *Staph.lactis* 2102, 2102R and *Staph.aureus* JHM showed that only the resistant strain grew on medium B solidified with 2% agar in the presence of $5 \times 10^{-5} \text{M}$ sulphathiazole within two days incubation. Plates of medium B containing sulphathiazole ($5 \times 10^{-5} \text{M}$) were inoculated with eight strains, including *Staph.lactis* 2102 and 2102R as controls, each on a separate sector of the surface, and incubated for two days. Growth which appeared in this time was subsequently tested in liquid culture for sulphathiazole-resistance. Control plates containing no



— Staph. lactis 2102 (left-hand scale)
 ---- Staph. lactis 2102R (right-hand scale)

Sensitivity of Staph. lactis 2102 and 2102R
 to sulphathiazole in medium B after increasing
 times of incubation.

sulphathiazole were used to check the viability of the inocula on medium B.

To prepare inocula, drops of sterile water were put on to the surface of a sterile paraffin wax plate. One drop was lightly touched with a platinum loop charged with organisms from an agar slope. Part of the inoculated water drop was used to inoculate an agar plate.

Five of the strains tested on plates were resistant to 5×10^{-5} M sulphathiazole. When tested in liquid medium B only one of the strains was resistant to 10^{-3} M sulphathiazole. This strain was called Staph.aureus R122.

For comparisons it was desirable to have a sensitive substrain of Staph.aureus R122. Attempts were made by two methods to select such sensitive organisms.

Staph.aureus R122 was subcultured daily for about 5 weeks in tryptose glucose broth. If the rate of growth of sensitive organisms were greater than that of resistant organisms, then under these conditions the resistant strain might be overgrown by sensitive organisms. However, at the end of the experiment no loss of resistance was found.

A replica-plating technique was also used. About 40 colonies of Staph.aureus R122 were grown on medium B. By using a nylon velvet pad replicas of this plate were made on to two fresh plates of medium B, one of which contained 10^{-2} M sulphathiazole. If a colony of sensitive organisms were

present on the original plate then a replica of this colony should appear on the plate which contained no sulphathiazole, but not on the plate in which sulphathiazole was present. About 250 colonies were tested in this way, but none showed any increased sensitivity to sulphathiazole.

Other attempts to select resistant organisms.

Attempts to select resistant organisms by single exposure of a large inoculum to sulphathiazole were unsuccessful. When inocula heavy enough to give confluent growth of sensitive strains in the absence of sulphathiazole were incubated on solid medium B containing inhibitory concentrations of sulphathiazole, ($5 \times 10^{-3} \text{M}$ - 10^{-4}M) the lag period before growth became visible was extended, but growth, when it appeared, was confluent. Individual colonies of increased resistance were never found. Organisms which had grown in the presence of sulphathiazole on a plate showed no increase in resistance when subcultivated on a plate or in liquid culture.

Two attempts were made to obtain resistant mutants of Staph. aureus JHM by ultra violet irradiation. A culture in medium B in the logarithmic phase of growth (BEL 25) was diluted 1:500 in medium B without glucose, and 5 ml was exposed in a quartz tube for 4 min. to a source of U.V. radiation. The quartz tube was shaken mechanically to spread its contents round the walls in a thin uniform film during

the irradiation. Counts before and after irradiation showed that in each experiment 99.90% of the organisms were killed.

Graded portions of the irradiated culture (0.25 ml - 1.0 ml) were used to inoculate tubes of medium B, some of which contained sulphathiazole (10^{-3} M, 10^{-4} M and 5×10^{-5} M). There was no growth in medium which contained sulphathiazole, and the cultures which grew in the absence of the drug showed no increase of resistance when subcultivated in medium B containing sulphathiazole.

AEROBIC GROWTH.

Conditions of good aeration were provided by incubating cultures in rocked T-tubes (Chapter IV) and growth curves for the various strains were determined. All strains of *Staph. aureus*, whether resistant or not, grew at about the same rate and to about the same final level in medium B without sulphathiazole. Typical growth curves, for *Staph. aureus* 402 and 4102, are shown in Figure 7. There was a lag of about 12 hr before logarithmic growth began, and growth reached its highest level after about 48 hr, at EML 40 to 45 (about 1 mg dry wt/ml). *Staph. lactis* 2102 and its resistant substrains grew to a lower final level; 2102 and 2102R2 grew to about EML 25 to 30, and 2102R grew to only EML 20. *Staph. lactis* 2102R2 consistently showed a lag before

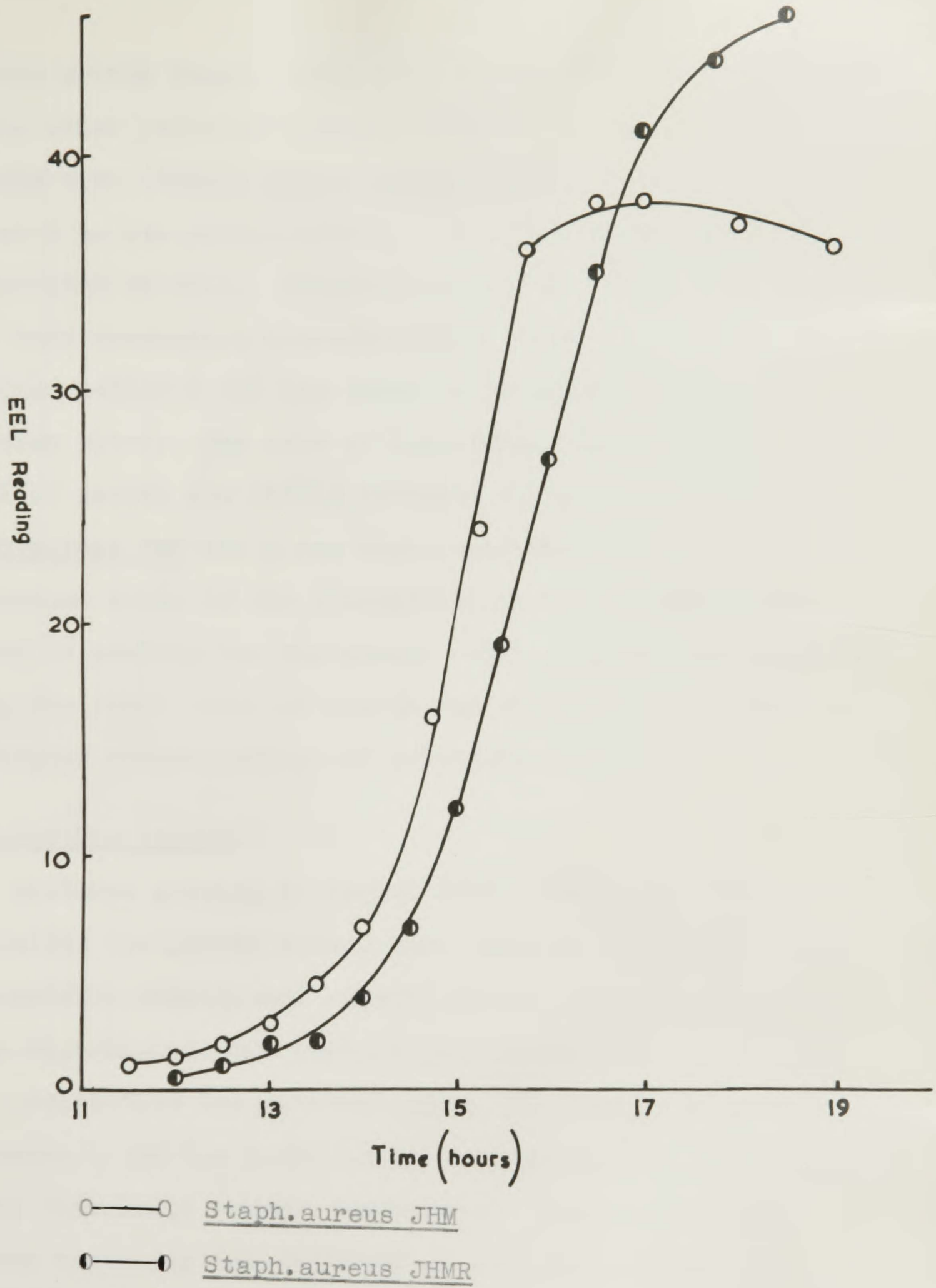
CHAPTER VI

Growth and inhibition

More complex media, such as tryptic meat broth or peptone water did not give much heavier or more rapid growth of staphylococci than medium B. Staph.lactis 2102 and its substrains grew less well than the strains of Staph.aureus in the media that were tested. Growth of the various strains of staphylococci was investigated in medium B under various conditions and in the presence of different inhibitors.

Aerobic growth.

Conditions of good aeration were provided by incubating cultures in rocked T-tubes (Chapter IV) and growth curves for the various strains were determined. All strains of Staph.aureus, whether resistant or not, grew at about the same rate and to about the same final level in medium B without sulphathiazole. Typical growth curves, for Staph.aureus JHM and JHMR, are shown in Figure 7. There was a lag of about 12 hr before logarithmic growth began, and growth reached its highest level after about 18 hr, at EEL 40 to 45 (about 1 mg dry wt/ml). Staph.lactis 2102 and its resistant substrains grew to a lower final level; 2102 and 2102R2 grew to about EEL 25 to 30, and 2102R grew to only EEL 20. Staph.lactis 2102R2 consistently showed a lag before



Growth of *Staph. aureus* JHM and JHMR in medium B.

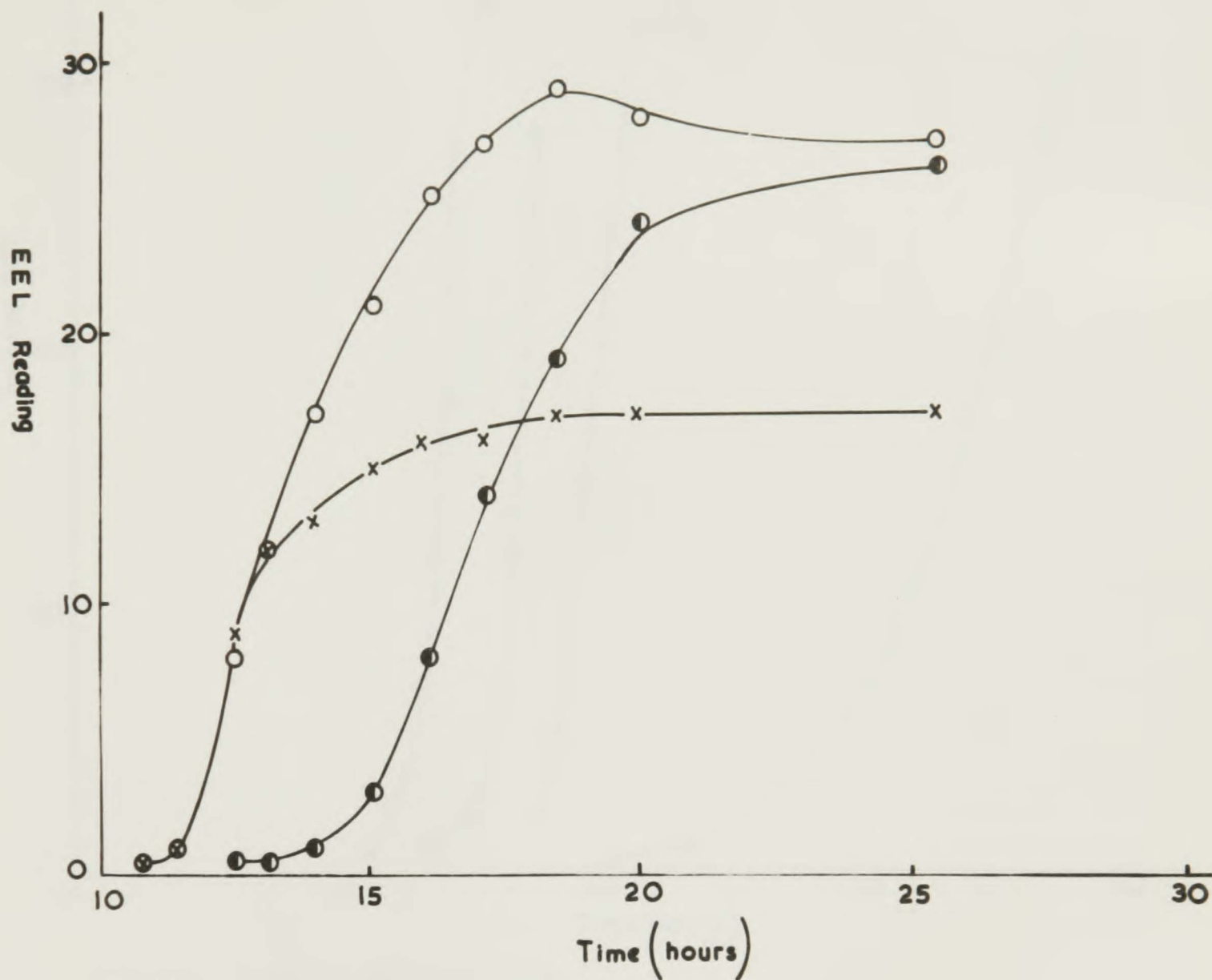
visible growth began, about 4 hr longer than the parent strain or the other resistant strain (Figure 8). At the beginning of this work (1955), Staph.lactis 2102R grew as heavily in medium B as its parent strain, but after three years it no longer grew as well. Compare Figures 11 (1955) and 8 (1958).

When increasing concentrations of sulphathiazole are added to medium B the lag phase is progressively lengthened (Figures 9-11). The rate of logarithmic growth or the final level of growth are little affected by sulphathiazole with Staph.aureus JHM and H and their resistant strains, the only difference being in the concentrations of sulphathiazole needed to prolong the lag phase. With Staph.lactis 2102 and 2102R the final level of growth was reduced in presence of the higher concentrations of sulphathiazole.

Semi-aerobic growth.

Cultures growing in sloped 6"x $\frac{3}{4}$ " tubes, the normal conditions for growth tests, were regarded as being under semi-aerobic conditions. Growth curves were not measured in these experiments, but certain generalizations may be made from qualitative inspections during the course of incubations. In medium B the lag phase before visible growth was rather longer than under aerobic conditions, about 15 hr, and maximum turbidity was not reached for about 30 hr. The highest level of growth of strains of Staph.aureus was EEL 30-35 and 20-25 for Staph.lactis 2102 and its substrains.

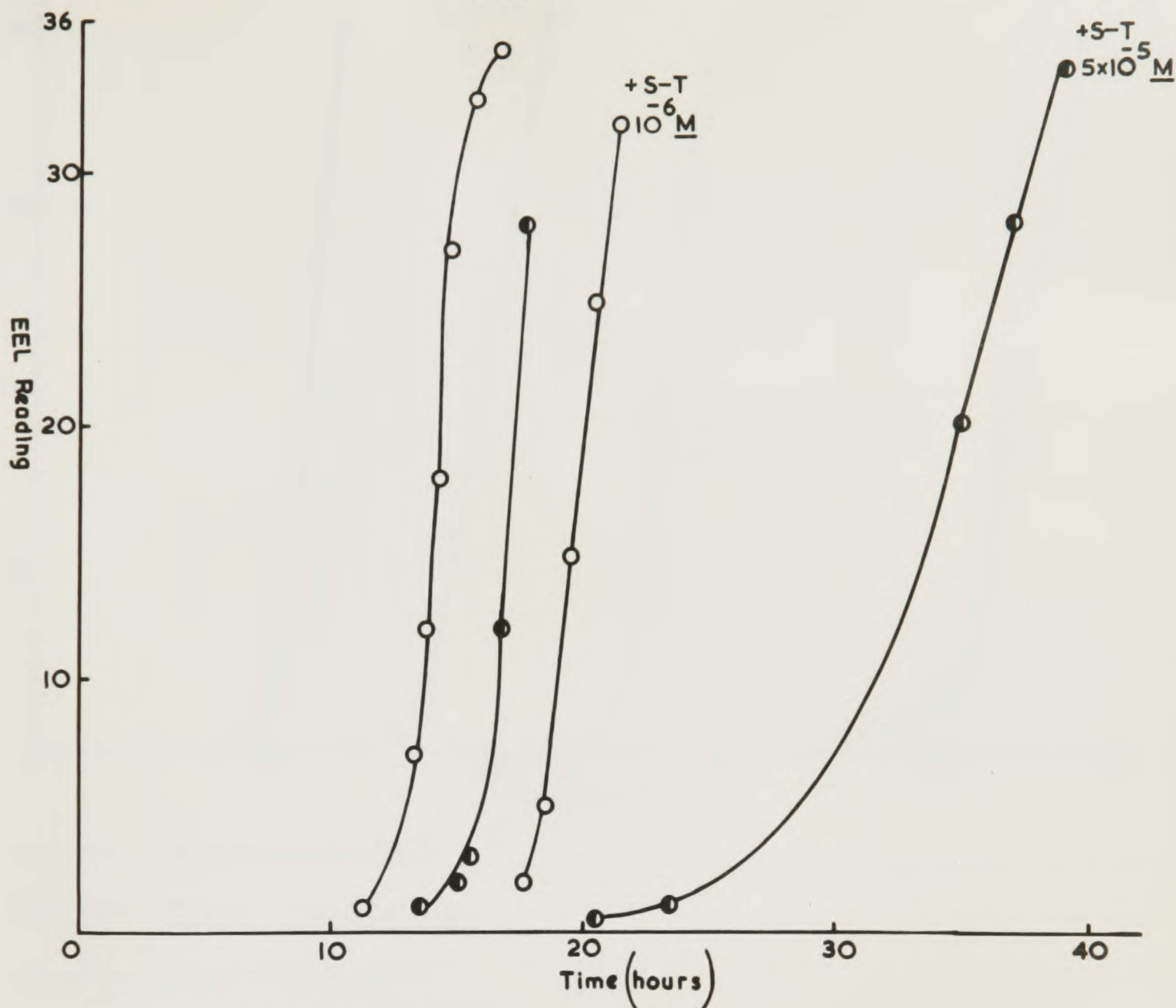
FIGURE 8



○ — ○ Staph. lactis 2102
 x — x Staph. lactis 2102R
 ● — ● Staph. lactis 2102R2

Growth of *Staph. lactis* 2102, 2102R and 2102R2 in medium B

FIGURE 9

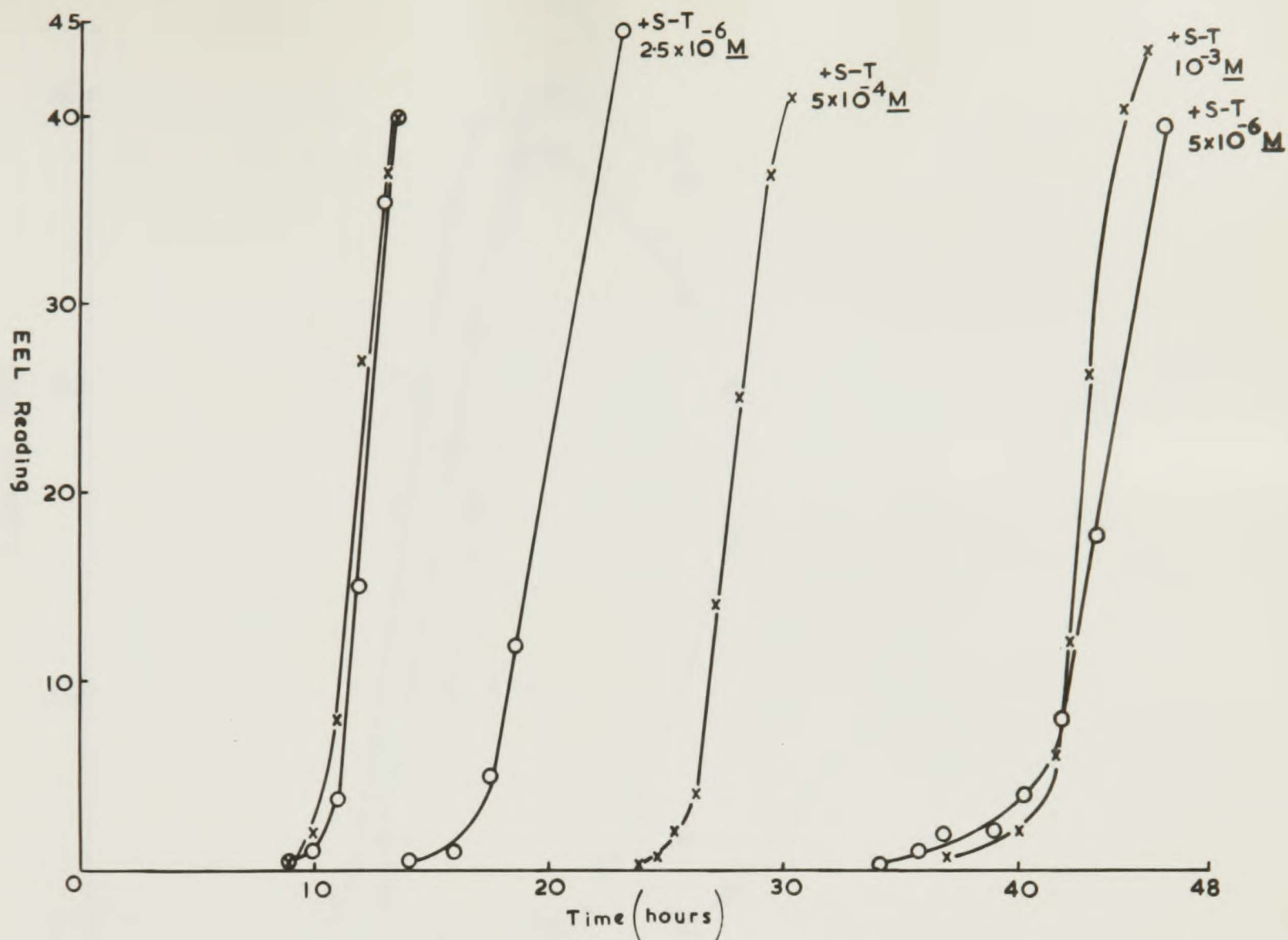


○—○ Staph. aureus JHM

●—● Staph. aureus JHMR

(S-T = Sulphathiazole)

Growth of Staph. aureus JHM and JHMR in medium B
± sulphathiazole.

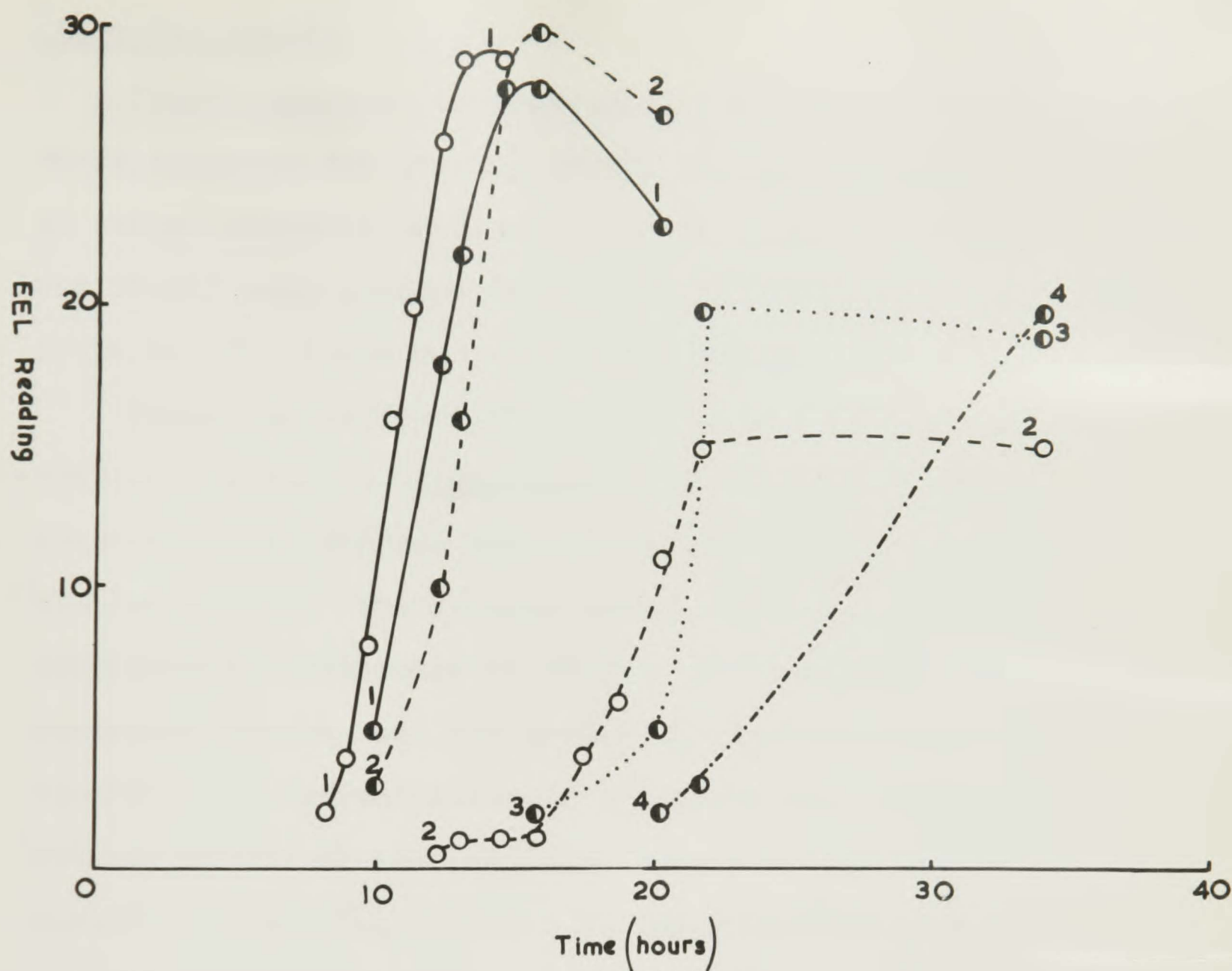


○ — ○ Staph. aureus H

x — x Staph. aureus HR2

(S-T = Sulphathiazole)

Growth of *Staph. aureus* H and HR2 in medium B $\pm$ sulphathiazole



○ — ○ Staph. lactis 2102

● — ● Staph. lactis 2102R

1 = - Sulphathiazole

2 = + $10^{-5}M$ "

3 = + $10^{-4}M$ "

4 = + $10^{-3}M$ "

Growth of Staph. lactis 2102 and 2102R in medium B
± sulphathiazole

Anaerobic growth.

Staph.aureus is a facultative anaerobe. A defined medium which supported the aerobic growth of Staph.aureus fails to do so under anaerobic conditions unless carbon dioxide, pyruvate and uracil were present (Fildes and Richardson, 1937). The presence of nitrate was also advantageous.

Sevag and Steers (1949) found that a sulphonamide-resistant strain of Staph.aureus was unable to grow anaerobically, whereas the parent strain could grow to a limited extent. The defined medium that was used lacked the supplements recommended by Fildes and Richardson for anaerobic growth, and the effect of their addition is not reported. A concentration of sulphathiazole which reduced aerobic growth of the sensitive strain by 40% completely prevented anaerobic growth. It was suggested that sulphathiazole inhibited anaerobic pathways of metabolism, and that these were completely lost in the resistant strain.

Staph.lactis 2102 and 2102R grew very poorly anaerobically in several media (Table 9). Only in medium B supplemented with yeast extract was there appreciable growth. Lascelles (1956) described a strain of Staph.aureus which grew anaerobically in presence of haematin, but addition of haematin to medium B did not permit anaerobic growth of Staph.lactis 2102, indeed aerobic metabolism was inhibited.

Table 9

Anaerobic growth of Staph. lactis 2102 and 2102R

Medium	2102		2102R	
	Anaerobic	Aerobic	Anaerobic	Aerobic
Medium B	0	11	0	7
" + anaerobic suppl.	0	15	0	12
" + yeast extract	6	24	6	23
" + anaerobic suppl.				
+ haematin	0	0		
Peptone water	3	10	0	10
Tryptic meat broth	2	20		
" " + anaerobic suppl.	2	20		

Numbers are EEL readings after about 100 hr incubation

Anaerobic supplement:- Uracil $5 \times 10^{-5} \underline{\text{M}}$ Na Pyruvate $1.6 \times 10^{-3} \underline{\text{M}}$ Na nitrate $2 \times 10^{-3} \underline{\text{M}}$ Na bicarbonate $2.5 \times 10^{-2} \underline{\text{M}}$

Yeast extract 1.25g "Bacto" yeast extract/l

Haematin 2 mg/l

An absolute requirement for the anaerobic supplement of uracil, pyruvate, bicarbonate and nitrate was not found in Staph.aureus JHM and H and their resistant strains in medium B, though addition of these compounds improved anaerobic growth (Table 10). Anaerobic growth was very poor in the absence of glucose. Both sensitive and resistant strains were able to grow in medium B in the presence of sulphathiazole (10^{-6} M and 10^{-4} M respectively).

After the amino acid requirements of the various strains of staphylococci had been established, a fully defined medium, (medium B₂), was prepared, suitable for the aerobic growth of Staph.aureus JHM and H and their resistant substrains.

Staph.aureus H grew anaerobically in this medium only when the supplements were present, and sulphathiazole (10^{-6} M) did not inhibit. Staph.aureus JHM and JHMR grew anaerobically in unsupplemented medium B₂ but growth was more rapid when the supplements were present. Neither Staph.aureus JHM nor H grew anaerobically if glucose was omitted from medium B₂. Under aerobic conditions, the anaerobic supplement was inhibitory to Staph.aureus JHM and JHMR in the fully defined medium.

Although there are differences between one sensitive strain and another in the requirements for anaerobic growth, there is no evidence that the development of resistance or the presence of sulphathiazole affect anaerobic metabolism.

The dependence of Table 10

Anaerobic growth of Staph. aureus JHM and H
and resistant substrains

Medium	JHM	JHMR	H	HR2
	a A	a A	a A	a A
Medium B	12 22	12 21	11 29	14 30
" + ST	13 28	8 26	7 29	13 28
Medium B + anaerobic suppl.	24 31	26 32	33 33	29 39
" + " + ST	30 28	29 31	30	28 40
Medium B + anaerobic suppl.-glucose	1 13	2 14	1 17	4 16
" + " - " + ST	1 13	2 15	2	4 14
Medium B ₂	9 17	11 18	3 19	
" + ST			3 18	
Medium B ₂ + anaerobic suppl.	19 0	20 0	27	
" + " + ST			26	
Medium B ₂ + anaerobic suppl.-glucose	1 0	1 0	2	
" + " - " + ST			2	

a = Anaerobic

Numbers are EEL readings after about 100 hr incubation.

A = Aerobic

ST = Sulphathiazole 10^{-6}M and 10^{-4}M for sensitive and resistant strains respectively in medium B
 $5 \times 10^{-7}\text{M}$ and 10^{-4}M in medium B₂

The dependence of anaerobic growth on the presence of glucose in addition to pyruvate is very striking, but has not been investigated further since it does not seem relevant to the present study of resistance.

Inhibition of growth by sulphonamides.

The sensitivities of the various strains of staphylococci towards sulphathiazole, sulphapyridine, sulphadiazine and sulphanilamide were determined in medium B (Table 11). The highest concentrations of sulphapyridine and sulphadiazine that could be used were limited by the low solubilities of these substances.

All strains were most sensitive to sulphathiazole. Sulphadiazine was the next most potent inhibitor and sulphanilamide was least effective. Resistant strains show cross-resistance to all the drugs in the group; resistance is not specific to the drug used to develop the resistant strain.

Duration of resistance to sulphonamides.

All the strains of staphylococci were subcultured once every month on agar slopes for two and a half years, a total of about thirty transfers in the absence of drug. The sensitivities of the organisms to sulphathiazole were unchanged at the end of this time.

Table 11

Cross-resistance of strains of staphylococci to sulphonamides in medium B.

Organism	Sensitivity towards			
	Sulphathiazole(M)	Sulphapyridine(M)	Sulphadiazine(M)	Sulphenilamide(M)
<u>Staph. lactis 2102</u>	10^{-5} (90)	$\times 10^{-4}$ (90)	$\times 10^{-4}$ (90)	5×10^{-4} (90)
" <u>2102R</u>	5×10^{-4} (60)	$\times 2 \times 10^{-4}$ (20)	$\times 2 \times 10^{-4}$ (20)	$\times 10^{-3}$ (20)
" <u>2102R2</u>	5×10^{-4} (50)			
<u>Staph. aureus JHM</u>	5×10^{-6} (80)	$\times 10^{-4}$ (80)	2×10^{-5} (80)	2×10^{-4} (80)
" <u>JHMR</u>	$\times 10^{-4}$ (60)	$\times 2 \times 10^{-4}$ (40)	$\times 2 \times 10^{-4}$ (40)	$\times 10^{-3}$ (40)
<u>Staph. aureus H</u>	10^{-5} (80)	10^{-4} (70)	5×10^{-5} (80)	$\times 4 \times 10^{-4}$ (70)
" <u>HR2</u>	$\times 10^{-3}$ (60)	$\times 2 \times 10^{-4}$ (30)	$\times 2 \times 10^{-4}$ (30)	$\times 10^{-3}$ (30)
<u>Staph. aureus R122</u>	5×10^{-4} (40)	$\times 2 \times 10^{-4}$ (30)	$\times 2 \times 10^{-4}$ (30)	$\times 10^{-3}$ (30)

Values in brackets are times (in hours) of incubation before growth occurred in the highest tolerated concentration of drug.

* Highest concentration tested.

Effect of aminopterin on growth.

The effect of aminopterin on growth of the various strains of staphylococci was tested in medium B, to find out whether sulphonamide-resistant strains also show decreased sensitivity to this drug, which is believed to inhibit the reduction of the pteridine ring of dihydropteroylglutamic acid to the tetrahydro level. (Futterman, 1957; Wright, Anderson and Herman, 1958).

However, there was no inhibition of any of the strains, even by the highest concentrations of aminopterin that were used (10^{-4} M). Aminopterin was still inactive in the presence of just sub-inhibitory concentrations of sulphathiazole.

Effect of p-nitrobenzoic acid on growth.

p-Nitrobenzoic acid is an analogue of PAB and it is inhibitory to some organisms, especially in the presence of aspartic acid (Davis, 1951). Its effect on growth of the various strains of staphylococci in medium B was investigated to find out whether there was any cross-resistance towards sulphonamides and p-nitrobenzoic acid (Table 12). The arguments of Davis and Maas (1952) about the mechanisms of resistance to antagonists of PAB operating in Esch.coli are based largely on the presence or absence of cross-resistance towards p-nitrobenzoic acid and sulphonamides (Chapter II).

Table 12

Sensitivities in medium B of strains of staphylococci
to p-nitrobenzoic acid

Organism	Maximum tolerated conc. of p-nitrobenzoic acid (M) after various times of incubation				
	20 hr	30 hr	40 hr	50 hr	80 hr
<u>Staph. lactis</u> 2102	10^{-4}	3×10^{-4}	10^{-3}		
" 2102R	10^{-4}	10^{-3}	10^{-3}		
" 2102R2	3×10^{-5}	3×10^{-5}	10^{-3}		
<u>Staph. aureus</u> JHM	10^{-6}	10^{-4}	10^{-4}	10^{-3}	
" JHMR	10^{-6}	10^{-4}	10^{-3}	10^{-3}	
<u>Staph. aureus</u> H	10^{-4}	10^{-3}	10^{-3}	2×10^{-3}	
" HR2	10^{-4}	10^{-3}	2×10^{-3}	2×10^{-3}	
<u>Staph. aureus</u> R122	$< 2.5 \times 10^{-4}$	$< 2.5 \times 10^{-4}$	$< 2.5 \times 10^{-4}$	2.5×10^{-4}	10^{-3}

CHAPTER VII

General biochemical properties and nutrition

All the strains of staphylococci were Gram positive, catalase positive organisms which grew in typical staphylococcal clusters. Colonies on solid media were round, 1-2 mm diameter, and usually became pigmented after keeping at room temperature for a few days. Staph.lactis 2102 and Staph.lactis 2102R2 gave an orange coloured growth on tryptic meat agar slopes, and the medium became a reddish-brown colour, apparently through the excretion of a porphyrin-like substance. Staph.lactis 2102R excreted less of this coloured material, and the growth was whiter in colour. Staph.aureus JHM, JHMR and R122 gave growth on agar slopes which was white, but which, after keeping at room temperature in darkness for a day or two became golden in colour. Staph.aureus H and HR2 never produced a pigment.

Staph.lactis 2102 and its substrains were coagulase negative. All the other strains were coagulase positive and were therefore considered to belong to the species Staph.aureus (Breed et al, 1957). A distinctive property of Staph.aureus is the ability to grow in the presence of high concentrations of sodium chloride (Chapman, 1945). Staph.lactis 2102 and its substrains grew poorly on Chapman's medium No.110 (Chapman, 1946), which contains 7.5% sodium chloride. The other strains all grew well on this medium.

The strains were examined by various biochemical tests (Table 13). These experiments failed to reveal any conspicuous differences between sensitive and resistant strains. The ability to reduce nitrate was irregularly distributed, but this property did not show a clear relation to sulphonamide-resistance.

Determination of amino acid requirements.

The procedure was essentially that of Gladstone (1937). The effect on growth was observed of omitting each amino acid in turn from a complete mixture (Appendix III) which supported growth. The usual conditions for growth tests were employed, with a washed inoculum.

The amino acids that were most often needed for growth were valine, arginine and histidine (Table 14). It was surprising that all strains were able to dispense with cystine, and use sodium thioglycollate as a main source of sulphur. Gladstone (1937) found that most strains of staphylococci tested required cystine, and that it was difficult to remove this requirement by "training". No marked changes in requirements for amino acids seemed to accompany resistance to sulphathiazole in strains of Staph.aureus.

Staph.lactis 2102 grew only after 30 hr when aspartic acid was omitted, and after 60 hr in the absence of glutamic acid. The growth of Staph.lactis 2102R was as rapid in the absence of either acid as it was in the complete medium (25 hr).

Table 13

Biochemical properties of strains of staphylococci

	2102	2102R	2102R2	JHM	JHMR	H	HR2	R422
Fermentation	Glucose	A	A	A	A	A	A	A
	Sucrose	A	A	A	A	A	A	A
	Galactose	A	A	A	A	A	A	A
	Fructose	A	A	A	A	A	A	A
	Mannose	-	-	-	A	A	A	A
	Lactose	A	A	A	A	A	A	A
	Maltose	A	A	A	A	A	A	A
	Mannitol	-	-	-	A	A	A	A
	Erythritol	-	-	-	-	-	-	-
	Glycerol	A	A	A	A	A	A	A
	Rhamnose	-	-	-	-	-	-	-
	Dulcitol	-	-	-	-	-	-	-
	Sorbitol	-	-	-	-	-	A	A
	Xylose	A	A	A	-	-	-	-
	Ribose	A	A	A	A	A	A	A
	Arabinose	A	A	A	-	-	-	-
	Indole form ⁿ .	-	-	-	-	-	-	-
	Voges-Proskauer	-	-	-	-	-	Tr	Tr
	Methyl red	-	-	-	+	+	+	+
	Nitrate reduction	++	+++	-	Tr	Tr	-	-
	H ₂ S form ⁿ .	-	-	-	-	-	-	-
	Litmus milk	A C	A C	A C	A C	A C	A C	A C
	Gelatin liquifact ⁿ .	-	-	-	+++	+++	+++	+++

A = Acid formation

C = Clot formation

No gas was formed from any of the substances used in fermentation tests

Table 14

Amino acid and vitamin requirements of strains of staphylococci

Omission	2102	2102R	2102R2	JHM	JHMR	H	HR2	R122
Tryptophan	0	0	0	0	0	+	+	+
Tyrosine	0	0	0	+	+	+	+	+
Valine	0	0	0	0	0	0	0	0
Alanine	0	0	0	+	+	+	+	+
Threonine	0	0	0	+	+	(+)	(+)	+
Leucine	0	0	0	+	+	0	0	(+)
Methionine	0	0	0	(+)	(+)	(+)	(+)	(+)
Phenylalanine	0	0	0	+	+	0	0	+
Arginine	0	0	0	0	0	0	0	0
Histidine	0	0	0	0	0	+	+	0
Aspartic acid	(+)	+	+	0	0	+	+	0
Glutamic acid	(+)	+	0	+	+	+	+	+
Proline	+	+	+	0	0	0	0	+
Glycine	+	+	+	(+)	(+)	+	+	(+)
Cystine	+	+	+	+	+	+	+	+
Biotin	0	0	0	+	+	+	+	+
Nicotinic acid	+	+	+	0	0	0	0	0
Thiamine	+	+	+	0	0	0	0	0

0 = No growth

+ = Growth

(+) = Growth delayed

 } When constituent is omitted
 } from the medium

If both amino acids were omitted at the same time, Staph.lactis 2102 failed to grow after six days, whereas 2102R grew after two days. Staph.lactis 2102R2 grew in the absence of aspartic acid as rapidly as in the complete medium, but glutamic acid was absolutely required for growth after four days.

Growth under semi-aerobic conditions in this defined medium was not so rapid as in medium B. There was a lag of 25-30 hr before growth became visible, and the level of highest growth was only about EEL 25 with the strains of Staph.aureus, and about EEL 15 with Staph.lactis 2102 and its substrains.

It was possible to grow each strain in a medium that contained only those amino acids that were found to be essential when they were omitted singly from a complete mixture of 15 amino acids. However, growth was always improved when non-essential amino acids were also present.

From the results of these experiments, a completely defined medium was devised which was suitable for growth of Staph.aureus JHM and Staph.aureus H and their sulphonamide-resistant substrains. This medium was called medium B₂.

Breakdown of aspartic and glutamic acids by Staph.lactis 2102 and 2102R.

Because of the differences in the requirements of Staph.lactis 2102 and 2102R for aspartic and glutamic acids, it was decided to investigate the oxidation of these acids by

washed suspensions of the two organisms. Organisms were grown on the surface of casein digest agar, harvested and washed as described by Gale and Rodwell (1948). The oxidation of the amino acids was followed in Warburg manometers as described by these authors. After an hour the manometers were removed from the bath, and the contents of the cups were centrifuged to remove the cells. Ammonia in the supernates was estimated in the apparatus of Markham (1942).

The results are given in Table 15. Aspartic acid was hardly oxidized at all, and the rate of attack on glutamic acid was not very high. There were no differences between the sensitive and resistant organisms in their aerobic metabolism of these amino acids. Growth of Staph.lactis 2102R in the presence of 10^{-2} M sulphathiazole did not affect its ability to oxidise aspartic or glutamic acid.

Vitamin requirements.

Qualitative.

All strains grew in medium B, which contains biotin, thiamine and nicotinic acid. The effect on growth was observed of omitting each vitamin in turn from this medium. Inocula were prepared as in the experiments on amino acid requirements.

All the Staph.aureus strains required nicotinic acid and thiamine but not biotin for full growth. Staph.lactis 2102 and its substrains required only biotin for full growth.

Quantitative.

Table 15

Oxidation of L-aspartic and L-glutamic acids by
Staph.lactis 2102 and 2102R

	<u>2102</u>		<u>2102R</u>		<u>2102R + sulphathiazole</u>	
	Q_{O_2}	Q_{NH_3}	Q_{O_2}	Q_{NH_3}	Q_{O_2}	Q_{NH_3}
*L-aspartic acid	1	1	1	0	1	0
^x L-glutamic acid	22	10	25	9	26	8
Blank (no substrate)	4	18	6	16	4	15

$$Q_{O_2} = \mu l O_2 \text{ uptake/hr/mg}$$

$$Q_{NH_3} = \mu l NH_3 \text{ liberated/hr/mg}$$

* Corrected for blank.

Quantitative.

The concentrations of nicotinic acid and thiamine required for growth of Staph.aureus H and HR2 in medium B₂ were titrated in the presence of sulphathiazole (5×10^{-7} M and 10^{-4} M respectively) and in its absence; 100 µg nicotinic acid/ml was sufficient to give full growth of both strains in the presence or absence of drug. There was no growth in the absence of nicotinic acid. Thiamine also was absolutely required; 0.5 µg/ml was sufficient to give full growth of both strains whether or not sulphathiazole was present.

EXTRACTS

Materials and Methods

Growth of *Escherichia coli* on p-aminobenzoic acid

Medium B (25 ml) after the addition of 1 ml of 1% p-aminobenzoic acid was inoculated with 2 ml of a suspension of *Escherichia coli* to a turbidity of about 0.5. The tubes were incubated for 15-16 hr at 37° in a rotary incubator. The suspensions were centrifuged out at 7000 rpm for 15 min. The culture supernatant with the culture

Experimental Results

Section 2

The metabolism of p-aminobenzoic acid and related compounds

Estimation of PAB

Two assay organisms were used, *Escherichia coli* and *Escherichia coli* 873/104, a PAB-resistant mutant. *Escherichia coli* 873/104 was one of the first organisms to be used for assay of PAB (Landy and Chase, 1954) and preliminary experiments were done with this organism to determine the optimum. However, its use involved some difficulties. 1. The concentration of PAB required for growth appeared to fall as the time of incubation was increased. Growth was read after 72 hr incubation, though growth is observed at concentrations of PAB as still remaining.

CHAPTER VIII

Materials and Methods

Growth of organisms for experiments with washed suspensions.

Medium B (25 ml after all additions) in a 7"x1 $\frac{1}{4}$ " tube was inoculated with 2 ml of a suspension of organisms made to a turbidity of about EEL 10. The tube was incubated for 14-16 hr at 37° in a sloped position. The organisms were centrifuged out at 3000 r.p.m. for 15 min. and washed once with the culture volume of 0.02M phosphate buffer pH 6.8 or 7.4. The washed organisms were resuspended in buffer or in the reaction mixture used for the experiment, to a density of about 0.5 mg dry wt per ml.

Estimation of PAB.

Two assay organisms were used, Acetobacter suboxydans 621 and Escherichia coli 273/384, a PAB-requiring mutant.

Acetobacter suboxydans was one of the first organisms to be used for assay of PAB (Landy and Dicken, 1942) and preliminary experiments were done with this organisms in preference to the mutant. However, its use involved some difficulties:

1. The concentration of PAB required for growth continued to fall as the time of incubation was increased. Assays were read after 72 hr incubation, though growth in sub-optimal concentrations of PAB was still proceeding.

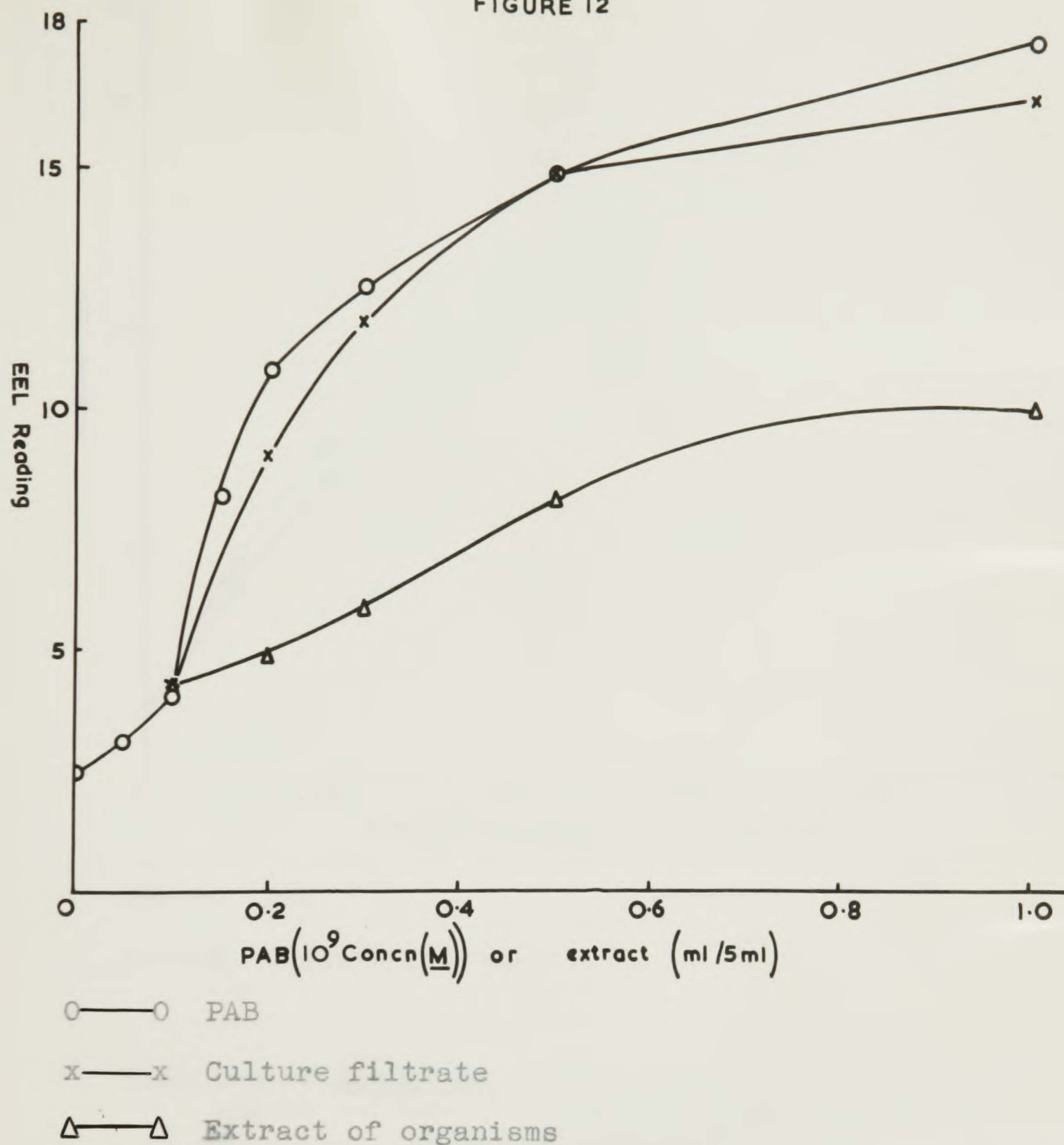
2. Graded portions of cell-free culture medium gave a response curve of the assay organism similar to that given by PAB, but extracts of staphylococcal cells gave a response curve quite unlike that produced by PAB (Figure 12) although this abnormal response was sensitive to inhibition by sulphathiazole. Since added PAB could be recovered quantitatively from cell extracts it was unlikely that an inhibitor of the assay organism was present.

3. The absolute requirement for PAB is very much decreased by a number of substances, some of which are products of PAB function (Lascelles and Cross, 1955).

Assays with Esch.coli 273/384 were more satisfactory. The medium was less complex, and kept better. (Appendix III). Assays could be read more quickly since growth in sub-optimal concentrations of PAB had ceased after 48 hr incubation. Cell extracts of staphylococci had no growth promoting effect at all; activity was found only in the culture filtrates. Response to graded quantities of culture filtrate, diluted 1:10 in water, followed the same curve as response to PAB (Figure 13) although growth in the presence of culture filtrates appeared rather sooner than did growth with PAB.

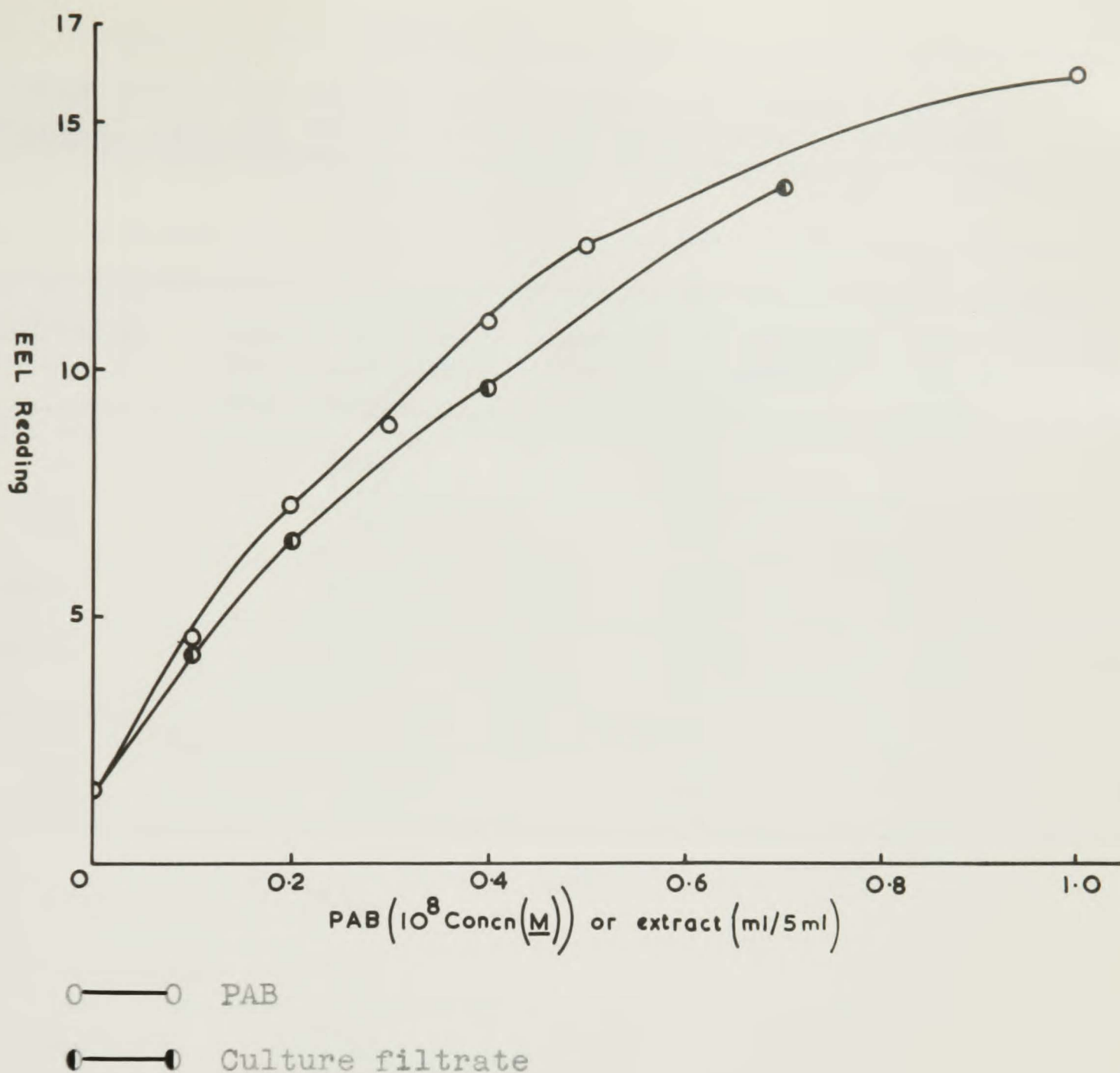
The requirement for PAB is reduced by the presence of methionine, xanthine, thymine and vitamin B₁₂ (Lampen, Jones and Roepke, 1949; de Haan, 1950). These sparing effects are illustrated in Table 16. If any of these compounds were added in sufficient concentration to the assay medium with

FIGURE 12



Response of Acetobacter suboxydans 621 to p-aminobenzoic acid and to culture filtrate of Staph.aureus JHM (grown in medium B) and to an extract of the organisms.

FIGURE 13



Response of Esch. coli 273/384 to p-aminobenzoic acid
 and to culture filtrate of Staph. aureus HR2
 (grown in medium B)

Table 16

Sparing effect of additions to medium on response of Esch. coli 273/384 to PAB and to staphylococcal extract.

Addition	Conc. of PAB(M) for half maxi- mal growth	Amount of extract for half maximal growth (ml)	Sparing effect of addition on PAB Extract	
-	3×10^{-9}	0.3		
Me	10^{-9}	0.1	3-fold	3-fold
Me+X	3×10^{-10}	0.02	10 "	15 "
Me+X+T	3×10^{-11}	0.003	100 "	100 "
Me+X+T+V+B ₁₂	No PAB needed		∞	∞
B ₁₂	10^{-9}	0.1	3-fold	3-fold

Me = Methionine (10^{-3} M)

X = Xanthine (10^{-4} M)

T = Thymine (10^{-4} M)

V = Valine (10^{-3} M)

B₁₂ = Vitamin B₁₂ (2 μ g/ml)

PAB in material under assay, then a falsely high value would be obtained for the PAB content of the extract. It was not practicable to overcome this difficulty by saturating the medium with these PAB sparing substances since they abolished the requirement for the factor. Instead, an attempt was made to show that these compounds were not present, in amounts sufficient to reduce the PAB requirement, in material under assay. This was done by demonstrating that the compounds gave equivalent sparing of PAB or of staphylococcal extract needed for half-maximal growth (Table 16). Had the compounds been present in extracts in amounts already sufficient to produce their PAB-sparing effects (which a rough calculation had shown to be unlikely), then it would not be expected that their further addition to the extract would give any sparing effect.

The minimal medium of Davis and Mingioli (1950) was used, modified by autoclaving the glucose in the medium. To double-strength medium (2.5 ml) in 6"x $\frac{3}{4}$ " tubes were added extract or PAB solution, and water to 4.9 ml. After autoclaving 10 lb for 7 min. the tubes were inoculated with 0.1 ml of a suspension of Esch.coli 273/384 which had been diluted in water to $\frac{1}{10}$ EEL 1 (about 5×10^5 organisms per ml inoculated assay medium). The tubes were incubated sloped at 37° for 48 hr, and growth was assessed in the EEL colorimeter. To prepare extracts for assay, cultures, including the organisms,

were heated for 5 min. in boiling water, and the supernates assayed after centrifugation.

Each assay included a standard response curve for PAB; each extract was assayed at two or three levels, and every tube was set up in duplicate. When more than one dose of extract fell in the range of the standard curve, the concentration of PAB in the extract was calculated from the results at each level, and an average was then taken. Good agreement ($\pm 10\%$) was found between the concentrations of PAB calculated from results at different levels of dilution of the extract. Very close duplication was found between similar tubes, and the assay showed no day-to-day variation in the range of response to PAB or in the level of growth in response to sub-optimal concentrations of PAB. Added PAB was recovered quantitatively from staphylococcal cultures.

An evaluation of the significance of the results of one assay was made by the method of factorial analysis described by Bliss (1956) for a non-randomized assay. Three points on the response curve to PAB and three on the response curve to an extract were used for this purpose. The precision of the assay was assessed by determining the statistic C, which measures the reliability of the assay slope; a value of 1.030 was obtained. The more nearly C approaches unity, the more precise is the assay, while an assay with C greater than two is of little value. The potency of the extract was determined

by factorial analysis, and the confidence limits of this value were assessed. This procedure gave a result of 144 ± 30 μg PAB per ml. Direct calculation from the assay readings in the ordinary way gave a value of 140 μg PAB per ml. The validity of the assumptions that are made in this factorial analysis, namely, a linear relation between the response and the log. dose, and agreement in slope of the response curves for different samples, was confirmed statistically.

Estimation of PAB in presence of sulphathiazole by chromatography followed by assay.

With PAB at the normal level used in assays (10^{-9}M - 10^{-8}M) the assay organism is inhibited by 10^{-7}M sulphathiazole. Consequently, PAB and sulphathiazole must be separated before PAB can be assayed.

A good separation of PAB and sulphathiazole was obtained on descending chromatograms run on Whatman No.1 paper for about 12 hr at room temperature, using as solvent n-butanol saturated with water.

R values	Sulphathiazole	0.50
f	PAB	0.15

The position of PAB on the chromatogram was found by running a marker strip which was subsequently cut off and examined by bioautography with Esch.coli 273/384. The region of the chromatogram which contained PAB was eluted with water, and the eluted material was assayed for PAB.

Recoveries of known amounts of PAB added to staphylococcal extracts were not quantitative, even when no sulphathiazole was present in the extracts, neither was recovery of PAB run alone quantitative. Washing the paper with 2N acetic acid before chromatography led to more rapid development (about 8 hr) but did not improve the recovery of PAB.

Assay of folic acid.

Lb.casei factor.

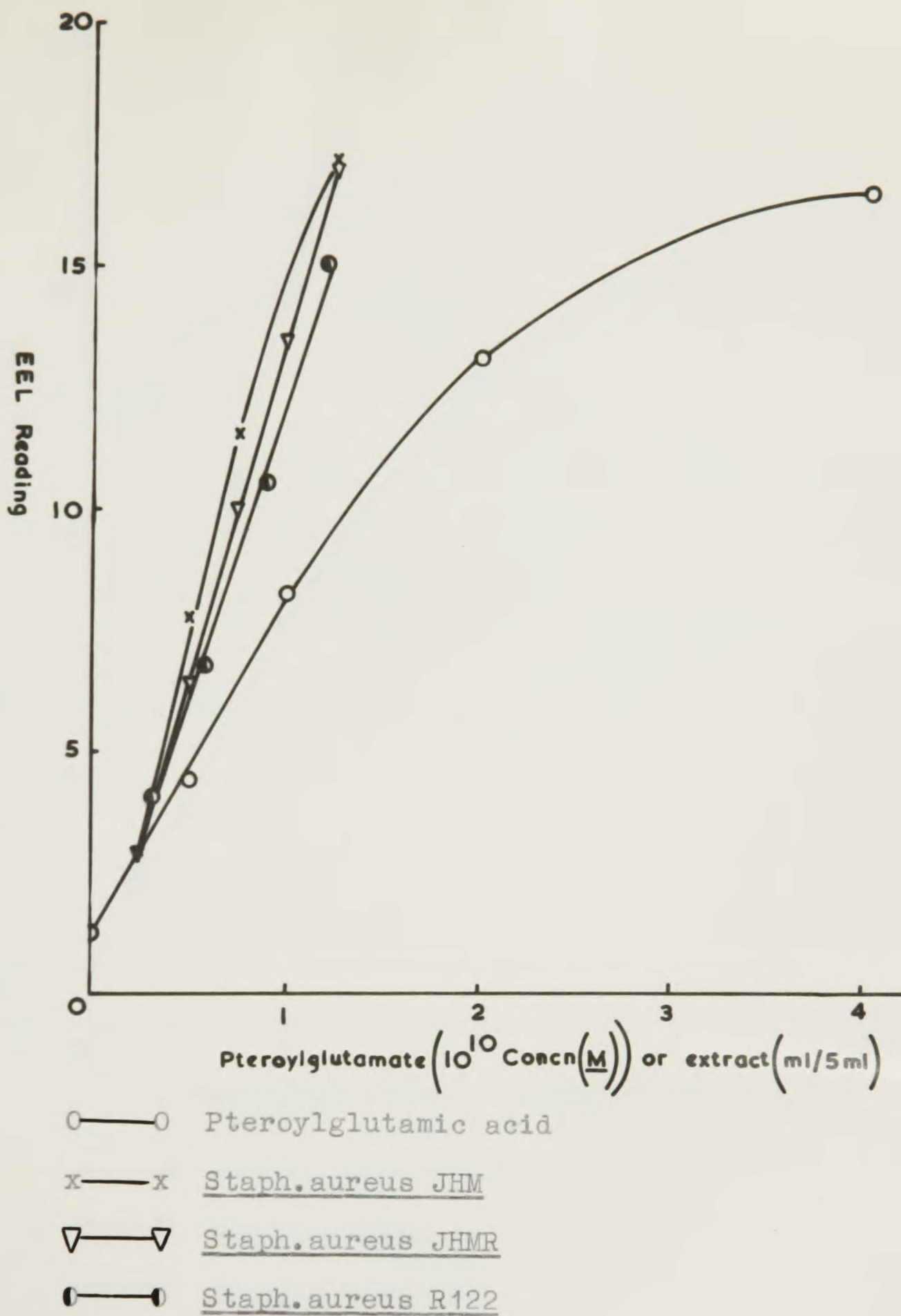
The assay technique of Nimmo-Smith, Lascelles and Woods (1948), as modified by Lascelles and Woods (1952), was used. A summary of the procedure is given in Table 17. Extracts of all the strains of Staph.aureus that were used gave a response curve which diverged from that given by pteroylglutamic acid (Figures 14 and 15). Consequently, the amounts of LF present in extracts was assessed against a reference extract as described by Lascelles and Woods (1952). The reference extract was obtained by pooling the residues of experimental extracts and storing at 2°. Each complete assay contained: (a) the "unknowns," each at two or three concentrations, (b) four levels of pteroylglutamic acid in the range 0.01-0.08 µg/ml, and (c) four levels of reference extract. This had previously been assayed against pteroylglutamic acid so that the response to the smallest quantity was approximately equal to that with 0.01 µg pteroylglutamic acid/ml. In plotting the results, the scale for the reference extract standard curve was

Procedures for assay of folic acid with different organisms.

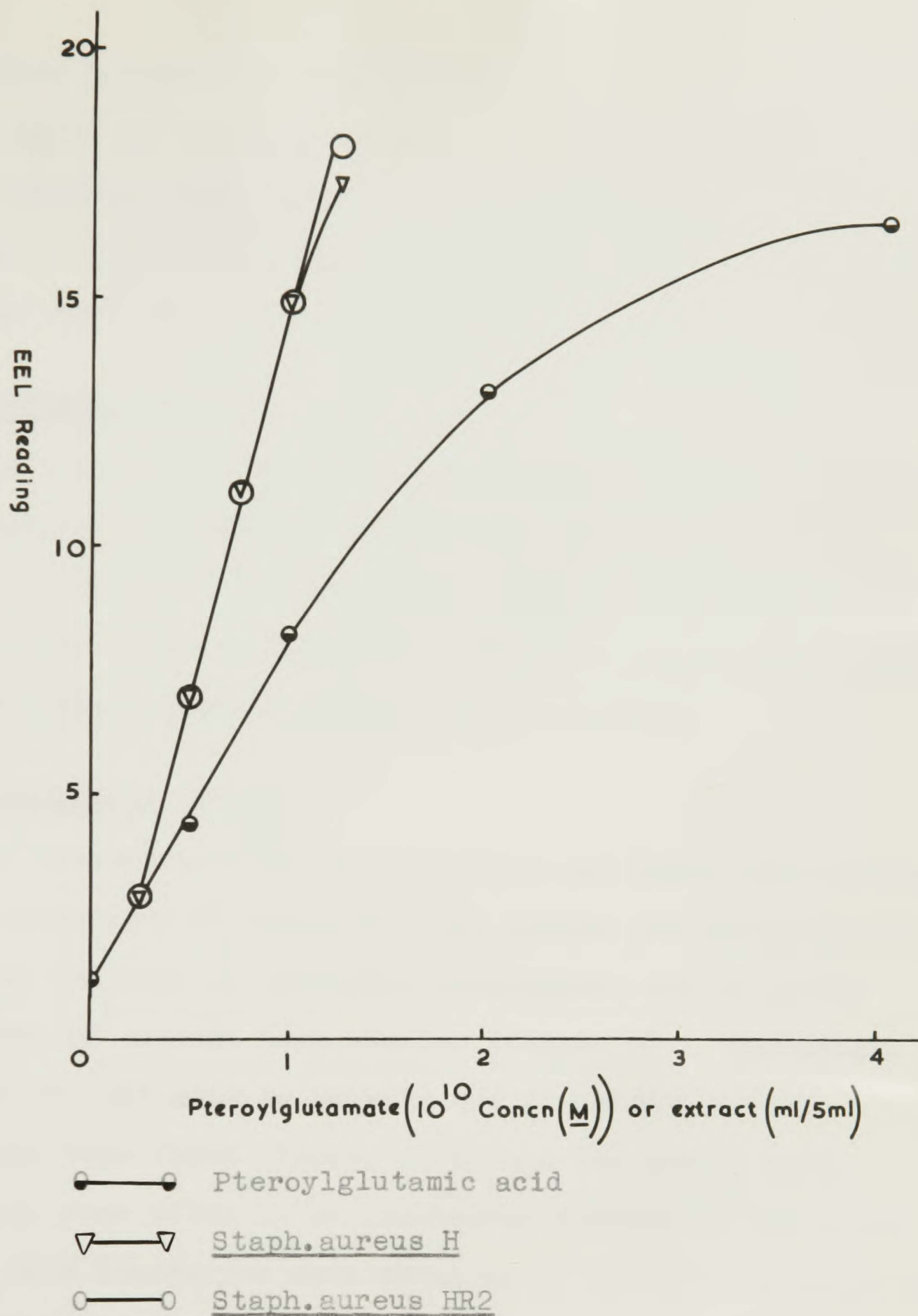
Details	Lb. casei	Lc. citrovorum	Strep. faecalis R
Medium	Lb. casei factor assay medium	Lc. citrovorum factor assay medium	Strep. faecalis R factor assay medium
Final vol.	5 ml	5 ml	5 ml
Inoculum per tube	0.5 ml of 1:50 dil ⁿ . of 24 hr inoculum development medium* culture from 18 hr tryptose glucose broth culture	0.1 ml of a $\frac{1}{50}$ EEL 1 washed suspension in 1% saline of organisms from a 16 hr tryptose glucose broth culture	0.5 ml of 1:100 dil ⁿ of 16 hr tryptose glucose broth culture
Incubation time	20-24 hr	72 hr	40-44 hr
Temperature	37°	37°	37°
Atmosphere	5% CO ₂ in H ₂	5% CO ₂ in H ₂	Air
Test organism responds to	Pteroylglutamic acid, pteroyltriglutamic acid, leucovorin	Leucovorin	Pteric acid, rhizopterin pteroylglutamic acid, leucovorin
Preparation of extract	Organisms and suspending fluid diluted with an equal volume of assay medium and autoclaved at 10 lb for 10 min. Supernate assayed		

* LF assay medium containing 10^{-7} M pteroylglutamic acid.

Table 17



Response of Lb.casei to pteroylglutamic acid and to extracts of Staph.aureus JHM, JHMR and R122



Response of Lb.casei to pteroylglutamic acid and to extracts of Staph.aureus H and HR2

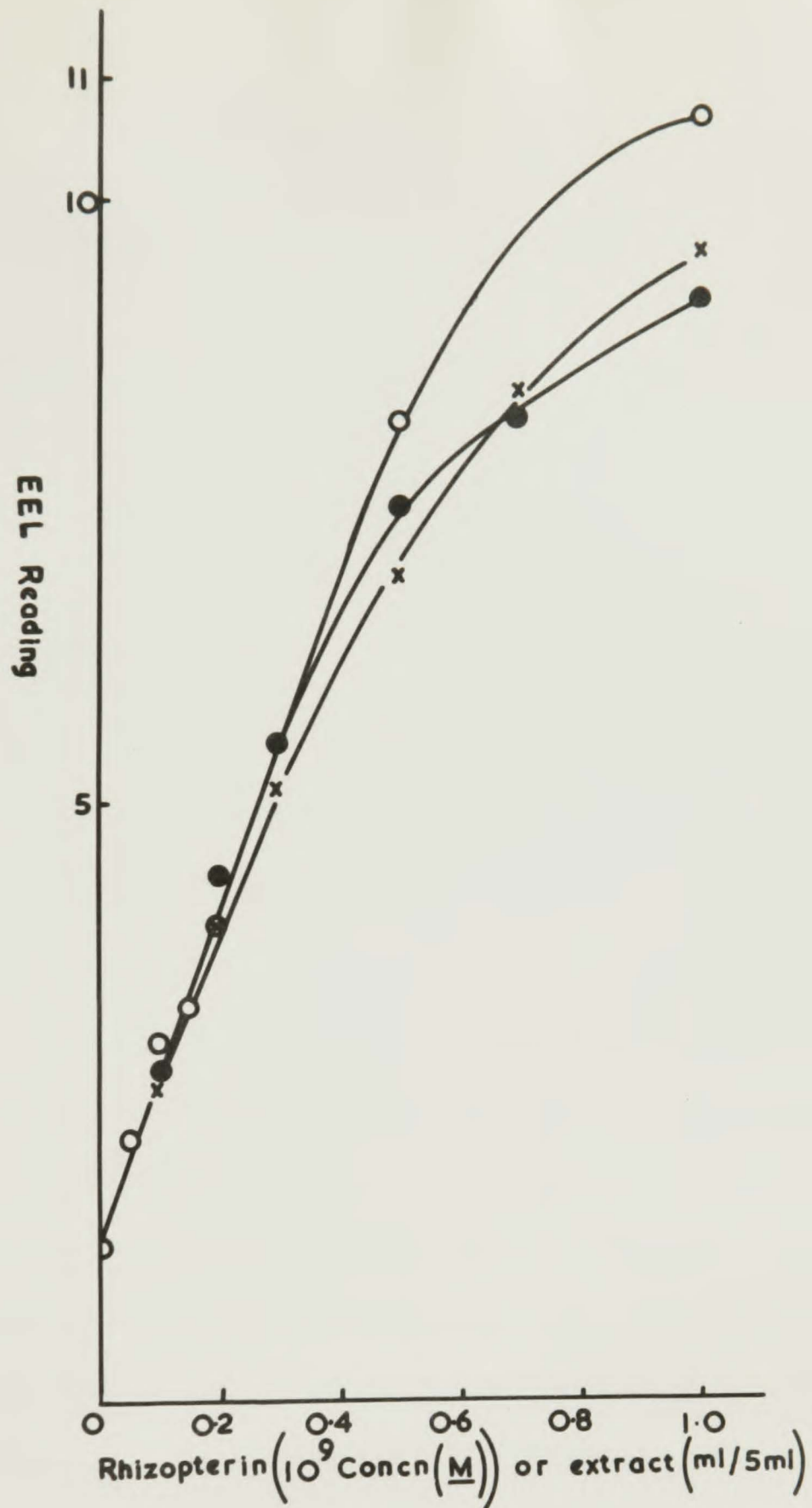
determined in terms of pteroylglutamic acid by equating the lowest level of reference extract to the concentration of pteroylglutamic acid which gave the same turbidity. The potency of the "unknowns" (in terms of pteroylglutamic acid) was then read off from the reference extract curve.

Lc.citrovorum factor.

The method of estimation was essentially that described by Sauberlich and Baumann (1948) with the modifications introduced by Lascelles and Woods (1952). A summary of the procedure is given in Table 17. Good recoveries of leucovorin added to staphylococcal extracts were obtained.

Strep.faecalis R factor.

The procedure is due to Lascelles and Woods (unpublished), and is summarized in Table 17. The curves for the response of the assay organism to synthetic rhizopterin and to graded quantities of extract were similar (Figure 16). The assay organism did not grow as heavily nor as quickly as had previously been found (Cross, 1956) and the assays were, therefore, read after 40 hr incubation instead of the usual 20 hr. Good recoveries were obtained of rhizopterin added to staphylococcal extracts. The potencies of "unknowns" were read off from the response curve in terms of rhizopterin.



O — O Rhizopterin
 x — x Staph. aureus H
 ● — ● Staph. aureus HR2

Response of Strep. faecalis R to rhizopterin and to extracts of Staph. aureus H and HR2

CHAPTER IX

The prevention of sulphonamide-inhibition by PAB and by the products of the function of PAB

The inhibition by sulphathiazole of the growth of staphylococci in medium B could be completely overcome by the addition of PAB. The inhibition index (Shive, 1950) is a useful concept in considering competitive inhibitions by analogues of metabolites. Shive derived the equation:

$\frac{[I]}{[S]} = K$, where K is a constant, the inhibition index, and is the molar ratio of the analogue concentration [I] to the metabolite concentration [S] in the medium which gives a defined inhibition within a constant period of time. The concentrations of I and S must be sufficiently high that they are not appreciably altered during the period of the experiment by the metabolic activities of the biological system; and other experimental conditions must not be varied. Davis and Maas (1952) found that the inhibition index for sulphathiazole: PAB was about 25 for a sulphonamide-sensitive wild-type strain of Esch.coli; 200 in a one-step sulphathiazole-resistant mutant, and 1000 in a multi-step resistant mutant. These indices were proportional to the levels of resistance of the three strains. Whether a similar relation occurred with the sulphonamide-sensitive and resistant strains of staphylococci was investigated.

Sulphathiazole-inhibition overcome by PAB.

The lowest concentration of PAB was determined in medium B that allowed at least half maximal growth in the presence of sulphathiazole (Table 18). The inhibition indices were calculated from the concentrations of PAB needed to overcome inhibition by $2 \times 10^{-3} \text{M}$ sulphathiazole after 40-45 hr incubation. As the time of incubation increased, so the concentration of PAB needed to overcome inhibition fell. Growth in these lower concentrations of PAB was as heavy as when sulphathiazole was absent, though there was a longer lag before growth began. With the higher concentrations of PAB, growth in the presence of sulphathiazole was as rapid as in the absence of the drug. The titration of PAB was not very close; there was a three-fold increase in concentration of PAB from one tube to the next. However, when the inhibition index was determined at more than one concentration of sulphathiazole, the results were in reasonable agreement.

The inhibition indices of the various strains were roughly proportional to their degrees of resistance (Table 19). Davis and Maas (1952) interpreted their results as suggesting that the resistant strains had an enzyme with an altered relative affinity for PAB and sulphathiazole. Before such an interpretation could be accepted for the present results it would be necessary to exclude other mechanisms of resistance discussed in Chapter II, since an increase in the inhibition index might also result from the development of other mechanisms of resistance.

Table 18

Prevention of sulphathiazole-inhibition by PAB in medium B

Organism	Conc. of sulphathiazole (M)	Conc. of PAB(M) for half maximal growth	Time (hr)	Inhibition Index
<u>Staph. aureus JHM</u>	10^{-3}	3×10^{-4}	15	10
		10^{-4}	45	
		3×10^{-5}	55	
<u>Staph. aureus JHM</u>	2×10^{-3}	5×10^{-4}	20	20
		10^{-4}	45	
		5×10^{-5}	60	
<u>Staph. aureus JHMR</u>	10^{-3}	6×10^{-6}	30	330
		3×10^{-6}	45	
	2×10^{-3}	10^{-5}	30	
<u>Staph. aureus H</u>	10^{-3}	10^{-4}	40	10
		3×10^{-4}	40	10
<u>Staph. aureus HR2</u>	10^{-3}	10^{-6}	40	1000
	2×10^{-3}	3×10^{-6}	40	660
<u>Staph. aureus R122</u>	2×10^{-3}	3×10^{-6}	40	660
<u>Staph. lactis 2102</u>	2×10^{-3}	10^{-4}	40	20
<u>Staph. lactis 2102R</u>	2×10^{-3}	10^{-5}	40	200
<u>Staph. lactis 2102R2</u>	2×10^{-3}	10^{-5}	40	200

Table 19

Relation between inhibition index and sensitivity to sulphathiazole (in absence of PAB) in medium B

Organism	Inhibition Index (K) after 40 hr	Change of K in resistant strain	Max. tolerated conc. of sulphathiazole(M)	Degree of resistance
<u>Staph.lactis 2102</u>	20		10^{-5}	
" <u>2102R</u>	200	x10	2.5×10^{-4}	x25
" <u>2102R2</u>	200	x10	2.5×10^{-4}	x25
<u>Staph.aureus JHM</u>	15		2.5×10^{-6}	
" <u>JHMR</u>	330	x20	5×10^{-5}	x20
<u>Staph.aureus H</u>	10		5×10^{-6}	
" <u>HR2</u>	660-1000	x66-100	5×10^{-4}	x100
<u>Staph.aureus R122</u>	660	x66*	5×10^{-4}	x100*

* Compared to Staph.aureus H

Prevention of sulphonamide-inhibition by products of PAB utilization.

When ultimate products of PAB utilization are present in the medium, the amount of PAB required to overcome sulphonamide-inhibition is reduced, and the inhibition index is increased (see review by Shive, 1950). A mixture of methionine, xanthine, thymine and valine, abolishes the PAB requirement for growth of Staph.aureus in the presence of sulphanilamide. (de Haan, Winkler and Baljet, 1949).

The resistance to sulphonamides of some or all of the strains of staphylococci may have been due to the synthesis of compounds, normally ultimate products of PAB function, by pathways which did not involve PAB. To determine whether this was so, the effect of addition of these compounds on the PAB requirement of both sensitive and resistant strains growing in the presence of sulphathiazole was investigated. If any substance reduced the PAB requirement of the sensitive strain, but not of the resistant strain, then this would suggest that the compound was synthesized in the resistant strain by a route which no longer involved PAB function.

To test the effect of methionine on the requirement for PAB, it was necessary to use medium B₂, the fully defined medium for growth of Staph.aureus, from which methionine is absent. The effect of valine could not be investigated because its presence is absolutely required for growth.

Results of experiments with Staph.aureus JHM and H and

their resistant substrains are shown in Tables 20 and 21. Addition of methionine led to an increase in the inhibition index with all the strains. Xanthine reduced the PAB requirement only in the presence of methionine, and thymine was active only in the presence of both methionine and xanthine. Serine and pteroylglutamic acid had no effect on the PAB requirement.

With Staph.aureus JHM and JHMR, methionine, xanthine and thymine showed similar PAB-sparing effects. Addition of vitamin B₁₂ led to a further reduction in the PAB requirement of Staph.aureus JHM and to earlier growth of Staph.aureus JHMR in the absence of PAB. In neither strain, however, was a mixture of methionine, xanthine, thymine and vitamin B₁₂ as effective as PAB in overcoming inhibition by sulphathiazole.

Staph.aureus H and HR2 behaved similarly to Staph.aureus JHM and JHMR, except that with Staph.aureus HR2 xanthine did not enhance the effect of methionine in sparing PAB. In a subsequent experiment (Table 22) methionine plus xanthine reduced slightly the amount of PAB needed for growth in the presence of sulphathiazole. Furthermore, methionine plus thymine was much less effective than methionine plus thymine plus xanthine in reducing the PAB requirement of Staph.aureus HR2.

Since, with the exception of xanthine in Staph.aureus HR2, the compounds tested produced similar PAB-sparing effects in sensitive and resistant strains of Staph.aureus JHM and H, it

Table 20

Effect of additions to medium B₂ on the inhibition index of Staph.aureus JHM and JHMR growing in presence of sulphathiazole.

Addition	Conc. of PAB needed for half maximal growth (M)		Inhibition Index	
	<u>JHM</u>	<u>JHMR</u>	<u>JHM</u>	<u>JHMR</u>
-	10 ⁻⁴	10 ⁻⁵	10	100
Me	3x10 ⁻⁵	3x10 ⁻⁶	33	330
X	3x10 ⁻⁴	10 ⁻⁵	3	100
T	3x10 ⁻⁴	10 ⁻⁵	3	100
Me+X	10 ⁻⁵	3x10 ⁻⁷	100	3300
Me+T	3x10 ⁻⁵	10 ⁻⁶	33	1000
X+T	3x10 ⁻⁴	10 ⁻⁵	3	100
Me+X+T	3x10 ⁻⁶	- (80 hr)	330	∞
Me+X+T+B ₁₂	3x10 ⁻⁶ (80 hr)	- (50 hr)	>330	∞

S.aureus JHM 10⁻³M sulphathiazole 135 hr incubation

S.aureus JHMR 10⁻³M sulphathiazole 110 hr incubation

Me = Methionine (10⁻³M)

X = Xanthine (10⁻⁴M)

T = Thymine (10⁻⁴M)

B₁₂ = Vitamin B₁₂ (2 µg/ml)

Table 21

Effect of additions to medium B₂ on the inhibition index of Staph.aureus H and HR2 growing in presence of sulphathiazole.

Addition	Conc. of PAB needed for half maximal growth (<u>M</u>)		Inhibition Index	
	<u>H</u>	<u>HR2</u>	<u>H</u>	<u>HR2</u>
-	3×10^{-4}	10^{-5}	3	200
Me	3×10^{-5}	3×10^{-6}	30	660
X	3×10^{-4}	10^{-5}	3	200
T	3×10^{-4}	10^{-5}	3	200
Me+X	10^{-5}	3×10^{-6}	100	660
Me+T	3×10^{-5}	3×10^{-6}	30	660
X+T	3×10^{-4}	10^{-5}	3	200
Me+X+T	3×10^{-6}	3×10^{-7}	300	6600

S.aureus H 10^{-3} M sulphathiazole 80 hr incubation

S.aureus HR2 2×10^{-3} M sulphathiazole 80 hr incubation

Me, X, T as in Table 20.

is, therefore, likely that these compounds are synthesized in both sensitive and resistant strains by mechanisms in which PAB participates.

Quantitative requirements for products of PAB function.

If a resistant strain has a decreased requirement for a given product of PAB function, then this should be detected by titrating the amount of product needed to reduce the amount of PAB required to overcome sulphonamide-inhibition.

The minimum concentration of methionine needed by Staph. aureus H and HR2 to spare PAB was determined by measuring the inhibition index in medium B₂ in the presence of $10^{-3}M$ sulphathiazole and decreasing amounts of methionine. The requirements for xanthine and thymine were then determined similarly in the presence of methionine and of methionine plus xanthine respectively. To reduce the number of tubes needed for an experiment the titrations were not close, each concentration level of the product of PAB utilization was one fifth of that preceding.

Incubation periods of only about two days were required to show the effects of additions with Staph. aureus HR2. With longer incubations in the presence of methionine, growth occurred in the absence of PAB or other additions. For comparison, inhibition indices for the sensitive strain are also quoted after two days incubation. Although greater PAB-sparing effects were found on longer incubation of Staph. aureus H,

the concentrations of the compounds needed to produce this was unchanged. (Table 22).

In both strains, $8 \times 10^{-6} \text{ M}$ methionine was the lowest concentration that gave a maximal PAB-sparing effect. For maximal sparing of PAB $2 \times 10^{-5} \text{ M}$ xanthine was required by Staph. aureus H, whereas $4 \times 10^{-6} \text{ M}$ was adequate for the resistant strain. Each strain showed a maximal PAB sparing effect with 10^{-4} M thymine.

The difference in the requirement for xanthine of the resistant and sensitive strain was confirmed by a closer titration. The significance of this observation is considered in the Discussion.

Table 22

Quantitative requirements for methionine, xanthine and thymine
to give PAB-sparing effect

Staph. aureus H in medium B₂

10^{-3} M sulphathiazole

Methionine (M)	Xanthine (M)	Thymine (M)	Inhibition index	
			50 hr	90 hr
-	-	-	1	3
10^{-3}	-	-	10	30
8×10^{-6}	-	-	10	30
2×10^{-6}	-	-	3	10
2×10^{-4}	10^{-4}	-	30	100
2×10^{-4}	2×10^{-5}	-	30	100
2×10^{-4}	4×10^{-6}	-	10	30
2×10^{-4}	10^{-4}	10^{-4}	100	1000
2×10^{-4}	10^{-4}	2×10^{-5}	100	300

Staph aureus HR2 in medium B₂

10^{-3} M sulphathiazole

-	-	-	200
10^{-3}	-	-	800
8×10^{-6}	-	-	800
2×10^{-6}	-	-	400
2×10^{-4}	10^{-4}	-	1000
2×10^{-4}	4×10^{-6}	-	1000
2×10^{-4}	8×10^{-7}	-	300
2×10^{-4}	10^{-4}	10^{-4}	3000
2×10^{-4}	10^{-4}	2×10^{-5}	>3000

CHAPTER X

The synthesis of p-aminobenzoic acid by staphylococci

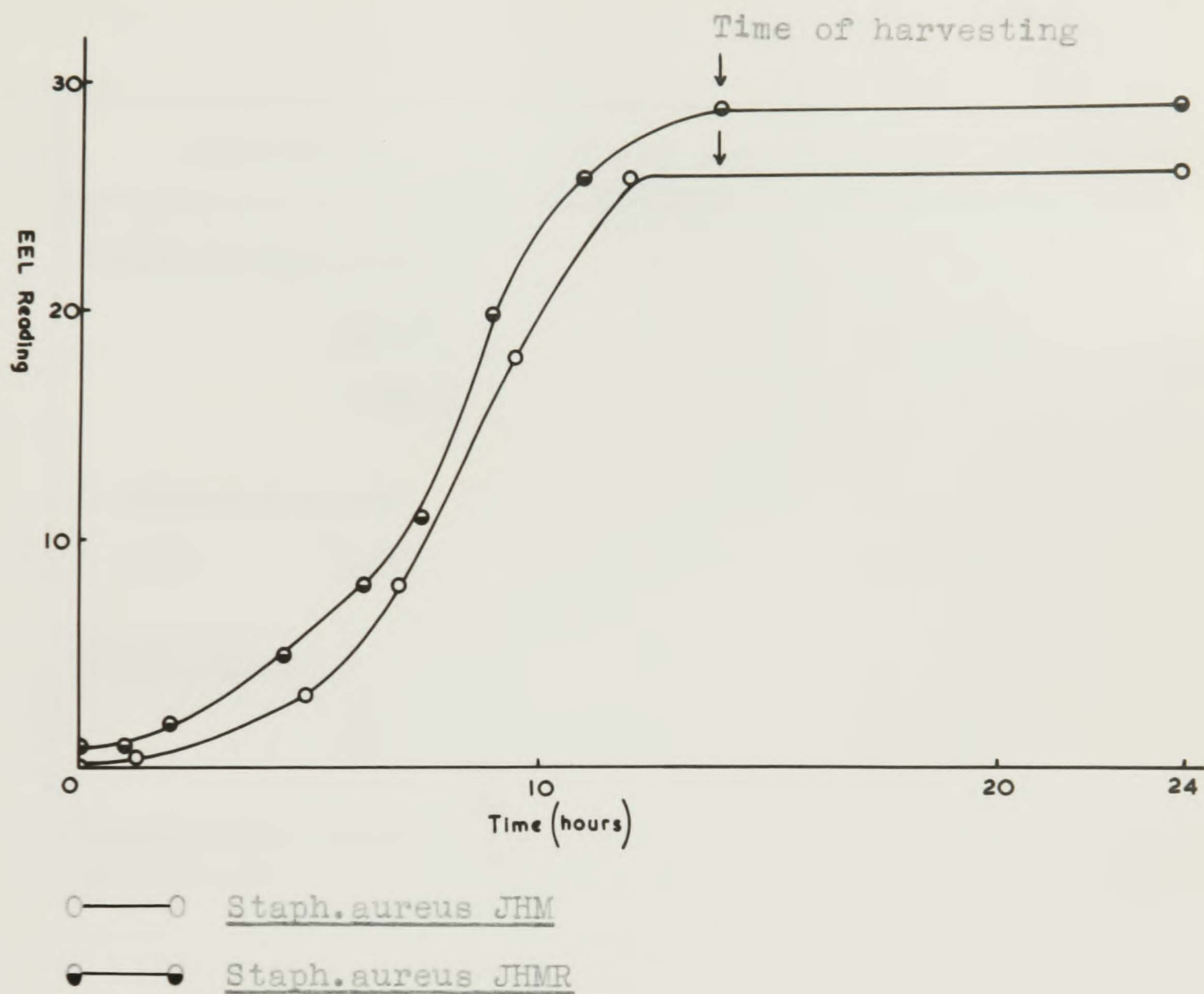
Some sulphonamide-resistant substrains of bacteria produce greater amounts of PAB during growth than their sensitive parent strains, but this is not found in all cases of sulphonamide-resistance (see Introduction). The synthesis of PAB during growth of the various strains of staphylococci in medium B in the presence and absence of sulphathiazole was investigated. Some work was also done on the synthesis of PAB by washed suspensions of organisms.

Synthesis of PAB by organisms growing in medium B.

Organisms were grown in 25 ml volumes of medium B from large inocula (Chapter VIII). Cultures were incubated 14-16 hr, by which time growth had just reached the end of the logarithmic phase (Figure 17). With the large inoculum used (2 ml of an EEL 10 suspension), growth of all strains was insensitive to inhibition by sulphathiazole.

The amount of PAB in these cultures was assayed with Esch.coli 273/384. (Table 23). The few assays that were done with Acetobacter suboxydans 621 gave results in substantial agreement. The growth attained by Staph.lactis 2102 and its substrains was less (about 0.2 mg/ml) than that of the strains of Staph.aureus (about 0.4 mg/ml). For comparison, therefore, results are expressed as μgPAB per mg dry wt of organisms in the culture.

FIGURE 17



Growth of Staph. aureus JHM and JHMR in medium B
from a large inoculum

Table 23

Synthesis of PAB by staphylococci growing 14-16 hr in medium B

Organism	µg PAB per ml culture	µg PAB per mg dry wt organisms
<u>Staph. lactis 2102</u>	7	35
" <u>2102R</u>	21	150
" <u>2102R2</u>	5	33
<u>Staph. aureus JHM</u>	75	170
" <u>JHMR</u>	88	180
<u>Staph. aureus H</u>	92	210
" <u>HR2</u>	100	240
<u>Staph. aureus R122</u>	1700	4900

To find out whether, with a longer period of growth, the greater differences between the synthesis of PAB in a sensitive strain and in a resistant strain still occur, the amount of PAB synthesis with time was measured in Staph. aureus JHM and JHMR (Figure 18). The synthesis followed a similar course in each strain.

Synthesis of PAB by organisms resistant to sulphonamides and tetracycline

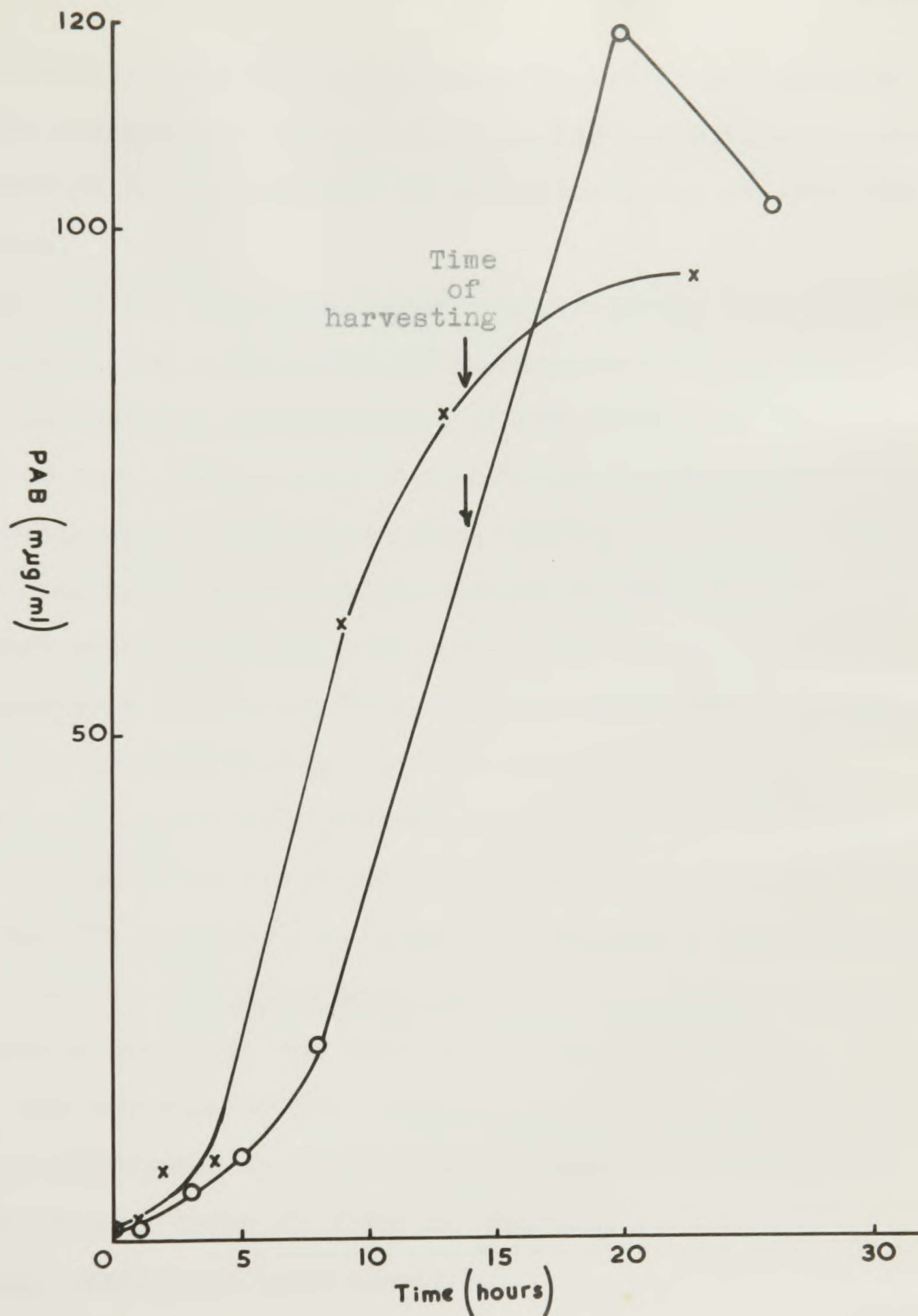
It may be argued that only strains grown in the presence of sulphonamides or the resistant strains synthesized smaller amounts of PAB than their sensitive parent strains. To test

The most striking feature of the results is the very high synthesis of PAB by Staph.aureus R122. The concentration of PAB in the medium is just over $10^{-5}M$. The other strains of Staph.aureus only synthesized about one twentieth of this amount of PAB. The resistant strains of Staph.aureus JHM and H did not synthesize appreciably different amounts of PAB from their sensitive parent strains. Still less PAB was synthesized by Staph.lactis 2102. In one of its resistant strains, 2102R2, the synthesis was the same as that in the parent, but in the second resistant strain, Staph.lactis 2102R, there was a five-fold increase in PAB synthesis, although this organism is no more resistant to sulphathiazole than is 2102R2. The synthesis of PAB by Staph.lactis 2102R, even though it is greater than that of its parent strain, is still no greater than that found in the sensitive strains of Staph.aureus.

To find out whether, with a longer period of incubation, greater differences between the synthesis of PAB in a sensitive strain and in a resistant strain might occur, the course of PAB synthesis with time was measured in Staph.aureus JHM and JHMR. (Figure 18). The synthesis followed a similar course in each strain.

Synthesis of PAB by organisms growing in medium B in the presence of sulphathiazole.

It may be argued that only during growth in the presence of sulphathiazole do the resistant strains synthesize greater amounts of PAB than their sensitive parent strains. To test



○ — ○ Staph. aureus JHM

x — x Staph. aureus JHMR

Synthesis of p-aminobenzoic acid during growth
from large inocula of Staph. aureus JHM and JHMR
in medium B

this possibility, organisms were grown as before in medium B, but in the presence of sulphathiazole. PAB and sulphathiazole were separated in the extracts by chromatography, and the PAB was assayed.

Since PAB was never recovered quantitatively from chromatograms, the reliability of these assays is uncertain. To check the assays, known amounts of PAB were added to portions of each extract before separating the sulphathiazole, and the recoveries of PAB from these extracts were determined. This gave some measure of the proportion of the total PAB in the extract that was being estimated by the assay. Extracts from a sensitive strain and from its resistant substrain were prepared in the same experiment, and sulphathiazole was separated from PAB in each extract on the same chromatogram, so that each would be subjected to similar losses of PAB before assay. Results are shown in Table 24. Sensitive and resistant pairs of strains of Staph.aureus synthesized similar amounts of PAB during growth in the presence of sulphathiazole. Although the accuracy of the figures is uncertain, a ten-fold or greater difference in PAB synthesis between sensitive and resistant strains (such as would be required to explain resistance) would have been detected.

Synthesis of PAB by washed suspensions of organisms.

A few experiments were done to investigate whether staphylococci could synthesize PAB in solutions more simple

Table 24

Synthesis of PAB by strains of Staph. aureus growing 14-16 hr in medium B in the presence of sulphathiazole

Organism	µg PAB per ml culture	µg PAB per mg dry wt organisms	% Recovery of PAB
<u>Staph. aureus JHM</u>	99	250	60
	73	180	40
<u>Staph. aureus JHMR</u>	99	250	60
	73	180	40
<u>Staph. aureus H</u>	11	37	30
" <u>HR2</u>	8	20	40
<u>Staph. aureus R122</u>	550	1400	100

Sensitive strains grown with $5 \times 10^{-6} M$ sulphathiazole

Resistant strains grown with $5 \times 10^{-5} M$ sulphathiazole

to a final volume of 5 ml were added to 5 ml water and

incubated upright at 37°C. usually for 1 hr. The supernatant was

then assayed for PAB using Staph. aureus R122.

Results are shown in Table 24. According to description:

all strains tested synthesized PAB in medium B as well as in

least as well as in the growth medium. With the sensitive

strains, synthesis of PAB is considerably better in medium B

medium than in the growth medium, where the synthesis is

markedly less than with the resistant strains. The finding was

than the growth medium. Most of this work was done with Staph.aureus JHM and JHMR but a few observations were made with other strains.

Synthesis of PAB by washed suspensions of organisms in the minimal medium of Davis and Mingioli (1950) was first investigated. It was anticipated that in this simple medium, without additions, synthesis of PAB would be slight. The effect of possible precursors on the synthesis could then be examined. Synthesis of PAB by washed suspensions in medium B was also examined. Here it was anticipated that synthesis of PAB would take place, and it was intended to find out the effect of omitting constituents of the medium on this synthesis.

Organisms were grown in 25 ml volumes of medium B from large inocula as already described. Washed suspensions were prepared in 0.02M phosphate buffer pH 6.8 or 7.4 to a concentration of about 0.5 mg dry weight per ml. Suspension (2.0 ml), double strength medium (2.5 ml), and other additions to a final volume of 5 ml were mixed in 5"x $\frac{5}{8}$ " tubes and incubated upright at 37°, usually for 5 hr. The suspensions were then assayed for PAB using Esch.coli 273/384.

Results are shown in Table 25. Contrary to expectation, all strains tested synthesized PAB in Davis' minimal medium at least as well as in the growth medium. With the sensitive strains, synthesis of PAB is considerably better in Davis' medium than in the growth medium, where the synthesis is markedly less than with the resistant strains. This finding was

Table 25

Synthesis of p-aminobenzoic acid by suspensions of Staph.
aureus in various media

Figures are μg PAB/ mg dry weight of organisms

Medium	<u>Strain</u>				
	<u>JHM</u>	<u>JHMR</u>	<u>H</u>	<u>HR2</u>	<u>R122</u>
Davis' minimal	120	120	130	150	1500
" - glucose	0	0	0	0	12
Medium B	30	100	60	140	1500
" - glucose	2	10	0	7	35
Medium B ₂	30	120			
" - glucose	0	0			
0.02M buffer pH7.4	0	0			
" + glucose [Ⓢ]	59	48			
" + casein hyd. [Ⓢ]	0	4			
" +glucose+NH ₃ [Ⓢ]	180	100			
" +glucose+casein hyd.	56	54			

Glucose[Ⓢ] 0.2% in medium

Casein hydrolysate[Ⓢ] Equivalent to 3g casein/l in medium

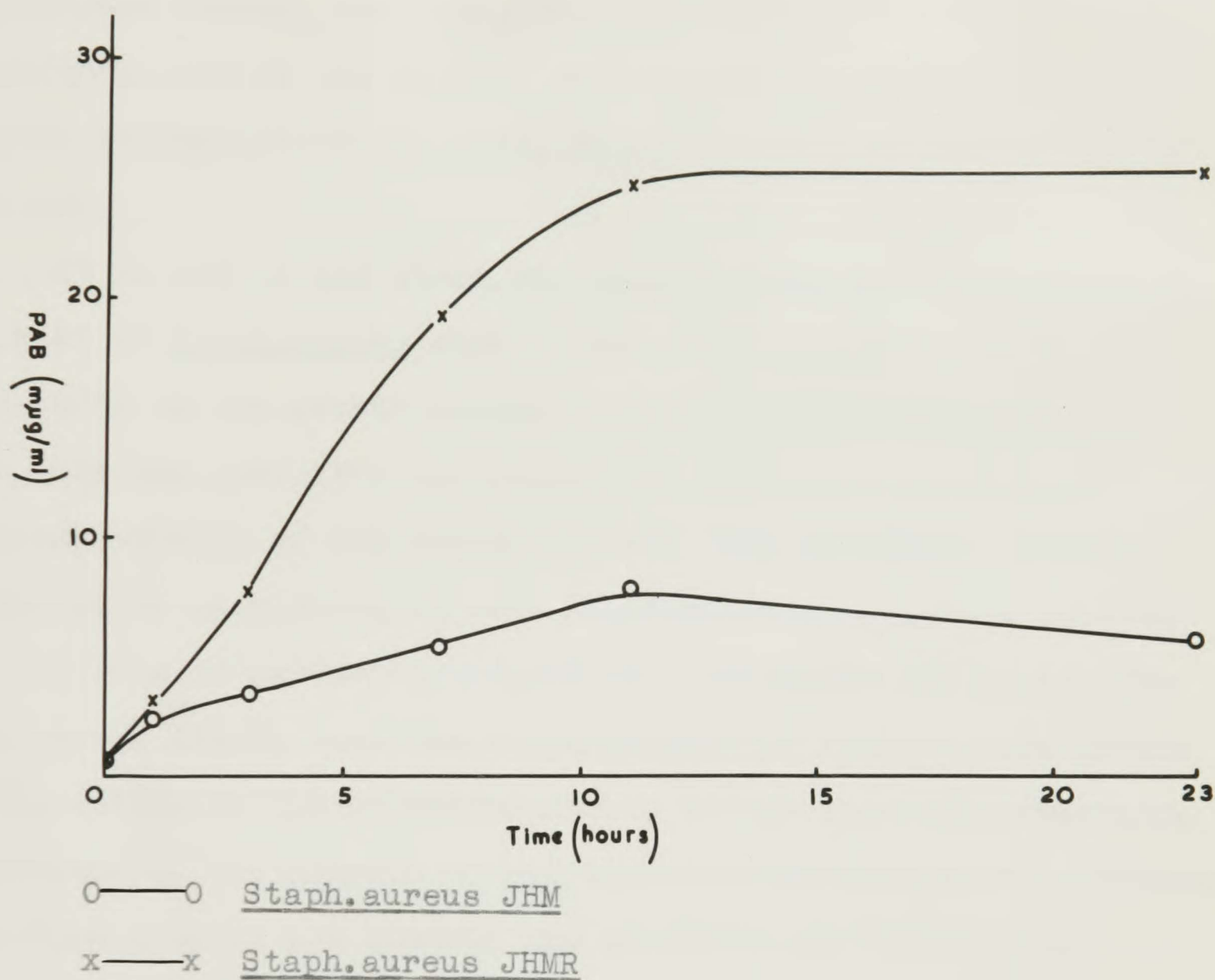
NH₃[Ⓢ] 0.1% ammonium sulphate in medium

surprising since growing cultures of both sensitive and resistant strains of Staph.aureus JHM and H in medium B had synthesized about the same amounts of PAB. This difference between sensitive and resistant strains was still shown when suspensions were incubated in medium B for different lengths of time, (Figure 19) and it was also shown by washed suspensions incubated in medium B₂, the completely defined growth medium. There was no synthesis in any of these media if glucose was omitted.

The amount of PAB formed by Staph.aureus JHM and JHMR in glucose and phosphate only, was about half that synthesized in Davis' medium; further addition of ammonia increased the synthesis to the same level as that in Davis' medium. Similar results have been obtained with Staph.aureus R122 (G.A.Rushton, unpublished results).

These findings indicate that washed suspensions of Staph.aureus JHM, while able to synthesize PAB from glucose and ammonia just as well as suspensions of the resistant strain are less capable of synthesizing PAB from glucose and amino acids. The resistant strains might be better able to produce ammonia from amino acids than can the sensitive strains. Lam and Sevag (1957) have some evidence that sulphathiazole inhibits transaminase activity in a sensitive strain of Staph.aureus. It is, therefore, possible that in resistant strains, transaminase activity is increased. However, with neither Staph.aureus JHM nor JHMR was the synthesis of PAB from glucose

FIGURE 19



Synthesis of PAB by washed suspensions (1 mg dry wt/ml)
of Staph. aureus JHM and JHMR in medium B

increased by addition of acid-hydrolyzed casein. This may be because tryptophan or cysteine, which are absent from acid-hydrolyzed casein, are among the requirements for this synthesis, or, it may be that the vitamin constituents of the growth medium (nicotinic acid, thiamine and biotin) are needed as well.

It is not at all clear why washed suspensions of sensitive strains of Staph. aureus show a decreased synthesis of PAB when incubated in the growth medium, while the resistant strains do not, because sensitive and resistant substrains synthesize similar amounts of PAB during growth. The synthesis of PAB seems to be proceeding in both sensitive and resistant strains at the time of harvesting (14-16 hr; see Figure 17) so it does not appear likely that the PAB-synthesizing enzymes have ceased to be active in the sensitive strain by the time of harvesting. Furthermore, the enzymes of the sensitive strains which synthesize PAB from glucose and ammonia are as active as those of the resistant substrains.

inhibition by sulphathiazole.

The synthesis of 15-hydroxy factor (157) in Staph. aureus

Organisms were grown in 25 ml volumes of medium in 250 ml Erlenmeyer flasks for 14-16 hr as described previously. The organisms were harvested, and washed once in 0.1M phosphate buffer pH 6.8. Suspensions of organisms in buffer were added to double strength incubation mixture 7. (Appendix 10) which

CHAPTER XI

The synthesis of folic acid by staphylococci

The synthesis of folic acid by washed suspensions and by growing cultures of Staph.lactis 2102 and 2102R has been investigated by Lascelles and Woods (1952). The findings will be briefly summarized as they form the basis of the work described in this chapter.

Folic acid formed by staphylococci gave a response curve with the assay organism (Lb.casei), which differed from that given by pteroylglutamic acid in that heavier growth was produced at optimal concentrations; the staphylococcal material was not identical with any member of the folic acid group that was tested. Folic acid was synthesized by washed suspensions of organisms in a mixture containing buffer, glucose, glutamate and PAB. In this mixture and during growth, the sulphathiazole-resistant strain synthesized about ten times as much folic acid as the parent strain, and this synthesis was less sensitive to inhibition by sulphathiazole.

The synthesis of Lb.casei factor (LF) by staphylococci.

Organisms were grown in 25 ml volumes of medium B from large inocula for 14-16 hr as described previously. The organisms were harvested, and washed once in 0.02M phosphate buffer pH 6.8. Suspensions of organisms in buffer were added to double strength incubation mixture F, (Appendix III) which

contained glucose, glutamate, buffer and PAB, to give a final concentration of about 0.2 mg dry wt of organisms per ml. Such suspensions, 2.5 ml final volume in 5"x $\frac{1}{2}$ " tubes, were incubated upright at 37° usually for 5 hr. The total amount of LF in the organisms and the suspending medium was assayed at the start and at the end of an incubation. Estimations on extracts of washed organisms and on suspending medium or culture filtrate, showed that all the LF was present in the organisms.

Initial LF content of harvested organisms.

The amount of LF in organisms harvested from medium B and suspended in mixture F is shown in Table 26. The LF content of all the sensitive strains is about the same, roughly 30 μ g/mg dry weight. In two of the resistant strains, Staph. lactis 2102R and Staph. aureus R122 there is a considerably greater amount of LF, but in three other resistant strains no more LF is present than in their parent strains.

Lascelles and Woods (1952) found that synthesis of LF by washed suspensions of Staph. lactis 2102 and 2102R occurred only in organisms which contained initially a low concentration of the factor. Such organisms were obtained by growth in the presence of sub-inhibitory concentrations of sulphathiazole, 5×10^{-6} M for sensitive strains, and 5×10^{-5} M for resistant strains. The content of LF of depleted organisms is shown in Table 27.

Table 26

Initial LF content of harvested organisms

Organism	LF (mg/mg) in harvested organisms	
	Normal	Depleted
<u>Staph.lactis 2102</u>	20-50	1-5
<u>Staph.lactis 2102R</u>	100-300	3-13
<u>Staph.lactis 2102R2</u>	48	1-9
<u>Staph.aureus JHM</u>	25-35	2-7
<u>Staph.aureus JHMR</u>	33	2-9
<u>Staph.aureus H</u>	25-32	4-7
<u>Staph.aureus HR2</u>	28	1-9
<u>Staph.aureus R122</u>	110-140	8

Figures show the range found, usually in at least three experiments, with each strain.

Normal organisms grown in medium B

Depleted organisms grown in medium B in the presence of sulphathiazole.

The effect of depletion with higher concentrations of

Synthesis of LF by suspensions of washed organisms in mixture F.

The amounts of LF synthesized in mixture F by initially depleted organisms is shown in Table 27. Undepleted organisms showed very slight synthesis of LF. The finding of Lascelles and Woods (1952) that Staph.lactis 2102 synthesizes about ten times as much LF as its parent strain was confirmed. Although 2102R2, the other resistant substrain did not synthesize much more LF than its parent, there was a significant and reproducible increase. Staph.aureus R122, like Staph.lactis 2102R, showed a relatively high content of folic acid after normal growth in medium B, and it also synthesized a comparatively large amount of LF in medium F after initial depletion. Unfortunately, no sensitive strain of Staph.aureus R122 was available for comparison. The resistant strains of Staph.aureus JHM and H synthesized no more LF than the small amount formed by their parent strains. Various procedures were used to attempt to increase this synthesis.

The time course of LF synthesis by depleted Staph.aureus H and HR2 in mixture F was studied to find out whether the synthesis was greater with a longer incubation (Figure 20). Synthesis of LF continued beyond the normal period of incubation (5 hr) and approached the level found in undepleted harvested organisms. Staph.aureus HR2 synthesized rather more LF after 10 hr incubation than its parent, but the difference was not striking.

The effect of depletion with higher concentrations of

Table 27

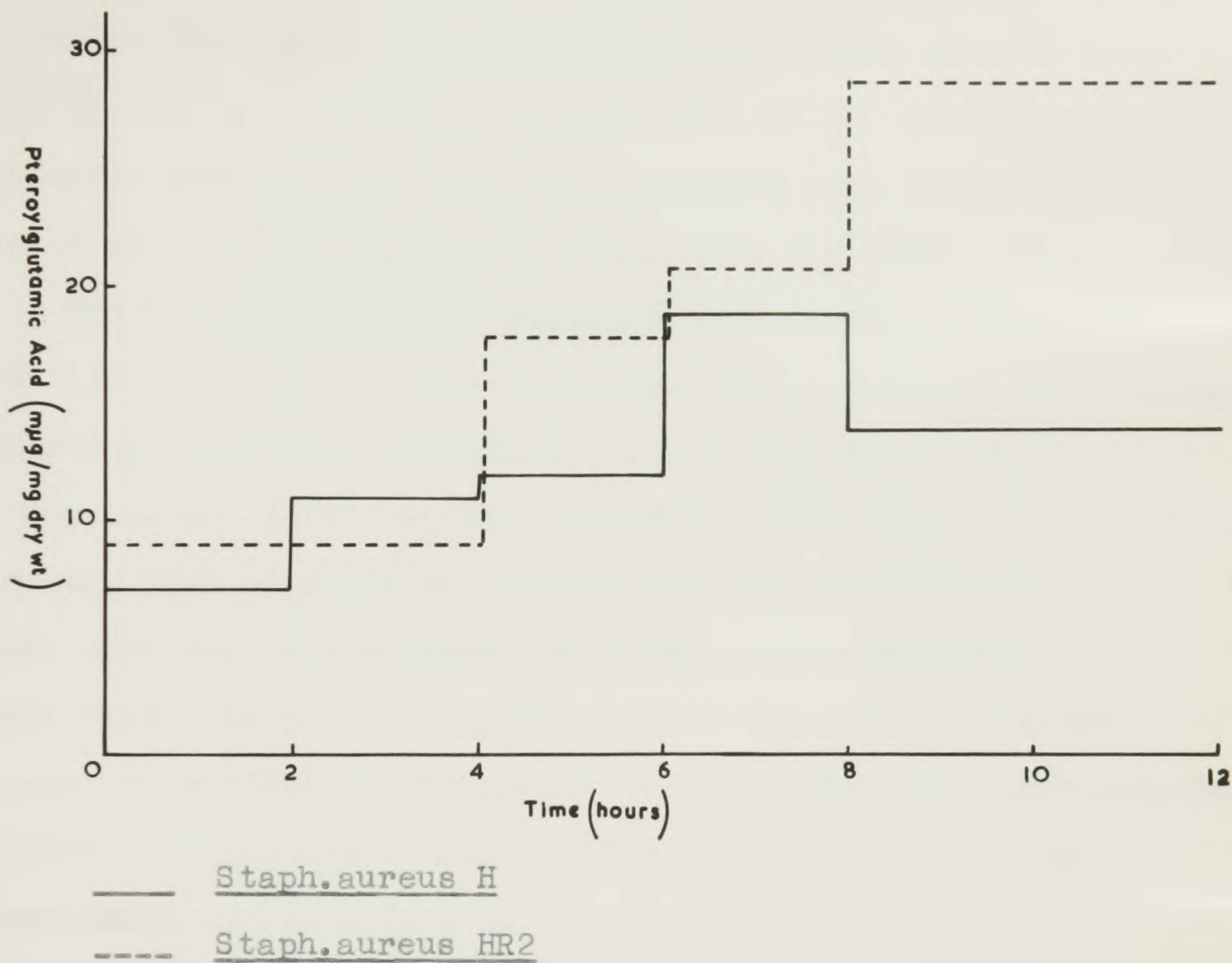
Synthesis of LF by depleted organisms in mixture F

Organism	LF (mg/mg)	
	Initial	Increase
<u>Staph.lactis 2102</u>	3	15
<u>Staph.lactis 2102R</u>	8	100
<u>Staph.lactis 2102R2</u>	4	26
<u>Staph.aureus JHM</u>	4	6
<u>Staph.aureus JHMR</u>	6	9
<u>Staph.aureus H</u>	6	7
<u>Staph.aureus HR2</u>	6	6
<u>Staph.aureus R122</u>	8	70

Mixture F contains buffer, PAB, glucose and glutamate. 0.2 mg dry wt organisms/ml.

Figures are averages of the results of several experiments.

FIGURE 20



Synthesis of folic acid in mixture F by Staph. aureus H
and HR2 (initially depleted) after various times of
incubation

sulphathiazole on the subsequent ability to synthesize LF was examined. In an experiment with Staph.aureus HR2, growing the organisms with concentrations of sulphathiazole greater than $5 \times 10^{-5} \text{M}$ did not cause greater depletion of LF, nor were the organisms subsequently able to synthesize more LF in mixture F than those grown with $5 \times 10^{-5} \text{M}$ sulphathiazole (Table 28).

The effect of growing cultures in a complex medium was next investigated. Medium B supports the growth of Staph.aureus JHM and H, and must, therefore, enable the organisms to synthesize at least adequate amounts of folic acid. It may be, however, that in a richer medium, more suitable precursors of folic acid may be available, enabling greater synthesis of folic acid. Also, in a complex medium the organisms might accumulate sufficient precursors of LF so that when subsequently incubated in mixture F they might synthesize more LF than organisms grown in medium B.

Organisms were grown in medium G, (Appendix III) which contains peptone, marmite and liver extract, and washed suspensions of the harvested organisms were prepared. The synthesis of LF by normal and depleted organisms in mixture F was investigated (Table 29). The initial content of LF and the synthesis in mixture F was about the same as with organisms grown in medium B.

Lascelles and Woods (1952) found that the synthesis of LF in mixture F by Staph.lactis 2102 and 2102R was inhibited by

Table 28

Synthesis of LF by organisms depleted by growth with various concentrations of sulphathiazole. Staph. aureus HR2 in mixture F

Conc. of sulphathiazole during growth (<u>M</u>)	<u>LF (μg/mg)</u>	
	Initial	Increase
-	10	0
5×10^{-5}	1	6
10^{-4}	1	3
2×10^{-4}	1	5

Table 29

Synthesis of LF in mixture F by organisms grown in medium G
with various concentrations of sulphathiazole.

Conc. of sulphathiazole during growth	JHM		JHMR		H		HR2	
	LF (mg/mg)	Initial Increase	LF (mg/mg)	Initial Increase	LF (mg/mg)	Initial Increase	LF (mg/mg)	Initial Increase
-	13	13	13	13	5	19	5	1
10^{-5}					2	7		
2.5×10^{-4}							2	8
5×10^{-4}							1	8
10^{-3}							1	8

sulphathiazole. Synthesis by the resistant strain was eight times more resistant to sulphathiazole than that of the parent; this compared with a fifty-fold difference when growth was the criterion of sensitivity. It was pointed out that this comparison is only realistic when it is known that all the reactants necessary for synthesis of LF by suspensions of organisms are present in optimal amounts.

The sensitivity of the synthesis of LF to inhibition by sulphathiazole in suspensions of Staph.lactis 2102R2 resembled that of 2102R. (Table 30). In the experiments with Staph.aureus JHM and H and their resistant substrains the amounts of LF synthesized were low, but there was a progressive inhibition of LF synthesis by increasing concentrations of sulphathiazole up to $10^{-3}M$, and the resistant strains were less sensitive to this inhibition than the parent strains.

The synthesis of Lc.citrovorum factor (CF) by staphylococci.

Lascelles and Woods (1952) showed that the difference in behaviour of pteroylglutamic acid and LF during assay with Lb.casei could not be attributed to the presence of CF (5-formyl-5,6,7,8-tetrahydropteroylglutamic acid, Figure 1). Typical extracts of Staph.lactis 2102R were very much more active in supporting the growth of Lb.casei than the growth of Lc.citrovorum. This was confirmed, and it was also found that none of the extracts of any of the strains of Staph.aureus contained detectable CF activity.

Table 30

Inhibition of synthesis of LF in mixture F by the presence
of sulphathiazole

Conc. of PAB $10^{-5}M$ throughout

Synthesis: μg LF/mg

Conc. of sulphathiazole (M)		-	10^{-6}	10^{-5}	10^{-4}	10^{-3}
<u>Staph. lactis 2102R</u>	Synthesis	160	150	64	9	
	% Inhibition		10	60	90	
<u>Staph. lactis 2102R2</u>	Synthesis	44	40	18	5	0
	% Inhibition		10	60	90	100
<u>Staph. aureus JHM</u>	Synthesis	6	6	4	2	1
	% Inhibition		0	30	60	80
<u>Staph. aureus JHMR</u>	Synthesis	8	7	5	5	3
	% Inhibition		10	40	40	60
<u>Staph. aureus H</u>	Synthesis	7	6	5	3	0
	% Inhibition		15	30	60	100
<u>Staph. aureus HR2</u>	Synthesis	10	6	6	6	4
	% Inhibition		40	40	40	60

recovery of ribisopterin from staphylococcal extracts (Chapter VIII) it seemed unlikely that the extracts inhibited the assay organisms. The most likely explanation seemed to be that the LF in the extracts was a form of folic acid to which Strep. faecalis does not respond. The same compound is pteroyltriglutamic acid (Figure 1), which on a molar basis is ten times as active for Staph. aureus as for Strep. faecalis (Jones and Stokstad, 1948). Pteroylheptaglutamic acid, a higher conjugate, is active for neither organism.

Use of *Strep. faecalis* R as assay organism for folic acid.

A possible reason for the low content of folic acid in extracts of *Staph. aureus* JHM and H and their resistant substrains is that some of the folic acid might be present in a form to which *Lb. casei* does not respond. *Strep. faecalis* R responds to two compounds of the folic acid group, rhizopterin and pteric acid (Figure 1) which are not active for *Lb. casei*. Accordingly, extracts of the various strains of staphylococci were tested for their ability to support the growth of *Strep. faecalis* R in a medium from which folic acid was omitted. (Appendix III).

Results of determinations of the initial content of rhizopterin of organisms grown in medium B and of the synthesis of rhizopterin in mixture F are shown in Table 31. The very low content of *Strep. faecalis* R factor in most of these extracts is surprising, as all had LF activity. Since there was good recovery of rhizopterin from staphylococcal extracts (Chapter VIII) it seemed unlikely that the extracts inhibited the assay organism. The most likely explanation seemed to be that the LF in the extracts was a form of folic acid to which *Strep. faecalis* R does not respond. One such compound is pteroyltriglutamic acid (Figure 1), which on a molar basis is ten times as active for *Lb. casei* as for *Strep. faecalis* R (Jukes and Stokstad, 1948). Pteroylheptaglutamic acid, a higher conjugate, is active for neither organism.

Table 31

Initial content of Strep. faecalis R factor in normal and depleted cells of staphylococci and synthesis of the factor in mixture F

Organism	Rhizopterins $\mu\text{g}/\text{mg}$ dry wt			
	Normal cells		* Depleted cells	
	Initial	Increase in mixture F	Initial	Increase in mixture F
<u>Staph. lactis</u> 2102	6	3	10	5
<u>Staph. lactis</u> 2102R	20	80	13	40
<u>S. aureus</u> JHM	0	5	2	1
<u>S. aureus</u> JHMR	0	5	1	1
<u>S. aureus</u> H	0	0	1	1
<u>S. aureus</u> HR2	0	5	1	1
<u>S. aureus</u> R122	3	5	2	7

* Grown as before in the presence of sulphathiazole

The results of acid hydrolysis of extracts of Staph.aureus JHM and H supported this view (Table 32). Extracts were diluted 1:1 with N hydrochloric acid, autoclaved at 10 lb for 10 min. and neutralized with N sodium hydroxide. The activity of the extracts was considerably increased by this procedure. Extracts prepared by direct treatment of organisms with N hydrochloric acid or 0.1M citrate buffer pH2 had no activity for Strep.faecalis R. A more quantitative conversion of material present in the extracts into Strep.faecalis R factor could probably be achieved by use of a conjugase enzyme (see Jukes and Stokstad, 1948), but this was not attempted.

Table 32

Effect of acid treatment on content of Strep. faecalis R
factor in staphylococcal extracts

Organism	Initial $\mu\text{g}/\text{mg}$	After acid treatment $\mu\text{g}/\text{ml}$
<u>Staph. aureus JHM</u>	0	8
<u>Staph. aureus H</u>	0	14

Materials and Methods

Growth of large quantities of *Escherichia coli* for experiments in this section was as follows.

Tryptic digest of peptone, or peptone water (10% w/v) solidified with 2% agar in a 250 ml. flask was inoculated with 1-5 ml. of a suspension of organisms in water made up to a colorimeter reading of about 0.5. The flask was incubated for 24 hr. at 37°C. The organism was then washed with about 20 ml. of water, filtered, and used to prepare plates of agar, and centrifuged down. The organisms were stored as a stock about 20 ml. water and resuspended in the reaction mixture.

Experimental Results

Section 3

The metabolism of sulphathiazole and the permeability to sulphathiazole and p-aminobenzoic acid

Chemical estimation of PAB in suspensions of *Escherichia coli* and *Staphylococcus aureus*.

The method of Bretton and Marshall (1959) was used to estimate PAB when it was present in concentrations of 10^{-5} M or higher. A standard curve was established (Figure 2) and concentrations of PAB were subsequently read from this curve. Samples were usually diluted to give readings from 5-30 on the scale of the EEL colorimeter with green filter (525 mμ).

This method was originally devised to estimate sulphonamides and is not specific for PAB. Any substance which can be diazotized and coupled in acid solution to the

CHAPTER XII

Materials and Methods

Growth of large quantities of organisms for experiments with washed suspensions.

Tryptic digest of casein, or peptone water (100 ml) solidified with 2% agar in a Roux bottle, was inoculated with 1.5 ml of a suspension of organisms in water made to a colorimeter reading of about EEL 20, and incubated for 20 hr at 37°. The organisms were washed off with about 20 ml water, filtered through glass wool to remove pieces of agar, and centrifuged down. The organisms were washed once in about 20 ml water and resuspended in the reaction mixture used for the experiment to a density of about 100 mg dry wt per ml.

Chemical estimation of PAB and sulphathiazole.

Bratton and Marshall method.

The method of Bratton and Marshall (1939) was used to estimate PAB when it was present in concentrations of 10^{-5} M or higher. A standard curve was established (Figure 21) and concentrations of PAB were subsequently read from this curve. Samples were usually diluted to give readings from 5-30 on the scale of the EEL colorimeter with green filter (OGR 1).

This method was originally devised to estimate sulphonamides and is not specific for PAB. Any substance which can be diazotized and coupled in acid solution to the

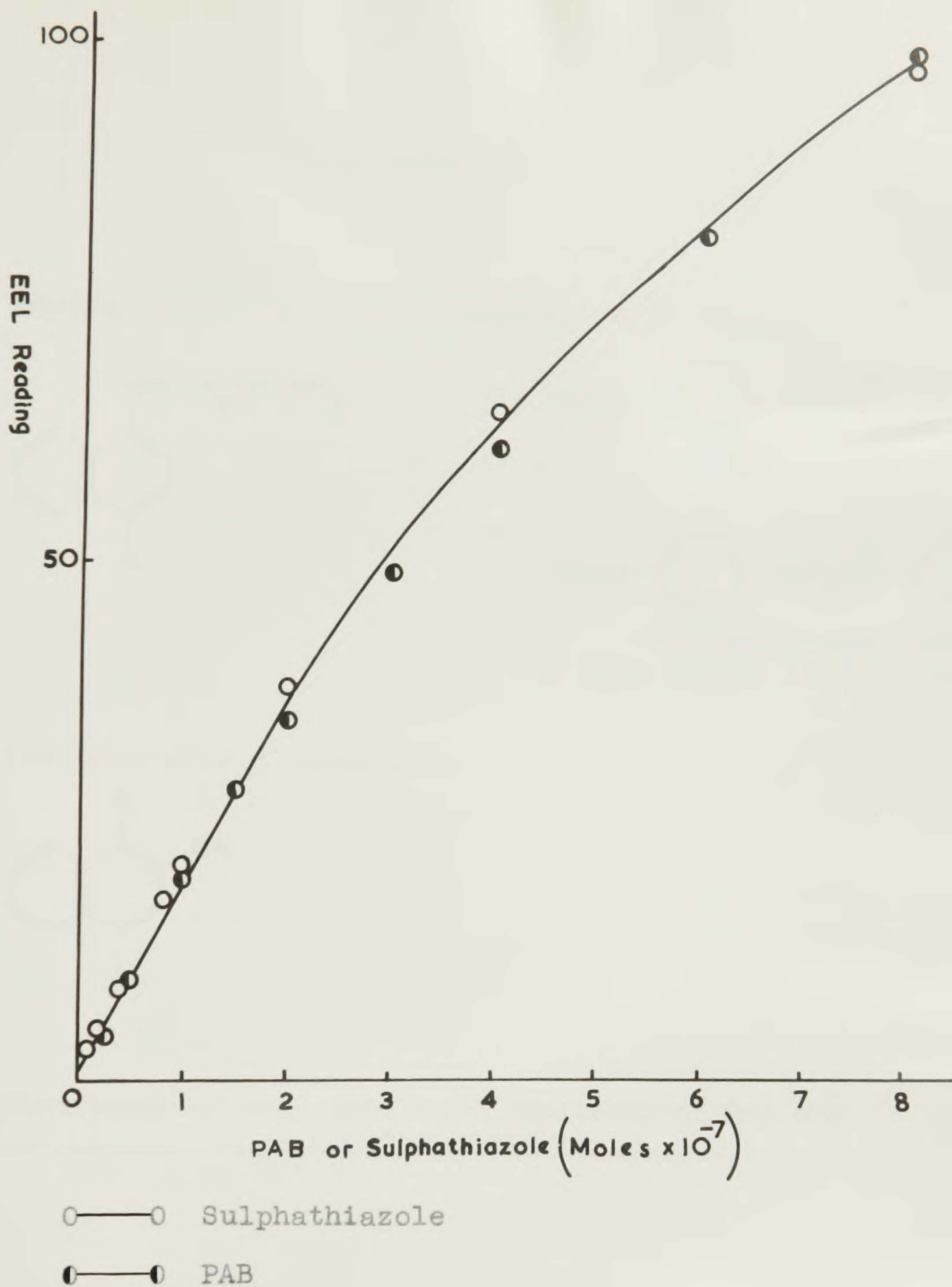
reagent N-(1-naphthyl) ethylenediamine dihydrochloride, will give a positive reaction. The same procedure was used to estimate sulphathiazole as has been described for estimation of PAB. (Figure 21). Good recoveries of both sulphathiazole and PAB were obtained from staphylococcal cultures by this method.

Havinga method.

Sulphathiazole and PAB could each be estimated in the presence of the other when both were present in concentrations greater than about $10^{-5}M$. The method is a modification of that of Havinga (1944) for the estimation of sulphapyridine in the presence of PAB.

The dye formed when PAB is diazotized and coupled with β -naphthol is more acidic than that formed by sulphathiazole under similar conditions (Figure 22). When a weakly alkaline (pH9) solution containing both dyes is extracted with chloroform, the sulphathiazole derivative passes into the organic layer, whereas that of PAB remains in the aqueous phase. The PAB derivative can readily be extracted at all pH's by n-amyl alcohol.

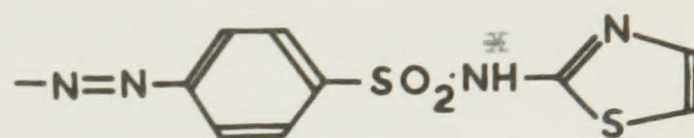
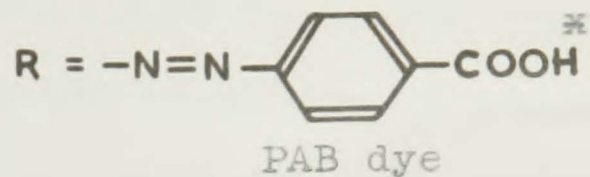
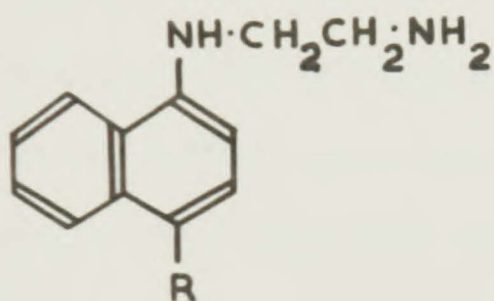
In estimations by this method, the sample and water were added to a 5"x $\frac{5}{8}$ " tube to a volume of 2 ml; 0.1N hydrochloric acid (0.2 ml) was added and the tube was cooled in ice-water before 1 drop sodium nitrite solution (5g/100 ml) was added. After half-an-hour the volume was made up to 10 ml



Estimation of sulphathiazole and p-aminobenzoic acid
(Bratton and Marshall method)

FIGURE 22

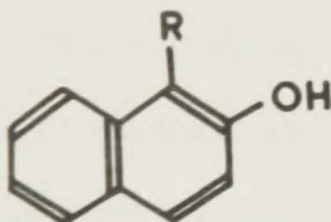
Reaction with the Bratton and Marshall reagent:



Sulphathiazole dye

$\ast$ H more acidic in PAB dye

Reaction with β -naphthol:



Structures of dyes used in estimations of PAB and
sulphathiazole

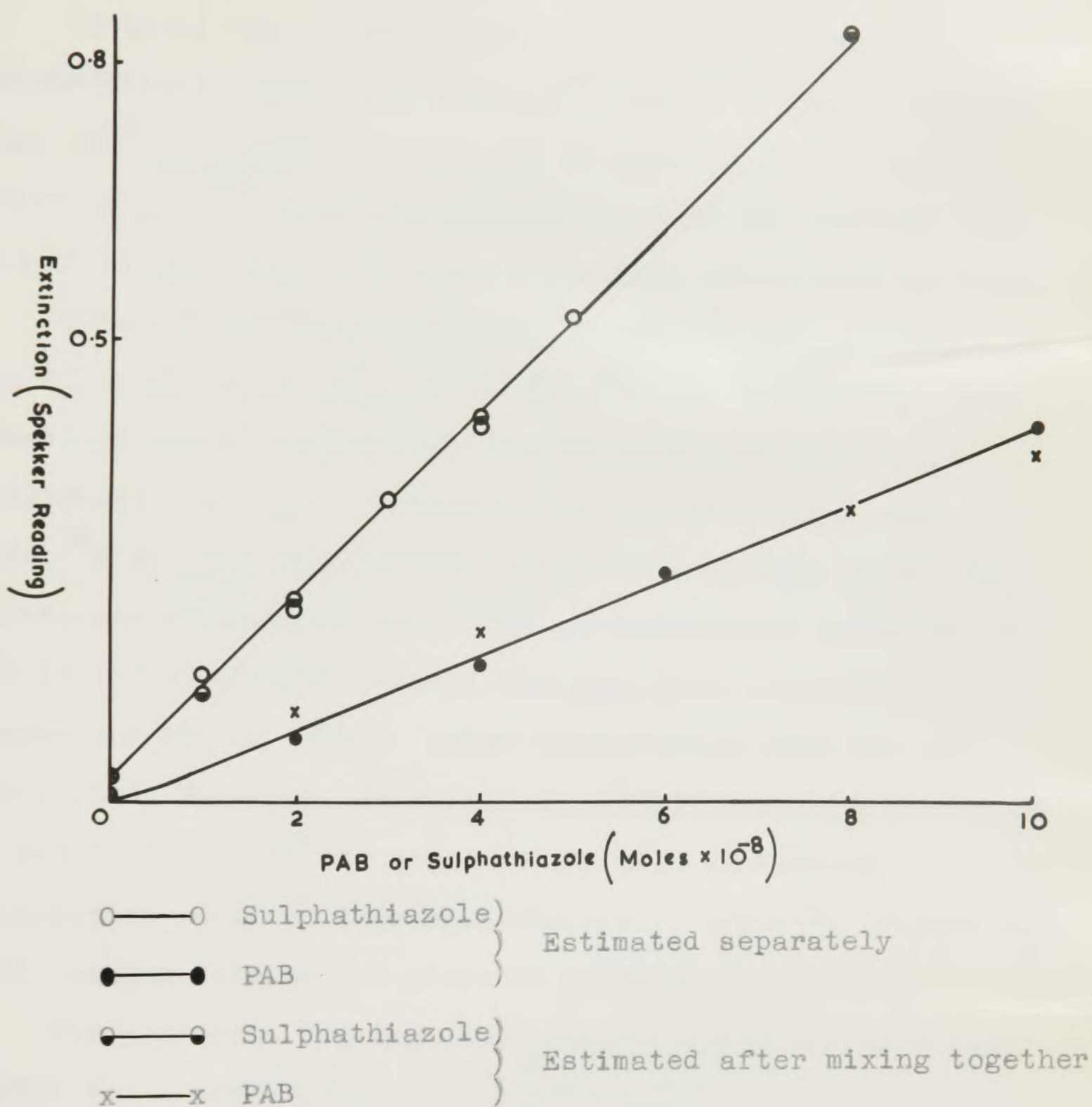
with cold water (2°) and 0.3 ml of a solution containing 20 mg β -naphthol in 10 ml 0.1N sodium hydroxide was added. The pH was adjusted to 9, using thymol blue as external indicator.

After adding 2 ml chloroform the tube was corked and shaken for about a minute. The chloroform layer was removed with a Pasteur pipette, and the aqueous layer was extracted twice more with chloroform. It was necessary to centrifuge the tube after each extraction to break the emulsion which formed. The combined chloroform extracts were centrifuged to clear, and the depth of colour (originating from sulphathiazole) was then measured in a Hilger Spekker photoelectric colorimeter (Hilger and Watts, Ltd, London) with spectrum blue filter (602) against a blank of chloroform.

The aqueous layer was extracted twice in the same way with 2.5 ml *n*-amyl alcohol. The combined extracts were cleared by centrifuging and read in the Spekker against *n*-amyl alcohol as zero, with spectrum blue filter.

Standard curves were established for PAB and sulphathiazole separately, relating Spekker reading to concentration of each dye (Figure 23). Sulphathiazole and PAB estimated in mixtures of various proportions gave results which fitted the standard curves well (Figure 23). The standard curves were subsequently used to calculate concentrations of PAB and sulphathiazole from Spekker readings.

FIGURE 23



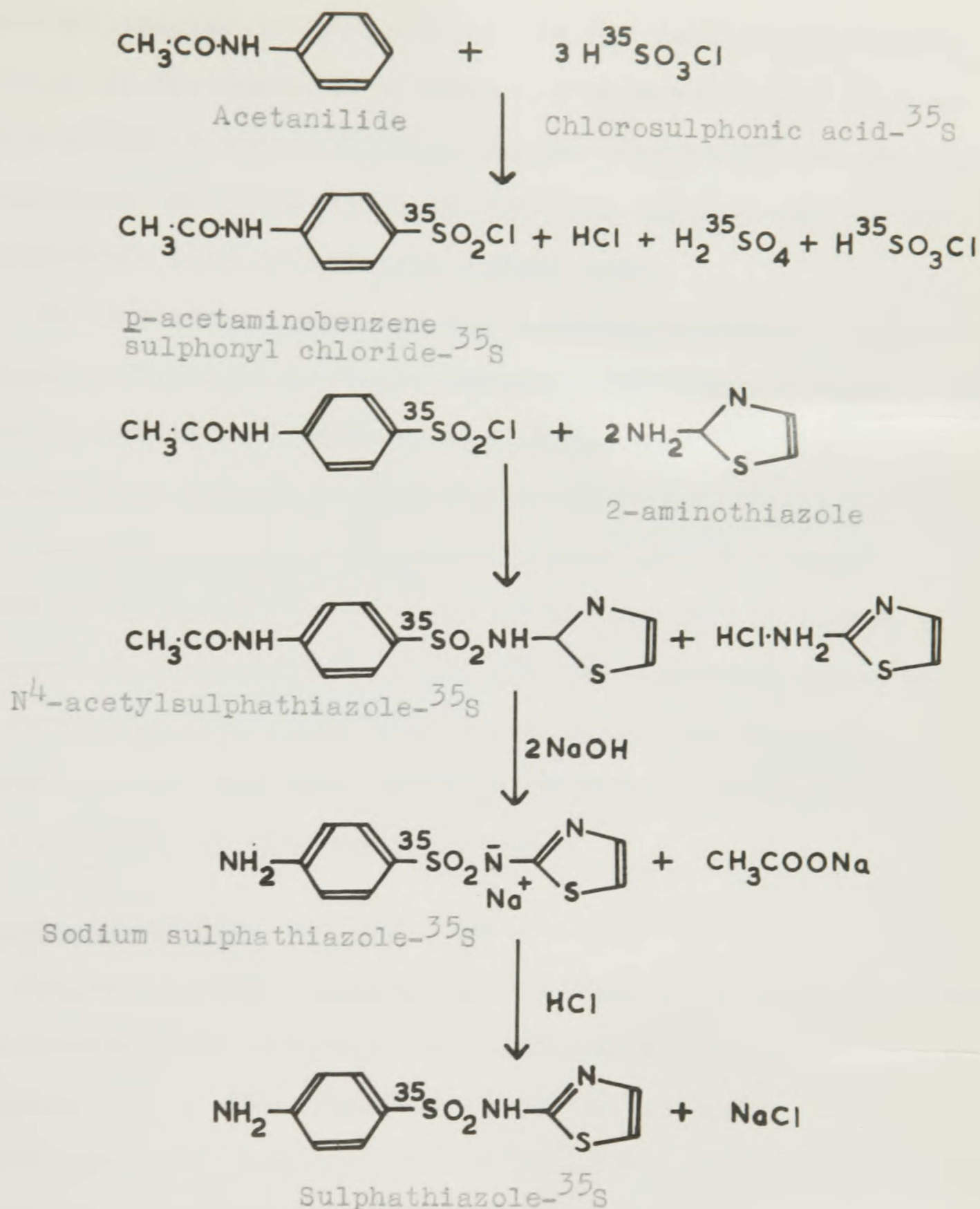
Estimation of sulphathiazole and p-aminobenzoic acid
(Havinga method)

Preparation of sulphathiazole labelled with ^{35}S .

Chemical methods would not measure quantities of sulphathiazole less than about 10^{-8} mole. It was calculated that 10^{-9} mole could be detected by measurement of radioactivity if 10^{-2} mole sulphathiazole could be labelled with one millicurie (mC), assuming a counting efficiency of 3-5%.

When the relative costs of ^{14}C and ^{35}S were compared it was clearly more economical to use ^{35}S for labelling. This labelling could be in either the thiazole ring or in the sulphonamido-group. A synthesis of sulphathiazole labelled with ^{35}S in the thiazole ring has been described (Noll, Bang, Sorkin and Erlenmeyer, 1951) and sulphanilamide labelled with ^{35}S in the sulphonamido-group has also been prepared (Wacker, Trebst and Simon, 1957). After consultation with May and Baker, Ltd, Dagenham, Essex, about several possible syntheses, it was decided that the simplest and most economical preparation would be from chlorosulphonic acid- ^{35}S (Figure 24). Full working details are given in Appendix I.

The procedure for the first step is described by Gilman (1932) for a preparation on a considerably larger scale than was required in this case. In tests on a small scale, it was found that the time needed to complete the reaction was only about an hour. The best yields in terms of chlorosulphonic acid converted to product (15%) were obtained with proportions of 3 moles chlorosulphonic acid to 1 mole acetanilide.



Synthesis of sulphathiazole- ^{35}S from chlorosulphonic acid- ^{35}S

Recrystallization of the product did not seem worthwhile, since if it was used immediately the crude material gave as good a yield in the next step, as the recrystallized product. Furthermore the recrystallization (from chloroform) led to considerable loss of material (about 40%).

The conditions used for the reaction between *p*-acetaminobenzene sulphonyl chloride and 2-aminothiazole were those described by Fosbinder and Walter (1939). No modifications were introduced other than a scaling down of the quantities of the reactants and the use of a slight excess of 2-aminothiazole. The procedures for isolation of *N*⁴-acetylsulphathiazole and for all the remaining steps in the synthesis were given by May and Baker Ltd (Private communication), and were modified only by a scaling down of the quantities of the reactants used.

Criteria of purity.

Sulphathiazole which was synthesized in a trial experiment was compared with authentic sulphathiazole ("Thiazamide," May and Baker Ltd). The synthetic sulphathiazole was a white crystalline solid, melting point 197°-199°. A mixture with authentic sulphathiazole melted at 198°-200°.

On chromatography, the *R_f* values of the synthetic sulphathiazole in *n*-butanol:ammonia and in methanol:benzene: *n*-amyl alcohol:ammonia were 0.45 and 0.40 respectively. A single spot was obtained on chromatograms. The authentic

sulphathiazole had similar properties.

Solutions of synthetic and authentic sulphathiazole ($1.25 \times 10^{-4} \text{M}$) were estimated by the Bratton and Marshall method. The concentrations of the two solutions were in agreement by this method.

The synthetic sulphathiazole at 10^{-3}M inhibited growth of Staph. aureus JHM in medium B. This inhibition was overcome by 10^{-4}M PAB after 40 hr incubation. Similar results were obtained with authentic sulphathiazole.

Measurement of radioactivity.

Measurement of radioactivity enabled 10^{-9} mole sulphathiazole to be estimated, at least while the sulphathiazole- ^{35}S was freshly prepared. (The half-life of ^{35}S is only 87 days). This estimation is free from interference from other substances, provided they are not present in amounts sufficient to increase the self-absorption of the sample under investigation. In equating measured radioactivity with concentration of sulphathiazole it is assumed that the ^{35}S remains part of the sulphathiazole molecule. The validity of this assumption will be discussed later (Chapter XIV, page reference 110).

Solutions containing sulphathiazole- ^{35}S were evaporated to dryness under an infra-red lamp on 15 mm internal diameter planchets 1.5 mm deep. The planchets were stamped out from aluminium sheet (0.1 mm thickness) and were washed in acetone

and water. A disc (15 mm diameter) of Green's lens tissue No. 105 was put on to a planchet before the solution was added. The presence of this disc led to the solution drying evenly over the bottom of the planchet, so that no correction for sample geometry was necessary.

Counts were measured with an Ekco automatic scaler, Type N530.D (Ekco Works, Southend-on-Sea, Essex). An end window Geiger-Müller tube Type EHM2/S (General Electric Co.) was connected to the scaler through a quenching probe unit, Ekco Type N558. The Geiger-Müller tube was enclosed in an ERD lead castle (ERD Engineering Co. Ltd, Slough, Bucks).

The counter was checked daily with a standard ^{14}C source, so that when its efficiency varied, correction could be made to results obtained on different days. Such correction was seldom necessary because all samples from an experiment were usually counted on the same day. For the same reason, it was not usually necessary to correct for the decay of the sulphathiazole- ^{35}S during an experiment. So that results of different experiments could be compared, a standard solution of sulphathiazole- ^{35}S was included in each experiment, to allow counts per min. (c.p.m.) to be converted to μmoles sulphathiazole. The background was counted daily, and was 9 c.p.m. throughout.

Each solution was plated on at least three planchets, each of which was counted. If there was considerable

variation between planchets of the same material, extra ones were counted. The sum of the counts of the individual planchets was large enough to ensure that the probable error of the counting was less than 2%. The number of counts needed to achieve this, for a given ratio of sample strength to background strength, was determined from published curves (Francis, Mulligan and Wormald, 1954).

There was no detectable effect of self-absorption provided the weight of dried sample was less than about 0.5 mg; if necessary samples were diluted to give weights not greater than this. The relation between c.p.m. and concentration of sulphathiazole was linear up to 3000 c.p.m. Active samples were diluted so that they did not give more than about 2000 c.p.m.

Chromatography.

Diazotizable amines were separated by this technique. Chromatograms were run on Whatman No.1 or No.3 paper at room temperature. The solvents used were: (figures are parts by volume)

n-butanol 80: water 20

n-butanol 80: 0.88 ammonia 3: water 17

After thorough shaking the upper organic layer was used. Methanol 35: n-amyl alcohol 17.5: benzene 35: 0.88 ammonia 4: water 8.5. This mixture forms a single phase at room temperature. This solvent is a slight modification of one used by Ekman (1948):

Methanol 35: n-amyl alcohol 17.5: benzene 35: 4% 0.88 ammonia added just to saturation.

Methanol 35: n-amyl alcohol 17.5: benzene 35: 2N hydrochloric acid added just to saturation. This solvent was also described by Ekman (1948).

To detect diazotizable substances the chromatogram was allowed to dry after development, and the paper was then exposed for 10 min. to nitrous fumes generated from sodium nitrite and hydrochloric acid. After a further 10 min. in air the chromatogram was sprayed with a solution of N(1-naphthyl) ethylenediamine dihydrochloride (100 mg/100 ml).

Metabolism of sulphathiazole by *Streptococcus faecalis*

Cultures were grown from large inocula in 250 ml. medium in the presence of sulphathiazole, usually 100 µg/ml. In a few cases, organisms were grown from smaller inocula in 5 ml. medium. At the end of the incubation the organisms were spun down and the supernatant was removed. The medium was then sprayed with 5 min. before the spray reagent was added. The medium was then removed by the method of Bratton and Marshall (1939) for the determination of sulphathiazole.

The results of the metabolism of sulphathiazole by *Streptococcus faecalis* are shown in Table 13. From these experiments it was concluded that the disappearance of sulphathiazole during growth of *Streptococcus faecalis* is due to the Bratton and Marshall reagent rather than to the action of the organism.

CHAPTER XIII

Metabolism of sulphathiazole

A possible way in which staphylococci might become resistant to sulphathiazole would be for the organisms to convert the drug into non-inhibitory substances. If any such metabolism of sulphathiazole did occur then it should be most apparent during the growth of cultures of resistant strains. Another formal possibility is that in sensitive strains, sulphathiazole is metabolized to a more powerful inhibitor, or converted to the actual inhibitor, while in resistant strains this takes place to a lesser extent.

Metabolism of sulphathiazole by growing organisms.

Cultures were grown from large inocula in 25 ml medium B in the presence of sulphathiazole, usually for 14 hr. In a few cases, organisms were grown from smaller inocula in 5 ml medium B. At the end of the incubation the organisms were spun down and the supernate was heated in boiling water for 5 min. before the sulphathiazole was estimated either by the method of Bratton and Marshall or microbiologically.

The results of estimations of sulphathiazole in culture filtrates by the method of Bratton and Marshall are shown in Table 33. From these experiments there appeared to be no disappearance of sulphathiazole during growth. However, since the Bratton and Marshall reagent tests only for the presence of

Table 33

Concentration of sulphathiazole at beginning and end of incubation of staphylococcal cultures in medium B.
(chemical estimation)

Organism	Time of incubation (hr)	Conc. of sulphathiazole (M) in culture filtrate	
		Start of incubation	Finish of incubation
<u>Staph. lactis</u> 2102	15	5×10^{-5}	6×10^{-5}
" <u>2102R</u>	15	5×10^{-5}	5×10^{-5}
" <u>2102R2</u>	15	5×10^{-5}	6×10^{-5}
<u>Staph. aureus</u> JHM	15	5×10^{-6}	4×10^{-6}
" <u>JHMR</u>	15	5×10^{-5}	4×10^{-5}
<u>Staph. aureus</u> H	15	5×10^{-5}	6×10^{-5}
" <u>HR2</u>	15	5×10^{-5}	5×10^{-5}
<u>Staph. aureus</u> R122	95	10^{-3}	1.6×10^{-3}

a diazotizable amino group, the possibility remains that sulphathiazole might be altered in a way which leaves its amino group unaffected, or, much less likely, that the sulphathiazole that is metabolized is quantitatively replaced by another substance containing a diazotizable amino group.

Culture supernates were, therefore, also tested quantitatively for their power to inhibit a sulphathiazole-sensitive strain, Staph. aureus H. The concentration of sulphathiazole in the diluted culture filtrate was taken to be the same as that of a standard solution of sulphathiazole which inhibited growth of the test organism for the same length of time. Results from two or three dilutions of the filtrate gave values in very close agreement for the concentration of sulphathiazole in the undiluted filtrate. In every case there was no change in the inhibitory effect of the sulphathiazole after organisms had grown in its presence (Table 34).

Chromatography of culture filtrates.

Supernates were examined by chromatography to detect diazotizable amines. The solvent most often used was the modification of that of Ekman (1948) which has already been described (Chapter XII). Chromatograms on Whatman No.1 or No.3 paper were developed for about 6 hr at room temperature by descending chromatography.

Chromatograms of supernates from cultures which had grown in the presence of sulphathiazole (usually $10^{-3}M$) showed two

Table 34

Concentration of sulphathiazole at beginning and end of
incubation of staphylococcal cultures in medium B
(microbiological estimation)

Organism	Time of Incubation (hr)	Concentration of sulphathiazole in culture filtrate (<u>M</u>)	
		Start of incubation	Finish of incubation
<u>Staph. lactis 2102</u>	14	10^{-3}	10^{-3}
" <u>2102R</u>	14	10^{-3}	10^{-3}
<u>S. aureus JHM</u>	44	10^{-3}	10^{-3}
" <u>JHMR</u>	98	10^{-4}	10^{-4}
<u>S. aureus HR2</u>	44	10^{-3}	10^{-3}
<u>S. aureus R122</u>	95	10^{-3}	10^{-3}

Experiments with sulphathiazole- ^{35}S

When supernates from cultures grown in the presence of sulphathiazole- ^{35}S (10^{-3}g) were chromatographed it was found that radioactivity was present in the supernatant in the same positions as the two fractions and normally purified spots, and nowhere else. The positions of the radioactive spots were

spots of a similar brownish-red colour when diazotized and sprayed with the Bratton and Marshall reagent. The larger spot had an R_f value of 0.40 and corresponded in position to that given by sulphathiazole alone; the second spot had an R_f value of 0.15. The latter was not obtained with supernates from cultures which had been grown in the absence of sulphathiazole, nor with the supernatant from sulphathiazole-containing medium immediately after inoculation. Chromatograms of uninoculated medium containing sulphathiazole which had been incubated at 37° for 14 hr gave only one spot of R_f 0.40 on chromatograms. The unknown was chromatographically distinct from 4-amino-5-imidazolecarboxamide, which has been reported to accumulate in sulphonamide-inhibited cultures of Esch.coli (Stetten and Fox, 1945; Shive, Ackerman, Gordon, Getzendamer and Eaken, 1947), and from PAB (see Table 35). As far as could be judged by eye from the chromatograms the amount of the unknown substance formed was proportional to the concentration of sulphathiazole present in the growth medium. All strains seemed to form about the same amount of the unknown.

Experiments with sulphathiazole- ^{35}S .

When supernates from cultures grown in the presence of sulphathiazole- ^{35}S (10^{-3}M) were chromatographed it was found that radioactivity was present on the chromatogram in the same positions as the two Bratton and Marshall positive spots, and nowhere else. The positions of the radioactive spots were

Table 35

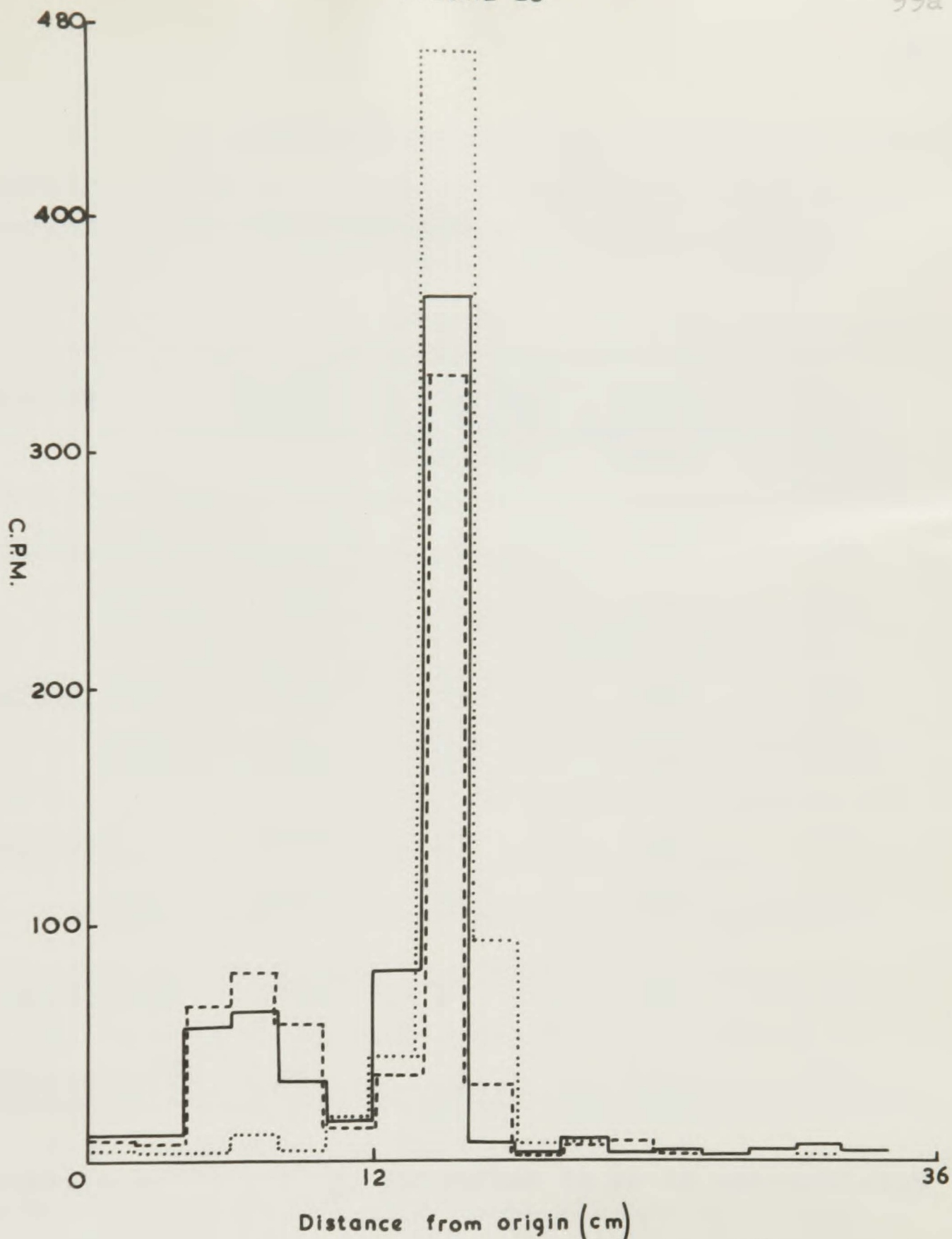
R_f values of sulphathiazole and other Bratton and Marshal positive substances in various solvents

Solvent	Substance	R_f
35 Methanol	Sulphathiazole	0.43
17.5 <i>n</i> -amyl alcohol	"Unknown"	0.19
35 Benzene	PAB	0.24
+ 8.5 Water	4-amino-5-imidazole-carboxamide	0.30
+ 4 0.88 ammonia	Sulphanilic acid	0.28
	Sulphanilamide	0.49
A		
+ 2N hydrochloric acid to saturation	Sulphathiazole	0.43
	"Unknown"	0.22
	PAB	0.23
	4-amino-5-imidazole-carboxamide	0.27
Butanol: water	Sulphathiazole	0.55
	"Unknown"	0.15
	PAB	0.10
Butanol: ammonia	Sulphathiazole	0.53
	"Unknown"	0.13
	PAB	0.10

determined with an unshielded mica-end window Geiger-Müller tube which was connected to a scaler unit via a probe unit. The end window of the Geiger-Müller tube was shielded by a thin polythene disc with a hole 2 cm. square in the centre. Squares of this size were lightly drawn down the whole length of a chromatogram and the radioactivity of each was measured for 2 min. by applying the counting tube window to the paper. A typical result obtained by plotting radioactivity against distance from the origin is given in Figure 25. The chromatograms were diazotized and sprayed after counting to confirm that the peaks of radioactivity corresponded to the positions of the spots.

When organisms had been grown with sulphathiazole-³⁵S two peaks of radioactivity were found, whereas sulphathiazole-³⁵S gave only one peak of activity after being incubated in the uninoculated medium. An approximate estimate of the proportion of the sulphathiazole that was converted to the unknown by various strains during growth was made by comparing the number of counts found in each of the two peaks (Table 36). There was no correlation between the activity found in the peak due to the unknown and the degree of resistance of the strain to sulphathiazole, though there seems to be a species variability. Staph.lactis 2102 and 2102R formed very much less of the unknown material than did the strains of Staph.aureus.

The unknown compound formed during growth of Staph.aureus HR2 in presence of sulphathiazole was investigated in more detail.



_____ Staph.aureus H + sulphathiazole-³⁵S
 - - - - - Staph.aureus HR2 + "
 Sulphathiazole-³⁵S incubated 14 hr in medium B

Chromatography of Staph.aureus H and HR2
 culture filtrates after growth in medium B
 in the presence of sulphathiazole-³⁵S (10^{-3} M)

Table 36

Radioactivity of Bratton and Marshall positive spots on chromatograms

Organism	c.p.m. Unknown	% Unknown (of total activity)	c.p.m. Sulphathi- azole	c.p.m. Total chroma- togram
<u>Staph. lactis</u> 2102	22	7	265	294
" <u>2102R</u>	14	4	313	333
<u>Staph. aureus</u> JHM	82	16	423	512
" <u>JHMR</u>	73	14	436	517
<u>Staph. aureus</u> H	155	23	457	687
" <u>HR2</u>	204	31	404	666
<u>Staph. aureus</u> R122	95	33	181	289
*Sulphathiazole- ³⁵ S	21	3	611	699

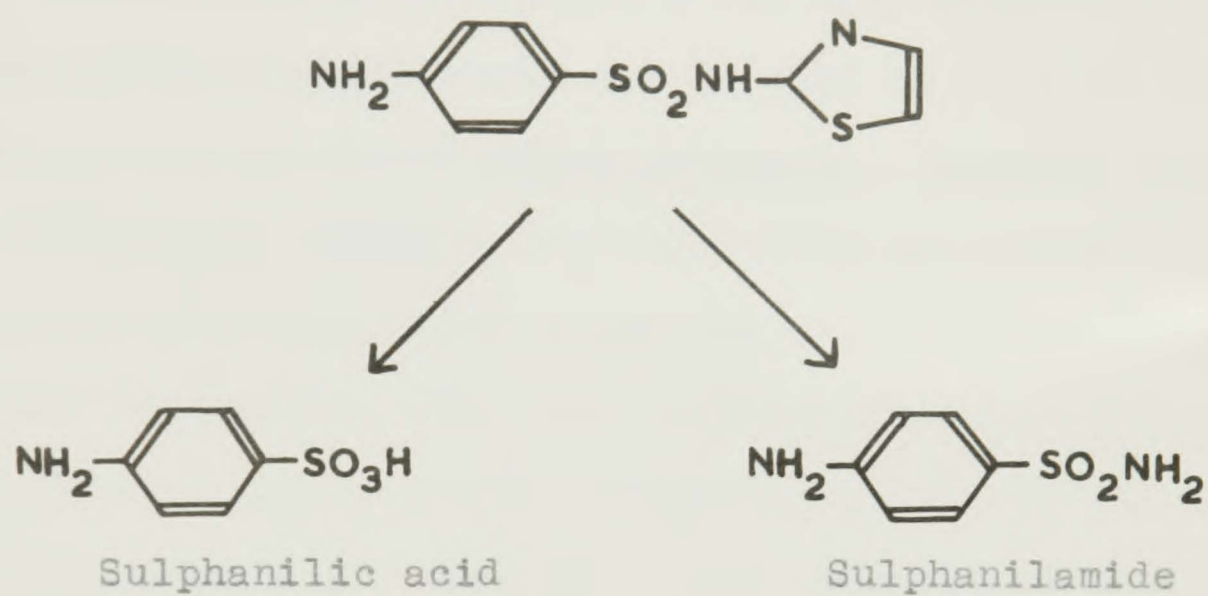
* Sulphathiazole-³⁵S (10^{-3} M) incubated 14 hr in uninoculated medium B. Inhibited in one experiment but not in another.

This may be because the positions of the unknown and PA2 on the stripe were close together. The unknown was eluted from a chromatogram with water. Measurement of the eluted material with a Geiger counter showed that radioactive material was present, but this eluent failed to inhibit the growth of Staph. aureus H in medium B. This again might be because PA2

The R_f values of the unknown and of possibly related compounds were determined in several solvents. No solvent tested gave a good separation of the unknown from PAB added to cultures after growth (Table 35). The unknown retains the diazotizable amino group and the ^{35}S atom of sulphathiazole- ^{35}S . Two substances which would fulfil these requirements, and which could be imagined to be derived from sulphathiazole, are sulphanilic acid and sulphanilamide (Figure 26). Both of these substances, however, were chromatographically distinct from the unknown compound (Table 35).

Attempts were made to determine whether the unknown had growth-inhibiting properties. Strips cut from chromatograms to include the unknown spot were put on the surface of agar plates of Davis' minimal medium which had been inoculated with either Esch.coli 518 or Esch.coli 273/384 (a PAB-requiring mutant). The main sulphathiazole spot inhibited growth of both organisms in its vicinity, whereas the unknown spot gave erratic results; Esch.coli 518 was not inhibited, but Esch.coli 273/384 was inhibited in one experiment but not in another. This may be because the positions of the unknown and PAB on the strips were close together. The unknown was eluted from a chromatogram with water. Measurement of the eluted material with a Geiger counter showed that radioactive material was present, but this eluant failed to inhibit the growth of Staph.aureus H in medium B. This again might be because PAB

FIGURE 26



Hypothetical breakdown products of sulphathiazole

was also being eluted from the chromatogram or because the eluted material had been diluted too much for its inhibitory properties to be shown when tested in liquid culture. An amount of eluant equivalent to 0.3 ml of the original supernate containing 10^{-3} M sulphathiazole was diluted to 5 ml in testing its effect on the growth of Staph.aureus H. Assuming that one molecule of unknown compound is formed from one molecule of sulphathiazole and that about a quarter of the sulphathiazole is converted to unknown, then the concentration of unknown would not be much greater than 10^{-5} M in the test of its power of inhibition even if it had been eluted quantitatively. The concentration of sulphathiazole required to inhibit this strain is 5×10^{-6} M.

Since similar amounts of the unknown compound were formed from sulphathiazole during growth of pairs of sensitive and resistant strains it seemed unlikely that the basis of resistance was the conversion of sulphathiazole to an inactive substance. For this reason, the chemical nature of the unknown compound was not investigated further.

Metabolism of sulphathiazole by washed suspensions of organisms.

Staph.aureus H and HR2 were grown in medium B (25 ml) from large inocula in the presence of 5×10^{-5} M sulphathiazole. After harvesting and washing in 0.02 M phosphate buffer pH7.4, organisms were suspended at a density of about 1 mg dry wt per ml in buffer and were incubated with sulphathiazole at various

concentrations (10^{-3}M - 10^{-5}M). After 5 hr sulphathiazole was estimated by the method of Bratton and Marshall in cell-free supernates. There was no disappearance of sulphathiazole, and chromatography revealed only one diazotizable component, which was sulphathiazole itself.

Supernates from heavy suspensions of Staph.aureus JHM and JHMR which had been incubated for an hour in medium B with sulphathiazole- ^{35}S ($2.5 \times 10^{-3}\text{M}$) were examined chromatographically (Figure 27). The unknown compound was found; with Staph.aureus JHM it comprised only 5% of the total radioactivity on a chromatogram, while with the resistant substrain it represented 23%. If the suspensions were boiled before addition of sulphathiazole then none of the unknown was formed.

Discussion of results.

There is no evidence to support the view that the resistance of any strain to sulphathiazole is due to the conversion of sulphathiazole into non-inhibitory products. Culture filtrates when tested chemically or microbiologically showed no disappearance of sulphathiazole following growth of organisms in its presence. The chemical estimation is not specific for sulphathiazole and only shows that the aromatic primary amine group in its molecule remains intact, but the microbiological test appears to be unequivocal in showing that the inhibitory effect of sulphathiazole is undiminished after staphylococci have grown in its presence. If this test had

FIGURE 27



Chromatography of supernates from thick suspensions of Staph. aureus JHM and JHMR incubated in medium B with sulphathiazole-³⁵S ($4 \times 10^{-3} M$)

shown an apparent decrease in the activity of the sulphathiazole, then this could have been interpreted as being due not only to destruction of the inhibitor but also to its inactivation by the synthesis of PAB. The fact that there is no decrease in the activity of sulphathiazole shows, therefore, that no strain is able to form as much as 10^{-4} M PAB in the presence of 10^{-3} M sulphathiazole. (The inhibition index of the test organism is 10, so a concentration of PAB one tenth that of the sulphathiazole would inactivate the drug towards the test organism).

Even though there is some conversion of sulphathiazole into an unknown compound by growing organisms, this takes place in sensitive and resistant pairs of strains to a similar extent. Also the amount of sulphathiazole converted to unknown is too small to explain the degree of resistance found in the sulphathiazole-resistant strains. In no strain was more than one third of the sulphathiazole present during growth converted to unknown. Clear evidence was not obtained as to whether the unknown substance was inhibitory after separation on chromatograms from sulphathiazole, perhaps because of contamination of the unknown with PAB, which was difficult to separate well from the unknown compound. Since culture filtrates showed the full inhibitory effect of the sulphathiazole added before incubation it does seem likely that the unknown compound has inhibitory activity similar to that of sulphathiazole.

It does not seem that inhibition by sulphathiazole is brought about in any of the present strains by conversion of

the drug to a more potent inhibitor. After incubation in growing cultures, sulphathiazole-³⁵S gave rise to only two substances on chromatograms, and of these, only the spot corresponding to sulphathiazole itself is definitely inhibitory when tested by bioautography. Furthermore, if the unknown compound were responsible for the inhibitory effect of addition of sulphathiazole to a medium, then the extent of its formation might be expected to be similar in strains of similar sensitivity to the drug.

The method of sulphathiazole-³⁵S growth in organisms after incubation with the drug was described previously. A similar indirect method was similar in principle to that described by Mitchell (1953) for the measurement of the permeability of staphylococci to phosphate. Results of these two methods were in reasonable agreement in view of the uncertainties and approximations made during the calculations over the actual observations.

Method 1. Experiments with thin suspensions of organisms.

Organisms were harvested after growth in medium 2, washed once in water and resuspended in medium 3 (double strength) to a density of about 2 mg dry wt per ml. Subsequent procedure has for clarity been shown schematically (Table 37).

CHAPTER XIV

The uptake of sulphathiazole by washed suspensions of staphylococci

The uptake of sulphathiazole by the various strains of staphylococci was investigated with the primary object of finding out whether there was any correlation between the amount of sulphathiazole taken up by a strain and its degree of resistance to the drug. The amount of sulphathiazole taken up by organisms during growth in medium B was found to be so small as to be barely detectable even when sulphathiazole-³⁵S was used. The uptake was more easily measured when the density of organisms was increased by using washed suspensions.

The amount of sulphathiazole-³⁵S present in organisms after incubation with the drug was measured directly. A second, indirect method was similar in principle to that described by Mitchell (1953) for the measurement of the permeability of staphylococci to phosphate. Results of these two methods were in reasonable agreement in view of the assumptions and approximations made during the calculations from the actual observations.

Method 1. Experiments with thin suspensions of organisms.

Organisms were harvested after growth in medium B, washed once in water and resuspended in medium B (double strength) to a density of about 2 mg dry wt per ml. Subsequent procedure has for clarity been shown schematically (Table 37).

Table 37

Procedure for experiments with thin suspensions of staphylococci incubated in medium B with sulphathiazole.

Suspension (1 ml) placed in weighed 5"x8" tube and 10^{-2} M sulphathiazole-³⁵S (1 ml) added

Incubated upright 1-6 hr at 37° Weighed

Organisms spun down at 3000 r.p.m. for 15 min.

Pad of organisms P
weighed

Supernate A removed with fine Pasteur pipette.

Organisms suspended in water (2 ml) Weighed

Heated 5 min in boiling water
Sulphathiazole-³⁵S counted

Organisms spun down at 3000 r.p.m. for 15 min.

Pad of organisms P₂
weighed

Supernate B removed.
Heated 5 min. in boiling water
Sulphathiazole-³⁵S counted

Organisms suspended in water (2 ml) W. Weighed

Heated 5 min. in boiling water

Spun down debris

Supernate C removed
Sulphathiazole-³⁵S counted

The amount of sulphathiazole-³⁵S associated with the organisms at the end of the incubation, x c.p.m. is given by:

$$x = A_C v_W + A_B v_B - \frac{(P_1 - 2.5D)A_A}{1.01}$$

where A_A , A_B and A_C are the specific activities (c.p.m./ml) of supernates A, B and C; v_B is the volume (ml) of supernate B, and v_W the volume (ml) of the suspension W of washed organisms in water. P_1 is the weight (g) of the pad of centrifuged organisms after the incubation and D is the dry weight (g) of organisms used in the experiment.

The values of v_B and v_W were taken to be equal to the weights of supernate B and suspension W since their relative densities are very close to unity. The concentrations of sulphathiazole in the washed suspension W and in supernate C were about the same. The organisms were removed before plating samples to avoid the charring which took place when suspensions were dried on planchets.

It was found with thick suspensions of organisms that the weight of a given amount of packed staphylococci (after correction for 25% of the volume being interstitial liquid) was 2.5 times their dry weight. Hence, in the present experiments the wet weight of the organisms at the end of the incubation was taken as 2.5 times their dry weight. The excess by which the weight of the pad of centrifuged organisms exceeded the calculated value was attributed to extracellular

supernate A. An amount of sulphathiazole equivalent to this weight of supernate A (relative density 1.01) was deducted from the amount of sulphathiazole calculated to be present in the pad of organisms. The calculation from the results of a typical experiment is illustrated in Table 38.

The amount of sulphathiazole-³⁵S associated with the organisms after washing, y c.p.m. is given by:

$$y = A_C v_W - (P_2 - 2.5D)A_B$$

where P_2 is the weight (g) of the pad of washed organisms. The calculation from results of a typical experiment is illustrated in Table 38.

Uptake of sulphathiazole-³⁵S from medium B.

Results are shown in Table 39. The effect of a single washing of the organisms after incubation with sulphathiazole is very striking; the sulphathiazole is very largely removed, which indicates that it is not firmly bound. The uptakes by the various strains were similar, increasing somewhat with time, but showing no obvious differences between sensitive and resistant strains. The only exception to this was Staph.aureus JHM which showed an uptake of sulphathiazole roughly double that of its resistant substrain.

Because of the large correction that was applied in the calculation and because agreement of results from similar preparations was not very good, the uptake of sulphathiazole was measured by a second method.

Table 38

Example of calculation of results for thin suspensions.

of staphylococci incubated with $5 \times 10^{-3} M$ sulphathiazole-358.

Staph. aureus H incubated 1 hr in medium B + $5 \times 10^{-3} M$ sulphathiazole-358.

A_C , Specific activity of supernate C	= 95 c.p.m./ml
v_W , Volume of suspension W	= 2.097 ml
$\therefore A_C v_W$ = 95x2.097	= 199 c.p.m.
A_B , Specific activity of supernate B	= 1290 c.p.m./ml
v_B , Volume of supernate B	= 1.826 ml
$\therefore A_B v_B$ = 1290x1.826	= 2356 c.p.m.
P_1 , Weight of centrifuged organisms	= 0.028g
D , Dry weight of organisms	= 0.002g
A_A , Specific activity of supernate A	= 88000 c.p.m./ml
$\therefore \frac{(P_1 - 2.5D)A_A}{1.01}$ = $\frac{0.023 \times 88000}{1.01}$	= 2004 c.p.m.
$\therefore x$, c.p.m. associated with organisms	= 199 + 2356 - 2004 = 551 c.p.m.
$\therefore$ c.p.m. per mg dry wt = $\frac{551}{2}$	= 276 c.p.m.
c.p.m. per μ mole sulphathiazole-358	= 17.6 (supernate A)
$\therefore$ μ moles sulphathiazole per mg = $\frac{276}{17.6}$	= 16
P_2 , Weight of centrifuged washed organisms	= 0.160g
$\therefore (P_2 - 2.5D)A_B$ = 0.155x1290	= 199 c.p.m.
$\therefore y$, c.p.m. associated with washed organisms	= 199 - 199 = 0

Table 39

Uptake of sulphathiazole from medium B by washed suspensions of staphylococci incubated with $5 \times 10^{-5} M$ sulphathiazole-35S.

Organism	Time of (hr) incubation	μ moles sulphathiazole taken up per mg dry weight After incubation	After washing
<u>Staph. lactis 2102</u>	1	10	0
	3	20	0
	9	53	0
<u>Staph. lactis 2102R</u>	1	25	0
	6	24	0
<u>Staph. lactis 2102R2</u>	1	15	0
	6	23	0
<u>Staph. aureus JHM</u>	1	77	19
	5	72	13
<u>Staph. aureus JHMR</u>	1	28	1
	5	33	2
<u>Staph. aureus H</u>	1	22	0
	3	30	0
	6	37	3
<u>Staph. aureus HR2</u>	1½	8	1
	3	29	3
	8	36	1
<u>Staph. aureus R122</u>	1	30	3
	6	48	8

0 means less than 0.5 μ moles/mg dry wt.

Method 2. Experiments with thick suspensions of organisms.

Organisms were grown in Roux bottles. After harvesting, washed suspensions in water were prepared and their dry weights determined. A given volume of this suspension was taken, and after sedimenting by centrifugation, the organisms were resuspended to a measured volume to give a density of about 100 mg dry wt in the medium (at double strength) used for investigation of permeability.

This suspension (1 ml) was placed in a weighed 5"x $\frac{3}{8}$ " tube, and sulphathiazole and other test solutions were added to a final volume of 2 ml. The tube was incubated upright at 37° for an hour and was then weighed. The organisms were spun out (15 min, 3000 r.p.m.) and the supernate (E) was removed as completely as possible with a fine Pasteur pipette into a clean tube. The tube containing bacteria was reweighed after removing the excess solution from the inner wall with filter paper. As part of the experiment, a tube was incubated in which the sulphathiazole was similarly diluted by the constituents of the incubation mixture, but from which the organisms were omitted.

The concentration of sulphathiazole in this solution (F) and in supernate E was estimated either by the method of Bratton and Marshall, or by measurement of radioactivity. Before these estimations, all solutions were heated for 5 min. in boiling water to kill the organisms. The tops of the tubes

were closed with glass bubbles to prevent loss by evaporation during heating.

The amount of sulphathiazole associated with the organisms at the end of the incubation, Z c.p.m. is given by

$$Z = A_F v_F - A_E v_E - \frac{v_{P_3} A_E}{4}$$

where A_E and A_F are specific activities (c.p.m./ml) of supernate E and solution F respectively; v_E is the volume of supernate E (ml), and v_F is the volume of solution F; v_{P_3} is the volume of the centrifuged pad of organisms.

The total volumes of solution F and supernate E were calculated from their weights and relative density (1.01). It was possible to remove the supernate from the pad of centrifuged organisms completely, as far as could be judged by eye, and the weights of the pads from the various tubes in an experiment agreed closely. The interstitial liquid was, therefore, assumed to be the only extracellular supernate E present. The interstitial volume for closely packed spheres was taken to be 25% of the total volume of the pad of organisms (Conway and Downey, 1950), which was calculated from its weight and relative density (1.13, measured in haematocrit tubes). An amount of sulphathiazole equivalent to this volume of supernate E was deducted as correction for interstitial liquid. The calculation from results of a typical experiment is illustrated in Table 40.

The results calculated in Table 40

Example of calculation of results for thick suspension.

Staph. aureus JHMR incubated 1 hr in medium B + $4 \times 10^{-3} M$ sulphathiazole-³⁵S

A_F , Specific activity of solution F = 25450 c.p.m./ml

V_F , Volume of solution F = 2.019 ml

$$\therefore A_F V_F = 25450 \times 2.019 = 51380 \text{ c.p.m.}$$

A_E , Specific activity of supernate E = 22450 c.p.m./ml

V_E , Volume of supernate E = 1.777 ml

$$\therefore A_E V_E = 22450 \times 1.777 = 39890 \text{ c.p.m.}$$

V_{P_3} = Volume of pad of centrifuged organisms = $\frac{0.289}{1.13} = 0.256 \text{ ml}$
(Wt of pad = 0.289g; relative density = 1.13)

$$\therefore \frac{V_{P_3} A_E}{4} = \frac{0.256 \times 22450}{4} = 1437 \text{ c.p.m.}$$

$$\therefore z, \text{ c.p.m. associated with organisms} = 51380 - 39890 - 1437 = 10053 \text{ c.p.m.}$$

Dry weight of organisms = 88 mg

$$\therefore \text{c.p.m. per mg dry wt} = \frac{10053}{88} = 114 \text{ c.p.m.}$$

c.p.m. per μ mole sulphathiazole ³⁵S = 6.4 (solution F)

$$\therefore \mu \text{ moles sulphathiazole per mg} = \frac{114}{6.4} = 18$$

μ moles sulphathiazole per $(0.256 - \frac{0.256}{4})$ ml organisms =
 μ moles sulphathiazole per 0.192 ml supernate E

$$\frac{10053}{22450 \times 0.192} = 2.3$$

The results calculated as above were checked in a few cases by showing that roughly the same amount of sulphathiazole was recovered from the pad of organisms by two washes with water (2 ml) as was calculated to be present. In one experiment the centrifuged organisms which had been incubated with sulphathiazole-³⁵S were washed either with water or with $10^{-3}M$ sulphathiazole. Each washing procedure gave a similar recovery of sulphathiazole-³⁵S, in agreement with the results calculated indirectly from the dilution of the sulphathiazole-³⁵S at the beginning of the experiment (Table 41). These results indicate that the sulphathiazole taken up by the organisms is not bound firmly, but can exchange very easily with the external medium. Chromatography of material extracted by boiling water from Staph. aureus HR2 after incubation with sulphathiazole showed the presence of only one diazotizable substance which was sulphathiazole itself.

The agreement between determinations of uptake of sulphathiazole by measurement of radioactivity ($-SO_2$ -group) or by chemical estimation (NH_2 -group) make it very probable that measurement of ³⁵S gives a valid estimate of concentration of sulphathiazole. Although sulphathiazole is partly converted to an unknown compound (Chapter XIII) during incubation of thick suspensions of organisms, the chromatographic evidence quoted above shows that sulphathiazole is taken up by the organisms.

Estimation by various procedures of uptake of sulphathiazole-³⁵S
by Staph.aureus JHM and JHMR.

Incubated 1 hr at 37° in medium B with $4 \times 10^{-3} \text{M}$ sulphathiazole-³⁵S.

Table 41

Organism	mm moles sulphathiazole/mg dry weight, estimated by:			
	Dilution of sulphathiazole by cell suspension		Washing in water	Washing in 10^{-3}M sulphathiazole
	Measurement of ³⁵ S	Bratton and Marshall est ⁿ .	Measurement of ³⁵ S	Measurement of ³⁵ S
<u>Staph.aureus JHM</u>	7	17	17	-
	13	15	-	18
<u>Staph.aureus JHMR</u>	20	14	17	-
	17	15	-	16

Dilution of sulphathiazole by cell suspension: Method 2

Washing in water: Organisms suspended after incubation in 2 ml water and spun down.
Resuspended in water (2 ml) and again spun down.
Supernates counted.

Washing in 10^{-3}M sulphathiazole: Procedure as above, using 10^{-3}M sulphathiazole (2 ml) instead of water.

Values are averages of duplicate determinations.

Uptake of sulphathiazole by thick suspensions of organisms in medium B.

Again the primary object was to find out whether there was a difference in the uptake of sulphathiazole between resistant and sensitive strains. With $5 \times 10^{-3} \text{M}$ sulphathiazole uptake by all the strains is about the same (Table 42). The uptake of sulphathiazole by Staph.aureus JHM and H and their resistant substrains is roughly proportional to the concentration of sulphathiazole in the medium during the incubation; the ratio

$$\frac{\text{mu moles sulphathiazole per } x \text{ ml organisms}}{\text{mu moles sulphathiazole per } x \text{ ml medium}}$$

is fairly constant at different concentrations of sulphathiazole in the medium. A value greater than unity for this ratio indicates that the concentration of drug associated with the organisms is higher than that in the medium.

Uptake of sulphathiazole by suspensions of organisms in medium B in the presence of PAB.

The purpose of these experiments was to find out whether the uptake of sulphathiazole- ^{35}S was reduced in the presence of PAB, which might displace the drug from an enzyme surface. Heavy suspensions were incubated as before in medium B for an hour at 37° with $2.5 \times 10^{-3} \text{M}$ sulphathiazole and various concentrations of PAB. The uptake of sulphathiazole- ^{35}S by the organisms was calculated from measurements of the radioactivity of supernates.

Uptake of sulphathiazole by thick suspensions of staphylococci

Organism	μμ moles sulphathiazole per mg dry wt.				μμ moles sulphathiazole per x ml cells μμ moles sulphathiazole per x ml medium			
	Molar conc. of sulphathiazole present during incubation				Molar conc. of sulphathiazole present during incubation			
	5x10 ⁻³	4x10 ⁻³	2.5x10 ⁻³	5x10 ⁻⁴	5x10 ⁻³	4x10 ⁻³	2.5x10 ⁻³	5x10 ⁻⁴
<u>Staph. lactis 2102</u>	20				1.3			
<u>Staph. lactis 2102R</u>	34				1.5			
<u>Staph. aureus H</u>	20		10		1.5			
<u>Staph. aureus HR2</u>	22		11		1.7			
<u>Staph. aureus JHM</u>	23	15	10	3	1.8	1.6	1.5	3
<u>Staph. aureus JHMR</u>	18	18	11		1.4	1.8	2.0	

Table 42

Incubated 1 hour at 37° in medium B, with sulphathiazole-³⁵S.

Values are averages of duplicate determinations.

No difference was found in the uptake of sulphathiazole-³⁵S whether PAB was present or not (Table 43). This observation, together with the fact that sulphathiazole is so easily removed from the cell by washing, strongly suggests that at least the greater part of the uptake of sulphathiazole is not due to binding at a particular site. This is considered further in the Discussion.

Uptake of sulphathiazole by suspensions of organisms in water and in buffer.

A few measurements were made of the uptake of sulphathiazole by suspensions in water and in buffer to find out whether the uptake was dependent on an external source of energy and nutrients, and whether under these conditions the uptake was the same in sensitive strains and their resistant substrains. Such comparison is perhaps not so relevant to the problem of resistance as is comparison of the uptake from the growth medium, in which the phenomenon of resistance is actually manifested.

Heavy suspensions of organisms were incubated with $5 \times 10^{-3} \text{M}$ sulphathiazole for 1 hr at 37° in water and in 0.02M phosphate buffer pH 7.4 at the same time as they were incubated in medium B. The same procedure and method of calculation were used as before. Results are shown in Table 44. The uptake from buffer was generally less than from the growth medium, and the uptake from water was still less. Even from water, the

sulphathiazole-³⁵S.

Values are averages of duplicate determinations.

Table 43

Effect of PAB on uptake of sulphathiazole-³⁵S by cell suspensions

Organism	Conc. of PAB during incubation (<u>M</u>)	μ moles sulphathiazole taken up per mg cells
<u>Staph.aureus JHM</u>	0	10
	2.5x10 ⁻⁵	12
	2.5x10 ⁻⁴	11
	2.5x10 ⁻³	12
<u>Staph.aureus JHMR</u>	0	11
	2.5x10 ⁻⁶	11
	2.5x10 ⁻⁵	10
	2.5x10 ⁻⁴	9
	0	13
	2.5x10 ⁻³	11
<u>Staph.aureus H</u>	0	10
	2.5x10 ⁻⁴	11
	2.5x10 ⁻³	13
<u>Staph.aureus HR2</u>	0	11
	2.5x10 ⁻⁵	14
	2.5x10 ⁻⁴	14
	2.5x10 ⁻³	12

Incubated 1 hour at 37° in medium B, + 2.5x10⁻³M sulphathiazole-³⁵S.

Values are averages of duplicate determinations.

Uptake of sulphathiazole from various suspension media (Method 2)

Incubated 1 hr at 37° with $4 \times 10^{-3} \text{M}$ sulphathiazole- ^{35}S

Organism	<u>$\mu\text{moles sulphathiazole}$ per mg dry wt.</u>			<u>$\mu\text{moles sulphathiazole per x ml cells}$ $\mu\text{moles sulphathiazole per x ml medium}$</u>		
	Water	0.02M Buffer pH7.4	Medium B	Water	0.02M Buffer pH7.4	Medium B
<u>Staph. lactis 2102</u>	10	16	20	0.5	1.0	1.3
<u>Staph. lactis 2102R</u>	26	28	34	1.2	1.3	1.5
<u>Staph. aureus JHM</u>	-	16	25	-	1.3	2.2
<u>Staph. aureus JHMR</u>	-	4	18	-	0.3	1.4
<u>Staph. aureus H</u>	9	10	20	0.6	0.7	1.5
<u>Staph. aureus HR2</u>	10	14	22	0.7	1.0	1.7

Table 44

uptake of sulphathiazole led to a concentration associated with the organisms about equal to that of the medium. The difference in uptake of sulphathiazole from buffer between Staph. aureus JHM and JHMR was not shown when the uptakes by thin suspensions of these organisms were compared.

The difference in uptake of sulphathiazole between Staph. aureus JHM and J and their resistant strains that might be responsible for the resistance, was also investigated. It was probable that the resistance might be due to an increased amount of a PAB-utilizing enzyme or to a change in the relative affinities of such an enzyme for PAB and sulphathiazole. It was thought possible that if a change in the properties or quantity of a PAB-utilizing enzyme had occurred in any of the resistant strains, then this might be reflected by a greater uptake of PAB from the growth medium by the resistant strains. However, since PAB is not normally required in the growth medium of staphylococci, which are able to synthesize this factor, the probability of finding such a change did not seem very great.

The uptake of PAB from the growth medium by thick suspensions of washed organisms was investigated. Only Staph. aureus JHM and J and their resistant strains were used in these experiments.

Uptake of PAB from medium

Organisms were grown in Bact bottles, and suspensions of washed organisms (100 mg dry wt per ml) were prepared in

CHAPTER XVThe uptake of p-aminobenzoic acid by washed suspensions of Staph. aureus

The failure to demonstrate any differences between Staph. aureus JHM and H and their resistant strains that might be responsible for the resistance, made it seem increasingly probable that the resistance might be due to an increased amount of a PAB-utilizing enzyme or to a change in the relative affinities of such an enzyme for PAB and sulphathiazole. It was thought possible that if a change in the properties or quantity of a PAB-utilizing enzyme had occurred in any of the resistant strains, then this might be reflected by a greater uptake of PAB from the growth medium by the resistant strains. However, since PAB is not normally required in the growth medium of staphylococci, which are able to synthesize this factor, the probability of finding such a change did not seem very great.

The uptake of PAB from the growth medium by thick suspensions of washed organisms was investigated. Only Staph. aureus JHM and H and their resistant substrains were used in these experiments.

Uptake of PAB from medium B.

Organisms were grown in Roux bottles, and suspensions of washed organisms (100 mg dry wt per ml) were prepared in

medium B. These suspensions were incubated for an hour at 37° with added unlabelled PAB. Subsequent procedure and calculation were the same as in the experiments on the uptake of sulphathiazole by thick suspensions (Chapter XIV). The method of Bratton and Marshall was used to estimate PAB.

To check the results, the pad of centrifuged organisms was resuspended in 5 ml. water and heated for 5 min. in boiling water. The debris was spun down and the concentration of PAB in the supernate was estimated. The amount of PAB recovered in this way from the pad of organisms was in good agreement with the amount of PAB that the first calculation had indicated to be present (Table 45). Very little material which gave a positive reaction in the Bratton and Marshall test remained in the cell debris.

The first experiments with Staph. aureus H showed that the amount of PAB taken up by the organisms was no greater after 3 hr incubation than after 1 hr, and all subsequent incubations were for 1 hr. The uptake of PAB was usually measured directly on the pad of centrifuged organisms as described above.

Results are shown in Table 46. The uptake of PAB was proportional to the concentration of PAB in the medium, and the concentration of PAB within the organisms was higher than in the external medium. With the same concentration of PAB in the medium all strains tested took up very similar amounts of PAB.

Table 45

Uptake of PAB by thick suspensions of washed cells of
Staph.aureus H incubated in medium B

Conc. of PAB in medium (<u>M</u>)	Time of incubation (hr)	Uptake of PAB (mμ moles/mg)	
		*Indirect	+Direct
10^{-3}	1	6	7
5×10^{-4}	1	4	4
2.5×10^{-4}	1	2	2
1.25×10^{-4}	1	1	2
-	1	0	1
5×10^{-4}	0		3
5×10^{-4}	1		5
5×10^{-4}	3		4
-	3		1

* Indirect estimation of PAB by calculation from concentration of PAB in supernate after removal of cells.

+ Direct estimation of PAB in pad of centrifuged organisms.

In both cases, correction was made for interstitial solution containing PAB.

Values are averages of duplicate determinations.

PAB uptake was estimated directly in the pad of centrifuged organisms.

Values are averages of duplicate determinations.

Table 46

Uptake of PAB by thick suspensions of washed cells of
Staph.aureus incubated for 1 hr in medium B

Organism	Conc. of PAB in medium(M)	Uptake of PAB	
		μ moles/mg	$\frac{\mu\text{ moles PAB/x ml cells}}{\mu\text{ moles PAB/x ml medium}}$
<u>S.aureus JHM</u>	10^{-3}	7	2.6
	5×10^{-4}	4	3.1
	2.5×10^{-4}	3	4.0
	-	1	
<u>S.aureus JHMR</u>	10^{-3}	6	2.3
	5×10^{-4}	3	2.5
	2.5×10^{-4}	2	2.5
	-	1	
<u>S.aureus H</u>	10^{-3}	7	2.9
	5×10^{-4}	4	3.4
	2.5×10^{-4}	2	3.6
	-	1	
<u>S.aureus HR2</u>	10^{-3}	6	2.8
	5×10^{-4}	3	3.2
	2.5×10^{-4}	2	3.9
	-	1	

PAB uptake was estimated directly on the pad of centrifuged organisms.

Values are averages of duplicate determinations.

Uptake of PAB from medium B in the presence of sulphathiazole.

Although there was no difference in the uptake of PAB by sensitive and resistant strains of Staph.aureus from medium B, it seemed possible that in the sensitive strains PAB might be displaced from the site of its function more easily by sulphathiazole than it would be in the resistant strains. Such behaviour has been observed in Enterococcus and Esch.coli by Wacker, Trebst and Simon (1957). This difference might be reflected by the uptake of PAB in sensitive and resistant strains in the presence of sulphathiazole.

Experiments were done with Staph.aureus JHM and JIMR with sulphathiazole as well as PAB added to some tubes. After incubation the organisms were spun out, and PAB and sulphathiazole were extracted with 4 ml water. The concentrations of PAB and of sulphathiazole in the extracts were determined by the modified method of Havinga described in Chapter XII. To check the accuracy of the sum of these two concentrations, the total diazotizable substance in the extracts was estimated by the method of Bratton and Marshall.

The presence of sulphathiazole did not lead to any decrease in the amount of PAB taken up by either strain (Table 47). The apparent slight increase in PAB uptake in the presence of sulphathiazole was probably due to incomplete separation of PAB from sulphathiazole during their estimation.

Table 47

Uptake of PAB by thick suspensions of washed cells of
Staph.aureus JHM and JHMR incubated for 1 hr in medium B
in the presence of sulphathiazole

Organism	Conc. of PAB in medium(<u>M</u>)	Conc. of sulphathi- azole in medium(<u>M</u>)	Uptake of PAB μ moles/mg
<u>Staph.aureus</u>	10^{-3}	-	6
<u>JHM</u>	10^{-3}	10^{-3}	8
	10^{-3}	5×10^{-4}	7
	-	-	1
<u>Staph.aureus</u>	10^{-3}	-	7
<u>JHMR</u>	10^{-3}	10^{-3}	9
	10^{-3}	5×10^{-4}	9
	-	-	1

Values are averages of duplicate determinations.

General biochemical properties.

The present work has been an investigation of the biochemical mechanisms of resistance to penicillin. The sensitivity of strains to penicillin was determined by changes in optical density, and comparisons have, therefore, been made of the biochemical properties of resistant and sensitive strains.

It should be borne in mind that some of the properties of resistant strains might have arisen by mutation unrelated to those which led to resistance. Thus, Hargreaves and Schmitz (1925) went so far as to divide bacteria which showed changed morphology and virulence as well as loss of sensitivity, into a group of "pseudo-resistant" forms, distinct from the genuinely resistant strains which retained the characteristics of the parent strains. One of the reasons for this distinction was that the changes found in "pseudo-resistant" organisms could be observed without the development of resistance.

DISCUSSION

However, at the present time, all the changes found are regarded as consequences of the development of resistance by mutation to penicillin and not as a property only of the variant (Dawson, 1955). The very extensive changes which have been observed are accompanied by the loss of negative sensitivity to penicillin and the presence of penicillinase. (Griggs, Crawford, Abraham and Glazebrook, 1957).

Even if all the differences between sensitive and resistant strains are a consequence of the development of resistance, it may still be difficult to decide which changes are primarily

General biochemical properties.

The present work has been an investigation of the biochemical mechanisms of sulphonamide-resistance. Decreased sensitivity to an inhibitor must result from biochemical changes, and comparisons have, therefore, been made of the biochemical properties of resistant and sensitive strains.

It should be borne in mind that some of the properties of resistant strains might have arisen by mutations unrelated to those which led to resistance. Thus, Morgenroth and Schnitzer (1925) went so far as to divide bacteria which showed changed morphology and virulence as well as loss of sensitivity, into a group of "pseudo-resistant" forms, distinct from the genuinely fast strains which retained the characteristics of the parent strains. One of the reasons for this distinction was that the changes found in "pseudo-resistant" organisms could be observed without the development of resistance.

However, at the present time, all the changes found are regarded as consequences of the development of resistance by mutations which do not necessarily affect one property only of the variant (Demerec, 1955). The very extensive changes which may accompany resistance are exemplified by the Gram negative bacilli obtained by growing Staph. aureus in the presence of penicillin. (Briggs, Crawford, Abraham and Gladstone, 1957).

Even if all the differences between sensitive and resistant strains are a consequence of the development of resistance, it may still be difficult to decide which changes are primarily

responsible for resistance, and which are secondary consequences of an altered metabolism. Knowledge of the mode of action of an inhibitor is of value in deciding the immediate relevance of observed changes to the mechanism of resistance. For example, the changes in amino acid requirements and in carbohydrate metabolism reported in sulphonamide-resistant organisms (Steers and Sevag, 1949; Sevag and Green, 1944) cannot account for the development of fastness on the basis of the Woods-Fildes hypothesis of sulphonamide action, and appear, therefore, to be secondary consequences of resistance.

In the present work, differences between sensitive and resistant strains, other than their degree of fastness, have been few. With a single exception, morphology and cultural appearance of all the strains remained unchanged; Staph.lactis 2102R produced less pigment and excreted less porphyrin than its parent strain growing on solid medium. The significance of this observation is not clear, because the second resistant substrain, 2102R2, resembled the parent rather than the first resistant strain. The virulence of all the strains has not been tested, but coagulase positive strains retained this property when resistance developed.

The rate of growth of sulphonamide-resistant staphylococci has been reported to be lower than that of sensitive organisms (Steers and Sevag, 1949; Beljanski, 1953). In the presence of glucose, growth of resistant strains was found by Steers and Sevag to lag 48 hr behind the sensitive strains even though no

drug was present, and the resistant strains showed a still longer relative lag if glucose was absent. This was not found with any of the present strains of Staph.aureus. Staph.lactis 2102R2 showed a lag 4 hr longer than did Staph.lactis 2102 and 2102R, though the rate of growth of all three strains was the same. The reason for this is not known; the synthesis of an essential metabolite may proceed more slowly in 2102R2. Another difference between 2102R and 2102R2 was that the former did not grow as heavily as the parent strain in the semi-defined medium B (based on acid-hydrolyzed casein). This difference was not shown when 2102R was first used, but has developed over a period of three years with monthly subcultivation; during this time the degree of resistance remained unchanged.

Comparisons of nutrition and general biochemical properties did not reveal differences between sensitive and resistant strains of Staph.aureus. However, some points of difference were again found between Staph.lactis 2102 and its resistant substrains.

Staph.lactis 2102 grew only after a lag if aspartic or glutamic acid were omitted from the medium, whereas 2102R grew normally in the absence of either acid, though not of both together. The increased ability of the resistant strain to synthesize glutamic acid might be related to the increase in the synthesis of folic acid, which contains the amino acid in its molecule. Both aspartic and glutamic acids are formed by

transamination, from oxaloacetic and α -ketoglutaric acids respectively. Lam and Sevag (1957) have some evidence that sulphathiazole inhibits transaminase activity in a sensitive strain of Staph.aureus. It is, therefore, possible that in 2102R transaminase activity has increased with the development of resistance, resulting in an increased rate of synthesis of aspartic and glutamic acids. Such an interpretation, however, is not valid for Staph.lactis 2102R2 since it has an absolute requirement for glutamic acid, although it also grows in the absence of aspartic acid.

Staph.lactis 2102 and 2102R reduced nitrate to nitrite whereas 2102R2 did not. The significance of this is not clear. Of the strains of Staph.aureus, only R122 was able to reduce nitrate.

Particular attention has been given to the metabolism of p-aminobenzoic acid (PAB) and related compounds, since it is here that changes are most likely to occur in sulphonamide-resistant strains. These results will be discussed in terms of the various mechanisms of resistance proposed by Davis and Maas (1952). (See Chapter II).

Synthesis of PAB.

The resistance of Staph.aureus R122 may be due, at least in part, to the synthesis of relatively large amounts of PAB. It synthesized twenty times as much PAB as did Staph.aureus JHM and H, and it was about 100 times less sensitive to sulphathiazole.

A better comparison would be with a sensitive strain of the same organism, but this could not be obtained. In other work where an increased synthesis of PAB has accompanied sulphonamide-resistance there has not always been a proportional relation between increase in PAB synthesis and change in resistance.

The sulphonamide-resistant staphylococci described by Landy et al. (1943) synthesized about seventy times more PAB than the parent strains, but showed a thousand-fold decrease in sensitivity.

Staph. aureus JHMR and HR2 and Staph. lactis 2102R2 synthesized the same amount of PAB as their parent strains during growth in medium B. Staph. lactis 2102R showed a four-fold increase in PAB synthesis over that of the parent strain, but its sensitivity to sulphathiazole was fifty times less.

The inhibition index of all the sensitive strains is about 10, whereas in all the resistant strains it is over 100. If increased synthesis of PAB were alone responsible for resistance, then an organism such as Staph. aureus HR2 which is able to grow in the presence of $5 \times 10^{-4} \text{M}$ sulphathiazole in medium B would have to synthesize $5 \times 10^{-5} \text{M}$ PAB, which it does not. Furthermore, the value of the inhibition index, measured at concentrations of drug appreciably higher than are needed to inhibit the resistant strain during the same time of incubation, would not be very different from that of the sensitive strain. Thus, to overcome the inhibition caused by $2 \times 10^{-3} \text{M}$ sulphathiazole,

$2 \times 10^{-4} \text{ M}$ PAB would have to be present. Even if the organism synthesized $5 \times 10^{-5} \text{ M}$ PAB, it would still be necessary to add $1.5 \times 10^{-4} \text{ M}$ PAB, so that the value of the inhibition index would only be 13.

Alternate pathways of metabolism.

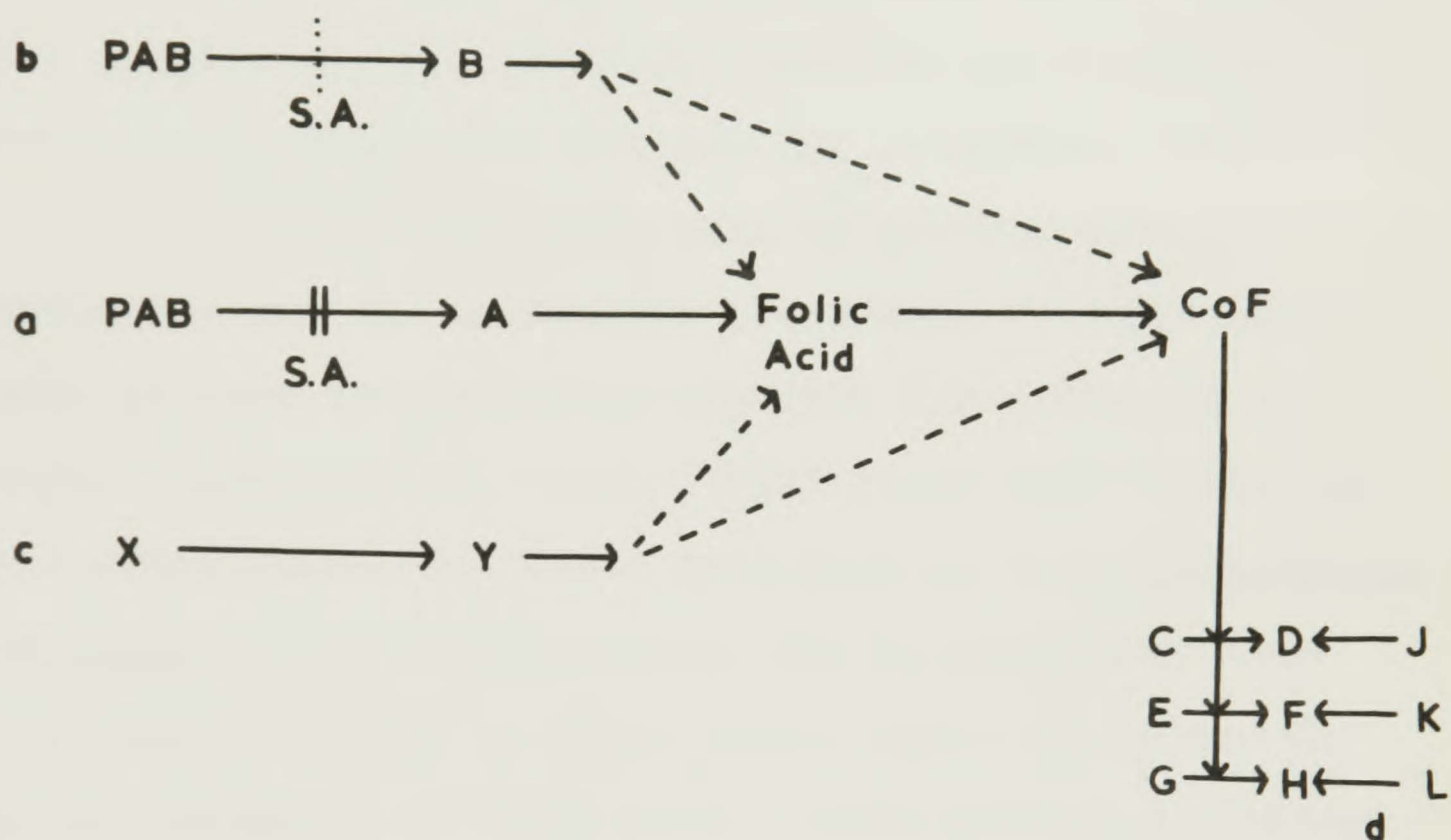
The probable route of folic acid synthesis, proceeding from PAB, by a reaction inhibited by sulphonamides, to coenzyme F, which is involved in a number of metabolic reactions ($C \rightarrow D$, $E \rightarrow F$ etc.) is illustrated in Figure 28a. The reaction inhibited by sulphonamides might be avoided in a number of ways:

1. Utilization of PAB may be by a pathway which is less sensitive to inhibition by sulphonamides (Figure 28b). This does not seem very likely, for if the PAB molecule were used as such, it is not easy to see why sulphonamides should fail to inhibit its utilization, whatever the pathway of its metabolism.

To demonstrate this possibility, it would be necessary to show that the reaction undergone by PAB was different in a sensitive and a resistant strain. To do this, work with cell-free extracts probably would be needed.

Bevag and Ishii (1958) have evidence for a changed pathway of folic acid synthesis induced by sulphathiazole. Growth of a PAB-requiring mutant of Esch.coli in the presence of sulphathiazole enabled the organisms to synthesize folic acid from 2-amino-4-hydroxy-6-carboxypterin and PAB. This ability was also shown by

FIGURE 28



Hypothetical pathways of synthesis of Coenzyme F

resistant organisms grown in the presence or absence of sulphathiazole, but not by sensitive organisms grown in the absence of sulphathiazole. It has not been shown whether this new pathway is less sensitive to inhibition by sulphathiazole.

Growth in the presence of sulphathiazole was necessary in the present work to obtain appreciable synthesis of folic acid by washed suspensions of organisms incubated in a mixture containing only buffer, PAB, glucose and glutamate. The organisms were depleted of folic acid by growing with sulphathiazole, and may accumulate precursors of folic acid since only glucose is absolutely required for synthesis of folic acid. Lascelles and Woods (1952) found that 2-amino-4-hydroxy-6-formylpterin increased synthesis by both Staph.lactis 2102 and 2102R in the above mixture, but it was rather less effective than a mixture of amino acids, which approximately doubled the synthesis of folic acid. Other pterins including 2-amino-4-hydroxy-6-carboxypterin had no effect. There is no evidence that sulphathiazole alters the pathway of folic acid synthesis. Although Staph.lactis 2102R synthesizes about ten times more folic acid than its parent strain, it does so from the same substrates and the difference in the amount of folic acid formed is still shown during growth in the absence of sulphathiazole.

2. Folic acid or coenzyme F may be synthesized by a route in which PAB is not an intermediate (Figure 28c). For example,

sulphonamide-resistant strains of Strep.mitis acquire the ability to utilize pteroylglutamate present in the medium (Harrison and Clapper, 1950). The sensitive strains are unable to utilize this compound. No compounds of the folic acid group are present in the staphylococcal growth medium used in the present work, and none of the strains responded to added pteroylglutamate.

This mechanism of resistance cannot be responsible for more than a part of the synthesis of folic acid in the resistant strains, because sensitivity to sulphonamides is only reduced, and is not lost entirely. The amount of folic acid formed by an alternative pathway which by-passed PAB could be ascertained by determining the incorporation of labelled PAB into folic acid. This has not been attempted owing to lack of time. This mechanism has already been discussed (Chapter VIII).

There is no evidence that resistance is due to the conversion of sulphathiazole into less inhibitory products. All strains synthesized by routes which no longer require the utilization of folic acid ($J \rightarrow D$, $K \rightarrow F$ etc. Figure 28d). It seems unlikely that such a mechanism could be responsible for a high degree of resistance because of the number of pathways that would have to be modified to abolish the requirement for folic acid. However, small changes in resistance might be caused by a reduction of the folic acid requirement for individual reactions.

Indirect evidence has been obtained that xanthine may, at least in part, be synthesized by Staph.aureus HR2 by a route in

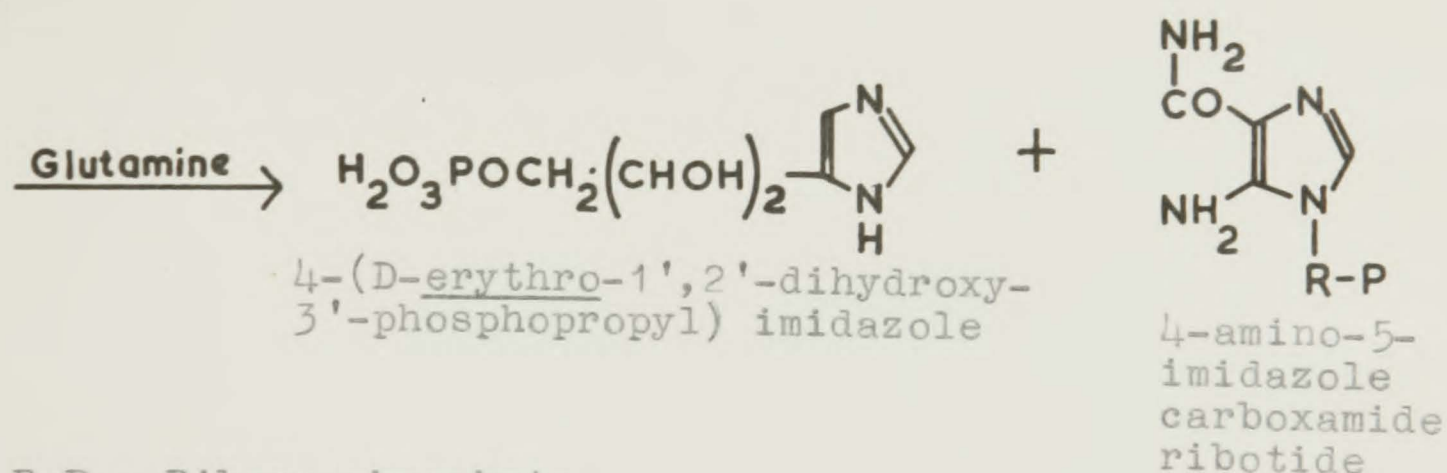
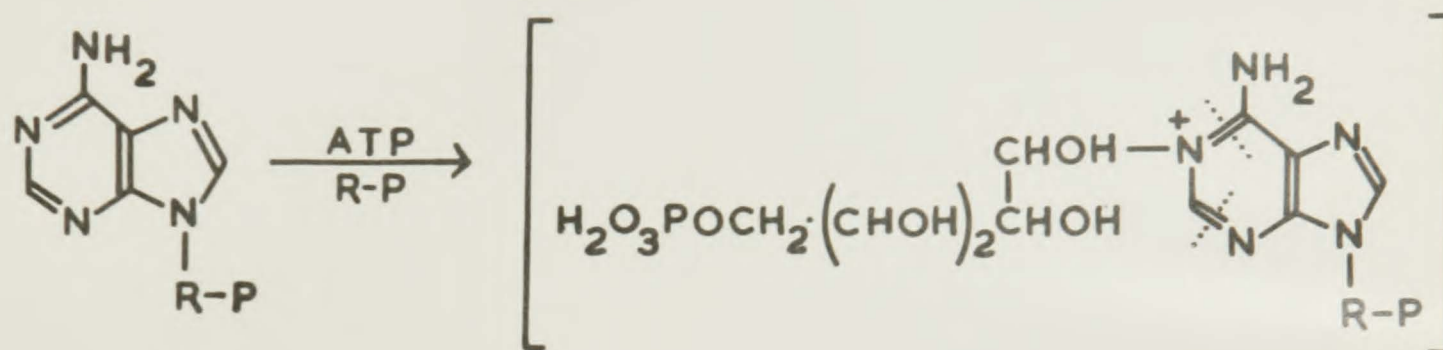
Even when the entire volume of the organisms was assumed to be permeable to sulphathiazole, the concentration of the drug in the organisms was 1-2 times that in the surrounding medium. Whether this uptake was at a particular site in the organism was not determined, but since the presence of PAB had no effect on the uptake, it seemed unlikely that an appreciable part of the sulphathiazole was combining with a site at which PAB also reacts.

Noll, Bang, Sorkin and Erlenmeyer (1951) found that the uptake of sulphathiazole by Esch.coli was reduced by the presence of PAB in the medium. There was a limit below which the uptake of sulphathiazole could not be reduced by high concentrations of PAB, so it was concluded that part of the drug was free in the organism, and that part was in competition for a receptor site with PAB.

Recent work has shown that the cell wall may account for a considerable part of the uptake of small molecules (Mitchell and Moyle, 1956; Weibull, 1955). Britt and Gerhardt (1958) have measured lysine uptake by cell walls, protoplasts and intact organisms of Micrococcus lysodeikticus, and have found that much of the uptake was localized, and concentrated at the cell wall.

In the present work the method of measuring uptake was not sufficiently refined for changes to be detected in the uptake by different parts of the organism. If a large part of the total sulphathiazole associated with the organism is adsorbed

FIGURE 29



Proposed reactions in histidine biosynthesis
 (Moyed and Magasanik, 1957)

Even when the entire volume of the organisms was assumed to be permeable to sulphathiazole, the concentration of the drug in the organisms was 1-2 times that in the surrounding medium. Whether this uptake was at a particular site in the organism was not determined, but since the presence of PAB had no effect on the uptake, it seemed unlikely that an appreciable part of the sulphathiazole was combining with a site at which PAB also reacts.

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In the present work the method of measuring uptake was not sufficiently refined for changes to be detected in the uptake by different parts of the organism. If a large part of the total sulphathiazole associated with the organism is adsorbed

at the cell wall, this would mask differences in the uptake of sensitive and resistant strains at other regions in the cytoplasm, especially if these differences concerned only a small proportion of the total drug taken up.

Other workers have reported that sulphonamide-resistant organisms do not take up smaller amounts of sulphathiazole than sensitive strains (Yegian and Budd, 1945). In fact, Wacker, Trebst and Simon (1957) found that a resistant strain of Enterococcus Stei took up more sulphanilamide than did a sensitive strain, although the binding of the drug was less firm in the resistant strain. Sulphathiazole was not bound firmly by any of the strains of staphylococci, and it was easily removed by washing with water. With other drugs also it is often impossible to correlate the degree of resistance and the amount of drug taken up by the bacteria (see Chapter II).

Decreased requirement for a product of the inhibited reaction.

The operation of this mechanism of resistance to sulphonamides would manifest itself in a decreased requirement for folic acid or for products of the function of folic acid.

With Staph.aureus H and HR2 it was found that the quantitative requirements for methionine and thymine were the same in both strains. The amount of xanthine needed for growth in presence of sulphathiazole, however, was about five times less in the resistant strain than in the sensitive strain. This may mean that some xanthine is synthesized by a route in which

folic acid is involved to a lesser extent or not at all. It is unlikely that the decreased requirement for xanthine is more than partly responsible for resistance. Staph.aureus HR2 is the most resistant of all the strains used in this work, and it is difficult to see how a five-fold decrease in xanthine requirement could account alone for its resistance.

It is difficult to assess the relative requirement for folic acid in sensitive and resistant organisms because staphylococci do not respond to any form of folic acid that is added to the medium. It was thought possible that more efficient utilization of folic acid for synthesis of coenzyme F might be reflected by a change in the inhibition index of the drug aminopterin, which is believed to inhibit the reduction of the pteridine ring of dihydropteroylglutamic acid to the tetrahydro-level. (Futterman, 1957; Wright, Anderson and Herman, 1958). However, none of the strains, sensitive or resistant to sulphathiazole, was inhibited by aminopterin. It is generally found that organisms which do not respond to folic acid are also insensitive to aminopterin (Woolley, 1952).

Increased amount of a PAB-utilizing enzyme or a changed relative affinity of an enzyme for PAB and sulphathiazole.

These two possibilities will be considered together, since experimental distinction between them is not easy. If an increased number of receptor sites for PAB were present in a resistant strain, then all analogues of PAB should become less

effective inhibitors, and cross-resistance to p-nitrobenzoic acid would be expected. In fact, no appreciable cross-resistance to p-nitrobenzoic acid was observed in any of the present sulphonamide-resistant strains.

The second possibility, a change in the relative affinities of an enzyme for PAB and sulphonamides, is compatible with all the experimental observations. It accounts for the increase in the inhibition index in the resistant strains, and for the resistance of the synthesis of folic acid in these strains to inhibition by sulphathiazole. The decreased affinity for sulphathiazole might well be specific (Davis and Maas, 1952) and the affinity for other inhibitors of PAB utilization might be unchanged. This would explain the absence of cross-resistance to p-nitrobenzoic acid.

The evidence for this mechanism of resistance is indirect, but its value is strengthened by the evidence that other mechanisms are not operating. Decisive proof that the relative affinity of a PAB utilizing enzyme for its substrate and for an inhibitor had changed in a resistant strain would require investigation of the properties of the isolated enzyme.

The values of the dissociation constants of an enzyme-inhibitor complex, K_i , and of an enzyme-substrate complex, K_s , may be calculated from the equation

$$I \frac{\alpha}{1 - \alpha} = \frac{K_i}{K_s} S + K_i \quad (\text{Hunter and Downs, 1945})$$

where I is the concentration of inhibitor, S is the concentration of substrate, and α is the ratio v/v_0 where v_0 is the velocity of the enzymic reaction without inhibitor, and v is the velocity in presence of inhibitor. If $I \frac{\alpha}{1-\alpha}$ is plotted against S , the graph will be a straight line of intercept K_i on the $I \frac{\alpha}{1-\alpha}$ axis, and of slope $\frac{K_i}{K_s}$.

Kinetic treatment has been applied to the inhibition of growth of Esch.coli by sulphonamides and its competitive reversal by PAB (Klotz and Gutmann, 1945). The validity of such procedure is very doubtful because of the number of substrates and enzymes which take part in the complex phenomenon of growth. It seems unlikely that it would even be profitable to make kinetic studies on folic acid synthesis from PAB in whole organisms. The rate of the reaction would depend on PAB concentration alone only if all the other substrates were present in optimal amounts, and we do not know fully the nature of these substrates, much less in what concentrations they are needed.

Recent work (Katunuma et al, 1957; see Chapter III) has indicated that in Mycobact.avium at least, the reaction of PAB with which sulphonamides interfere is



and a cell-free enzyme has been obtained which catalyses this reaction. If a similar reaction occurs in staphylococci,

kinetic studies on its inhibition by sulphonamides might reveal differences in K_s and K_i between the enzymes of sulphonamide-sensitive and resistant strains.

To summarize the conclusions as to the mechanisms of their resistance, the various resistant strains will be briefly considered individually.

The resistance of Staph. aureus R122 may be due to an ability to synthesize relatively large amounts of PAB.

Staph. lactis 2102R has a greater ability than its parent strain to synthesize folic acid, and this synthesis is more resistant to inhibition by sulphathiazole. Staph. lactis 2102R2 is derived from the same parent strain, but it is clearly a distinct mutant, as it differs in several ways from 2102R. This strain does not synthesize much more folic acid than the parent, but the synthesis is more resistant to sulphathiazole. It seems probable that resistance of both these strains is due to a changed enzyme: substrate affinity, which leads to more efficient synthesis of folic acid in the case of 2102R.

A similar mechanism of resistance seems to occur in Staph. aureus JHMR and HR2. An alternate pathway of xanthine biosynthesis may also contribute to the resistance of Staph. aureus HR2.

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The conclusions from the present work support the idea that qualitative changes in enzymes may be important in the

development of resistance. Such changes may be responsible for resistance in four out of the five strains examined.

The importance of the investigation of drug-resistance at an enzymic level is thus indicated. Recent developments in the study of folic acid synthesis make this approach more promising than hitherto. It appears that the work described in this thesis could most profitably be continued by investigating the synthesis of folic acid in cell-free extracts to confirm the results of the Japanese workers, and to seek for differences between sensitive and resistant strains at this level.

APPENDIX I

Chemicals.

Synthesis of ^{35}S -labelled salicylic acid.

1. 2-Acetaminobenzoic acid chloride. Gilman (1930)
7.2g H^{35}SO_4 of specific activity 7.5Ci/gm on 22/2/57
was transferred to a 25 ml round-bottomed, two-necked flask,
and the total weight of chlorosulphonic acid was made up to
3.5g (30ml) by adding freshly distilled unlabelled
chlorosulphonic acid. The flask was cooled in an ice-water
bath and the temperature was kept at $10^\circ\text{--}15^\circ$ while 1.35g
acetanilide (10ml) was added over about 30 min. The mixture
was stirred mechanically throughout.

APPENDICES

When the addition of acetanilide was complete, the flask
was heated on a sand bath and was kept at 60° for about 30 min.
until no more small bubbles were visible in the reaction
mixture. The temperature then was raised to 80° to complete
the reaction. During this heating the mixture was stirred
mechanically.

After cooling, the reaction mixture was cautiously added
to 25 ml ice-water with continuous stirring. The solid which
separated was collected on a Buchner funnel and washed with a
small volume of cold water. The product was dried overnight
with P_2O_5 .

Yield 0.5g (7% conversion of chlorosulphonic acid).

This yield was lower than had been obtained in trial

APPENDIX I

Chemicals.

Synthesis of ^{35}S -labelled sulphathiazole.

1. p-Acetaminobenzene sulphonyl chloride. Gilman (1932)

7.2mC $\text{H}^{35}\text{SO}_3\text{Cl}$ of specific activity 7.9mC/mM on 29/7/57 was transferred to a 25 ml round-bottomed, two-necked flask, and the total weight of chlorosulphonic acid was made up to 3.5g (30mM) by adding freshly distilled unlabelled chlorosulphonic acid. The flask was cooled in an ice-water bath and the temperature was kept at $10^\circ\text{--}15^\circ$ while 1.35g acetanilide (10mM) was added over about 30 min. The mixture was stirred mechanically throughout.

When the addition of acetanilide was complete, the flask was heated on a sand bath and was kept at 60° for about 30 min. until no more small bubbles were visible in the reaction mixture. The temperature then was raised to 80° to complete the reaction. During this heating the mixture was stirred mechanically.

After cooling, the reaction mixture was cautiously added to 25 ml ice-water with continuous stirring. The solid which separated was collected on a buchner funnel and washed with a small volume of cold water. The product was dried overnight with P_2O_5 .

Yield 0.5g (7% conversion of chlorosulphonic acid).

This yield was lower than had been obtained in trial

experiments, so for the next step the radioactive product was diluted with freshly prepared unlabelled p-acetaminobenzene sulphonyl chloride, to permit the reaction to be done on the same scale as in the trial experiments.

2. N^4 -acetylsulphathiazole. Fosbinder and Walter (1939) and May and Baker Ltd, Dagenham, Essex (Private communication).

1g p-acetaminobenzene sulphonyl chloride- ^{35}S (4.3mM) was dissolved in 4.2 ml dry ethyl acetate in a 25 ml round-bottomed flask.

A solution of 1g 2-aminothiazole (10mM) in 8.8 ml dry ethyl acetate was added in portions over half an hour while the mixture was cooled in water and stirred mechanically. After 6 hr the stirring was stopped, and the reaction mixture was left to stand overnight at room temperature.

The ethyl acetate was removed on an oil bath at 80° . 0.25 ml water was added and the mixture was stirred. 0.21 ml 80% acetic acid and 1.9 ml water were added, and the mixture was warmed to 50° and kept for 30 min. at that temperature. The suspension of N^4 -acetylsulphathiazole was cooled to room temperature and left for 4 hr. The solid was then collected at the pump and washed with cold water.

Yield 0.9g (70% conversion of p-acetaminobenzene sulphonyl chloride).

3. Sodium sulphathiazole. May and Baker Ltd, Dagenham, Essex.

(Private communication).

0.36g sodium hydroxide was dissolved in water in a 5"x $\frac{5}{8}$ " tube graduated at 3.5 ml. 0.9g N⁴-acetylsulphathiazole-³⁵S was added, and 30 mg "Norite", and the volume was made up with water to 3.5 ml. A small piece of porous pot was added, and a glass bulb was put in the mouth of the tube. The mixture was boiled vigorously for an hour, allowing the volume to fall to 3.1 ml.

The mixture was filtered, and the charcoal washed with 0.15 ml water. The temperature of the filtrate was brought to 80° and 0.7g sodium chloride was added with stirring. The mixture was then cooled to 20° with occasional shaking. The sodium sulphathiazole which precipitated was collected, and washed twice with 0.3 ml cold saturated brine.

4. Purification and isolation of sulphathiazole. May and Baker Ltd. (Private communication).

Damp sodium sulphathiazole-³⁵S was dissolved in water and the volume made up to 7 ml. 30 mg "Norite" was added, and the mixture was stirred at room temperature for an hour.

The mixture was filtered and the charcoal washed with 0.3 ml water. Concentrated hydrochloric acid was added slowly with stirring until the initial precipitate redissolved. 15 mg citric acid added and 15 mg "Norite", and the mixture was stirred for an hour. The charcoal was filtered off, and washed with 0.3 ml water.

A solution of ammonia was prepared from ammonia solution S.G. 0.88 (6.4 ml) and water (12 ml), and was added to the filtrate with stirring, until the solution was acid to litmus and alkaline to Congo red. The suspension of sulphathiazole-³⁵S was cooled and filtered.

Yield 450 mg (58% conversion of N⁴-acetylsulphathiazole)

Melting Point 197° - 199°

All four compounds were obtained from May and Baker Ltd.

Chlorosulphonic acid-³⁵S was obtained from the Radiochemical Centre (Ministry of Supply), Amersham.

2-Aminothiazole - Kindly supplied by May and Baker Ltd as specially purified material.

Sources of other special chemicals.

PAB derivatives:

PAB - (Roche Products Ltd). Purified by recrystallization from water, twice as the hydrochloride, and twice as the free base, by Mr R.W. Wakelin.

Rhizopterlin - (Merck and Co. Inc.) A stock solution (10^{-3} M) was provided by Dr R.G. Tucker.

Pteroylglutamic acid - (Lederle Laboratories Division of the American Cyanamide Co.) To make 10 ml of a 10^{-3} M stock solution, 4.4 mg was mixed with about 10 mg NaHCO₃ and the mixture dissolved in water.

v

Leucovorin - (Lederle Laboratories). A stock solution (10^{-3} M) was provided by Dr R.G. Tucker.

Aminopterlin - (Lederle Laboratories). A stock solution (10^{-3} M) was prepared as with pteroylglutamic acid.

Sulphonamides:

Sulphathiazole and sulphanilamide stock solutions were 10^{-2} M.

Sulphapyridine and sulphadiazine stock solutions were 10^{-3} M.

All four compounds were obtained from May and Baker Ltd.

Vitamins:

Vitamin B₁₂ - dilutions were made from a stock 2 mg/ml solution in physiological saline. ("Cyanocobalamin", B.D.H., Ltd).

Pyridoxal HCl - (Merck and Co. Inc.). A stock solution (10^{-3} M) was provided by Dr J.G. Morris.

Other compounds:

4-Amino-5-imidazolecarboxamide - (California Foundation for Biochemical Research)

Sodium pyruvate - Prepared by Mr R.W. Wakelin.

Haematin - A solution (100 µg/ml) was provided by Dr J. Lascelles.

The haematin was prepared as described by Lascelles (1956).

Escherichia coli 273/354 A.T.C.C. No. 9723a. Obtained from Dr S. Berlin. An X-ray induced mutant, requiring PAB. Cultured on tryptic meat agar slopes, incubated for 15 hr at 37°.

Agrobacterium suboxydans 621 A.T.C.C. No. 621. Obtained from S.C.T.C. Cultured on yeast glycerol agar slopes, incubated for 24 hr at 30°.

APPENDIX II

Organisms

- Staphylococcus lactis 2102 A laboratory strain. Obtained from Sir Paul Fildes.
- Staphylococcus lactis 2102R A sulphonamide-resistant substrain developed by Dr J. Lascelles from Staph.lactis 2102
- Staphylococcus lactis 2102R2 A second sulphonamide-resistant substrain developed from Staph.lactis 2102.
- Staphylococcus aureus JHM A laboratory strain. Isolated from a boil by Dr J.H. Marshall.
- Staphylococcus aureus JHMR A sulphonamide-resistant substrain developed from Staph.aureus JHM.
- Staphylococcus aureus H "Oxford strain" N.C.T.C. No.6571.
- Staphylococcus aureus HR2 A sulphonamide-resistant substrain developed by Dr J. Lascelles from Staph.aureus H.
- Staphylococcus aureus R122 A laboratory strain. Obtained from the Radcliffe Infirmary, Oxford. Sulphonamide-resistant.

All strains of staphylococci were cultured on slopes of tryptic meat agar, incubated for 15 hr at 37°.

- Escherichia coli 518 A.T.C.C.9723. Obtained from Dr R. Roblin. Parent strain of E.coli 273/384. Cultured on tryptic meat agar slopes, incubated 15 hr at 37°.
- Escherichia coli 273/384 A.T.C.C. No.9723a. Obtained from Dr R. Roblin. An X-ray induced mutant, requiring PAB. Cultured on tryptic meat agar slopes, incubated for 15 hr at 37°.
- Acetobacter suboxydans 621 A.T.C.C. No.621. Obtained from N.C.T.C. Cultured on yeast glycerol agar slopes, incubated for 24 hr at 30°.

Streptococcus faecalis R A.T.C.C. No.8043. Cultured in tryptose glucose agar stab. Incubated for 24 hr at 37°.

Lactobacillus casei (helveticus) A.T.C.C. No.7469. Obtained from Dr Barton Wright. Cultured in tryptose glucose agar stab. Incubated for 24 hr at 37°.

Leuconostoc citrovorum (Pediococcus cerevisiae) A.T.C.C. No.8081. Cultured in tryptose glucose agar stab. Incubated for 24 hr at 37°.

preservative.

Tryptic meat agar.

Tryptic meat broth 500 ml

Water 500 ml

Agar 20g

pH adjusted to 7.6

Penicillin water.

Evans' peptone 20g

NaCl 5g

Water 1000 ml

pH adjusted to 7.4

Tryptic digest of casein.

Prepared from B.N.R. casein, treated with pancreatic extract, and stored at treble strength with chloroform as preservative.

APPENDIX IIIMediaTryptic meat broth.

Prepared from minced beef heart, treated with pancreatic extract and stored at double strength with chloroform as preservative.

Tryptic meat agar.

Tryptic meat broth	500 ml
Water	500 ml
Agar	20g
pH adjusted to	7.6

Peptone water.

Evans' peptone	20g
NaCl (w/v) Oxoid liver extract in water.	5g
Water	1000 ml
pH adjusted to	7.4

Tryptic digest of casein.

Prepared from B.D.H. casein, treated with pancreatic extract, and stored at treble strength with chloroform as preservative.

Tryptic casein agar.

Tryptic digest of casein	300 ml
Water	600 ml
Agar	18g
pH adjusted to	7.4

Tryptose glucose broth.

Bacto tryptose	10g
Glucose	10g
K_2HPO_4	5g
Bacto yeast extract	2g
*Liver extract	10 ml
+Salts B	5 ml
Water to	1000 ml
pH adjusted to	6.8

* 16% (w/v) Oxoid liver extract in water.

+ $MgSO_4 \cdot 7H_2O$	20g
$MnSO_4 \cdot 4H_2O$	1g
NaCl	1g
$FeSO_4 \cdot 7H_2O$	1g
Conc. HCl	0.5 ml
Water to	500 ml

Tryptose glucose agar.

Bacto tryptose 10g
 Glucose 10g
 K_2HPO_4 2g
 $CaCO_3$ 3g
 Liver extract 10 ml
 Salts B 5 ml
 Agar 15g
 Water to 1000 ml

Glycerol yeast agar.

Glycerol 40 ml
 Bacto yeast extract 5g
 Agar 15g
 pH adjusted to 6.0
 Water to 1000 ml

General procedure for preparation of synthetic media.

Media were generally prepared at double the final working concentration, i.e. to half the stated volume. Constituents other than vitamins and Tween 80 were dissolved in a volume of water a little less than the final volume required. The solution was boiled gently for 3-5 min, cooled, and the remaining additions were made. The pH was adjusted if necessary, and checked on a meter, and the volume was made up to that required. Media were filtered through Whatman No.50 paper and stored at 2° with carbon tetrachloride: toluene (1:1 v/v) as preservative.

Thiamine	200 µg
Nicotinic acid	1000 µg
Biotin	1 µg
Water to	1000 ml
pH adjusted to	7.4

* Added as a sterile solution to the autoclaved medium.

* Prepared after the method of Snell and Hansen (1945).
(Equivalent to 3g original casein).

For anaerobic growth, a supplement was added:

NaNO_3	170 mg
Urea	5 mg
* Na pyruvate	220 mg
* NaHCO_3	1.7g

* Sterile-filtered and added to the autoclaved medium.

Medium B (modified from Lascelles and Woods, 1952)

* Glucose	7g
+ Acid-hydrolysed casein	3g
DL-tryptophan	40 mg
L-cysteine HCl	100 mg
$\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$	10g
KH_2PO_4	700 mg
NaCl	2g
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	100 mg
$\text{FeSO}_4(\text{NH}_4)_2\text{SO}_4 \cdot 6\text{H}_2\text{O}$	6 mg
Thiamine	200 μg
Nicotinic acid	1000 μg
Biotin	1 μg
Water to	1000 ml
pH adjusted to	7.4

* Added as a sterile solution to the autoclaved medium.

+ Prepared after the method of Snell and Rannefeld (1945).
(Equivalent to 3g original casein).

For anaerobic growth, a supplement was added:

NaNO_3	170 mg
Uracil	5 mg
* Na pyruvate	220 mg
* NaHCO_3	1.7g

* Seitz-filtered and added to the autoclaved medium.

Medium for investigation of amino acid requirements of staphylococci. (Gladstone, 1937).

N	NaOH	26 ml
	KH_2PO_4	4.5g
	$\text{FeSO}_4(\text{NH}_4)_2\text{SO}_4 \cdot 7\text{H}_2\text{O}$	6 mg
	$\text{MgSO}_4 \cdot 6\text{H}_2\text{O}$	100 mg
*	Glucose	2.5g
	DL-aspartic acid	180 mg
	DL-glutamic acid	180 mg
	DL-phenylalanine	80 mg
	DL-tyrosine	100 mg
	DL-histidine HCl	100 mg
	DL-lysine HCl	90 mg
	DL-tryptophan	40 mg
	Glycine	50 mg
	DL-alanine	120 mg
	DL-isoleucine	180 mg
	DL-threonine	160 mg
	DL-serine	140 mg
	DL-leucine	170 mg
	DL-valine	150 mg
	DL-proline	140 mg
	DL-methionine	6 mg
	L-hydroxyproline	80 mg
	L-arginine HCl	50 mg

(Cont'd)

Medium for investigation of amino acid requirements (cont'd)^{xiv}

+ DL-cystine	50 mg
+ Na thioglycollate	120 mg
Nicotinic acid	1000 µg
Thiamine HCl	200 µg
Biotin	1 µg
Water to	1 l
pH adjusted to	7.4

* Added as a sterile solution to the autoclaved medium.

+ Sodium thioglycollate was only included when cystine was omitted.

In order that the amino acid content could be varied, each amino acid was made up in concentrated solution, so that the addition of 0.1 ml of each gave the final concentration required in 5 ml of the completed medium. Cystine and tyrosine were made up in N/10 HCl; aspartic and glutamic acids were made up in N/10 NaOH; tryptophan in N/20 NaOH; other amino acids were dissolved in water. All amino acids were added to the medium before autoclaving.

Nicotinic acid	1000 µg
Thiamine	200 µg
Biotin	1 µg
Water to	1000 ml
pH adjusted to	7.4

* Added as a sterile solution to the autoclaved medium.

Medium B₂ (cont'd)

Medium B₂. *For growth, a supplement was added:*

* Glucose	170.7g
N sodium hydroxide	26 ml
KH ₂ PO ₄	4.5g
FeSO ₄ (NH ₄) ₂ SO ₄ 7H ₂ O	1.76 mg
MgSO ₄ 6H ₂ O	100 mg
DL-isoleucine	180 mg
DL-leucine	170 mg
DL-valine	150 mg
DL-proline	140 mg
DL-phenylalanine	80 mg
L-arginine HCl	50 mg
Glycine	50 mg
DL-threonine	160 mg
DL-tryptophan	40 mg
DL-aspartic acid	180 mg
DL-histidine HCl	90 mg
DL-cystine	50 mg
Nicotinic acid	1000 µg
Thiamine	200 µg
Biotin	1 µg
Water to	1000 ml
pH adjusted to	7.4

* Added as a sterile solution to the autoclaved medium.

(Cont'd)

For anaerobic growth, a supplement was added:

NaNO ₃	170 mg	32
Uracil	5 mg	100 mg
* Na pyruvate	220 mg	100 mg
* NaHCO ₃	1.7g	50 mg

* Seitz-filtered and added to the autoclaved medium.

CaCl ₂ · 2H ₂ O	500 mg
MgSO ₄ · 7H ₂ O	100 mg
K ₂ HPO ₄ · 3H ₂ O	500 mg
FeSO ₄ (H ₂ O) ₇ · 10H ₂ O	50 mg
MnSO ₄ · 2H ₂ O	50 mg
NaCl	50 mg
Adenine	10 mg
Guanine	5 mg
Nicotinic acid	1000 mg
Ca pantothenate	1000 mg
Vitamin B ₁₂	3 mg
Tween 80 (5% (v/v) in water)	4 ml
pH adjusted to	6.0
Water to	1000 ml

Medium for assay of PAB, using *Acetobacter suboxydans* 621. 1951).

Acid hydrolysed casein	3g
Glycerol	40 ml
Glucose	100 mg
DL-tryptophan	50 mg
L-cysteine HCl	50 mg
KH_2PO_4	500 mg
$\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$	930 mg
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	200 mg
$\text{FeSO}_4 (\text{NH}_4)_2 \text{SO}_4 \cdot 6\text{H}_2\text{O}$	10 mg
$\text{MnSO}_4 \cdot 2\text{H}_2\text{O}$	10 mg
NaCl	10 mg
Adenine	14 mg
Guanine	4 mg
Nicotinic acid	1000 μg
Ca pantothenate	1000 μg
Vitamin B ₁₂	2 μg
Tween 80 (5% (v/v) in water)	4 ml
pH adjusted to	6.0
Water to	1000 ml

Medium for assay of *Lactobacillus casei* factor (Lascelles, 1951).

* Tryptic digest of casein	500 ml
L-cysteine HCl	130 mg
Glucose	10g
Adenine	7 mg
Guanine	2 mg
MgSO ₄	800 mg
FeSO ₄ (NH ₄) ₂ SO ₄ ·6H ₂ O	56 mg
MnSO ₄ ·4H ₂ O	160 mg
Thiamine	1000 µg
Nicotinic acid	1000 µg
Riboflavin	400 µg
Ca pantothenate	1000 µg
Pyridoxine HCl	1000 µg
Biotin	1 µg
Water to	1000 ml
pH adjusted to	6.8

The stock medium was made up to 700 ml. 3.5 ml of this was diluted to 5 ml in each tube of an assay.

* See below.

Tryptic digest of casein for *Lactobacillus casei* factor assay medium. (Lascelles, 1951).

100 g Glaxo vitamin free casein was suspended evenly in 1400 ml 0.8% sodium bicarbonate solution, which had been warmed to 40°. A suspension of 1g Difco trypsin in 20 ml water and 5 ml toluene were added. The mixture was shaken, and then put in the incubator at 37° for 18-20 hours. The mixture was shaken occasionally.

The mixture was steamed for 40 minutes in the autoclave to remove toluene, and cooled. 4.4 ml acetic acid added, and undigested casein was removed by centrifuging. 54 ml acetic acid was added to the supernate.

40g "Norite" was added, and the mixture was shaken frequently. After 30 minutes the suspension was filtered as quickly as possible through two layers of Whatman No. 54 paper with suction. The residue on the paper was washed twice with 20 ml water. 20g "Norite" was added to the filtrate and the mixture was shaken frequently. After 30 minutes the suspension was filtered as quickly as possible through a Ford's Sterimat S/GS/22 filter mat.

40g KH_2PO_4 was added and 40g sodium hydroxide. The pH was adjusted to 6.8, and the volume of the digest was made up to 2 l with water.

Medium for assay of *Le.citrovorum* factor.

DL-alanine	200 mg
L-arginine HCl	240 mg
DL-aspartic acid	200 mg
DL-asparagine	300 mg
L-cysteine HCl	60 mg
DL-glutamic acid	600 mg
Glycine	100 mg
DL-histidine HCl	130 mg
DL-isoleucine	250 mg
DL-leucine	250 mg
DL-lysine di HCl	600 mg
DL-methionine	100 mg
DL-phenylalanine	100 mg
DL-proline	200 mg
DL-serine	50 mg
DL-threonine	200 mg
DL-tryptophan	40 mg
DL-tyrosine	200 mg
DL-valine	250 mg
Glucose	25g
Sodium acetate (anhydrous)	20g
Ammonium chloride	3g
KH_2PO_4	600 mg
$\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$	1.2g

(Cont'd)

Lc.citrovorum factor assay medium (cont'd)

MgSO ₄ ·7H ₂ O	200 mg
FeSO ₄ ·7H ₂ O	10 mg
MnSO ₄ ·4H ₂ O	20 mg
NaCl	10 mg
Adenine	8 mg
Guanine	8 mg
Uracil	10 mg
Xanthine	10 mg
Thiamine HCl	500 µg
Ca pantothenate	500 µg
Riboflavin	500 µg
Nicotinic acid	1000 µg
Biotin	1 µg
*Pyridoxal	2×10^{-7} M in final medium
pH adjusted to	6.8
Water to	1000 ml

* Pyridoxal added as Seitz-filtered solution to autoclaved medium.

* Pyridoxal added as a sterile solution (filtered) after autoclaving.

Medium for assay of Streptococcus faecalis R factor.
(Rabinowitz and Snell, (1947)).

Acid hydrolysed casein	5g
DL-tryptophan	100 mg
L-cysteine HCl	120 mg
Glycine	200 mg
DL-aspartic acid	100 mg
Na citrate	20g
Glucose	10g
KH_2PO_4	6g
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	200 mg
NaHCO_3	100 mg
$\text{FeSO}_4 (\text{NH}_4)_2 \text{SO}_4 \cdot 6\text{H}_2\text{O}$	15 mg
$\text{MnSO}_4 \cdot 2\text{H}_2\text{O}$	10 mg
Thiamine HCl	200 μg
Riboflavin	200 μg
Nicotinic acid	600 μg
Ca pantothenate	400 μg
Biotin	0.8 μg
pH adjusted to	6.8
Water to	1000 ml
* Pyridoxal	$5 \times 10^{-8} \underline{\text{M}}$

* Pyridoxal added as a sterile solution (filtered) after autoclaving.

Minimal glucose-salts medium (modified from Davis and Mingioli, (1950)).

K_2HPO_4	7g
KH_2PO_4	3g
Na citrate	0.5g
$MgSO_4 \cdot 7H_2O$	100 mg
$(NH_4)_2SO_4$	1g
Glucose	2g
Water to	1000 ml
pH adjusted to	7.0

Incubation mixture for folic acid synthesis by washed suspensions of organisms. (Lascelles and Woods, (1952)).

Glucose	180 mg
L-glutamic acid	147 mg
$10^{-3}M$ PAB	1 ml
$Na_2HPO_4 \cdot 12H_2O$	1.79g
KH_2PO_4	0.68g
pH adjusted to	6.8
Water to	100 ml

Medium G.

Evans' peptone	10g
Marmite	2g
Oxoid liver extract	0.8g
K_2HPO_4	2.5g
Glucose	10g
Salts B	2.5 ml
pH adjusted to	6.8
Water to	500 ml

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Sulphathiazole-resistant strains. *Staph. lactis* 21022 and 210222. *Staph. aureus* J102 and *Staph. aureus* J12 were developed by subcultivation in increasing concentrations of sulphathiazole from the sensitive strains *Staph. lactis* 2102. *Staph. aureus* J102 and *Staph. aureus* J12. *Staph. aureus* B122 was a sulphathiazole-resistant organism obtained by screening strains of staphylococci isolated from clinical infections. Various

ABSTRACT

Introduction.

An investigation has been made of the biochemical mechanisms of resistance to sulphonamide drugs in five strains of staphylococci. Resistant strains of micro-organisms for comparative studies are usually derived from sensitive parent organisms by a number of procedures, of which the most frequently used is repeated subcultivation in the presence of increasing concentrations of inhibitor.

There are several possible mechanisms of drug-resistance, and some of these have been demonstrated in sulphonamide-resistant organisms. More is known about the mode of action of the sulphonamides than of most antibacterial substances, and this information directs attention to the metabolism of p-aminobenzoic acid (PAB) and folic acid, where changes seem likely to lead to the development of resistance.

Growth, nutrition and general biochemical properties.

Sulphathiazole-resistant strains, Staph.lactis 2102R and 2102R2, Staph.aureus JHMR and Staph.aureus HR2 were developed by subcultivation in increasing concentrations of sulphathiazole, from the sensitive strains Staph.lactis 2102, Staph.aureus JHM and Staph.aureus H. Staph.aureus R122 was a sulphathiazole-resistant organism obtained by screening strains of staphylococci isolated from clinical infections. Various

attempts to get a sensitive strain of this organism were unsuccessful.

The resistant strains, compared with the parent strains showed a 20-200-fold increase in tolerance to sulphonamides in a semi-defined medium based on acid-hydrolyzed casein. None of the resistant or sensitive organisms were inhibited by aminopterin. The sulphathiazole-resistant strains did not show cross-resistance to p-nitrobenzoic acid, which, however, was not a very powerful inhibitor of the sulphathiazole-sensitive organisms.

The rates of growth of the resistant strains under aerobic conditions in a semi-defined medium were the same as the rates of growth of the parent strains, except that Staph. lactis 2102R2 showed a lag phase some four hours longer than that of the parent. No differences between sensitive and resistant strains were found under anaerobic conditions whether or not sulphathiazole was present.

Various biochemical tests, such as sugar fermentations, formation of indole, and Voges-Proskauer, failed to reveal changes accompanying resistance. The ability to reduce nitrate to nitrite was distributed irregularly among the resistant strains. All except Staph. lactis 2102 and its substrains were coagulase positive and grew on 7.5% salt agar.

The requirements for vitamins and amino acids were determined in a defined medium. Staph. lactis 2102 and its

substrains required only biotin, whereas all the other strains needed thiamine and nicotinic acid but not biotin. The quantitative requirements of Staph.aureus H and HR2 for nicotinic acid and thiamine were the same, whether or not sulphathiazole was present. The amino acids required varied among the sensitive strains but in no case were there more than slight differences between resistant and parent strains.

Metabolism of p-aminobenzoic acid and related compounds.

Inhibition by sulphathiazole was overcome competitively by PAB. The inhibition indices of all the sensitive strains were about 10, and 100-1000 for the resistant strains, in proportion to their degree of resistance.

Methionine, xanthine, thymine and vitamin B₁₂ reduced the amount of PAB acid needed for growth of Staph.aureus JHM and JHMR in the presence of sulphathiazole while pteroylglutamic acid and serine had no effect. With Staph.aureus HR2 addition of xanthine had less effect on the requirement for PAB than with the parent strain. Also the amount of xanthine needed to produce an effect was five times less in the resistant strain than in the parent strain. These observations suggest that some xanthine may be synthesized by a pathway that does not involve function of folic acid. There was no evidence in Staph.aureus HR2 of changed pathways of methionine or thymine biosynthesis.

The amount of PAB synthesized by growing organisms was estimated by microbiological assay, using a mutant, Esch.coli 273/384 which requires PAB for growth. The greatest synthesis was found with Staph.aureus R122 which formed 4900 µg PAB per mg dry weight of organisms. Staph.aureus JHM and H and their resistant strains all formed about 200 µg PAB per mg. With Staph.lactis 2102 and its substrains only about 30-100 µg PAB per mg was formed. The resistant strains did not synthesize more PAB than their parent strains during growth in the presence of sulphathiazole.

Washed suspensions of strains of Staph.aureus in buffer synthesized PAB from glucose and ammonia. In the sensitive strains but not the resistant strains, the mixture of amino acids and glucose provided in the growth medium gave a lower synthesis of PAB.

Folic acid was assayed as Lactobacillus casei factor (LF). The response of Lb.casei to extracts of every strain examined was the same, but it differed from that given by known forms of folic acid. The active material had very little growth-promoting activity for Leuconostoc citrovorum or Streptococcus faecalis R.

Synthesis of folic acid by washed suspensions of organisms, occurred only in organisms depleted of folic acid by growth in presence of sulphathiazole. Such suspensions of Staph.lactis 2102R formed about 150 µg LF per mg dry weight of

organisms from PAB, glucose and glutamate whereas the parent formed only about 20 $\mu\text{g}/\text{mg}$. This behaviour was not found in all resistant strains, and Staph.lactis 2102R2, a second resistant substrain, formed only 50 μg LF/mg though it had the same resistance to sulphathiazole as Staph.lactis 2102R. The amount of folic acid synthesized by other strains was not increased when resistance was acquired. However, the resistant strains could carry out this synthesis in the presence of higher concentrations of sulphathiazole than was possible in the sensitive strains.

Metabolism and uptake of sulphathiazole.

Disappearance of sulphathiazole during growth of cultures was not detectable by chemical or microbiological tests. Chromatographic evidence showed that sulphathiazole is partly converted by growing organisms into a second substance. This unknown comprised about a quarter of the total radioactivity of supernatants of cultures of Staph.aureus grown in the presence of sulphathiazole labelled with ^{35}S . Resistant and sensitive pairs of strains formed similar amounts of the unknown, so it seems unlikely that its formation has to do with resistance.

The uptake of sulphathiazole- ^{35}S by washed suspensions of staphylococci was investigated. Suspensions of organisms were incubated in the growth medium with sulphathiazole- ^{35}S and the uptake of radioactivity by the organisms was measured directly

on the cells or indirectly from measurements of the radioactivity of the supernatants after the organisms had been removed by centrifugation. All strains, whether sensitive or resistant, took up about the same amount of drug per mg dry weight. The concentration of sulphathiazole associated with the organisms was 1-2 times that of the medium. This uptake was not altered by the presence of PAB. The attachment of the drug seemed to be weak because it could very easily be removed by washing with water.

The amounts of PAB taken up from the growth medium by thick washed suspensions of Staph.aureus JHM and H and their resistant substrains were similar. The presence of sulphathiazole did not reduce the uptake of PAB.

Discussion.

The resistance of Staph.aureus R122 may be due to an ability to synthesize relatively large amounts of PAB.

Staph.lactis 2102R has a greater ability than its parent strain to synthesize folic acid, and this synthesis is more resistant to inhibition by sulphathiazole. Staph.lactis 2102R2 is clearly a distinct mutant as it differs in several ways from 2102R. This strain does not synthesize much more folic acid than the parent, but the synthesis is more resistant to sulphathiazole. It seems probable that resistance of both these strains is due to a changed enzyme: substrate affinity,

which leads to more efficient synthesis of folic acid in the case of 2102R.

A similar mechanism of resistance seems to occur in Staph.aureus JHMR and HR2. An alternative pathway of xanthine biosynthesis may also contribute to the resistance of Staph. aureus HR2.

This work indicates the importance of investigation of resistance to sulphonamides at an enzymic level. Recent developments in the study of folic acid biosynthesis make this approach more promising than hitherto.