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THE PHYSIOLOGICAL SIGNIFICANCE

of

p-AMINOBENZOIC ACID.

by

B.M. Bloomberg.

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Thesis Submitted for the Degree of

Doctor of Medicine

in the

University of the Witwatersrand,

Johannesburg.

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INTRODUCTION.

The interest of the biochemist in para-aminobenzoic acid is very recent and, indeed, only goes back about five years, but in this time quite a voluminous literature has accumulated on the biological aspects and importance of this aniline derivative.

Attention was originally focussed on it indirectly as a result of the intensive research devoted to the understanding of the mode of action of the various sulphonamides, which were shown during the last decade to be very powerful chemotherapeutic agents against many bacteria. Fildes (1940) propounded the hypothesis that p-aminobenzoic acid was an essential metabolite for bacteria, that it was normally associated with an enzyme system in the bacterial cell, and that sulphanilamide, being structurally similar to p-aminobenzoic acid, was capable in sufficient concentration of displacing p-aminobenzoic acid from its enzyme and stopping this essential line of metabolism. Fildes further suggested that a substance which was found to be an essential metabolite for bacteria would also be essential in the animal kingdom, so that such a substance might be found to act as a vitamin in the higher animals and even in man.

In 1941 interest in p-aminobenzoic acid was intensified with the announcement by Ansbacher (1941) that p-aminobenzoic acid was actually a vitamin and should be included in the vitamin B complex.

In this thesis, studies on the absorption and excretion of p-aminobenzoic acid are reported, the estimation of p-aminobenzoic acid being based on its property of antagonising the

sulphonamides. Evidence is presented that p-aminobenzoic acid is excreted as p-acetylaminobenzoic acid, and that its conjugation with the acetyl radical probably takes place in the liver. Further it is suggested that the experiments performed do not lend support to the view that p-aminobenzoic acid is a vitamin for man.

Finally the various physiological effects of p-aminobenzoic acid are discussed and an attempt is made to gauge its function in the living organism. Preliminary experiments indicating a new, hitherto unreported, role of p-aminobenzoic acid are recorded, namely its ability in large doses to increase the resistance of animals to disease.

CHAPTER I.

THE RELATION OF p-AMINOBENZOIC ACID TO THE SULPHONAMIDES.

Since the earlier work on p-aminobenzoic acid consisted entirely of investigations into its effect of antagonising sulphonamides, and since the experimental method used here is based on these considerations, it has been thought advisable to discuss this relationship before describing the actual experimental procedures used.

FILDES' "ESSENTIAL METABOLITE" HYPOTHESIS.

Stamp (1939) isolated from cultures of haemolytic streptococci a fraction capable of antagonising the bacteriostatic action of sulphanilamide and sulphapyridine in vitro. He found that this fraction could be extracted from the bacterial cell by means of dilute alkali, that it was resistant to heat, free from protein (gave a positive ninhydrin reaction, indicating the presence of free amino-acids), and consisted of material of relatively low molecular weight. It was active in low concentrations. All of these properties are compatible with the active substance being para-aminobenzoic acid, although Stamp did not identify the active substance in his extract. This author, however, discussed the possibility of an enzyme system affected by the sulphonamide drugs, thus anticipating Fildes' (1940) hypothesis.

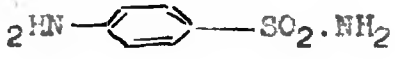
Woods and Fildes (1940) observed in in vitro experiments that para-aminobenzoic acid exerted an anti-sulphanilamide effect. Woods (1940) also showed that yeast extracts contained a substance which reversed the inhibitory action of sulphanilamide on the growth of haemolytic streptococci, and suggested on the basis of the chemical properties of this substance and

its behaviour in growth tests that it might be chemically related to sulphanilamide (p-aminobenzenesulphonamide). Since p-aminobenzoic acid had high activity in antagonising sulphanilamide inhibition, he concluded that the evidence, though circumstantial, indicated that the yeast factor was in fact p-aminobenzoic acid, and further suggested that p-aminobenzoic acid and sulphanilamide had a common point of action on some enzyme system or systems in the bacterial body. Fildes' hypothesis has already been mentioned, and although he offers no proof for his statement that essential metabolites are common to most or all cells, whether bacteria or not, his hypothesis has been chiefly valuable in that it has indicated a rational approach to research in chemotherapy. This, however, cannot be discussed here.

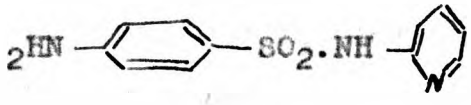
The following formulae show the relationship between these compounds:-



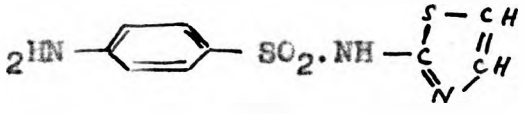
p-aminobenzoic acid.



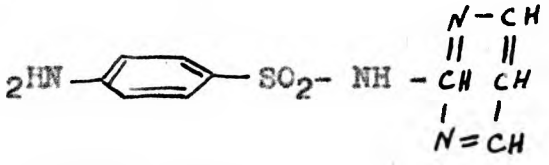
p-aminobenzene-sulphonamide. (sulphanilamide)



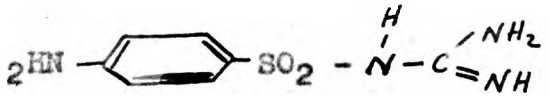
sulphapyridine.



sulphathiazole.



sulphadiazine.



sulphaguanidine.

INHIBITION BY P-AMINOBENZOIC ACID OF SULPHONAMIDE ACTION AGAINST VIRUSES, BACTERIA FUNGI AND PROTOZOA.

Selbie (1940) also showed that the therapeutic effect of sulphanilamide in mice infected with streptococci could be inhibited by p-aminobenzoic acid. Findlay (1940) confirmed that p-aminobenzoic acid inhibited the chemotherapeutic activity of sulphanilamide on haemolytic streptococci in mice, and also showed that p-aminobenzoic acid could inhibit the chemotherapeutic action of sulphanilamide on the virus of lymphogranulona venereum. He suggested that p-aminobenzoic acid or some closely allied compound formed from it in the tissues, would thus appear to be an essential metabolite for the virus of lymphogranulona venereum. Since this virus and that of trachoma are the only ones known to be acted upon by sulphanilamide, Findlay suggests that these viruses might be regarded as the only two which require p-aminobenzoic acid for their metabolism. For all other viruses p-aminobenzoic acid would thus not be an essential metabolite. However, since as yet viruses cannot be cultivated in the absence of living cells, and since there is also some doubt as to whether viruses are living agents at all, this suggestion is a very difficult one to prove or disprove. . There is, for instance, the possibility that the presence of p-aminobenzoic acid in, or its synthesis by, the different cells in which the development and growth of

viruses occurs, determines whether viruses will grow in such cells and whether sulphonamides have an action against such viruses. This question requires investigation and is of great practical significance. For example, Yeomans et al (1944) in showing that p-aminobenzoic acid has a therapeutic effect in typhus fever suggests that its effect might be due to its action on the cells of the host in which the causative organism, Rickettsia prowazeki, multiplies. This organism, like filterable viruses, cannot be cultivated in vitro. Preliminary experiments conducted here on typhoid fever in mice and reported on p.137 indicate that p-aminobenzoic acid has some effect on the resistance of the mammalian organism to infection, possibly acting on the R-E system. A further paper of interest in this connection is the finding of Marshall, Litchfield and White (1942) that p-aminobenzoic acid had a slight but definite activity against duck malaria, caused by Plasmodium lophurae - a protozoan parasite which in its early development also multiplies in the R-E system.

Woods (1940) demonstrated the activity of p-aminobenzoic acid in antagonising sulphanilamide inhibition against bacteria. He used Escherichia coli as the test organism and an entirely synthetic basal medium to culture the organism. McLeod (1940), using also an entirely synthetic basal medium and Escherichia coli as the test organism, demonstrated the presence of an unidentified sulphonamide inhibitor in extracts of fresh normal muscle, pancreas, and spleen of certain animals. The amount of this inhibitor was greatly increased when autolysis of the tissue had taken place, and it could

then be demonstrated in the livers of beef, rabbit and guinea-pig, and in the rabbit kidney. Similar results were obtained after acid hydrolysis of these tissues. A modification of McLeod's medium and method has been used to estimate p-aminobenzoic acid in the experiments reported in this thesis. Under the conditions of these experiments, p-aminobenzoic acid antagonises the inhibitory action of sulphapyridine, the sulphonamide derivative used by McLeod, which indicates the probability that McLeod's inhibitor was actually p-aminobenzoic acid. It is also probable that the inactive form of this inhibitor was the acetyl derivative of p-aminobenzoic acid - namely p-acetyl aminobenzoic acid - and an experiment confirming this point is described later (Experiment 2; p .63.) McLeod, however, reported the presence of sulphonamide inhibitor in normal human urine after acid hydrolysis, but this fact I have been unable to confirm (Expts. 3 - 9; pp. 75/82) Sulphonamide inhibitor was also found in some, but not all, sterile serous effusions occurring during certain diseases, and in pus but not in blood serum. It is probable that where negative findings were obtained, the explanation is that the technique used was not sufficiently sensitive to demonstrate the minute amounts of p-aminobenzoic acid present. These points are further discussed later, (p. 44 and Expt.7), where it is shown that when normal urine was concentrated sufficiently, the presence of p-aminobenzoic acid could be demonstrated. The development of sulphapyridine fastness in a strain of Diplococcus pneumoniae Type I was also shown by McLeod to result in a greatly increased production of

sulphonamide inhibitor. This fact has been confirmed by Spink, Wright, Vivino and Skeggs (1944), who state that sulphonamide-resistant strains of staphylococci produce more diazotizable substance, i.e. aromatic primary amines, than do sulphonamide-sensitive strains. Since exposing this diazotizable substance to Mirick's (1941) soil bacillus, which is specially adapted to oxidize p-aminobenzoic acid, destroys it, it can be inferred that this diazotizable substance is probably p-aminobenzoic acid. However, these workers point out that although in general sulphonamide-resistant strains produce more p-aminobenzoic acid than sulphonamide-sensitive strains, these results are inconstant.

This may be due to the inherent errors present in the micro-biological assay methods employed or may in fact indicate that the development of sulphonamide-resistance is not very closely related to p-aminobenzoic acid synthesis as claimed by Bevag and his co-workers in a series of papers discussed below.

Landy, Larsum, Oswald and Streightoff (1943), who present evidence that sulphonamide-resistant strains of staphylococcus aureus produce significantly more p-aminobenzoic acid (as much as 70 : 1 in some experiments, than the corresponding sensitive strains, were unable, however, to show by their method of p-aminobenzoic acid assay, significantly increased p-aminobenzoic acid production by sulphonamide-resistant strains of Diplococcus pneumoniae and other organisms, Escherichia coli, Vibrio cholerae and

and Shigella dysenteriae. They suggest on the basis of this evidence that the development of ability to synthesize p-aminobenzoic acid in excess of the normal metabolic requirements, as a result of continued exposure to sulphonamides, explains the phenomenon of sulphonamide fastness in S. aureus. Their experiments are, however, of greater significance in that these demonstrate very clearly the intimate nature of the antagonism between p-aminobenzoic acid and sulphonamides in the metabolism of bacteria.

Rubbo and Gillespie (1940, showed that p-aminobenzoic acid and certain of its derivatives act as growth factors for the anaerobic bacillus Clostridium acetobutylicum. They also showed that the growth factor in yeast was p-aminobenzoic acid which they extracted from yeast and isolated as the benzoyl derivative. They also found that one mol of p-aminobenzoic acid was able to antagonise 23,000 mols of sulphanilamide. Further, Rubbo, Maxwell, Fairbridge and Gillespie (1941, reported that the growth factor required by Cl. acetobutylicum conformed to the structural pattern of a di-substituted benzene ring in which the substituents occupy the 1 :4 positions. Optimal activity is developed when the substituents are an amino-group on the one side and a carboxyl or carboxymethyl on the other. Thus p-aminobenzoic acid stimulated growth in as small a concentration of 1.46×10^{-10}M, and p-aminophenylacetic acid in 1.32×10^{-11}M concentration. Substances which yield p-aminobenzoic acid by hydrolysis, reduction or oxidation, or by a

combination of these reactions, also function as growth factors. In a later paper, Rubbo and Gillespie (1942) showed that the bacteriostatic effect of the sulphonamide drugs is countered by increasing the concentration of growth factor 10- to 100- fold above the amount necessary to promote growth, and that 1 part by weight of p-aminobenzoic acid reverses the bacteriostasis caused by 26,000 parts by weight of sulphanilamide. They agree with Fildes' hypothesis, that the structural configuration of a chemotherapeutic agent must conform to the structural pattern of an essential metabolite for the organism.

During 1941 and 1942 several further reports confirming the antagonism between p-aminobenzoic acid and the sulphonamide group of substances, and supporting Fildes' hypothesis, appeared in the literature. Strauss, Lowell and Finland(1941) found a rough linear relationship between the concentrations of sulphonamides with bacteriostatic action on pneumococci in blood broth and the minimum concentration of p-aminobenzoic acid required to inhibit that action. The action of p-aminobenzoic acid was most marked against sulphanilamide, less against sulphapyridine and least marked against sulphathiazole. Mirick (1941) utilised a suspension of a soil bacillus which he adapted to oxidize p-aminobenzoic acid specifically, and found that this suspension rapidly destroyed the sulphonamide-inhibiting substance present in the filtrate prepared from a sulphonamide-resistant strain of pneumococcus. He showed, further, (Mirick, 1943) that sulphapyridine is bacteriostatic for this bacillus and this effect is inhibited by p-aminobenzoic acid. Loomis, Hubbard and Neter (1941) investigating

the inhibition of the bacteriostatic action of sulphanilamide by yeast extracts, found that there were at least two factors in their extracts which had anti-sulphanilamide activity. The one had properties which closely resembled p-aminobenzoic acid, but the other factor or factors which were not identified, had very different chemical properties. No further reports have appeared on the nature of these other substances, but later work confirms that p-aminobenzoic acid is not the only compound which antagonises sulphonamide activity and some interesting hypotheses have been developed to account for this antagonism. Dimond (1941) recorded the application of Woods' (1940) findings to the action of sulphanilamide on a dermatophyte, Trichophyton purpureum. The addition of sulphanilamide in 1 : 1000 concentration caused complete inhibition of growth, this inhibition being completely nullified by the addition of p-aminobenzoic acid in concentrations as low as 1 : 500,000. P-aminobenzoic acid alone, however, had no effect in increasing the rate of growth. Landy and Gyeno (1941) confirmed the bacteriostatic effect, as measured in vitro, of sulphanilamide, sulphapyridine and sulphathiazole on streptococci, pneumococci and staphylococci, and found this effect was completely neutralised by p-amino benzoic acid. They also demonstrated the inactivity of the ortho- and meta-isomers of p-aminobenzoic acid, from which they concluded, perhaps hastily, that p-aminobenzoic acid specifically neutralises sulphonamide activity. In the following year, Landy and Dicken (1942) showed that yeast growth was completely inhibited by sulphonamides, and that this inhibition was neutralised by p-aminobenzoic acid. They

found that yeast synthesizes either p-aminobenzoic acid or its biologically active equivalent and, therefore, suggest that p-aminobenzoic acid is of importance in yeast metabolism, possibly as the "essential metabolite" of Fildes.

Wiedling (1941) conducted experiments in the bacteriological laboratory of the Swedish State at Stockholm from which he concluded that p aminobenzoic acid is of the same importance to autotrophic plants as it is to the heterotrophically living bacteria. In experiments with two strains of the small fresh-water diatom Nitzschia palea var. debilis, which were grown on an agar made up with tap-water and mineral salts, an inhibition of the effect of sulphanilamide and its derivatives (sulphapyridine and sulphathiazole) was produced by p-aminobenzoic acid.

In a series of experiments attempting to elucidate the mode of action of sulphanilamide, Green and Bielschowsky (1942a), (1942b), concluded that the bacterial anti-sulphanilamide factor showed chemical and biological properties similar to those of p-aminobenzoic acid and considered that the hypothesis advanced by Fildes and Woods provided the best explanation of the facts. They suggested that p-aminobenzoic acid is utilised by all bacteria, and synthesized by most, and that the rate of its synthesis determines the degree of sensitivity of the bacterial cell to sulphanilamide. They also found that some of the bacterial anti-sulphanilamide factor is present in a conjugated form which only became ether-soluble (p-aminobenzoic acid is ether-soluble) after acid hydrolysis, thus confirming in bacteria what MacLeod (1940) had found to hold for mammalian tissues. On the

basis of in vitro findings with a number of aromatic sulphur compounds, these authors suggested that the active principle of compounds with a sulphanilamide-like action is the  radical, and that the sensitivity of bacteria to sulphanilamide is probably determined by the rate at which p-aminobenzoic acid is synthesized in the bacterial cell or is released into the surrounding medium. These authors also suggested that the more effectively a compound blocks the bacterial enzyme whose natural substrate is p-aminobenzoic acid, the greater is its bacteriostatic action.

Maier and Riley (1942) extended the observations on the antagonism of p-aminobenzoic acid and sulphonamides in viruses and bacteria by investigating this relationship in the plasmodial class of protozoa. They studied the inhibition of the antimalarial action of sulphonamides by p-aminobenzoic acid, using Plasmodium gallinaceum as the test infection in chicks, and found that whereas p-aminobenzoic acid itself produced no increase in the incubation period and no change in the infection itself, at certain dosage levels it antagonised completely the inhibitory effect of sulphanilamide, sulphathiazole and sulphadiazine. It failed, however, to alter the effect of the commonly used antimalarial drugs, quinine and atebrine. Their results suggest that the mechanism of inhibition by sulphonamides is similar for bacteria, viruses and plasmodia. Marshall, Litchfield and White (1942), independently made similar observations on malaria in ducks, and their experiments showed that p-aminobenzoic acid antagonised the antimalarial action of sulphaguanidine. They

also considered it probable that this antagonistic effect is intimately associated with the mode of action of sulphonamides in plasmodia as it is in bacteria.

Wood (1942) reported studies adding direct evidence in favour of Woods' and Fildes' theory. He observed that, over a wide range of concentrations, p-aminobenzoic acid neutralised the bacteriostatic properties of all of the six sulphonamide compounds studied, sulphanilamide, sulphaguanidine, sulphapyridine, sulphathiazole, sulphadiazine and diamino-diphenylsulphone, regardless of the differences in their chemical structure. It was found also that the amount of p-aminobenzoic acid needed to prevent bacteriostasis was directly proportional to the bacteriostatic potency of the drug, provided all other variables were held constant. Both of these observations suggested to the author that the bacteriostatic mechanism of the sulphonamide drugs works mainly, if not entirely, through the p-amino nucleus which is common to p-aminobenzoic acid and to all the drugs tested. The experimental finding that an organism made "drug fast" to sulphathiazole is found to be resistant to the action of other sulphonamide compounds also substantiates this concept.

If p-aminobenzoic acid is essential for bacterial growth, and the sulphonamide drugs, through their chemical similarity to this essential metabolite, succeed in blocking the enzyme system normally involved in its utilisation, it obviously follows that the bacteria will not grow. The experimental finding that one mol of p-aminobenzoic acid

will antagonise the action of several thousand mols of sulpha drugs - 1000 to 26,000 mols in different reports - does not invalidate this theory as to the mechanism of bacteriostasis; it may only indicate that the essential metabolite has a far greater affinity for the enzyme than has the drug, in which case a great excess of drug will be required to block the essential metabolite from its bacterial enzyme, or that there are variable amounts of sulphonamide antagonists already present in the culture medium which have also to be neutralised by the sulphonamide added. The wellknown lag in the bacteriostatic action of these drugs may be explained by assuming that the supply of p-aminobenzoic acid already in the medium (and possibly combined with bacterial enzyme, must become inadequate before the rate of bacterial growth will be noticeably affected.

IDENTIFICATION OF ENZYME SYSTEMS INVOLVED IN UTILISATION OF P-AMINOBENZOIC ACID

Several studies on this problem have already been reported. As an example of the competition between sulphanilamide and p-aminobenzoic acid in some essential enzymatic reaction in the cells, Lipmann (1941, called attention to the experimental fact that peroxidase catalysed the oxidation of p-aminobenzoic acid by hydrogen peroxide and that this reaction is inhibited by sulphonamides. No reaction takes place between p-aminobenzoic acid and H_2O_2 without the enzyme, the addition of which causes a red dye to be formed, H_2O_2 disappearing in amounts proportional to the colour formed. In the presence of sulphanilamide, less of the red dye is

produced and proportionately less hydrogen peroxide disappears. Sulphathiazole and sulphapyridine are similarly active. However, Lipmann also found that peroxidase catalysed the reaction between H_2O_2 and other substrates, pyrogallol and tyramine, and that these reactions were likewise inhibited by sulphanilamide. Further, in the presence of catalytic amounts of catechol, p-aminobenzoic acid is oxidised by a phenol oxidase, a red dye again resulting. This reaction, however, was not inhibited by sulphanilamide. It is difficult to correlate these enzymatic reactions and the biological activity of p-aminobenzoic acid and sulphanilamide since McLeod (1939) showed that many organisms susceptible to the action of sulphanilamide are unable to decompose hydrogen peroxide. This makes it difficult to imagine any general inter-relation between a peroxidase reaction and sulphanilamide activity. However, connection between the metabolism of phenolic substances and p-aminobenzoic acid is confirmed by Martin and Ansbacher's (1941) findings that the graying of the fur of animals produced by hydroquinone, first shown by Gettel (1936) and later confirmed by Martin could be cured by administering p-aminobenzoic acid. The amount of p-aminobenzoic acid required was 0.75 mg. daily to prevent hydroquinone achromotrichia in mice weighing 10 to 12 gm. - a relatively large quantity. Later, Martin, Ichniowski, Wisansky and Ansbacher (1942) showed that the oxidative destruction of adrenaline in a tyrosinase-adrenalin system is promoted by O-substituted phenol and inhibited by aminobenzoic acids, including p-amino-
benzoic acid.

SEVAG'S THEORY OF "INHIBITION OF BACTERIAL RESPIRATION" BY SULPHONAMIDES.

Sevag and Shelburne (1942,, could not support the "anti-catalase theory" of the action of sulphonamides, and considered that sulphanilamide acted primarily by blocking the respiratory enzymes rather than by interfering with the synthetic processes necessary for growth, as stated by Fildes. Further, Sevag, Shelburne and Rudd (1942, investigated the inhibition of bacterial and yeast carboxylases by sulphonamide drugs structurally related to cocarboxylase, and concluded that their results supported the hypothesis that sulphonamides exert their bacteriostatic action through chemical affinity for the carrier proteins of certain respiratory enzymes of the bacterial cell, and that this affinity may in part be related to structural similarity between components of the drugs and the corresponding respiratory coenzymes. Sevag and Shelburne's results (1942, in addition provide strong criticisms of Fildes' hypothesis. On Fildes' view, the "sensitivity" of a microbe to sulphanilamide would depend at least in part upon whether it could synthesize p-aminobenzoic acid readily or not. An organism whose synthetic powers were poor should be more sensitive than one with greater powers. Similarly a large number of bacteria should be less affected by a certain concentration of sulphanilamide than a small number. Inhibition or not would become a question of the proportion of sulphanilamide to p-aminobenzoic acid affecting the enzymes of each cell. Sevag and Shelburne's findings are at variance with the above

view expressed by Fildes regarding the role of p-aminobenzoic acid. They found that 0.012 to 0.035 M p-aminobenzoic acid, in the absence of sulphanilamide, inhibits the aerobic and anaerobic respiration of streptococci to the extent of 10 - 50% during a 1- to 3-hour period. In growth experiments, observed after 2-, 8-, 19- and 23-hour periods, 0.035 M p-aminobenzoic acid per se inhibited the growth 22, 34, 63, 67% respectively, and with 0.006 M concentration this effect was, respectively, 0, 0, 23 and 20%. Lower concentrations neither inhibited nor accelerated growth. 0.012 to 0.35 M p-aminobenzoic acid exercises a continuous additive inhibiting effect on the respiration of streptococci in the presence of sulphanilamide. The inhibiting effect of sulphanilamide on respiration may sometimes be nearly completely or partially reversed by 0.006 to 0.0006 M p-aminobenzoic acid, but at other times it has no effect whatsoever.

This inhibition of respiration of E.coli, S. aureus and Streptococcus pyogenes has been confirmed by Wyss, Strandskov, and Schmelkes (1942), but these latter workers, however, also reported that the inhibition of respiration could not be correlated with the bacteriostatic potency of sulphanilamide since its inactive isomers, meta- and ortho-aminobenzenesulphonamide also inhibits respiration and, in addition, sulphanilamide inhibited respiration equally of a resistant strain of E. coli, but did not affect growth, which throws some doubt on the validity of Bevag's criticism of Fildes' hypothesis, since it indicates that sulphonamides may act on different enzyme systems in the bacterial

cell to produce effects both on respiration and growth. Wyss, Strandskov and Schmelkes concluded that the inhibition of bacterial respiration by sulphonamides should not be regarded as typical of sulphonamide activity and that it was not a suitable criterion of the presence or absence of true sulphonamide activity.

THE "NARCOTIC HYPOTHESIS" OF JOHNSON.

Johnson (1942) presented an entirely new theory of the mechanism of p-aminobenzoic acid and sulphonamide action. He drew attention to the fact that narcotics and many toxic materials have stimulatory effects in low, and inhibitory effects in high concentration, and that both p-aminobenzoic acid and sulphonamides acted in the manner of narcotics. He found that ethyl carbonate (urethane), as well as p-aminobenzoic acid, exerted an antisulphanilamide effect on luminous bacteria. The results were more striking in relation to luminescence than to growth, although both were influenced. Johnson considered that structural similarities between the molecules of urethane and sulphanilamide were so remote as to rule out competitive action and urethane could hardly be considered an essential metabolite. All three compounds, urethane, p-aminobenzoic acid and sulphanilamide, could, however, act in the manner of narcotics on luminous bacteria, stimulating growth and luminescence in low, while inhibiting in high concentrations. Lamanna (1942) also reported a stimulatory effect on growth of some bacteria and some yeasts by sulphanilamide in low concentrations. Many narcotics have been found to have similar effects.

A further point emphasised by Johnson is that the stimulatory effects of one narcotic may antagonise or completely

overcome the inhibitory effects of another that is simultaneously present. If the inhibitor is sulphanilamide, the antagonist is naturally looked upon as "anti-sulphanilamide". The anti-sulphanilamide action of both urethane and p-aminobenzoic acid might well belong in this category, and the molecular structure of the antagonist narcotics need not be closely related, as would be required for competitive inhibition in the physico-chemical sense. Johnson's argument, however, cannot gainsay that it is equally feasible for p-aminobenzoic acid to antagonise sulphonamides by virtue of its being an "essential metabolite", while urethane neutralises sulphonamide in an entirely different way. His work, therefore, cannot be considered to have conclusively disproved Fildes' hypothesis. Support for this view is offered by McIlwain (1942).

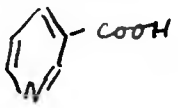
McILWAIN'S MODIFICATION OF FILDES' HYPOTHESIS.

Working in Wood's laboratory, McIlwain examined Johnson's contentions, and pointed out an important difference between the antagonistic actions of urethane and p-aminobenzoic acid on sulphonamide inhibition of the growth of other bacteria. McIlwain found that urethane, under certain conditions, antagonised growth inhibition of S. haemolyticus and E. coli caused by sulphanilamide, but that these conditions were limited, and illustrated well the reason for Fildes' and Wood's hypothesis. He found that much more urethane (between 1-100 mols) than p-aminobenzoic acid (1/5000 mol) was necessary to antagonise the antibacterial action of a given quantity of sulphanilamide (1 mol) and that this effect was not reproducible with certainty. McIlwain, therefore, considered that it was difficult to picture the mode of

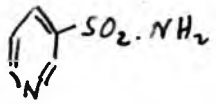
action of so small a proportion of p-aminobenzoic acid except by a specific enzyme mechanism, but that this did not necessarily apply to urethane. Further, the urethane antagonism was shown only towards low concentrations of sulphanilamide, which were just anti-streptococcal. whereas Fildes found that the ratio C_s/C_p, where C_s was the minimal concentration of sulphanilamide necessary for bacteriostasis in the presence of a concentration C_p of p-aminobenzoic acid,was constant, which is consistent with the view that sulphanilamide acted by inhibition of an enzyme normally reacting with p-aminobenzoic acid. The non-competitive type of antagonism shown by urethane is also known to be given by methionine, the action of which is harmonised with the p-aminobenzoic acid theory by an interesting extension of Wood's and Fildes' hypothesis by Harris and Kohn in their series of papers discussed below. McIlwain deduced further evidence against Johnson's "narcotic hypothesis" by a study of the action of other antibacterial agents, which were, like sulphanilamide, related to growth essentials, and whose action could be readily explained in terms of competitive enzyme inhibition. Thus the inhibition of pyridine-3-sulphonamide was unaffected by the presence of p-aminobenzoic acid or pantothenic acid, but was antagonised by a definite fraction of its concentration of nicotinic acid; pantothenic acid, but not nicotinic acid or p-aminobenzoic acid, revereed the inhibition due to pantoyltaurine. However, some other publications discussed later have appeared more recently, querying Fildes' and Wood's theory. Hirsch (1942) found that the antibacterial effects of p-aminobenzamide and p-aminophenylarsinic

acid (or atoxyl) on E. coli, which is not as strong as sulphanilamide, are also antagonised by p-aminobenzoic acid.

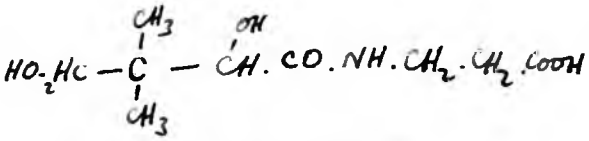
nicotinic acid.



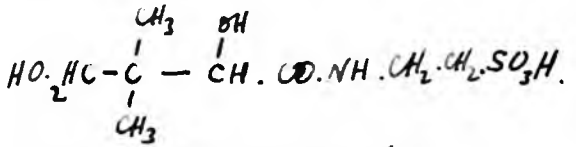
pyridine-3-sulphonamide.



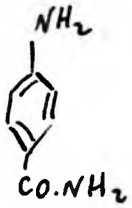
pantothenic acid.



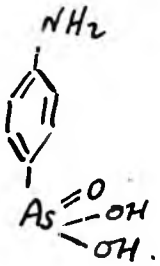
pantoyltaurine.



p-aminobenzamide.



p-aminophenylarsonic acid. (atoxyl.



Wood and Austrian (1942), however, found that, using cultures of staphylococcus aureus in a synthetic medium, nicotinamide and cozymase (diphosphopyridine-adenine-nucleotide, blocked the bacteriostatic action of chemically unrelated sulphonamide drugs as well as the chemically related compound sulphapyridine, and further that the antagonistic action of the pyridine-containing coenzyme, cozymase, was no greater against sulphapyridine than against other structurally dissimilar sulphonamide compounds. They observed that the antidrug effects of nicotinamide and cozymase in staphylococcal cultures were directly proportional to their ability to stimulate the growth of the organism in the synthetic medium. When tested in cultures of E. coli in which

they failed to accelerate bacterial growth, these same substances failed to influence the bacteriostatic action of the sulphonamide drugs. Another observation made by these workers was that the in vitro action of the coenzyme, cocarboxylase, as measured in the Warburg respirometer, was unaffected by the chemically related drug, sulphathiazole, even when the latter was present in great excess. These observations fail to confirm McIlwain's experiments and do not support the theory that sulphapyridine, sulphathiazole and sulphadiazine prevent bacterial growth by interfering with the functioning of the chemically related coenzymes, cozymase and cocarboxylase. The greater antibacterial powers of the substituted sulphonamide compounds may be explained by assuming that the chemical group attached to the sulphonamide radical enables the compounds to interfere with the metabolism of p-aminobenzoic acid more effectively than does the simpler sulphanilamide molecule. According to this "unitarian theory", the only metabolic function of the bacterial cell interfered with by the sulphonamide compounds is that concerned with the utilisation of p-aminobenzoic acid, the degree to which this function is disturbed determining the relative bacteriostatic potency of the drug.

KOHN AND HARRIS' MODIFICATION OF FILDES' HYPOTHESIS.

Kohn and Harris (1941) formulated a new theory which transferred p-aminobenzoic acid from the role of a substrate to that of a catalyst. Their theory was based upon the fact that several other compounds had been shown to have an anti

sulphonamide effect. For example, the same authors showed that methionine antagonised the action of sulphanilamide, sulphapyridine, sulphadiazine and sulphathiazole on E. coli. But methionine, unlike p-aminobenzoic acid, was only effective against low concentrations of the sulphonamides, and did not exhibit a simple relationship in the concentrations necessary to antagonise increasing amounts of the sulphonamides. Commercial peptone, used as an ingredient for many routine culture media was also shown to have an anti-sulphonamide action.

Kohn and Harris (1941) schematised sulphonamide action as follows: among the syntheses in the cell necessary for growth and multiplication there is a special group X (termed secondary reactions) into which enter substances (including methionine and peptone) the production of which is catalysed by p-aminobenzoic acid (termed primary reactions). When the stores of X fall below a critical concentration, growth rate decreases. This state of affairs is brought about by sulphonamides. Methionine exhibits an antisulphonamide action when the sulphonamide concentration is small, since the primary reaction involving methionine synthesis only is inhibited by such low concentrations. Therefore, both methionine and p-aminobenzoic acid are effective. As the drug concentration is increased more primary reactions become inhibited, and the synthesis of the other X components, in turn, is inhibited. P-aminobenzoic acid still remains effective as an antagonist since it restores the primary reactions and all the secondary reactions then follow. This theory thus proposes an alternative extension to Fildes' and Wood's

hypothesis of the fundamental antagonism between p-amino benzoic acid and sulphonamides.

Henry (1943, p.212), in a review on the mode of action of sulphonamides, criticised Kohn and Harris' theory on the following grounds. The essential facts underlying Kohn and Harris' interpretation were the following: First, that a latent period existed before sulphonamide action manifested itself in vitro. Second, methionine was able to counteract sulphonamide action, but only at low concentrations of sulphonamides. Third, ethionine, norvaline and norleucine inhibited bacterial growth and synergised sulphanilamide action, possibly by competing with and displacing methionine in the cell, since addition of methionine (or peptone), but not p-aminobenzoic acid, abolished these inhibitions. This was interpreted as placing the methionine antagonism in a reaction secondary to primary reactions involving p-aminobenzoic acid. Henry considered that two objections can be raised to this theory: firstly, the delay in sulphonamide action, though reported by many workers, was not always found, and secondly, there was no known example where an excess of enzyme produced an inhibition, and it was well-established that p-aminobenzoic acid acted as an inhibitor in concentrations above those which antagonised sulphonamide activity. This finding incidentally was confirmed here under quite different experimental conditions. (p.)

Henry (1943, p.220) summed up the present status of sulphonamide antagonists as follows: A great number of substances with a great diversity of source antagonised sulphonamide inhibition. The first question to be answered was whether

their antagonistic activity was due to their p-aminobenzoic acid content. This obviously could not be the case with methionine, urethane, glucose and other substances of known composition. For the remainder of the antagonists, those of unknown composition, it was thought, after the appearance of the Woods-Fildes theory, that they owed their activity to their p-aminobenzoic acid content. This assumption still persisted largely to-day, although there was no absolutely conclusive proof that p-aminobenzoic acid was present in any of these substances. Such evidence as the demonstration of the presence of diazotizable compounds in these substances indicated the presence of a primary aromatic amine, which might not necessarily be p-aminobenzoic acid. Further, sulphonamide antagonists had been shown to act either by specific interference or by non-specific growth-stimulation, or by inactive complex formation, whereas p-aminobenzoic acid had been conclusively shown to act by the first method, certainly not by the second, and only possibly by the third method.

It must be admitted that the antagonism of the sulphonamide inhibition of the carboxylase enzyme system by p-aminobenzoic acid could scarcely be explained on the basis of the latter's functioning as an essential metabolite or substrate. Secondly, in the experiments of Sevag and his co-workers with live bacteria, the experimental conditions were such that growth could not take place, yet antagonism of respiratory inhibition by sulphonamides was obtainable in the presence of p-aminobenzoic acid. Since, therefore, this antagonism took place in the absence of growth, the contention of these workers that the counteraction of the growth-inhibiting effect of sulphonamides

with p-aminobenzoic acid could occur through the pathway of respiratory enzymes appears reasonnable. But, again, these enzyme systems have not as yet been identified with certainty.

More recently, Sevag and Green (1944a) reported that the formation of an arylamine, i.e. a primary aromatic amine, by a strain of staphyloccus aureus which had been rendered resistant to sulphonamides, depended on the presence simultaneously of glucose and tryptophane, or pyruvate and tryptophane, in the medium, and that in the absence of glucose, pyruvate and tryptophane, growth took place without the formation of p-aminobenzoic acid.

Later Sevag and Green (1944b) reported that the arylamine found in the culture fluids of staphyloccus aureus was derived from tryptophane. They also found, unlike Landy et al (1943), that any degree of growth of a resistant strain of S. aureus could take place without the formation of arylamine. A susceptible strain could also be made to produce large amounts of arylamine without a corresponding change in resistance to sulphonamides. The development of resistance and increased arylamine formation were, therefore, considered by the authors to be unassociated processes. This finding that a micro-organism could become resistant to sulphonamides, without this resistance being due to increased synthesis of p-aminobenzoic acid, constitutes perhaps the severest blow to the Woods-Pildes' theory.

A possible link between the development of resistance and increased arylamine formation, if this does occur, might arise from the interference of sulphonamides with tryptophane metabolism, and the conversion of tryptophane to

arylamine. Sevag and Green (1944c) stressed that there was as yet no adequate, rigorous, chemical or physiological evidence that p-aminobenzoic acid synthesis occurred at all under normal conditions of bacterial growth. It should also be noted that the Woods-Fildes' hypothesis of the mode of action of sulphonamides, and those concepts which were formulated as extensions or adaptations of their hypothesis were primarily based on the results obtained with p-aminobenzoic acid added to the bacterial growth systems, whereas the results of Sevag and Green indicate that this concept was not applicable to conditions of normal bacterial growth, in particular to that of S. aureus. Their results, moreover, show that the development of resistance to sulphonamides was associated with the development of a sulphonamide-resistant type of glucose metabolism and not to an increased synthesis of p-aminobenzoic acid. In the presence of glucose, growth of a resistant strain of S. aureus occurred after the addition of sulphonamides. In the absence of glucose, the growth of the resistant S. aureus was inhibited. This was in striking contrast to the high degree of inhibition of the growth of susceptible strains in the presence and absence of glucose. These observations indicate that during the development of resistance to sulphonamides, S. aureus had acquired the specialised ability of metabolising glucose in a manner which was not susceptible to the inhibitory action of sulphonamides. Sevag and Green found that they could, by numerous subcultures of their sulphonamide-resistant strain of S. aureus on a medium without added glucose (and sulphonamides), so

alter the strain that it assumed a high degree of resistance to sulphonamides, even in the absence of glucose. Since the addition of tryptophane to the original sulphonamide-resistant strain (i.e.showing resistance only in the presence of glucose), antagonised inhibition by sulphonamides in the absence of glucose, the authors considered that the synthesis of growth-essential tryptophane from other amino-acids was inhibited by sulphonamides, but that this was counteracted in the presence of glucose. The inter-relationship, however, of glucose and amino-acid metabolism in such sulphonamide-resistant organisms is still obscure, although in a further paper Sevag and Green (1944d) reported that pantothenic acid mediated (a) in the metabolism of glucose leading to, or involved in, the synthesis of tryptophane essential for the gowth of the exacting strains of staphylococcus aureus; (b) in oxidation of tryptophane when glucose is present to one or more arylamines (not p-aminobenzoic acid,; and (c) in the glucose-tryptophane metabolism in counteracting the inhibitory action of sulphonamides on the utilisation of added tryptophane. Hence they finally decided that the inhibition of the synthesis of tryptophane from glucose and amino-acids, and not the synthesis of p-aminobenzoic acid, appeared to be the critical action of sulphonamides on S.aureus.

In other papers, Sevag and his co-workers have reported further studies on their "inhibition of respiration theory", as the mode of action of sulphonamides, which they put forward in 1942 and to which reference has already been made.(p.17) ((Sevag and Shelburne (1942); Sevag, Shelburne and Rudd, (1942).

They found (Sevag, Shelburne and Mudd, 1945) that sulphathiazole inhibited the carboxylase activity of whole yeast, and that one molecule of cocarboxylase added to the reaction system was capable of counteracting the inhibitory effect of from 8,088 to 53,400 molecules of sulphathiazole. In a second paper, Sevag, Henry and Richardson (1945) reported that p-aminobenzoic acid was incapable of reversing the inhibition, exercised on the carboxylase activity of E. coli, by acetaldehyde, the decarboxylation product of sodium pyruvate. The authors accepted as self-evident the fact that acetaldehyde combined specifically with the active site of the carboxylase enzyme and so prevented its activity. In contrast, p-aminobenzoic acid certainly counteracted the inhibition exerted on carboxylase by sulphathiazole, but its inhibitory effect was shown to be 10- to 20-fold less effective as a sulphathiazole antagonist than was the coenzyme, cocarboxylase. While antagonising the inhibitory effect of sulphathiazole on carboxylase, p-aminobenzoic acid at the same time, unlike cocarboxylase, also maintained a certain degree of inhibitory action on its own. In other words, p-aminobenzoic acid was thus shown to function as an inhibitor, as well as an anti-inhibitor, which is inconsistent with the view that p-aminobenzoic acid is an essential metabolite. Various other investigators have reported similar observations. Further, in all the other experiments performed by Sevag and his collaborators, no evidence was found that p-aminobenzoic acid ever participated actively in the metabolic activities of the organisms studied to account for its sulphonamide-antagonising property.

This was particularly true in those cases in which resting cells, and isolated enzyme systems, were tested. It has already been indicated (p.28 of this thesis) that no direct, conclusive evidence has been obtained by other investigators for Wood's and Wildes' "essential metabolite" theory. In a subsequent chapter the available literature on the status of p-aminobenzoic acid as a member of the vitamin B complex is critically reviewed, from which it is concluded that here, too, no certain proof of the essential part played by p-aminobenzoic acid in the metabolism of higher or lower animals has been obtained.

RELATIONSHIP BETWEEN P-AMINOBENZOIC ACID DERIVATIVES AND SULPHONAMIDES.

Many p-aminobenzoic acid derivatives are of great pharmacological importance as local anaesthetics. Keltch, Baker, Krahl and Clowes (1941) investigated the anti-sulphapyridine and anti-sulphathiazole effect in vitro against E. coli of several local anaesthetics derived from p-aminobenzoic acid, including benzocaine or anaesthesin (ethyl-p-amino-benzoate) and novocaine or procaine (beta-diethylamino-ethyl-p-amino-benzoate) and several other local anaesthetics not derived from p-aminobenzoic acid. They found that each of the p-aminobenzoic acid derivatives tested, partially or completely blocked the bacteriostatic effect of the sulphonamide, whereas none of the non-p-aminobenzoic acid derivatives had any effect. Their experiments also indicated that the blocking effect was reduced but not eliminated by substitution in the amino-group or esterification of the carboxyl group of p-aminobenzoic acid. Peterson and Finland (1944)

found that procaine, in amounts ordinarily employed for local anaesthesia, may be absorbed into the circulation in sufficient concentration to exert an inhibiting effect on the action of sulphonamide drugs that might be present in the blood.

EFFECT OF P-AMINOBENZOIC ACID ON THE TOXIC EFFECTS OF SULPHONAMIDES IN ANIMALS AND MAN

McCarty (1941) reported that p-aminobenzoic acid had no observable effect upon the immediate fatal toxicity of sulpha pyridine for mice. Strauss and Finland (1941) found that fevers and rashes due to sulphathiazole could neither be overcome nor prevented by the administration of p-aminobenzoic acid, even when given in amounts sufficient to overcome the antibacterial action of the sulphonamide drug in the blood and urine. These findings lend additional support to the view that the action of the sulphonamide drugs are not invariably related to a displacement of p-aminobenzoic acid as an essential metabolite for bacterial development.

S U M M A R Y.

Fildes (1940) focussed attention on p-aminobenzoic acid by suggesting that it was an essential metabolite for bacteria and that it was normally associated with an enzyme system in the bacterial cell. He propounded the hypothesis that the sulphonamide group of drugs owed their bacteriostatic action to their structural similarity to p-aminobenzoic acid which enabled them to displace it from its enzyme and stop an essential line of metabolism in the bacterial cell. Experimental support for this hypothesis has been provided by several investigators who have established that p-aminobenzoic acid could antagonise the inhibitory effect of the sulphonamides on the growth of viruses, bacteria, fungi and protozoa.

Though Fildes' hypothesis is generally accepted today, attempts to identify the enzyme system involved in the utilisation of p-aminobenzoic acid have led to inconclusive results. In addition, several other compounds have been found to have an anti-sulphonamide action. Other theories have, therefore, been proposed to explain the action of sulphonamides. These include the "narcotic hypothesis" of Johnson and the "sulphonamide inhibition of bacterial respiration" theory of Sevag. The latter and his co-workers have perhaps struck the severest blow to Fildes' theory by their demonstration that micro-organisms could develop resistance to sulphonamides without synthesizing an increased amount of p-aminobenzoic acid.

To explain the experimental finding that totally unrelated compounds could under certain circumstances antagonise sulphonamide action on bacteria, Kohn and Harris developed an interesting modification of Fildes' hypothesis. They suggested that p-aminobenzoic acid catalysed the production of various substances (primary reactions), and that these were necessary to promote cell growth and multiplication (secondary reactions). Sulphonamides acted by inhibiting the catalysis of the primary reactions and so led to a drop in the concentration of the substances required for the secondary reactions. Under certain conditions, such as a low concentration of the drug, the direct addition of one of the compounds promoting the secondary reactions might be sufficient to antagonise it. When, however, the drug concentration was high, only p-aminobenzoic acid was effective since it restored the primary reactions and all the secondary reactions then followed.

CHAPTER II.

THE DETERMINATION OF P-AMINOBENZOIC ACID.

The methods described for the estimation of p-aminobenzoic acid can be divided into two types:

A. Chemical methods.

B. Microbiological methods.

A. Chemical Methods.

Since p-aminobenzoic acid is a primary aromatic amine, it is diazotizable, and such a reaction can be used as the basis of one method of estimating its concentration in body fluids and foodstuffs. For example, the method of Bratton and Marshall (1939, for the estimation of sulphonamides, which are also primary aromatic amine derivatives, has been used for the estimation of p-aminobenzoic acid. In this method the sulphonamide is diazotized and coupled in acid solution with dimethyl-x-naphthylamine or with N-(1-naphthyl) ethylenediamine dihydrochloride to form a red pigment, which is determined colorimetrically. Although this test is non-specific since any primary aromatic amine will react, it is stated that the test can be made specific provided that the different reaction rates of the various diazotized substances, e.g. diazotized aniline, p-aminobenzoic acid and its isomers, sulphonamides and related compounds (Ansbacher, 1944, p.240), are taken into account. When, however, an assay of biological fluids for p-aminobenzoic acid is made after feeding or otherwise administering this compound, the objection of non-specificity is not a great one,

since a control estimation before feeding p-aminobenzoic acid will serve to indicate the presence of any other primary aromatic amine or derivative in that organism. Blanchard (1941) has employed this method for the quantitative determination of p-aminobenzoic acid in yeast. Eckert (1943) has determined the free and conjugated p-aminobenzoic acid in blood, using a modification of Bratton and Marshall's method, which he claims to be extremely sensitive, and capable of detecting 1 microgram (γ) of p-aminobenzoic acid in 0.5 c.c. blood, or 200 micrograms per 100 c.c. blood. More recently, Rose and Bevan(1944) have described a simplified method for the estimation of sulphonamide drugs in blood, urine and other body fluids, using a new coupling component N-β-sulphatoethyl-m-toluidine. None of these methods could be tried here since the reagents were unobtainable.

Another colour reaction for paminobenzoic acid was described by Tauber and Lauffer (1941). They showed that on the addition of p-dimethylaminobenzaldehyde to p-aminobenzoic acid in glacial acetic acid a deep yellow colour developed. They considered that the coloured compound was probably a Schiff base, and found that the isomers of p-aminobenzoic acid and their alkyl esters also gave this test.

The use of p-dimethylaminobenzaldehyde for the determination of sulphonamide drugs had, however, already been advocated by Werner (1939) and by Morris (1941). This compound has since been employed by Fuller (1942), using a test-paper method for the estimation of sulphonamides in

laked blood, and the method further modified by La Rose (1943) for the direct analysis of free sulphonamides and other primary amines, including p-aminobenzoic acid, in serum. However, Mawson (1942) pointed out difficulties in the use of this reagent for the estimation of sulphonamides in blood and has described a method to overcome them. I have tried out his method here and found it satisfactory for the concentrations of sulphonamides present in blood during treatment with these drugs (i.e. 5 - 15 mg. per cent.). It has not been used for the estimation of p-aminobenzoic acid in the experiments reported in this thesis since the minute amounts of p-aminobenzoic acid to be assayed made it essential to use a much more sensitive method, but Mawson's method is suitable for the detection of large quantities of p-aminobenzoic acid (5 - 10 mg.%), and is, therefore, being used in experiments investigating the effects of p-aminobenzoic acid in increasing the resistance of animals and man to infections, which are at present in progress.

Kirch and Bergeim (1943) described a method for the determination of p-aminobenzoic acid based on a colour reaction with diazotized thiamine. The smallest amount of p-aminobenzoic acid found to give a colour was about 10 micrograms. This method is applicable to urine, and complete recoveries of known amounts of p-aminobenzoic acid added to urine have been obtained. Free and conjugated p-aminobenzoic acid can be estimated even in the presence of most of the sulphonamides by a variation in this technique.

A procedure which makes use of tyrosinase may also

be employed (Wisansky, Grattan, Gawron and Ansbacher, unpublished data, quoted from Ansbacher 1944, p.240). When this enzyme catalyses the aerobic oxidation of catechol, a transient pale yellow-green colour is observed. If the medium contains p-aminobenzoic acid, this colour is not noticed, but a red colour is formed. The reaction is sensitive for the colorimetric determination of p-aminobenzoic acid in amounts of as low as 5 -10 micrograms. The same colour is apparently developed, when the system tyrosinase-oxygen is replaced by ceric sulphate, which is said to be easier to obtain and less expensive than the enzyme.

B. Microbiological Methods.

These methods fall into two groups. Group I methods are based upon the antagonism of p-aminobenzoic acid to sulphonamide bacteriostasis, and consist of determining in a simple, entirely synthetic, basal, bacteriological culture medium, to which a known quantity of a sulphonamide has been added, the amount of p-aminobenzoic acid required to allow growth to proceed. Using this as a standard, the amount of p-aminobenzoic acid in the material assayed is then determined. These methods have been criticised on the grounds (a) that sulphonamide antagonism is not due to p-aminobenzoic acid alone, but may result from the presence of many other compounds, as has already been discussed in Chapter I, and (b) also that these methods are relatively insensitive compared to Group II methods, discussed below. As methods of assay of p-aminobenzoic acid in unknown materials, animal body tissues, fluids, yeasts, etc., they are certainly

unsuitable. Nevertheless, in certain circumstances this objection does not hold. For example, the concentration of p-aminobenzoic acid in a pure solution of this substance can be reliably estimated by such methods. Secondly, after p-aminobenzoic acid has been administered, the increase of sulphonamide-antagonistic substance in, say, the urine can be justifiably considered to be p-aminobenzoic acid. Since the experiments reported in this thesis are mainly concerned with such circumstances, it is claimed that the method of assay used, and described in detail below, is in fact a reliable measure of the p-aminobenzoic acid present. In addition, these methods, based on sulphonamide antagonism, have the great advantage that the constituents of the culture mediums used are inexpensive and easily obtainable in any laboratory. It should also be noted that the more elaborate methods of the second group described below, for which specificity has been claimed, can also be strongly criticised on the basis of recent reports. In fact, no method yet devised for the assay of p-aminobenzoic acid, either chemical or microbiological, has been conclusively proved to be absolutely specific.

McLeod (1940) demonstrated the presence of sulphonamide-antagonists in extracts of various animal tissues and body fluids. His method consisted of determining the effect of different amounts of the various materials upon the growth of Escherichia coli observed in an inhibitor-free synthetic medium, to which was added an amount of neutral solution of sulphapyridine sufficient to prevent the visible growth of a standard inoculum of the organisms. The usual concentration

of sulphapyridine required was 1/20,000 - 1/60,000. Growth was estimated by observing the presence or absence of gross turbidity after incubation for 24 hours at 37°C. The synthetic medium alone, plus inoculum of E. coli, served as the control. By varying the concentration of sulphapyridine, differences in the amount of antagonist present could be estimated.

A modification of McLeod's method has been employed in these experiments. McLeod gives no figures for the sensitivity of his method since, as has already been mentioned, he did not know that the sulphonamide-antagonists he measured consisted mainly, if not entirely, of p-aminobenzoic acid. The modification used here permitted the detection of 1 microgram of p-aminobenzoic acid in the volume of material assayed, and a later change in the basal medium used increased the sensitivity to 0.2 micrograms.

Group II methods are based upon the fact that p-aminobenzoic acid is a growth factor for certain bacteria which are unable to synthesize p-aminobenzoic acid and which will not grow unless p-aminobenzoic acid is added to the medium. Rubbo and Gillespie (1940) showed that p-aminobenzoic acid and its derivatives acted as growth factors for an anaerobic bacillus, Clostridium acetobutylicum. In a further paper Rubbo, Maxwell, Fairbridge and Gillespie (1941) developed this finding into a microbiological assay for p-aminobenzoic acid. They found that Cl. acetobutylicum required a factor for growth conforming to the structural pattern of a di-substituted benzene ring in which the substituents occupied the 1:4 positions. Optimal activity was developed when the

substituents were an amino-group on the one hand and a carboxyl-group on the other. P-aminobenzoic acid was found to stimulate growth in concentrations of 1.46×10^{-10}M, i.e. approximately.002 micrograms per 100 c.c. of solution. Park and Wood (1942), however, found that p-aminobenzoic acid alone was insufficient to cause growth of Cl.acetobutylicum and that biotin was required in addition. McIlwain (1942a) used the same organism in a different medium (plus biotin) and found the method of assay satisfactory. He showed that methionine, which is known to antagonise sulphonamide-inhibition of E. coli, did not promote growth of Cl.acetobutylicum.

Landy and Dicken (1942) developed a new microbiological assay method based upon the growth response of Acetobacter suboxydans to p-aminobenzoic acid, which they claimed to be rapid, accurate and specific. The turbidity resulting from the growth of the bacteria was measured in a photoelectric colorimeter. They reported, however, assay values of a group of compounds derived from or related to p-aminobenzoic acid, which showed that the method could not be considered strictly specific for p-aminobenzoic acid, although relatively very large quantities of the latter compounds were needed to give a positive result. This method has been used by several other investigators.

In the same year Lewis (1942) described another assay method based upon the growth factor activity of p-aminobenzoic acid for Lactobacillus arabinosus. The Lactobacillus methods, which are used also for the assay of various members of the vitamin B complex, offer the great advantage that a chemical

titration can be substituted for an estimation of growth and turbidity. At the end of the growth period allowed the lactic acid which is formed by the bacillus during growth is titrated and affords a simpler, more sensitive and exact index of the quantity of p-aminobenzoic acid present than does turbidity estimated visually. The reliability of Lewis' method is indicated by reproducibility of assays, by agreement of assay values obtained with various levels of added samples, and by recoveries of p-aminobenzoic acid added to various samples. The method is very sensitive, the assay range being 0.00015 to 0.0005 micrograms.

Beadle and Tatum (1941) discovered and maintained X-ray induced mutant strains of the mould Neurospora crassa which were characterised by their inability to carry out specific biochemical processes. One of these mutant strains was apparently unable to synthesize p-aminobenzoic acid and could not grow until p-aminobenzoic acid was added to the medium. Hence p-aminobenzoic acid becomes a growth factor for the "aminobenzoic-less" mutant strain of Neurospora. Tatum and Beadle (1942), following up these very interesting findings, showed that the growth of the mutant strain of Neurospora was a function of the amount of p-aminobenzoic acid supplied. They consider that the mutant differed from the normal strain only by a single gene which must, therefore, control an essential step in the synthesis of p-aminobenzoic acid, and which was presumably primarily concerned only with the synthesis of p-aminobenzoic acid. A number of substances related to p-aminobenzoic acid were, however, able to replace it, but their activities were much less than that of p-aminobenzoic acid

itself. These authors further described a method of measuring the growth of the mould. Mitchell, Isbell and Thompson (1943) employed this mutant strain in a microbiological assay method for p-aminobenzoic acid, and determined the p-aminobenzoic acid content in various natural substances. Their results suggest that the method of Landy and Dickeh (1942) determined only a fraction of the total amount of p-aminobenzoic acid present. In a further paper, Thompson, Isbell and Mitchell (1943) claimed that their method had a high specificity and a sensitivity of .004 - .04 micrograms. The method is rapid (20 hours' incubation at 30°C.) and the results reproducible. These authors employed a different method of estimating the growth of Neurospora crassa to that of Tatum and Beadle.

Mirick (1943) presented still another method for quantitatively estimating small amounts of p-aminobenzoic acid, utilising his soil bacillus of the Pseudomonaceae family, which was specifically adapted to produce enzymes capable of oxidising p-aminobenzoic acid apparently to carbon dioxide, water and ammonia (Mirick, 1941). For this bacillus, p-aminobenzoic acid was neither a growth factor nor was it released into the medium in which the bacillus was cultured, but on the other hand it was completely destroyed by the bacillus. The quantity of primary aromatic amine in the solution was determined by adiazotization technique before and after the action of the soil bacillus, the difference being the quantity of p-aminobenzoic acid present. The specific adaptive enzymes of this soil bacillus may be used for the identification of quantities down to 1c micrograms of p-aminobenzoic acid.

benzoic acid, shows that the simple basal medium used does

The limitation in sensitivity of the method was shown to be due to the relative insensitivity of the diazo reaction as compared to the other microbiological methods. Nevertheless, this method appears to possess a specificity greater than any yet described. Mirick also discussed in his paper the other microbiological techniques of this group already described and severely criticised their so-called specificity.

SELECTION OF ASSAY METHOD FOR P-AMINOBENZOIC ACID.

The microbiological method used for the estimation of p-aminobenzoic acid in the experiments described in this thesis has been selected for the following reasons:-

(1) Availability of the reagents required for the culture media. For every other method described above a few, usually several, of the reagents required could not be obtained despite strenuous efforts.

(2) Despite the fallacy of considering all sulphonamide-antagonists to be p-aminobenzoic acid, it was felt that this objection was not a valid one when pure solutions of p-aminobenzoic acid and its derivatives were measured or when its excretion in the urine after feeding was estimated, since a control experiment on the material assayed was always carried out before p-aminobenzoic acid was administered. A consideration of the work reported so far (a) on substances which act synergistically with p-aminobenzoic acid in antagonising sulphonamides and (b) on sulphonamide-antagonists which are produced in a medium on the addition of p-aminobenzoic acid, shows that the simple basal medium used does

not contain any substance which may introduce an error on these grounds. In fact, many of the more elaborate microbiological experiments described under B, Group II above, employ media containing various amino-acids, purines and members of the vitamin B complex, some of which have already been shown, particularly by Bevag et al , to influence the antagonism of p-aminobenzoic acid to sulphonamides, and others have not as yet been tested. It is probable, therefore, that these substances influence the action of p-aminobenzoic acid as a growth factor on the test organism used, so that this objection holds throughout all the assay procedures yet described, with the possible exception of Kirick's (1943) method.

The greatest objection to the method used in these experiments, which could only be partly remedied, is its relative insensitivity as compared to the other group of microbiological methods described. During the course of these experiments the method used was improved so as to increase its sensitivity from measuring a minimum of 1 microgram in the volume of material added to the medium to 0.2 micrograms, but this is not sufficiently sensitive to measure the quantity of p-aminobenzoic acid in normal urine which is of the order of 0.02 micrograms/c.c. However, by concentrating normal urine to 1/10 and 1/100 of its volume, the quantity of p-aminobenzoic acid present could be estimated. Nevertheless, this technique is too laborious to make it practicable as a routine procedure and it has only been used in a few experiments.

DESCRIPTION OF MICROBIOLOGICAL METHOD USED FOR THE ESTIMATION OF P-AMINOBENZOIC ACID.

/Modified from McLeod (1940)/.

Principle

A synthetic culture medium which supports the growth of Escherichia coli is prepared. To it is added sufficient sulphapyridine to prevent growth of the organism. The material to be assayed for p-aminobenzoic acid is added and the amount required to neutralise the action of sulphapyridine is compared with a standard solution of p-aminobenzoic acid. The quantity of p-aminobenzoic acid present can then be calculated.

Basal Culture Medium.

The synthetic medium used was that developed by Cahyum, Beard, Schultz, Snow and Cross (1936), and consists of:-

Ammonium sulphate	4.72 grammes.
Asparagine	2.0 grammes.
Sodium Chloride	5.0 grammes.
Glucose	2.0 grammes.
KH_2PO_4 (M/5)	100 c.c.
NaOH, (N/1)	16.4 c.c.
$MgCl_2$; $FeCl_2$; $CaCl_2$ (½% mixture)	1.0 cc.
Distilled water to	1000 c.c.

pH adjusted to 7.6. Tubed in 4.c.c. quantities and sterilised in the autoclave at 5 lbs. pressure for 30 minutes.

Culture of E. coli.

A strain of E. coli obtained from a specimen of faeces was transferred on to Loffler's serum medium. Stock cultures

were kept in the smooth phase on this medium, stored in the refrigerator and usually subcultured on to fresh Loffler's medium at 4-weekly intervals. Since on two occasions 8-week old cultures were used, experiments were carried out to determine whether the duration of cold storage of the stock culture had any deleterious effect on the growth of the organism. These experiments are not reported in detail, but within the maximum period (8 weeks) at which subcultures were made from the stock culture, no difference was noted.

At the outset, this strain of E. coli would not grow when transferred from the stock Loffler's medium to the above basal medium. McLeod (1940) apparently experienced a similar difficulty since he later added a casein hydrolysate known to be free of sulphonamide-antagonistic material to his medium and claimed that this modification ensured an optimum growth rate. An attempt was first made here to improve the basal medium used in the same way, namely by the addition of a vitamin-free casein hydrolysate, (kindly supplied by Dr. L. Goldberg of the B.A.I.M.R.) Unfortunately this preparation was not free of sulphonamide-antagonistic substances, and produced variable growth in the basal medium even after the addition of considerably larger amounts of sulphapyridine than that used below. The problem was, therefore, tackled in other ways and was finally solved by subculturing the strain of E. coli used several times from one Loffler's medium to another after 24 hours incubation each time, and inoculating tubes of basal medium with heavy suspensions of organisms from each subculture. The basal

medium tubes were then incubated for 24 hours at 37°C. and subcultures were then again made from each basal medium tube on to fresh Loffler's medium. From the first three of these basal mediums no growth developed on the Loffler's subculture, showing that the organisms had died out, but from the fourth basal medium tube, a poor growth was obtained on the Loffler's medium after 24 hours incubation. This growth was now inoculated back and forth from basal medium to Loffler's medium at 24-hourly intervals (during which time it was kept at 37°C) and it was noted that successive inoculations into the basal medium produced heavier and heavier growths. Finally, maximal growth in the basal medium was produced after 24 hours with the size of inoculum detailed below, this quantity then being used as a standard inoculum throughout the experiments reported. The organism thus apparently had adapted itself to an existence on a poorly nutrient medium and had accustomed itself to the use of relatively simple nutrients. Simultaneously with this adaptation, another phenomenon was observed. With repeated subcultures, the quantity of p-aminobenzoic acid required by the organism to neutralise added sulphonamide gradually diminished. In experiment I a few results of typical experiments done are recorded. These show that whereas at the outset of this work 2.5 micrograms of p-aminobenzoic acid were required to neutralise the 1/25,000 sulphapyridine added (= 200 micrograms of sulphapyridine) and allow growth of the organism to proceed, only 1 microgram of p-aminobenzoic acid was required six months later. When the basal medium used was modified at about

this time by the inclusidn of ammonium phosphate $(NH_4)_2HPO_4$ as the source of nitrogen in place of asparagine, which became unobtainable, (glucose was retained as the source of carbon), the sensitivity of the method was further improved, and 0.2 micrograms of p-aminobenzoic acid was found to neutralise the 200 micrograms of sulphapyridine added. From this stage no further alteration in the p-aminobenzoic acid requirements was noted. It should be noted that when calculating the quantity of p-aminobenzoic acid present in the solution or urine assayed, the figures quoted in the various experiments recorded were derived by reference to a known p-aminobenzoic acid solution simultaneously tested.

Inoculum.

A subculture on Loffler's serum was made and incubated at 37°C. for 24 hours. The resulting growth was washed off with 10 c.c. of sterile normal saline and centrifuged at 3000 r.p.m. until the sediment was firmly packed down. The supernatant saline solution was decanted off completely and the sediment resuspended in 20 c.c. sterile saline. This washing of the bacterial suspension was carried out as a routine since, if the original suspension of organisms from the Loffler's slope was used, growth with the size of inoculum used sometimes occurred in the basal medium with 1/25000 sulphapyridine added. This was considered to be due to one or both of the following causes: (a) the bacteria growing on the Loffler's medium synthesize p-aminobenzoic acid which is present in the saline suspension and neutralise the sulphapyridine, hence allowing growth to proceed and (b) that other sulphonamide-antagonist substances present in the Loffler's

serum medium or water of condensation, e.g. peptones, described in Chapter I, neutralise the sulphapyridine and hence allow growth to proceed. When the saline used to wash the organisms off the Loffler's slope was decanted off the well-centrifuged organisms, and the latter re-suspended in fresh, sterile, normal saline, trouble was only very occasionally encountered. One drop of this washed bacterial suspension (approximately 0.05 c.c.) was added to the culture medium (4 c.c.). After addition of the volume of the material to be assayed, sterile, normal saline was added to the medium to make a total volume of 5 c.c. At the outset attempts were made to standardise, as most workers have done, the volume of bacterial inoculum, i.e. the number of organisms added by comparing the turbidity of the suspension with Brown's standard opacity tubes, but preliminary experiments (not quoted in detail) indicated that the number of bacteria could be varied fairly considerably without influencing the end result, since estimations of presence or absence of growth was made visually and not by a photo-electric method. Kohn and Harris (1941) obtained quantitative data on the growth of E. coli in different media in the presence and absence of sulphonamides, and reported that the size of the inoculum had no real effect on the results. The apparent effect they found to be due to the limitations of the medium and the time course of the inhibition. Large inocula (above 76,000 organisms) exhaust the medium and cease growth within 6 - 7 hours. The inoculum used in the following experiments exceeded this figure, as estimated by comparison with Brown's opacity tubes. Further, since the degree of growth was only

estimated roughly as slight, moderate or maximal growth, or no growth, it was decided not to measure very accurately the size of the inoculum used. All volumes were added from sterile pipettes kept in a sterile copper pipette container.

Addition of Sulphapyridine.

The optimal concentration of sulphapyridine required was estimated in preliminary experiments and this concentration kept constant throughout the experiments. It was found that a final concentration of 1/25,000 sulphapyridine was the minimal quantity required to ensure that no growth would occur with the above inoculum until p-aminobenzoic acid was added. Kohn and Harris (1941) plotted the relationship between rate of growth and drug relationship, and found this to be a simple one in a salt-glucose medium such as the above. A stock 0.025% solution of sulphapyridine in distilled water was then prepared and 200 c.c. added to the constituents of the basal medium above, the final volume being again made up with distilled water to 1000 c.c. This medium was again tubed in 4.c.c.-quantities, and these tubes distinguished from tubes of the basal medium by inserting a blue glass bead in each tube, whereas the basal medium tubes contained a yellow glass bead. The concentration of sulphapyridine in the basal medium is, therefore, 1/20,000. After addition of the test solution, the final volume was made up to 5.cc. in which, therefore, the sulphapyridine concentration is 1/25000 (i.e. 200 micrograms in 5.cc.)

Active and Inactive forms of P-aminobenzoic acid.

Mc-eod (1940) noted in his search for sulphonamide-antagonists in various tissues, that much of the antagonistic

substance is present in some bound, inactive form, since its amount apparently increased greatly when autolysis of the tissue was allowed to take place. He also found that some tissues, for example fresh liver and kidney, were free of active substance, although this became demonstrable after autolysis of these organs had taken place. Similar results were obtained if acid hydrolysis of the tissue was first performed. The "bound" sulphonamide antagonist was, therefore, split by such procedures. It should be noted that McLeod also found that, whereas normal human urine contained little or no active antagonist, this was uniformly present after acid hydrolysis. I have been unable to confirm this observation despite numerous assays of normal urine in the course of the experiments reported. Indeed, it is difficult to agree with McLeod's observation, since the technique he adopted is certainly not sensitive enough to react to the presence of 0.02 micrograms of p-aminobenzoic acid in the urine, which is the upper limit of the range found by the more elaborate and very sensitive micro-biological techniques described above. When the urine was concentrated 100 times, as described in experiment 7 , p-aminobenzoic acid in the conjugated form was found to be present in an amount similar to that found by other investigators. (Landy and Dicken (1942), Lewis (1942), Thompson, Isbell and Mitchell (1943). With the development of Wood and Fildes' essential metabolite theory and the correlation of p-aminobenzoic acid with sulphonamide antagonism, the probability that the bound, inactive form of sulphonamide-antagonist was an acetylated derivative of p-aminobenzoic acid was shown by various workers. It has been

known for a long time that metabolic acetylation of amino-groups, probably by the liver, is a common "detoxication" reaction carried out by the body, the acetylated derivative being then excreted in the urine.

One must, however, consider the possibility that p-aminobenzoic acid could be excreted in the urine combined in other ways. On theoretical grounds, in addition to the acetylated derivative, p-acetyl-aminobenzoic acid, it could be excreted, conjugated either with glycuronic acid as p-aminobenzoyl glycuronate or with glycine as p-amino-benzoyl glycine or p-amino hippuric acid. Harrow, Mazur and Sherwin (1933) fed relatively large quantities of p-aminobenzoic acid to a rabbit (1-2 gms) and by extraction of the urine were able to recover sufficient material to show that about 25% of the p-aminobenzoic acid fed was excreted by the rabbit in the acetylated form. They also found that some of the p-aminobenzoic acid was excreted as the glycuronate, since after removing the acetylated form with ether, the residue gave a very striking colour test with naphthoresorcinol. Quick (1932) had already drawn attention to the fact that nearly all workers had either underestimated or entirely ignored the possibility that aromatic acids may be combined with glycuronic acid. The glycuronic acid union with an aromatic acid is comparatively labile and unless special precautions are taken it is readily hydrolyced. No reports have appeared in the literature, to my knowledge, concerning the possible conjugation of p-aminobenzoic acid with glycine, although the excretion of p-amino hippuric acid and p-acetyl-aminohippuric acid has been shown by Finkelstein, Aliminosa

and Smith (1941) to be very rapid.

As far as acetylation is concerned, many other studies have appeared during the last 30 years in addition to those by Sherwin and co-workers, investigating this phenomenon. Many of these studies have been published in German journals. Ansbacher (1944, p.226) has reviewed this field and concluded that the major part of the acetyl groups employed for detoxication of p-aminobenzoic acid seemed to be obtained from metabolic processes, combined with phosphate as acetylphosphate. When acetic acid itself was fed, very little or none was used for acetylating purposes.

Strauss, Lowell and Finland (1941) found that following ingestion of 1 - 4 g. of p aminobenzoic acid by human subjects, the conjugation began early and progressed rapidly. Urinary excretion was rapid and largely completed in 12hours, about 2/3 of the drug being excreted in the "free" form. Other workers have found that the proportion of free or active p-aminobenzoic acid was very much less. The discrepancy may lie in the fact that Strauss, Lowell and Finland (1941) fed massive doses which probably exceeded the ability of the organism to conjugate it. When minute amounts are fed, which are more in keeping with the possible vitamin character of p aminobenzoic acid, the experiments quoted below indicate that p-aminobenzoic acid could only be recovered in the conjugated form. Lewis (1942) reported that in the case of urine the percentage of inactive p-aminobenzoic acid may be as high as 96%.

Methods of Converting the Inactive to the Active Form.

Before hydrolysis, the inactive form of p-aminobenzoic

acid in the urine neither supports growth of the micro-organism used nor is it diazotizable, indicating that the primary amine group is not free. Acid or alkaline hydrolysis has been employed by various workers to convert the inactive to the active form. The method used in this thesis was modified from that of McLeod (1940). In preliminary experiments, McLeod's technique was followed and sufficient HCl was added to the solution to be hydrolysed so that the final concentration of HCl was 0.2 N HCl. (This meant the addition of 1 drop concentrated HCl to 10 c.c. solution or urine). The solution was then heated in a boiling water-bath for 45 minutes, neutralised with 40% NaOH, and then sterilised by heating in boiling water-bath for another 30 minutes. However, it soon became obvious that further quantities of p-aminobenzoic acid could be liberated by making the solution more acid, this finally becoming maximal when the hydrolysis was carried out at a pH of 0.5-1. The modified technique then employed for all experiments was as follows: To 10 c.c. of solution or urine enough concentrated HCl was added to bring the pH to 1 or less. Usually 5-6 drops of acid were sufficient. It was then heated in a boiling water-bath for 60 minutes, cooled, neutralised carefully with 40% NaOH to a pH of approximately 7 (4-5 drops usually required) and the neutralised solution then sterilised by heating in a boiling water-bath for 30 minutes. The volume of acid and alkali added introduces an error of approximately 5-10%, for which no correction was made, since the method of assay itself has certainly a greater error. While these experiments were in progress, other methods of hydrolysis were reported.

Thus Lewis (1942, used alkaline hydrolysis, McIlwain (1942) found that the optimal conditions for liberating the bound acid was autoclaving the solution at 120° for 20 minutes, using 0.1 ml. of concentrated HCl for every 1 gm. of solution or solid material, and Thompson, Isbell and Mitchell (1943, reported that hydrolysis with 6N sulphuric acid was required to produce a maximal yield by their method. Their procedure was to add 5 ml. of 6N H_2SO_4 per g. of material, autoclave the mixture for 1 hour at 15 lbs. pressure and then nearly neutralise the sulphuric acid with barium carbonate. The precipitated barium sulphate and the undigested material they removed by filtration and adjusted the pH to neutrality with ammonium hydroxide. They also reported that during hydrolysis there was a partial destruction of pure p-aminobenzoic acid averaging ±15% by all methods. For uniformity in the results reported in this thesis, it was decided to retain the original method since the limits of sensitivity of the method are such that maximum hydrolysis appeared to have been reached as shown experimentally by the procedure used.

ACTIVE AND INACTIVE FORMS OF P-AMINOBENZOIC ACID EXCRETED IN NORMAL URINE.

It was thought of interest to determine in what form the minute amounts of p-aminobenzoic acid are excreted under normal conditions or when very small amounts are fed as may occur, for example, in consuming an ordinary diet. No direct observations have been carried out on this problem. Marrow, Mazur and Sherwin's (1933, observations were made after feeding large, almost toxic, doses and under "physiological conditions there is no certainty that p-aminobenzoic acid is excreted as the acetyl-derivative, although this has

been tacitly assumed by all workers. Since it is impracticable to extract the minute amounts present under normal conditions and since this would require very large amounts of urine and extraction solvents, it was thought that an indirect procedure based on the following considerations might throw light on the problem. If pure solutions, of the order of 1 microgram/c.c. of p-aminobenzoic acid and its derivatives, were subjected to the conditions of hydrolysis and assay procedure described above, that derivative which duplicates most closely the results obtained when urine is tested after administering p-aminobenzoic acid by mouth should be identical with or closely related to the form in which p-aminobenzoic acid is excreted under natural conditions. Therefore, several derivatives of p-aminobenzoic acid were tested as described below in experiment 2. On theoretical grounds, p-acetyl-aminobenzoic acid, p-amino-hippuric acid and p-amino-benzoyl glycuronate are the most likely derivatives excreted. The former two were synthesized, but the glycuronate could not be prepared since glycuronic acid could not be obtained, and the difficulties involved in synthesizing it were considered to be too great here. This compound could not, therefore, be tested, but Quick's (1932) observations indicate, however, that glycuronates are so unstable that they are hydrolysed on boiling the test solution even in neutral solution. Since neutralised samples of all the urines tested were always heated as controls when acidified samples were hydrolysed, and since no p-aminobenzoic acid was found in such specimens, the inference is drawn that very little, if any, of the

p-aminobenzoic acid is excreted as the glycuronate. On the other hand, it should be noted that the amount of "free" p-aminobenzoic acid found by other more sensitive methods — e.g. Lewis' (1942) method showed that 4. of the p-aminobenzoic acid excreted in the urine was in the "free" form — could actually be p-aminobenzoic acid "bound" as glycuronate, since autoclaving of the urine for sterilisation is, according to Quick, sufficient to hydrolyse the glycuronate.

It seems to me, therefore, that the possibility that <u>all the p-aminobenzoic acid excreted in the urine under normal conditions is actually "bound" or conjugated</u> is a very real one, and one which has been overlooked by all other workers in this field. This theory would explain a point that puzzled me from the outset of this work - namely, the reason for the fact that with the minute quantities of p-aminobenzoic acid excreted in a normal individual, part of it should have escaped the detoxication mechanisms of the body. My suggestion is that under physiological conditions all the p-aminobenzoic acid excreted is "bound", the so-called "free" p-aminobenzoic acid being p-aminobenzoic acid combined as p-aminobenzoyl glycuronate and the so-called "bound" p-aminobenzoic acid being combined as p-acetylaminobenzoic acid, since experiment 2 shows that p-acetylaminobenzoic acid behaves exactly like the form in which p-aminobenzoic acid is excreted naturally. The other derivatives of p-aminobenzoic acid tested in experiment 2 were readily obtainable since they are used as local anaesthetics, and have been included for comparative purposes. When large quantities are fed as by Harrow, Mazur and Sherwin (1933) it is probable that some

p-aminobenzoic acid is excreted actually as p-aminobenzoic acid. It is significant, however, that these workers were able to demonstrate that some of the p-aminobenzoic acid fed was actually excreted, nonjugated with glycuronic acid, so that there seems to be every reason that similar conditions hold for small quantities of p-aminobenzoic acid.

Under diseased conditions it becomes possible that even minute amounts of p-aminobenzoic acid may be excreted unchanged, and since this is most likely to occur in diseases of the liver, some cases of liver disease were investigated and reported in experiment 9. It will be seen that the liver damage must be extreme before the proportion of "free" p-aminobenzoic acid increases when 25 mgms. are fed. In such patients presumably the detoxicating power of the liver has become impaired. Incidentally, the value of applying this finding to the development of a liver function test is nil for practical purposes, since the presence of liver disease is obvious clinically when the liver can still conjugate the small amounts of p-aminobenzoic acid administered. The use of larger doses is not helpful since, firstly, these doses may be toxic and, secondly, when doses even of 100 mg. are given, "free" p-aminobenzoic acid is excreted by the normal individual.

Preparation of P-Aminobenzoic Acid.

Since only a few grammes of p-aminobenzoic acid were available, the preparation of sufficient quantities for these experiments was undertaken. Fortunately, esters of p-aminobenzoic acid, which are local anaesthetics, could be obtained and two of these have been hydrolysed - namely

procaine hydrochloride which was available at the outset of this work, and benzocaine which became available later. The preparation from benzocaine is much simpler, more rapid and gives a much higher yield of a purer product. Most of the p-aminobenzoic acid used in the experiments to be described was prepared from procaine and this method is, therefore, described first.

Preparation of P-Aminobenzoic Acid from Procaine Hydrochloride.

225 g. of procaine hydrochloride (novocaine hydrochloride, p-amino-benzoyl-diethyl-amino-ethanol)

$NH_2.C_6H_4.C.O.O.CH_2CH_2N(C_2H_5)_2.HCl.$

500 c.c. water.

1000 c.c. rectified spirits.

300 g. sodium hydroxide,

were mixed in a 4-litre flask. On addition of the NaOH a solid mass of procaine separated out. The mixture was boiled under reflux and the solid mass gradually redissolved, and the solution took on a yellowish-brown colour. The hydrolysis was considered complete when all the solid matter had redissolved and required 4 days. (The prolonged boiling in an alkaline solution probably caused considerable destruction of the p-aminobenzoic acid and would account for the relatively poor yield). The solution was then allowed to cool and the bulk of the ethyl alcohol distilled off. A heavy, oily liquid separated from the solution in a layer at the bottom. This layer, which was removed in a separating funnel, was probably largely the diethylamino-ethanol fraction. The remainder of the solution was neutralised with concentrated HCl, and when it was acid to litmus a yellowish-brown

precipitate separated out. This was filtered off on a Buchner with suction, the filtrate again acidified with a small quantity of acid, when a further precipitate separated out, and this was again filtered off. These procedures were continued until no further precipitate formed. The combined precipitates were washed three times with cold water, dried and then extracted with hot alcohol which dissolved the p-aminobenzoic acid, leaving solid NaCl which was filtered off. The filtrate was evaporated to dryness, the crude p-aminobenzoic acid redissolved in a dilute sodium bicarbonate solution and filtered off. The filtrate was then again acidified with concentrated HCl, and the precipitated p-aminobenzoic acid filtered off on a Buchner with suction and crystallised from hot alcohol. The product was finally re-crystallised from hot alcohol. Yield 45 gms. (Theoretical 113 gms.) M.P. (uncorrected) 184-185°C. A mixed melting-point with a small quantity of pure p-aminobenzoic acid available (M.P. 185-6°) was done. Mixed M.P. (uncorrected) 184°C. Hence considered that product was pure enough, although it was darker in colour than pure product available and product obtained by the hydrolysis of benzocaine described below, probably due to prolonged initial hydrolysis in alkaline solution.

Preparation of P-Aminobenzoic Acid from Benzocaine.

Quantities of

200 g. benzocaine (ethyl-p-amino-benzoate, $NH_2 . C_6H_4 . COOC_2H_5$,

1000 c.c. water.

1000 c.c. rectified spirits.

30 g. sodium hydroxide

were mixed in a 4-litre flask and boiled under reflux for 1-2 hours. The solution soon changes to a light yellow colour, hydrolysis being very rapid. The solution is then cooled and neutralised with concentrated HCl until just acid to litmus. This required 30-40 c.c. of conc. HCl. The alcohol was then distilled off under greatly reduced pressure until the volume was ± 1/3 of original. The boiling point was kept low so as to minimise destruction of the p-aminobenzoic acid. On cooling the concentrated solution, p-aminobenzoic acid crystallised out in long needle-like crystals which were filtered off on the Buchner funnel with suction, and then dried in the oven at about 70°C. The filtrate from this first crystallisation was brownish in colour and still contained by far the greater part of the p-aminobenzoic acid. This was precipitated out by making the solution progressively more acid, filtering off the solid separating out each time on the addition of a volume of 25-30 c.c. of concentrated HCl, and continuing thus to a final pH of 3-4 after which no more p-aminobenzoic acid separated out. The combined precipitates were dried in an oven at about 70°C. and the p-aminobenzoic acid separated from the solid NaCl, which had also partly precipitated out, by dissolving it in hot alcohol, and filtering. Impure p-aminobenzoic acid crystallised from the filtrate and was re-crystallised from a minimum volume of hot water leaving behind an oily residue, probably aniline. Yield 60-70%. Melting point (uncorrected) 185-6°C. Mixed M.P. with a known sample of pure p-aminobenzoic acid 185-186°C. (uncorrected). The

re-crystallised product was, therefore, pure p-aminobenzoic acid.

Experiment I.

Estimation of Sensitivity of Assay Method.

Varying quantities of a solution of pure p-aminobenzoic acid were added to 4 c.c. basal medium plus sulphapyridine, and volume made up to 5 c.c. with sterile saline. Final concentration of sulphapyridine 1/25,000. A suspension of E. coli., prepared as described above, added and solution incubated for 24 hours. Source of nitrogen in basal medium was asparagine.

Typical Experiments.

31.3.44. P-Aminobenzoic acid solution used 10micrograms/c.c.
All tests in duplicate.

After 24 hours Incubation, Controls. Basal medium alone - maximum growth.

Quantity of p-aminobenzoic acid added to Basal Medium + 1/25000 Sulphapyridine.	Growth after 24 hours.
0	Nil.
0.1 c.c. = 1 microgram	"
0.25c.c. = 2.5 "	Moderate.
0.5 c.c. = 5 "	"
0.75c.c. = 7.5 "	Maximal.
1 c.c. =10 "	"

17.10.44. P-aminobenzoic acid solution used 5 micrograms/cc.
All tests in duplicate.
Experimental details as before.

A - Source of nitrogen in basal medium was asparagine.

B - " " " " " " " ammonium phosphate.

Quantity of p-aminobenzoic acid added to Basal Medium + 1/25000 sulphapyridine.	Growth after 24 hours. A.	B.
0	Nil	Nil.
0.1 micrograms	Slight	Slight.
0.2 "	Slight	Maximal.
0.3 "	"	"
0.5 "	"	"
0.7 "	"	"
1.0 "	Maximal	"
1.5 "	"	"
2.5 "	"	"

Experiment 2. Behaviour of p-aminobenzoic acid and various of its derivatives when tested under the essay conditions used.

Test Medium: Basal medium + 1/25000 sulphapyridine.

Inoculum: As described above.

Method of Hydrolysis: As described above.

Incubated for 24 hours.

Substances tested: (1) P-aminobenzoic acid.

$NH_2.C_6H_4.COOH$. Mol.weight. 137.

✗ (2) p-acetylamino-benzoic acid.

$CH_3.CO.NH.C_6H_4.COOH$. Mol.weight.179.

✗ (3) p-amino-hippuric acid.

$NH_2C_6H_4.CO.NH.CH_2.COOH$. Mol.weight 194.

✗ (4) Benzocaine-ethyl p-amino-benzoate.

$NH_2.C_6H_4.COOC_2H_5$ Mol.weight 165.

x (5) p-aminobenzoyl-diethyl-amino-ethanol hydrochloride (Novocaine, Procaine). $NH_2.C_6H_4.CO.O.CH_2.CH_2.N(C_2H_5)_2.HCl.$ Mol. weight 272.5

x (6) 4-butyl-amino-benzoyl dimethyl-amino-ethanol hydrochloride (Decicaine, Antocaine) $C_4H_9.NH.C_6H_4.CO.O.CH_2.CH_2.N(CH_3)_2\ HCl.$ Mol. weight 300.5

Two sterile solutions of each of above substances (1) - (6) prepared so that their concentrations, estimated as p-aminobenzoic acid, were 5 micrograms/c.c. and 1 microgram/c.c. Varying quantities as below added to test medium (basal medium + 1/25000 sulphapyridine) and volumes made up to 5c.c.

Preparation of p-Acetyl-aminobenzoic acid.

3 g. of p-aminobenzoic acid was dissolved in a small volume of pyridine, 2 mols of acetic anhydride added and the mixture boiled gently under reflux for 10 minutes. It was then poured into a beaker of melting ice and acidified with conc. HCl until the solution was acid to litmus. By vigorous scratching of the sides of the beaker with a glass rod, a precipitate of p-acetylaminobenzoic acid separated out which was filtered off, and after washing with cold water, was re-dissolved in hot water, and a little charcoal added to decolorise the solution, which was then again filtered. After evaporating the filtrate on a steam bath until the p-acetylaminobenzoic acid began forming a layer on top of the solution, it was set aside to cool slowly. Long needle-like crystals of p-acetylaminobenzoic acid formed. These were filtered off on the suction pump, dried at 37°C. overnight, and the melting-point determined. Yield 1 gramme. M.P. (uncorrected) 252°C. (The M.P. given for p-acetylaminobenzoic acid by various authors is 250-252°C.)

x p-Amino-hippuric acid kindly supplied by Dr.L.Colberg of U.S.A. Institute of Medical Research.

Unhydrolysed Substance.	Growth on adding quantity in micrograms stated to test medium.								
	0.2	0.4	0.6	0.8	1	2	3	4	5
p-Amino-benzoic acid	Maximal.	Max.	Max.	Max.	Max.	Max.	Max.	Max.	Max.
p-Acetyl-aminobenzoic acid.	Nil.	Nil	Nil	Nil	Nil	Nil	Slight	Slight.	Nil Slight.
p-Amino-hippuric acid.	Nil	Nil	Slight	Slight	Slight	Nil	Nil	Nil	Slight.
Benzocaine	Slight	Slight	Slight	Nil	Moder-ate.	Mod.	Max.	Max.	Max.
Novocaine	Slight	Nil	Max.	Max.	Max.	Max.	Max.	Max.	Max.
Decicaine	Nil.	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil.

Hydrolysed Substance.	Growth on adding quantity in micrograms stated to test medium.															
	0.2		0.4		0.6		0.8		1.0		2		3		4	5
p-Amino benzoic acid	Max.		Max.		Max.		Max.		Max.		Max.		Max.		Max.	Max.
p-Acetyl-amino-benzoic acid.	Slight		Max.		Max.		Max.		Max.		Max.		Max.		Max.	Max.
p-Amino-hippuric acid.	Nil		Nil		Slight		Nil		Nil		Max.		Max.		Max.	Max.
Benzocaine	Moder-ate.		Max.		Max.		Max.		Max.		Max.		Max.		Max.	Max.
Novocaine	Slight		Max.		Max.		Max.		Max.		Max.		Max.		Max.	Max.
Pelcaine	Slight		Nil		Nil		Nil		Nil		Slight		Mod.		Nil	Nil.

SUMMARY.

Several chemical and microbiological methods have been described for the determination of p-aminobenzoic acid. A modification of McLeod's (1940) microbiological method has been employed in the experiments described in this thesis. The method of assay consists in comparing the amount of material which is required to neutralise the inhibitory action of sulphapyridine on Escherichia coli growing in a synthetic medium, with that of a standard solution of p-aminobenzoic acid under identical conditions. This micro-biological method was selected since it is more sensitive (0.2 micrograms/cc.) than the chemical methods and the reagents are readily obtainable. The objection that sulphonamide-antagonism need not be due only to p-aminobenzoic acid, does not hold when the excretion of the latter after ingestion is measured since a control estimation is always carried out before the p-aminobenzoic acid is fed.

The methods of converting the inactive forms of p-aminobenzoic acid in so far as sulphonamide-antagonism is concerned, to the active form are discussed, and the method used in these experiments is described. From a study of the behaviour of various derivatives of p-aminobenzoic acid under the assay conditions used, the finding of other workers is confirmed that the inactive form excreted in the urine, and constituting by far the greater part, is p-acetylaminobenzoic acid. The suggestion is, however, made that under physiological conditions all the p-aminobenzoic acid is conjugated and excreted either as p-acetylaminobenzoic acid or p-aminobenzoyl glycuronate.

CHAPTER III.

THE ABSORPTION AND EXCRETION OF P-AMINOBENZOIC ACID.

Review of Previous Studies on Absorption and Excretion of p-aminobenzoic acid.

Very few studies have appeared in the literature regarding the absorption and excretion of p-aminobenzoic acid, although several workers have noted that this is very rapidly completed when p-aminobenzoic acid is taken by mouth. No information regarding the factors controlling absorption of p-aminobenzoic acid from the gastro-intestinal tract is available, nor indeed do we know from which part the absorption actually occurs. Strauss, Lowell and Finland (1941) found that maximum blood levels are reached in 1-2 hours, and further that the excretion is rapid and is practically completed in 12 hours. Their experiments were carried out using relatively large quantities of p-aminobenzoic acid (1-4 grammes by mouth, and they determined the p-aminobenzoic acid content of the blood and urine by Bratton and Marshall's (1939, method for sulphonamides which, as has already been indicated, can be applied to p-aminobenzoic acid estimations. Conjugation of the p-aminobenzoic acid apparently begins early and progresses rapidly; 4 hours after doses of 1-2 grams the compound is no longer detected in the blood. After single 4-gram doses, small quantities are still present in the blood after six hours. Urinary excretion is rapid and is largely completed in 12 hours. Following absorption, this compound apparently enters the blood cells but

is not equally distributed between cells and extracellular fluid. The concentration of p-aminobenzoic acid in blood plasma is about three times as great as its concentration in red cells. Snyder (1942, p.60), in a review on liver physiology, makes the statement that the bile salts greatly facilitate the absorption of p-aminobenzoic acid from the intestine. The source of the statement is not, however, acknowledged.

Lustig, Goldfarb and Gerstl (1944) studied the problem by using p-aminobenzoic acid in which the nitrogen had been replaced by the isotope N^{15}, and found that no storage or utilisation of the labelled N^{15} of p-aminobenzoic acid was demonstrable. A male mouse weighing 16 g., depleted by placing it on a p-aminobenzoic acid-free diet for 8 - 12 weeks, received a single intraperitoneal injection of 5mg. of p-aminobenzoic acid containing the isotope N^{15}. The mouse was sacrificed 24 hours after the injection and the N and N^{15} content of all organs was determined. The p-aminobenzoic acid, if retained and uniformly distributed in the body of the mouse, would have corresponded to a concentration of 0.010% excess atom N^{15}. The determination of heavy nitrogen, however, revealed that the excess N^{15} values of all organs, except the kidney, were below 0.007%. The kidney showed traces of excess atom N^{15}(0.009%). To study the influence of larger quantities of p-aminobenzoic acid, a male mouse weighing 22 g. received three subcutaneous doses of 10 mg. within a 24-hour period. This amount of labelled p-aminobenzoic acid, if equally distributed in the organism, would have resulted in an excess atom N^{15}

concentration equivalent to 0.045%. Nineteen hours after the last injection, however, only traces were found in the organs, but 227 micrograms N^{15}, corresponding to 82% of the injected amount, were present in the excreta.

Several workers have reported that p-aminobenzoic acid is excreted in the urine partly in the free form and partly, as already discussed, conjugated as the acetyl derivative. Doisy and Westerfeld (1943) believe that acetyl phosphate, formed through a chain of reactions from pyruvate through acetoin and diacetyl, and not acetate, is the acetylating agent. Young (1939), in a review of the subject, stated that p-aminobenzoic acid is excreted unchanged by the dog, whereas it is converted to the acetylamino-derivative by man and the rabbit.

ABSORPTION AND EXCRETION OF P-AMINOBENZOIC ACID IN MAN AFTER FEEDING MINUTE DOSES.

The studies so far reported in the literature have concerned themselves with the absorption and excretion of p-aminobenzoic acid after relatively large doses have been fed. In view of the possible significance of p-aminobenzoic acid as a member of the vitamin B complex (which is more fully discussed in Chapter IV of this thesis), it was thought of interest to determine the excretion of this substance in man after minute doses are fed. From the reaction of the body to doses of p-aminobenzoic acid of the order likely to be involved if this substance was a vitamin, it was hoped to prove or disprove indirectly that p-aminobenzoic acid was a member of the vitamin B complex. This argument is developed later on p. 106, but here the disposal of small amounts of p-aminobenzoic acid, after being ingested by several

were attempted at the outset, but the technique employed and described in the previous chapter was found to be neither sufficiently sensitive nor accurate, and since such estimations do not provide more information than can be obtained from the urine, they were not continued.

Certain differences in these results to those already reported are easily apparent. Thus, Harrow, Mazur and Sherwin (1933) reported that only about 25% of 1 or 2 gm. of p-aminobenzoic acid fed to rabbits could be isolated from the urine in the acetylated form. Strauss, Lowell and Finland (1941) found that about 33% of doses of 1-4gm. fed to man was excreted in the conjugated form. They were also unable to detect p-aminobenzoic acid in the urine 12 hours after ingestion. Kirch and Bergeim (1943), after noting that whereas early reports in the literature [Gibbs and Hare (1889); Hildebrand (1903)] indicated that p-aminobenzoic acid passed through the animal organism unchanged, and that Ellinger and Hensel (1914) were the first to report the excretion of p-aminobenzoic acid partly conjugated as the acetyl derivative, the excretion continuing over a period of 1 - 4 days, interpret their experimental findings as indicating that some excretion of the acetyl derivative continues after 24 hours in human subjects, whereas no free p-aminobenzoic acid could be detected after 12 - 15 hours. Lewis (1942) found, using his microbiological procedure, that as much as 96% of the p-aminobenzoic acid in normal urine existed in an inactive form - probably the acetyl derivative.

Experiments 3, 4 and 5 indicate that when an amount of 100 mgms. of p-aminobenzoic acid is fed, approximately 70 - 75% of the p-aminobenzoic acid excreted is present as the acetyl derivative, whereas when amounts of 25 mgms. and less are fed, no p-aminobenzoic acid can be detected in the normal individual in the free form at all, apparently 100% of the p-aminobenzoic acid being acetylated. The evidence for acetylation being the method of "detoxicating" p-aminobenzoic acid has already been discussed in Chapter III. That the liver is the main site of the acetylation is accepted by all workers on metabolic detoxication reactions. It can, therefore, be said that the liver of the normal human organism is certainly able to deal adequately with p-aminobenzoic acid up to the amount of 25 mgms. The effect of disease of the liver was investigated (Experiment 9), from which it is apparent that when gross disease of the liver is obvious clinically, the individual is unable to detoxicate 25 mgms. of p-aminobenzoic acid and a slight amount of free p-aminobenzoic acid appears in the urine.

Experiments 3 - 6 also indicate that p-aminobenzoic acid is rapidly absorbed and excreted and that the excretion is apparently completed within 16 hours, within the limits of error of the experimental technique used.

The curious fact also emerges that even when a minute dose of 1 mg. is fed by mouth, p-aminobenzoic acid appears in the urine in the first 8-hour specimen collected after the dose is given, indicating that if the human

organism requires p-aminobenzoic acid, it is already saturated. This is shown in Experiment 6. However, the amount excreted is approximately proportional to the amount that could be recovered when the larger amounts were fed and may indicate that not more than approximately 50% of p-aminobenzoic acid fed is absorbed. The accuracy of the method used is not sufficiently great to investigate this point in greater detail. Some of the other investigators mentioned above claim to have recovered, by more refined techniques, all the p-aminobenzoic acid fed so that the poor recovery here may be due to experimental error. On the other hand, it is quite possible that when such minute quantities are fed the absorption may not be quantitative. Another explanation must also be considered - namely, that the tissues of the subjects tested were unsaturated with respect to p-aminobenzoic acid, so that a proportion of the p-aminobenzoic acid absorbed is retained. This alternative is considered unlikely for the reasons (a) that the amount recovered is approximately the same even when larger doses were fed, (b) continued doses of 1 mg. of p-aminobenzoic acid at 8-hourly intervals do not increase the amount excreted and (c) Experiment 7 shows, as reported by other investigators [Lewis (1942), Mitchell, Isbell and Thompson (1943), Landy and Dicken (1942)], that normal urine does contain minute amounts of p-aminobenzoic acid. To show such a quantity by the technique used, it was necessary to concentrate the urine 100-fold. Unfortunately, this procedure is too laborious to use as a routine.

It is, therefore, concluded that the human subjects

tested here are saturated with respect to p-aminobenzoic acid, if indeed this substance is a vitamin. However this may be, experiment 6 also shows that continued 1 mg. quantities at 8-hourly intervals does not increase the amount excreted, from which it appears permissible to deduce that all the p-aminobenzoic acid fed is absorbed rapidly from the intestine - certainly within 8 hours.

The fact that practically no foodstuff (see Table in Chapter V) has more than minute amounts of p-aminobenzoic acid, and that normally only minute traces (approximately 0.1 - 0.2 micrograms) are excreted in the urine throwe some doubt on whether p-aminobenzoic acid is a vitamin at all. Further, the reaction of the human organism to p-aminobenzoic acid, as shown in experiments 3 - 6, appears to me to be more compatible with p-aminobenzoic acid belonging to the group of "toxic" substances rather than to the group of vitamins. Since this view would be strengthened if it could be shown that the minute amounts of p-aminobenzoic acid excreted normally in the urine as the conjugated form were not derived from the diet taken, but were formed by the bacteria in the large intestine and absorbed — this being feasible since firstly, the quantity normally excreted is of the order of bacterial synthesis and, secondly, as already indicated (p.48) p-aminobenzoic acid can be synthesized by E. coli, for which it is an essential metabolite — these points were investigated. Experiment 8 shows that p-aminobenzoic acid can be absorbed from the large intestine, although the amount absorbed is much less than from the small intestine,

and appears to be continued over a longer period. It must be noted, however, that experiment 8 actually demonstrates the absorption of p-aminobenzoic acid from the rectum and perhaps the pelvic colon, which parts of the large intestine have probably a much smaller absorbing power than have the ascending, transverse and descending colons. If p-aminobenzoic acid was introduced into the upper reaches of the large bowel, it would probably be found that a much larger percentage absorption of p-aminobenzoic acid would be obtained. The bacterial flora of the large intestine is, of course, very numerous throughout, the large intestine. Experiment 8 establishes, however, that p-aminobenzoic acid can be absorbed through the large intestine and, therefore, lends support to the hypothesis that the p-aminobenzoic acid excreted in the urine is actually formed by bacterial action in the large intestine and, when absorbed, is treated by the body as a "toxic" substance, acetylated by the liver and excreted. Experiment 10 (reported in Chapter V) provides experimental confirmation of this hypothesis.

Experiment 3. Excretion of p-Aminobenzoic acid after
9.5.44. ingestion of 100 mgms. which was taken at 8 a.m. after emptying the bladder. All the urine passed for following 24 hours was collected at 4-hourly intervals until 12 midnight, and the final specimen at 8 a.m. the following morning. The volume of each specimen was noted and its p-aminobenzoic acid content estimated, from which the total quantity of p-aminobenzoic acid excreted was calculated.

	Subject E.M.B.			Subject J.A.M.		
	p-Aminobenzoic acid excreted in milligrams.			p-Aminobenzoic acid excreted in milligrams.		
	Free	Acetylated.	Total.	Free.	Acetylated.	Total.
Before administering 100 mgms.	Nil	Nil	Nil	Nil	Nil	Nil
After 4 hours	10.5	10.5	21	5.5	11.0	16.5
After 8 hours	12.3	19.5	31.8	11.0	16.5	27.5
After 12 hours	-	-	-	12.8	25.3	38.1
After 16 hours	13.7	33	46.7	12.8	26.9	39.7
After 24 hours	13.7	35.8	49.5	12.8	29.2	42

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Experiment 4. Excretion of p-aminobenzoic acid after ingestion of 25 mgms.

Experimental details as in previous experiment.

Subject.	p-Amino benzoic acid excretion before feeding (Control		p-Aminobenzoic acid excreted in mgms. after:-									
			4 hrs.		8 hrs.		12 hrs.		16 hrs.		24 hrs.	
	Free	Acetylated.	Free	Acetylated.	Free.	Acetylated.	Free	Acetylated.	Free	Acetylated.	Free	Acetylated.
J.H.	Nil	Nil	-	-	Nil	12	-	-	Nil	12.18	Nil	12.18
L.B.	Nil	Nil	-	-	Nil	9.4	-	-	Nil	9.4	Nil	9.4
S.B.	Nil	Nil	Nil	6.8	Nil	10.8	Nil	11.4	Nil	11.5	Nil	11.5
J.C.	Nil	Nil	Nil	9.4	Nil	9.5	Nil	9.9	Nil	9.9	Nil	9.9
P.B.	Nil	Nil	Nil	9.5	Nil	11.4	Nil	11.5	Nil	11.5	Nil	11.5
M.L.	Nil	Nil	Nil	12.3	Nil	12.8	Nil	13.0	Nil	13.0	Nil	13.0
B.R.	Nil	Nil	Nil	1011	Nil	13.0	Nil	13.3	Nil	13.6	Nil	13.6
J.M.	Nil	Nil	Nil	12.5	Nil	14.0	Nil	14.3	Nil	14.4	Nil	14.4

6.10.44.

Experiment 5. Excretion of p-aminobenzoic acid after ingestion of 10 mgms.

Experimental details as in previous experiments.

Subject	p-Amino benzoic acid excretion before feeding (Control).		p-Aminobenzoic acid excreted after:- in mgms.									
			4 hrs.		8hrs.		12 hrs.		16 hrs.		24 hrs.	
	Free	Acetylated.	Free	Acetylated.	Free	Acetylated.	Free	Acetylated.	Free	Acetylated.	Free	Acetylated.
B.B.	Nil	Nil	-	-	Nil	4.7	-	-	Nil	4.9	Nil	4.9
B.R.	Nil	Nil	-	-	Nil	3.6	-	-	Nil	3.2	Nil	3.2.
J.M.	Nil	Nil	-	-	Nil	3.3	-	-	Nil	3.4	Nil	3.4.

16.10.44. Experiment 6. Excretion of p-aminobenzoic acid after ingestion of 1 mg. doses at 8-hourly intervals for 4 days (96 hours).

Urines tested at 8-hourly intervals for 24 hours preceding first dose and for 40 hours after last dose.
First dose taken at 24th hour
Last " " 120th "

	Subjects: B.B.		J.M.		B.R.	
	Free	Acet.	Free.	Acet.	Free.	Acet.
p-Aminobenzoic acid excreted in mgms.						
From 0-8 hours	Nil	Nil	Nil	Nil	Nil	Nil
8-16 "	"	"	"	"	"	"
16-24 "	"	"	"	"	"	"
24-32 "	"	0.37	"	0.23	"	0.18
32-40 "	"	0.35	"	0.19	"	0.08
40-48 "	"	0.26	"	0.29	"	0.41
48-56 "	"	0.47	"	0.53	"	0.22
56-64 "	"	0.19	"	0.32	"	0.05
64-72 "	"	0.17	"	0.15	"	0.43
72-80 "	"	0.50	"	0.26	"	0.19
80-88 "	"	0.15	"	0.17	"	0.26
88-96 "	"	0.16	"	0.08	"	0.34
96-104 "	"	0.37	"	0.34	"	0.34
104-112 "	"	0.15	"	0.25	"	0.01
112-120 "	"	0.22	"	0.20	"	0.30
120-128 "	"	0.18	"	0.13	"	0.20
128-136 "	"	Trace less than 0.05	"	Nil	"	Nil
136-144 "	"	Nil	"	"	"	"
144-152 "	"	"	"	"	"	"
152-160 "	"	"	"	"	"	"

3.11.44.

Experiment 7. The p-Aminobenzoic acid content of normal urine.

24-hour specimen of urine (B.B.) volume 1130 c.c. concentrated down on a steam-bath to the consistency of a thick syrup with separated-out solid matter. The residue was made acid to a pH of 3 and the p-aminobenzoic acid extracted with an approximately equal volume of ether (about 125 c.c.) 5 times. The ether extract is evaporated to dryness and the p-aminobenzoic acid in the residue taken up in 10 c.c. of ½% $NaHCO_3$ and filtered. The pH was then adjusted to 7 and the volume of the filtrate made up to 11.3 c.c., thus concentrating the urine 100 times. The content of p-aminobenzoic acid was then determined as in the previous experiments.

Free p-aminobenzoic acid was not detectable in 1 c.c. of the extract, whereas the smallest volume of extract in which conjugated p-aminobenzoic acid was found was 0.1 c.c.

Since 0.2 micrograms of p-aminobenzoic acid was required to allow growth of E. coli under the conditions of this experiment, it follows that there was less than 0.2 micrograms free p-aminobenzoic acid present in 1 c.c. of extract or in 100 c.c. of urine, i.e. less than 0.002 micrograms free p-aminobenzoic acid per c.c. of urine, and that there was 0.2 micrograms of p-acetylaminobenzoic acid present in 0.1 c.c. of extract = 100c.c. of urine, i.e. 0.002 micrograms of p-acetylaminobenzoic acid per c.c. of urine.

17.7.45.

Experiment 8. Absorption of p-aminobenzoic acid from the large intestine.

25 mg. of p-aminobenzoic acid was dissolved in 100 c.c. of saline and injected intra-rectally with a syringe and enema tube and retained for 8 hours. Urine specimens collected before, and at 8-hourly intervals after, the administration of p-aminobenzoic acid and the p-aminobenzoic acid excretion estimated as already detailed.

	Subject B.B.		Subject B.R.	
	Free	Aceby.	Free.	Acet.
Mgms.p-aminobenzoic acid excreted in urine before p-aminobenzoic acid administered.	Nil	Nil	Nil	Nil
Mgms.p-aminobenzoic acid excreted in urine after:-				
8 hours	"	0.86	"	2.30
16 "	"	1.33	"	2.98
24 "	"	1.41	"	3.06

July, 1944.

Experiment 9. Excretion of p-aminobenzoic acid by African patients with liver disease after oral ingestion of 25 mgms. p-aminobenzoic acid.

Patient.	Diagnosis.	Quantity of p-aminobenzoic acid in urine before p-aminobenzoic acid fed (Control)		p-aminobenzoic acid excreted in mgms. after:									
				4 hours.		8 hours.		12 hours.		16 hours.		24 hours	
		Free.	Acet.	Free.	Acet.	Free.	Acet.	Free.	Acet.	Free.	Acet.	Free.	Acet
S.	Carcinoma of liver (proved by Biopsy).	Nil.	Nil	Nil	7.90	0.18	9.34	0.29	10.19	0.40	10.67	0.40	10.67
Al.	? Generalised Miliary T.B. + T.B. of liver. ? Carcinoma of liver.	Nil	Nil	0.02	6.80	0.03	10.20	0.05	12.70	0.07	12.80	0.07	12.85
Ab.	Jaundice. ? Infective Hepatitis.	Nil	Nil	0.01	4.20	0.03	11.10	0.03	11.13	0.03	11.13	0.03	11.13

SUMMARY.

The studies previously reported in the literature have concerned themselves with the absorption and excretion of p-aminobenzoic acid after relatively large doses have been fed. In view of the possible significance of p-aminobenzoic acid as a member of the vitamin B complex, the absorption and excretion of this substance in man after feeding minute doses were studied. When 100 mg. was fed, approximately 25-30% of the amount excreted was present in the "free" form, whereas when 25 mg. or less were fed, all the p-aminobenzoic acid excreted was acetylated, indicating that the liver of the normal human organism is able to"detoxicate" or "conjugate" p-aminobenzoic acid up to an amount of 25 mg. The experiments also indicate that p-aminobenzoic acid is rapidly absorbed — within 8 hours, although only to the extent of about 50%, and that the excretion is completed within 16 hours or less.

A curious fact emerging from these experiments is that even when such a minute dose as 1 mg. was fed by mouth, p-aminobenzoic acid appeared in the urine in the first 8-hour specimen collected. This could be interpreted as indicating that the subjects tested must have already been saturated with p-aminobenzoic acid if indeed it is a vitamin at all. It is, however, claimed that these results indicate rather that the human organism treats p-aminobenzoic acid as it does other toxic aniline derivatives, and not as if it is a vitamin. This view is strengthened by the finding that p-aminobenzoic acid can be absorbed from the large intestine, which indicates that the minute quantity of p-aminobenzoic acid excreted normally in the urine could be formed by bacterial synthesis in the large intestine from which, when it happens to be absorbed, it is

immediately "detoxicated" by the liver and excreted.

This hypothesis is further developed in chapter V.

CHAPTER IV.

IS P-AMINOBENZOIC ACID A VITAMIN?
DEFINITION OF THE TERM "VITAMIN".

Before we can profitably discuss the claims of p-aminobenzoic acid to be a vitamin and, in fact, one of the components of the vitamin B complex, it is essential to define what we mean by the term "vitamin". Immediately we come upon considerable difficulties. In the early days of vitamin research, the implications of the term "vitamin" were fairly clear and the division of foodstuffs into (a. the energy-producing, tissue-building materials, and (b. the accessory food factors which were indispensable to growth, maintenance and reproduction of the human and animal world, was generally acceptable. The latter group of substances was further defined as being compounds which were effectivein minute amounts, provided no energy to the body, acted as organic catalysts, and, the most important point of all, could not be synthesized in the animal body, but had to be provided in the food.

Hopkins called the nutritional elements, which are required by the animal organism in addition to proteins, fats, carbohydrates, minerals and water, the "accessory food factors". Funk in 1911 proposed the generic term "vitamine" for these substances because they were essential to life and because he believed the anti-beriberi

factor to be an amine. Finally in 1920, when it was shown that these substances were not amines, Drummond proposed that the terminal "e" of "vitamine" be dropped and that this group of substances be referred to by the generic term "vitamin". This suggestion was generally adopted by workers in this field and is still accepted and in current use, although considerable discussion has now arisen as to whether this generic term should be retained or changed to a term which would not indicate any relationship to the chemical or physiological properties of these compounds, since it is becoming more and more difficult to separate the vitamins into a distinct, well-defined group which does not overlap with other groups.

Recent intensive research in this field has revealed the existence of compounds which have been arbitrarily added to the list of known vitamins, but which do not fulfil in some respect or other what is generally accepted as characteristic of this class of substances. As an example may be quoted such substances as choline and related compounds which supply the essential transferable methyl group, behave very similarly and have similar functions to the vitamins, but which can be synthesized in the animal body. It has also now been demonstrated that known and accepted vitamins can be synthesized in the animal body. Thus, parts of the vitamin B complex and vitamin K can be synthesized by intestinal bacteria, and even vitamin C can be synthesized by rats, which do not develop

scurvy on an ascorbic acid-deficient diet. If then choline is excluded from the group of vitamins, why should some members of the vitamin B complex, vitamin K and vitamin C be retained? Other compounds have also been arbitrarily excluded from the group of vitamins although they conform to the general definition given above. Such compounds are the essential amino acids and essential unsaturated fatty acids, which are constituents of enzyme systems involved in the metabolism of energy-bearing foods. These have apparently been excluded since our earlier knowledge of these substances showed them to be energy-producing and structure-building substances, in which respects they obviously differed from vitamins. ~~[illegible] other respects they behaved exactly like vitamins.~~ Rosenberg (1942, pp. 5-6) proposed a new name for such substances - "<u>vitagens</u>" which he defined as a broad group to include compounds which have a similarity to vitamins, but which differ in one or more important functional aspects from them. The term "vitagen" emphasises that the compounds of this class are concerned with the production and maintenance of life. Rosenberg recommends that this terminology be adopted until the time when more precise information is available concerning the physiological action of these compounds and the vitamins. It appears to me that, beyond acknowledging the fact that the group of "vitamins" is not capable of accurate definition, this term has no real value. In fact, it appears more suitable to transfer most of the accepted "vitamins" from their own group and

place them in the group of "vitagens".

Let us now examine some of the suggested recent definitions of the term "vitamin". It should be noted in passing that most textbooks and treatises dealing with vitamins, content themselves with a brief historical introduction and discreetly evade any attempt at a definition. I would humbly suggest that no conference of experts and workers in this field of nutrition could reach unanimity on this question at all, and it is probable that the use of the term "vitamin" will sooner or later have to be abandoned and the various members systematised according to their chemical structures and their physiological effects. Some of them, for example, belong more properly among intracellular enzyme systems and, when these are classified and grouped among such compounds, the blurred distinctions between vitamins, essential amino-acids, fatty acids and choline would be removed.

Stepp, Kuhnau and Schroeder (1938), in their introduction, refer to vitamins as components in very small quantities of the plant body sharing in the regulation of the processes of the plant. They can, therefore, also be considered as hormones of the plant world. After they have been taken up by the animal body in the food, they carry out important tasks, partly in regard to vital phenomena in the cell itself, and partly in regulating remote processes. From the standpoint of quantity, they can hardly be considered as energy producers. In general, since the animal is not able to synthesize them, it is

obliged to ingest the vitamins in its diet - directly in vegetable food and indirectly in animal nutriments - either already synthesized or as provitamins (from which the specific substance or vitamin is produced, by radiant energy or by catalytic co-operation of other diet factors). The vitamins must be grouped among the primitive substances of all organic life, since they are found in the lowest forms of life (bacteria, algae and fungi). They play a decisive role in the animal world, even among the organisms possessing neither hormonal glands nor hormones. In higher animals, where internal secretion first appears as a physiological arrangement, there are at once close reciprocations established with the vitamins.

Rosenberg (1942,pp.3-7) defines vitamins as organic compounds which are required for the normal growth and maintenance of life of animals, including man, which as a rule are unable to synthesize these compounds by anabolic processes that are independent of environment other than air, and which compounds are effective in small amounts, do not furnish energy and are not utilised as building units for the structure of the organism, but are essential for the transformation of energy and for the regulation of the metabolism of structural units. Rosenberg classifies as hormones those compounds which are produced anabolically, but which otherwise conform to his definition of vitamins. He claims that the above definition clearly differentiates this group of nutrients from all other food constituents. He dismisses the fact that

the apparent independence of cattle for certain members of the vitamin B complex and for vitamin K (which is due to bacterial synthesis of these vitamins in the rumen) requires that these members of the vitamin B complex and vitamin K should be excluded from the group of vitamins by definition, arguing that bacterial synthesis in the gut is not an anabolic process. The fact that one of the vitamins D can be produced in the skin by an anabolic process is also glossed over since this anabolic process is not independent of the environment but requires energy from the outside - namely ultraviolet light. Further, as long as at least one animal species is known to be unable to synthesize a particular compound, that compound, according to Rosenberg, should be considered a vitamin provided it conforms with the definition for vitamins in other particulars. Rosenberg, however, admits that the definition of vitamins, as given above, can and has been disputed. The most severe criticism according to him has arisen from the fact that the ingested essential nutrients exert no vitamin activity as such, but are active only after chemical transformation into other compounds. According to these views, the ingested compounds should be called "provitamins" unless it is established that they do not undergo transformation in the body. On this basis, compounds like nicotinamide and riboflavin which are constituents of a number of different enzymes would require that a multitude of different vitamins has to be recognised. Since Rosenberg holds that the term

"vitamine" is required for the science of nutrition which needs some name for this group, he proposes the term "vitazyme" for enzymes which contain vitamine. As has already been mentioned, another term "vitagens" also becomes necessary, according to Rosenberg, to describe compounds which act as suppliers of energy or as structural building units, but fulfil all the other qualifications of the vitamins. It seems to me that it would be more logical to dispense with the term "vitamins" altogether in scientific works describing or evaluating the significance of new compounds in nutrition and metabolism, since the rapid increase of knowledge in this field has led to insuperable difficulties in deciding what criteria a compound must satisfy to qualify as a vitamin.

To sub-divide the constituents of a diet by their chemical structure, discussing for each, thereafter, whether it provides energy, whether it is required for development and growth of body cells and tissues, whether it is incorporated into enzyme systems and so forth, would be more logical.

One cannot help feeling, when studying Rosenberg's elaborate definition, that he has first decided which substances should be included in the definition, these being the substances already called vitamins, and then has been at great pains to mould his definition so that it would include all of them, even though this has meant introducing such fine hair-splitting distinctions as to

make one wonder why all this should be necessary at all. Having made his definition, Rosenberg finds that he must exclude certain compounds which otherwise would be included on the general grounds of what one understands by a vitamin, and hastens to introduce new terms, vitagens and vitazymes. Further snags also come easily to mind. Some of these are discussed below, namely what distinctions, if any, must we draw between vitamins and the "essential metabolites" and "growth factors" of bacteria, other than the fact that vitamins refer to animals and the latter two to lower forms of life, in fact to vegetable life?

Although the term "vitamin" has become incapable of exact definition and, moreover, has lost most of its significance with the development of our knowledge of cellular physiology, it still has considerable value in popular discussion. The term "vitamin" should therefore be retained in such descriptions since it still serves some purpose, and since the impossibility of exact definition is not here of great importance.

Other illustrations of the difficulties into which our retention of the term "vitamin" has led us can be found in remarks made by various writers and reviewers on the significance of this term. György (1942, defined vitamins as food constituents of organic origin, minute quantities of which have specific biological effects and lack of which in the food produces pathological disturbances, occasionally only under special conditions. He

remarked then that in the light of recent research, two objections can easily be raised to this definition: (a. Should choline, because it is used not in "minute quantities" but in relatively high doses, be excluded from the group of water-soluble vitamins and should it, in consequence, be called only a "dietary essential"? If the answer is in the affirmative, why should ascorbic acid, pantothenic acid and nicotinic acid, all of which are also administered in rather large doses, be called vitamins, and what is the objective reason for the erection of such an artificial barrier? And if choline should be considered a vitamin - a view with which György agrees although choline can be anabolised in the animal body - why, then, should methionine, which supplies methyl-groups to choline and which, therefore, has choline-like properties, be excluded from the group of vitamins? In further consequence there would then be raised the problem of classifying essential amino-acids as close relatives of vitamins. (b) In the light of recent studies, it is unnecessary to make the classification of a vitamin depend on its character as a food constituent. Some vitamins, such as various members of the vitamin B complex, can be synthesized in the rumen of certain animals by bacteria. Moreover, it appears very probable that biotin, at least in mammals, is supplied mainly by the activity of the intestinal bacteria. The same process might apply to "folic acid". The presence of these factors in the ingested food would not necessarily then

be a prerequisite for adequate nutrition.

Williams (1943) believed that it was not possible to delimit the meaning of the term "vitamin" without taking into consideration the historical fact that at one time the nutritional requirements of animals were thought to be satisfied by minerals, carbohydrates, lipides and proteins alone. At the same time certain "building stones" entering into the make-up of fats and proteins (e.g. fatty acids and amino acids) were known to be utilised by the animal body in the synthesis of these major tissue constituents. Vitamins, historically, were something new and different. There has been a resistance to the classification of nutritionally important fatty acids (linoleic, linolenic acids) as vitamins (vitamin F) because they are too closely related to fats which had long been recognised as important food constituents. For the same reason, there is resistance to including new amino-acids or choline in the vitamin group. Their relationship to food constituents recognised long ago is too close. On the other hand, there has never been any resistance towards the inclusion of nicotinic acid among the vitamins. While it has been known as a compound, and even as an occasional constituent of tissues for several decades, it constitutes something new, historically, on the nutritional scene. Inositol and p-aminobenzoic acid, according to Williams, belong with nicotinic acid among the vitamins because they, too, are

not directly related to the classical groups of foodstuffs.

RELATIONSHIP OF "ESSENTIAL METABOLITES" AND "GROWTH FACTORS" TO "VITAMINS".

It is necessary to examine the relationship of the terms "essential metabolite" and "growth factor" to that of "vitamin", since p-aminobenzoic acid first came into prominence as an "essential metabolite". Fildes (1940) introduced the terms "essential metabolite" and "growth factors" and his views on the method of growth and multiplication of bacteria have already been described. (p.1,. He considered that what applied to bacteria, applied also to all cells. Briefly, Fildes defined an "essential metabolite" as a substance or chemical group which took an essential part in a chain of syntheses necessary for bacterial growth. A "growth factor" is an essential metabolite which the cell cannot synthesize and which must, therefore, be supplied in the nutrients. The literature identifying p-aminobenzoic acid as an "essential metabolite" and in some organisms as a "growth factor" has already been reviewed. Fundamentally, the terms "growth factor" which is applied to bacterial nutrition and "vitamin" which is applied to animal nutrition appear, therefore, to be analogous. On Rosenberg's definition, the terms "essential metabolite" and "vitagen" are also analogous, so that it may be possible in the animal world as in bacteria that the same substance may in some cases be a vitamin, and in others a vitagen.

Ansbacher (1941) was the first to claim that p-aminobenzoic acid was a vitamin and a member of the vitamin B complex, which he defined (Ansbacher 1944) as (a) a natural constituent of yeast, liver and/or cereals; (b) water-soluble; (c) a growth-promoting substance for bacteria, yeasts, fungi, and/or moulds; (d) a co-enzyme or activator of enzymatic processes; (e) physiologically effective in minute amounts; (f) a substance which causes a deficiency disease when lacking in the diet. He reported experiments showing that p-aminobenzoic acid was a chromotrichia factor for the rat and a growth-promoting factor for the chick. He fed black and piebald rats on a basal diet consisting of cerelose, casein, salts, agar, soybean oil, crisco and cod liver oil, to which was added thiamine hydrochloride, riboflavin and pyridoxine hydrochloride, calcium pantothenate, nicotinic acid, inositol and choline chloride. On this diet, the fur of the animals showed definite graying, and when this had become apparent, 70% of the animals received a second daily supplement containing p-aminobenzoic acid. A bluish discoloration of the skin, a typical first sign of growth of normally pigmented hair, was seen in from two to three weeks and black hair appeared within a month. The control animals continued to show typical achromotrichia. Chicks reared on a heated vitamin-K deficient diet, previously described by Ansbacher, were found to show only a small gain in weight and to die within about a month, even when ample amounts of calcium pantothenate and of the vitamin K-active 2-methyl-1,

4-naphthoquinone were fed, but when p-aminobenzoic acid was added to the ration, better growth resulted and the survival times were longer.

In the following sections, Ansbacher's claim is reviewed, and it is concluded on the basis of available evidence that p-aminobenzoic acid's place among the vitamins appears to rest on very insecure foundations.

p-Aminobenzoic Acid and Achromotrichia.

The relation of the vitamin B complex to graying of the hair in experimental animals goes back several years. Among the earliest reports in this field was one by Morgan, Cook and Davidson (1938), indicating that experimental achromotrichia was due to a deficiency in a factor or group of factors belonging to the vitamin B complex. Although several factors have since been incriminated, it is intended here to review only those reports on the relationship of p-aminobenzoic acid to achromotrichia. Following Ansbacher's announcement (1941), Martin and Ansbacher (1941) studied the effect of p-aminobenzoic acid on the graying of the fur of mice produced by hydroquinone. Most of the animals showed achromotrichia within 4-20 weeks which could be cured either by feeding a rice polish concentrate as a daily supplement or p-aminobenzoic acid (0.75 mg. daily). After about a week of the p-aminobenzoic acid treatment, some of the animals showed definite signs of blackening of the fur, and at the end of two weeks all the mice were cured. In fact, their fur appeared to be more deeply pigmented than that of the control animals on the stock diet without the addition of hydroquinone. The authors concluded that the

achromotrichia produced by hydroquinone poisoning was due to a vitamin deficiency and that p-aminobenzoic acid was the chromotrichia factor. In the same year Martin, Wisansky and Ansbacher (1941) reported a study on the influence of p-aminobenzoic acid and other substances on reactions yielding melanin, namely in tyrosine-tyrosinase, dopa-tyrosinase and catechol-tyrosinase systems, using potato tyrosinase as catalyst. The data were interpreted as showing conclusively that under identical experimental conditions, calcium pantothenate has no influence and that p-aminobenzoic acid and, interestingly enough, sulphanilamide were effective in modifying melanin formation. Thus when p-aminobenzoic acid was added, the intermediate red stage seen with tyrosine-tyrosinase systems did not develop and the black melanin precipitate was not formed, but instead a brownish reaction mixture was produced. In view of this, the authors considered that p-aminobenzoic acid modified in the animal organism the type of melanin produced from the oxidation of dioxyphenylalanine (or dopa) by dopa oxidase, an enzyme which is present in the skin, but the nature of which has not been clearly defined. Using the Warburg apparatus, Wisansky, Martin and Ansbacher(1941) also determined the effect of p-aminobenzoic acid on the kinetics of tyrosinase action, and found that it retarded the aerobic oxidation of tyrosine and dopa. How these findings can be correlated with the chromotrichia effect of p-aminobenzoic acid, however, was not discussed. It appears to me that these experiments contradict the

finding that p-aminobenzoic acid is a chromotrichia factor, since they seem to indicate an interference and not an assistance with melanin synthesis. Since aniline has the same effect, this action of p-aminobenzoic acid is probably a toxic one. The similar effect of sulphanilamide is, however, of interest as providing an example of an enzyme system which both p-aminobenzoic acid and sulphanilamide appear to attack at the same point although in this case their actions are not antagonistic.

The above conclusions are still further confused by a report of Emerson (1941). On the appearance of Ansbacher's paper, a considerable number of characteristically grayed rats were available to Emerson for experimentation. They had been raised on a somewhat different ration from that employed by Ansbacher, for they received a vitamin B deficient diet supplemented only with thiamine hydrochloride, riboflavin and pyridoxin hydrochloride, instead of thiamine hydrochloride, riboflavin, pyridoxin hydrochloride, calcium pantothenate, nicotinic acid, inositol and choline chloride. After 25 to 30 days, a marked darkening of the fur was noted in the group supplemented with calcium pantothenate alone and with calcium pantothenate plus p-aminobenzoic acid. The animals receiving p-aminobenzoic acid alone were not altered in appearance and were indistinguishable from the controls. There was, however, an evident stimulus to growth as well as cure of the graying in the animals supplemented with the calcium pantothenate along or the pantothenate and p-aminobenzoic acid. The

p-aminobenzoic acid alone, which had no influence on the graying, also evoked no growth response. In spite of these negative curative effects, Emerson further investigated the possibility that graying could be produced with the exact Ansbacher diet regardless of the fact that it was supplemented with 500 micrograms of calcium pantothenate daily and that such graying could be prevented or cured with p-aminobenzoic acid, but after more than two months on Ansbacher's diet, no evidence of graying had appeared in any of these control animals. Hence p-amino benzoic acid, which it was planned to give when graying developed, was not even necessary.

Unna, Richards and Sampson (1941) found that young black rats maintained on a highly purified diet free from vitamin B complex, but supplemented with thiamine, riboflavine, nicotinic acid, pyridoxine and choline regularly developed conspicuous graying of the fur within 3 to 7 weeks. A daily supplement of 100 micrograms of calcium pantothenate, representing approximately the optimum daily amount for growth, prevented the occurrence of gray hair and restored within 4 - 6 weeks the black pigmentation of the fur in rats which had been rendered gray on the deficient diet. Frequently some scattered gray hairs persisted in spite of an ample supplement of pantothenic acid. These results agreed with findings of other investigators, but it must be mentioned that not all workers agree on the relationship of pantothenic acid to achromotrichia although it appears to be one of the required

factors. Other B-complex factors such as biotin and possibly choline, other dietary deficiencies, notably iron, copper and manganese, and perhaps hormonal factors, in particular the sex hormones, have been suggested as playing some part in the development of rat achromotrichia. A disturbed relationship between the B-vitamins and the sex hormones, for example, has even been suggested as a cause of faulty pigmentation, but the relevant literature cannot be reviewed here.

Unna, Richards and Sampson (1941) also administered p-aminobenzoic acid in relatively large doses of 3 mg. daily to rats either from the beginning of their dietary regimen or after they had been rendered grey. 3 mg. of p-aminobenzoic acid to a rat must be considered a very large dose, beyond the maximum physiological utilisation since, as has already been pointed out (Experiment 6), 1 mg. doses to man result in its rapid excretion in the urine. It is, therefore, reasonable to assume that such a dose would be adequate to produce pigmentation if, indeed, p-aminobenzoic acid had such a physiological property, but Unna and his co-workers report that no such effect was observed in either group of experimental animals. Daily administration of 3 mg. of p-aminobenzoic acid over periods extending to 6 weeks were also unsuccessful in influencing the scattered gray hairs which frequently persist following the addition of pantothenic acid as described above. These authors also failed to obtain graying of the fur in rats receiving adequate amounts of pantothenic acid and hence were unable to confirm Ansbacher's

(1941) work. Henderson, McIntyre, Zaiman and Elvehjem (1942) were also unable to confirm Ansbacher's report, finding that p-aminobenzoic acid was not effective in relieving the graying of hair noted in rats fed low levels, 0-40 micrograms, of pantothenic acid. On higher levels of pantothenic acid, graying did not develop.

Martin (1942a), attempting to bring these various conflicting observations into harmony, repeated the work of the above investigators and developed an interesting thesis. He confirmed the results of Unna et al. and of Emerson that the six basic factors, thiamine, riboflavine, nicotinic acid, pyridoxine, choline and calcium pantothenate, were adequate for seemingly normal nutrition. They were, however, not adequate if either p-aminobenzoic acid or inositol was added to the diet.

Ansbacher (1941) and Martin and Ansbacher (1941) had inositol in their basic supplements and thus noted p-aminobenzoic acid deficiency. Neither Unna et al nor Emerson included inositol in their basic diet. Martin therefore suggested that the possible explanation lay in a stimulation and/or inhibition of bacterial growth in the intestinal tract and hence the bacterial synthesis of vitamin factors, known or unknown in nature. Both inositol and p-aminobenzoic acid have been demonstrated to be growth factors for certain bacteria. Further, the synthesis of certain factors, particularly biotin and inositol, by organisms present in the intestinal tract has been reported. That stimulation of the growth of micro-organisms by one

member of the vitamin B complex causes those micro-organisms to synthesize an increased quantity of another member of the B complex is certain. Martin's thesis then is that inositol stimulates the growth of organisms which utilise and destroy some member of the vitamin B complex, known or unknown, and precipitates a deficiency of that factor. p-Aminobenzoic acid either through stimulation or inhibition of bacterial growth (which it is capable of doing in large amounts, precipitates an inositol deficiency. Inositol may stimulate the growth of organisms which destroy pantothenic acid, whereas the addition of p-aminobenzoic acid may inhibit the growth of these organisms. Martin reports that Proteus vulgaris is absent from the gastro-intestinal tracts of rats fed excessive amounts of p-aminobenzoic acid daily, and that there is a tendency for the lactic-acid-forming organisms to overgrow. The seeming inhibition of Proteus organisms may, therefore, be actually due to overgrowth on the part of the acid-forming organisms. In a further communication, Martin (1942b) suggested that the problem of nutritional achromotrichia assumed a less controversial aspect with the knowledge of the role of folic acid, the powerful growth-stimulating effect of which on rats, first shown by Nielsen and Elvehjem (1942), Martin confirmed. In addition, he showed that "folic acid" is also a chromotrichial factor for rats. Physicians have often noted that graying of the hair occurred following a protracted illness associated with gastro-intestinal disease, and

Martin deduced from this observation, evidence of the importance of the bacterial flora of the intestine in achromotrichia. As it is impossible to produce a "folic acid" deficiency in the rat without the use of sulphonamides to reduce the intestinal synthesis of this factor, Martin suggests that this type of achromotrichia would only be produced without sulphonamides if vitamin balance were altered in a manner to affect the composition of the intestinal flora, thus altering the synthesis of "folic acid", and explains in this paper the discordant results reported on the role of p-aminobenzoic acid in achromotrichia by concluding that p-aminobenzoic acid plays a role in nutritional achromotrichia only in so far as it alters the intestinal flora and by so doing alters the intestinal synthesis of "folic acid". Briggs, Lucky, Mills, Elvehjem and Hart (1943, later showed experimentally in chicks that p-aminobenzoic acid can stimulate "folic acid" production by, and growth of, the micro-organisms found in the intestine of chicks.

There is some disagreement as to the value of pantothenic acid in the treatment of human gray hair, but the majority of workers appear to favour the view that its value as a human anti-gray hair factor is not adequately proved. A similar conclusion appears to be justified as far as p-aminobenzoic acid is concerned. Sieve (1941, was the first to announce that p-aminobenzoic acid caused a marked darkening of the hair of man. Fifty patients varying in age from 21 - 55 years, with definite achromo-

trichia were picked at random. In 30 cases p-aminobenzoic acid was the sole therapy, and in 20 cases, endocrine products in conjunction with the acid were administered. After about two months of treatment, a marked darkening of the hair was noted in all cases. The recently grown shafts appeared to be normally pigmented. Sieve considered that a dose of 100 mg. of p-aminobenzoic acid twice daily was ample to give results. In a second report, Sieve (1942) described the results of treatment of 800 patients of both sexes, varying from 16 - 74 years of age with p-aminobenzoic acid. He noted that after 3 to 8 weeks of treatment the gray hair was characterised by a yellowish cast which gave a dirty appearance to the hair, and in those cases in which this yellow hue was noted, a dark dusty gray colour was observed which gave the hair generally a darker sheen. After this there was a gradual increase in the darkening, which varied considerably in time, apparently depending upon the general physiological condition of the patient. Sieve drew attention to the fact that the clinical picture of achromotrichia is often complicated by a concurrent endocrine disturbance, but found that in such cases too p-aminobenzoic acid had a beneficial effect. Normal pigmentation of parts, such as the nipples and mucous membranes of the mouth, vagina and anus were observed during treatment with p-aminobenzoic acid for achromotrichia. Particularly in young girls just beyond puberty, there was noted a definite darkening of the

aerolae mammae, labia and vaginal mucous membranes. p-Aminobenzoic acid was also tried in cases of alopecia areata and vitiligo. In these cases a definite return of colour occurred in depigmented areas of the skin and white hair growing in areas of pigmentation took on a dirty yellow colour. The doses of p-aminobenzoic acid given were 100 mg. three times daily for 5 to 20 weeks.

Similar effects were noted by Banay (1942). Some 20 inmates of a penal institution received p-aminobenzoic acid in the form of a 100 mg. tablet three times daily for periods varying from 6 to 8 months as the sole therapy and without any change in the daily routine and dietary regimen. A progressive gradual darkening of the hair occurred, starting at the back of the head from the vertex down to the occipital region, and returning in geometric designs or islands instead of following a general distribution.

Eller and Diaz (1943) administered p-aminobenzoic acid to 88 persons of different ages and sexes in various stages of achromotrichia. The patients were given 100mg. tablets 3 or 4 times daily over 3 to 5 months with no untoward effects. There were only a small number (4, who showed definite changes in the colour of their hair, and in no patient in the entire group of 88 patients was there a complete change from white or gray to the normal, original colour of the patient's hair. Since the increase in pigmentation of the hair occurred in so few cases in the treated group, the authors are not certain that the p-aminobenzoic acid was the causative factor, in the

darkening of the hair, and are of the opinion that the use of p-aminobenzoic acid for achromotrichia needs further investigation and corroboration before being expounded to the public as a "cure" for gray hair.

A possible explanation of the discrepancies in the results of previous investigators is given by Brandaleone, Main and Steele (1943) in a critical evaluation of the criteria employed for deciding whether pigmentation of the hair was produced by either calcium pantothenate or p-aminobenzoic acid. These workers administered vitamin preparations for eight months to a group of elderly men and women with white or graying hair who were confined to hospital with chronic diseases such as rheumatoid arthritis, general arteriosclerosis and parkinsonism. Of the group of 19 patients, 7 received 100 mg. of calcium pantothenate, 200 mg. of p-aminobenzoic acid and 50 g. of brewer's yeast daily; 5 received the yeast and p-aminobenzoic acid, and 7 the yeast and calcium pantothenate. Three methods for judging change in hair colour were used: (1) photographs were taken before, during and at the end of medication, (2) samples of hair were clipped from a given area at the time the pictures were made and (3) all patients were seen by the same two observers at least twice a month and notes taken of any change observed. Photographs were found to be useless; slight changes in distance or lighting made considerable difference in the apparent colour of the hair. The subjective opinions were claimed to be more accurate than photographs and less

discouraging than hair samples. Several changes were noted in the hair of the treated subjects. The most common was the appearance of a yellow or greenish cast to the gray hair. Growth of scattered wiry black hair also became apparent. In several patients there was thought to be greater lustre without change in colour. In only 2 patients, however, was there unequivocal change in colour. Both were men with brown hair and the change tended towards a return to the original colour. This change became apparent after both calcium pantothenate and p-aminobenzoic acid had been administered daily for a period of 2 to 3 months, and increased slowly in intensity until the experiment was terminated. In a group of 6 younger individuals who received the above dosage for 6 months, 2 who received the p-aminobenzoic acid and calcium pantothenate only, and 6 who received 20 mg. calcium pantothenate and 3.5 g. of concentrated yeast daily, Brandaleone, Main and Steele (1944) found that not one showed any decrease at all in the gray colour of their hair.

The conclusion appears justified that no adequate evidence of the value of p-aminobenzoic acid as an achromotrichia factor in both animals and man is yet available. These studies cannot, therefore, be quoted in support of the inclusion of p-aminobenzoic acid among the vitamins. The rapidity of excretion of p-aminobenzoic acid when 100mg. is taken by mouth (Experiment 3, pp.75-76 of this thesis) suggests that the human organism deals with this substance as if it were a foreign toxic substance, rather than a

vitamin. Further, it should be noted that the doses used in these experiments are relatively high for a physiological action (Emerson, 1941), and that only about 6 mg. of p-aminobenzoic acid is present in 100 g. of brewer's yeast, which is about its richest source. Martin's work - that p-aminobenzoic acid acts only indirectly on the animal organism through its intestinal flora, so that even although p-aminobenzoic acid cures achromotrichia it may not be an achromotrichia vitamin but merely stimulate bacteria to produce it - must also be borne in mind. He considers that in rats this vitamin, produced by p-aminobenzoic acid, is "folic acid". This subject is further considered in Chapter V.

Finally, it is worth recording that an editorial in the New England Journal of Medicine (1945) deplores the tendency in America for clinical exploitation of the results of laboratory investigations before those findings have been adequately substantiated, and quotes the widely publicised use of p-aminobenzoic acid for the restoration of the original dark colour of graying hair in man as an outstanding example. The position is summed up in the statement that in animals achromotrichia is probably a multiple deficiency of p-aminobenzoic acid, pantothenic acid and inositol, and that the complete and rapid elimination of the p-aminobenzoic acid without practically any storage, as shown by various investigators, has not been taken adequately into account by some of those who advocate its use.

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p-Aminobenzoic acid and Chick Nutrition.

Ansbacher (1941) reported that p-aminobenzoic acid was a growth factor for the chick and prolonged their survival times on a heated grain mixture supplemented with the fat-soluble vitamins A, D and K, the basic B-vitamins and inositol. It is to be noted that this diet was deficient in biotin, folic acid and other, as yet unidentified, factors as well. [Waisman, Mills and Elvehjem (1942)]. Ansbacher (1941a) further reported that the addition of p-aminobenzoic acid to a heated diet low in vitamin K, advanced the time of occurrence of the haemorrhagic diathesis typical of the hypoprothrombinaemia of the baby chick. Briggs, Luckey, Mills, Elvehjem and Hart(1943) confirmed the fact that p-aminobenzoic acid produced growth responses in chicks receiving purified rations low in the unknown vitamins but complete in all other respects, but found that very high levels 5 - 15 mg. of p-aminobenzoic acid per 100 g. of diet were required. In addition, these workers found that solubilised liver had similar and, in fact, greater growth-promoting effects. Since the content of p-aminobenzoic acid in liver is only about 0.25 mg. per 100 g. (Mitchell, Isbell and Thompson, 1943). Briggs and his co-workers considered that p-aminobenzoic acid produced its effects indirectly by synthesizing necessary unknown factors, and that the most logical manner of such production is through intestinal synthesis. This possibility was tested out in vitro by inoculating Mitchell and Snell's medium for the determination of "folic acid".

plus added amounts of p-aminobenzoic acid, with a mixed culture of organisms obtained from the duodenum of chicks. No attempt was, however, made to determine the various kinds of organisms contained in the mixed cultures. A medium for the determination of "folic acid" was chosen for this purpose because the properties and occurrence of "folic acid" are quite similar to the properties of the unknown factor or factors. The results showed that as the amount of p-aminobenzoic acid was raised, bacterial growth increased. This cannot be considered surprising since it is known that p-aminobenzoic acid is a growth factor for various micro-organisms. What is of greater significance is that as the growth increased, the synthesis of "folic acid" increased approximately 3-fold. It is conceivable that other unknown factors may also be produced. Thus, when p-aminobenzoic acid is fed to chicks on diets low in such factors, one can again conclude that it also causes the production by intestinal synthesis of "folic acid" and perhaps other unknown factors, and that these factors and not p-aminobenzoic acid are the essential, specific growth-promoting factors for the chick.

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p-Aminobenzoic acid, Fertility and Lactation.

Sure (1941a,1941b) reported that there was complete failure of lactation in albino rats fed on a diet in which the vitamin B complex was supplied by pure thiamine, ribo-

flavin, pyridoxine, choline chloride, calcium pantothenate, nicotinic acid and a "W" factor from liver extracts. Apparently, therefore, some dietary factor was missing that was essential for lactation. The missing factor, called Bx, was found in rice polishings, defatted wheat embryo, dried grass and brewer's yeast, but was most abundant in liver and rice bran extracts, and when added to the diet resulted in an increase in the lactation efficiency from 5% to 67%, and a decrease in the percentage of stillbirths. Sure claimed that p-aminobenzoic acid was a component of the factor Bx although liver, which is one of the richest sources of Bx, has a very low p-aminobenzoic acid content, and despite the fact that, in his earlier experiments, he obtained negative results when supplementing the diet with daily doses of 15 mg. of p-aminobenzoic acid. The relatively high dose is noteworthy. His earlier work also suggested that inositol may be a component, since it was effective in producing a lactation response. However, when the "W" factor was removed and only known components of the vitamin B complex fed, the same dose of p-aminobenzoic acid appeared to have beneficial results, but when p-aminobenzoic acid + inositol were added the lactation efficiency increased, although inositol on its own appeared to effect no improvement. In a later report (Sure, 1943) the same author reported that inositol had actually a pronounced injurious influence on lactation of the albino rat, which is counteracted by p-aminobenzoic acid, and that p-aminobenzoic acid itself had a markedly favourable in-

fluence. p-Aminobenzoic acid was fed in 3 mg. daily doses per animal for 4 weeks, then in 7.5 mg. daily doses during breeding and 15 mg. daily doses during pregnancy and lactation. The author remarks that on Martin's theory the injurious effect of inositol may be due to its stimulation of the growth of micro-organisms which utilise and destroy another member of the vitamin B complex, or that it may interfere with the synthesis of biotin, folic acid or some other unknown member of the B complex. The response elicited by p-aminobenzoic acid could then be interpreted as supplying a factor essential for synthesis of such substances as biotin and folic acid. These have, however, to date not been shown to influence lactation. However, the evidence advanced by Sure of a lactation-inefficiency due to the absence of p-aminobenzoic acid in the diet cannot be considered conclusive and thus here again the status of p-aminobenzoic acid as a vitamin has not been established. It must also be recorded that Climenko and McChesney (1942, were unable to confirm all of Sure's results. They observed that 15mg. of p-aminobenzoic acid per rat per day delayed initiation of lactation; on the other hand, it did slightly decrease the mortality rates of newly-born rats.

Sieve (1942, also made a study of the effect of p-aminobenzoic acid in sterility in women. A group of 22 women who had been sterile for a minimum of 5 years were chosen. Their cases had been thoroughly studied and endocrinological deficiencies apparently excluded.

All had had utero-tubographs and Rubin tests, with normal findings. The husbands were found to be normal in every respect. All attempts at treatment had failed. p-Aminobenzoic acid was then given in doses of 100 mg. 4 times daily for 3-7 months and conception occurred in 12 of these cases - 54.5%. In 10 cases there was no response, but of these Sieve stated that 6 did not take medication as prescribed, 1 was complicated by old rheumatic heart disease, in another there was a possible tubal obstruction (although as mentioned above tests gave normal findings, and in still another a subtotal thyroidectomy for toxic goitre was done during p-aminobenzoic acid treatment. In only 1 case was p-aminobenzoic acid treatment considered to be an absolute failure. In view of the small number of cases and the manifold difficulties in the investigation of sterility, particularly the satisfactory evaluation of endocrine and general metabolic factors. it appears justifiable to accept the claims of p-aminobenzoic acid to be an anti-sterility vitamin with considerable reserve.

SUMMARY

Since it is becoming more and more difficult to classify the vitamins into a distinct and well-defined group of compounds, the time appears to have come to discontinue the artificial separation of these compounds from other constituents of the diet and to classify them according to their chemical structure and functions in the body. Nevertheless the claims of p-aminobenzoic acid to be included among the vitamins are considered on the basis of various definitions including that of Ansbacher, the pioneer worker in this field, who considered it to be a member of the vitamin B complex since it has a similar distribution in nature.

That p-aminobenzoic acid produced deficiency symptoms was first reported by Ansbacher who found that its absence from the diet of rats resulted in achromotrichia. Various workers have, however, been unable to confirm Ansbacher's observations. Martin attempted to harmonise the conflicting observations by suggesting that p-aminobenzoic acid acted only indirectly on the animal organism through its intestinal flora, which implies that even although p-aminobenzoic acid cures achromotrichia it is not the achromotrichic vitamin but merely stimulates intestinal bacteria to produce it. As far as graying of the hair in man is concerned, there is again disagreement and the conclusion appears justified that no adequate evidence of the value of p-aminobenzoic acid as an achromotrichial factor in both animals and man is yet available. Further, it should be noted that the doses used in these experiments are relatively high, and that the complete and rapid elimination of p-aminobenzoic acid without practically any storage suggests that the human

organism deals with it as if it were a foreign toxic substance rather than a vitamin. Ansbacher also reported that p-aminobenzoic acid was a growth factor for the chick and delayed the time of development of the haemorrhagic diathesis following vitamin K deficiency. However, these effects also require very high levels of p-aminobenzoic acid, and other workers have again concluded that p-aminobenzoic acid produces its effects indirectly by stimulating the synthesis of necessary unknown factors including "folic acid". Sure reported that an absence of p-aminobenzoic acid in the diet produced lactation inefficiency in rats and increased the percentage of stillbirths. The results have, however, not been adequately confirmed and again appear to be an indirect effect, requiring relatively large doses, on the intestinal micro-organisms to produce other compounds influencing lactation. Sieve also claimed that p-aminobenzoic acid had beneficial effects on sterility in women but in view of the small number of cases and the manifold difficulties in the investigation of sterility, it appears justifiable to accept the claims of p-aminobenzoic acid to be an anti-sterility vitamin with considerable reserve.

The evidence that p-aminobenzoic acid produces deficiency symptoms — perhaps the most important criterion of a vitamin — is, therefore, inconclusive and conflicting. Any action that it appears to have is believed by most workers to be indirect, and on the intestinal micro-organisms rather than on the animal itself. This explanation accords well with the firmly-established effects of p-aminobenzoic acid on micro-organisms for many of which it has been shown to be either an essential metabolite or a growth factor.

Regarding the physiological effectiveness of p-aminobenzoic acid in minute amounts — another generally accepted characteristic of vitamins — the reported work shows that the quantities required to remove deficiency effects are far greater than those ingested in a normal diet. Further, the fact that it appears to be formed by the intestinal organisms, (as well as acting on them), indicates that it may not even be necessary in the diet, and that its occurrence in various organs and body fluids is due to its absorption from the intestinal tract.

It appears legitimate to conclude that p-aminobenzoic acid is a growth-factor for bacteria, yeasts and fungi, but that it does not fulfil the requirements for a vitamin as given in any of the definitions in this chapter.

CHAPTER V.

THE PHYSIOLOGICAL SIGNIFICANCE OF P-AMINOBENZOIC ACID IN HIGHER ANIMALS AND MAN.

Although the status of p-aminobenzoic acid as a vitamin for higher animals and man cannot be considered as established, there is no doubt that it is of considerable physiological significance and occurs widely in nature.

Occurrence.

p-Aminobenzoic acid is apparently as widely distributed as the vitamin B complex. The quantities present, however, are small in all foodstuffs including yeast, which is by far the richest source, and from which it was first isolated by Bubbo and Gillespie (1940) as the benzoyl derivative. Ansbacher (1944,p.242) claimed that p-aminobenzoic acid occurred in three forms - "unbound p-aminobenzoic acid" or "free p-aminobenzoic acid"; "conjugated p-aminobenzoic acid", which was mainly, if not entirely, p-acetylaminobenzoic acid; and "bound p-aminobenzoic acid" which represented p-aminobenzoic acid associated with carriers, such as proteins, or p-aminobenzoic acid combined with other compounds, possibly peptides or

amino acids. As far as I am aware, however, no direct evidence exists for the occurrence of "bound p-aminobenzoic acid" in these forms. In the following table, the occurrence of p-aminobenzoic acid in the commoner foodstuffs and in some animal tissues and fluids is listed. It should be noted that only very few workers have reported assays of p-aminobenzoic acid in different materials.

Material.	p-Aminobenzoic acid in micrograms per gramme. Free.	Total.	Reference.
Milk	0.08	0.1-0.4	Mitchell, Isbell and Thompson, (1943).
Egg yolk	0.8		Lewis (1942).
Egg albumin	0.06		"
Rice bran	2-3	9-16	"
Wheat, whole	0.25	0.8	Mitchell, Isbell and Thompson (1943).
Wheat germ	0.5	1.8	"
Asparagus (Dried juice concentrate.	1.15	2.0	Lewis (1942).
Dried Cabbage.	9.7	13.9	
Dried carrots.	0.18	0.43	
Potato	0.3	0.4	Kisansky and Ansbacher, quoted from Ansbacher (1944, p.239).
Spinach	0.12	0.6	
Yeast (cake)	3.6	4.0	Mitchell, Isbell and Thompson (1943).
" (Baker's)	-	5.0	Blanchard (1941)

Material.	p-Aminobenzoic acid in micrograms per gramme. Free.	Total.	Reference.
Yeast (Brewer's)	6.6-61.0	9.3-59.0.	Lewis (1942).
Yeast (Autolyzed)	7.4	12.0	"
Yeast (Extract)	157	156	"
Blood (Human)	0.035	-	Landy and Dicken (1942).
Blood (Rat)	0.06	0.27	Mitchell, Isbell and Thompson (1943).
Blood (Ox)	0.0004		Lewis (1942).
Urine (Human)	0.014-0.021	0.35-0.49	"
	0.02	0.5	Mitchell, Isbell and Thompson (1943).
	0.015	-	Landy and Dicken (1942).
Beef Liver	0.2	2.5	Mitchell, Isbell and Thompson (1943).
Beef muscle	0.3	0.6	"
Rat "	0.15	1.7	"
Rat brain	0.14	0.7	"
Rat heart	0.15	1.35	"
Rat kidney	0.13	1.8	"
Pork	0.3	0.8	"

The table shows that only minute amounts of p-aminobenzoic acid occur in various foodstuffs. The great disparity between the quantity of p-aminobenzoic acid in yeast and in other sources has been ascribed to the fact that yeast, like other micro-organisms, synthesizes p-aminobenzoic acid which is an essential metabolite for its growth and development. The question thus arises whether the negligible amounts of p-aminobenzoic acid which have been found in various animal tissues and fluids do not simply represent an absorbed part of the p-aminobenzoic acid synthesized by the intestinal bacteria. As far as I am aware, this possibility has not been considered heretofore. Point is lent to this hypothesis by Landy's finding that dried human faeces contained 4-12 micrograms of free p-aminobenzoic acid/gramme of faeces, more than 100 times the quantity in the urine (quoted from Ansbacher 1944, p.241). It is probable that this p-aminobenzoic acid was synthesized by the intestinal organisms since the quantities in foods are small and what p-aminobenzoic acid is present would be very rapidly absorbed from the small intestine. Further, Ahlström, v. Euler and Wallerström (1942) noted that after a dose of sulphapyridine, the liver and urine of rats contained no p-aminobenzoic acid.

INTESTINAL SYNTHESIS OF P-AMINOBENZOIC ACID.

The possibility that the negligible amounts of p-aminobenzoic acid excreted in the urine is formed in the intestine during bacterial growth and is not derived from the food, has been overlooked by other workers.

The following experiment, which also appears to be the first in vivo observation of the synthesis of p-aminobenzoic acid by intestinal organisms, was therefore undertaken in an attempt to confirm this hypothesis.

medium.

2.2.45.

Experiment 10. Synthesis of p-Aminobenzoic Acid by Intestinal Bacteria of Man.

Principle:

Sulphaguanidine, which is a known bacteriostatic drug on intestinal bacteria and is only very slightly absorbed from the intestine, was taken by mouth for several days, during which time 24-hour specimens of urine were collected, concentrated and assayed for p-aminobenzoic acid. After a few days on sulphaguanidine, specimens of faeces were tested for sterility daily.

Subject B.B.

2.2.45 to 5.2.45. } 24-hour specimens of urine collected for 3 days before sulphaguanidine treatment commenced = Urines A, B, C.

5.2.45. Sulphaguanidine course commenced with 0.1g./Kg. body weight, followed by 0.05g./Kg. 4 times daily.

Subject's weight: 135 lbs. - approximately 61 Kilogrammes. Hence doses of sulphaguanidine taken were 6 g. followed by 3 g. 4 times daily. Continued for 7 days to 11.2.45., during which time 24-hour specimens of Urine D, E, F, G, H, J collected. Following cessation of sulphaguanidine treatment, urine collected for further 24 hours = Urine K. Faeces tested for sterility from 7.2.45 to 11.2.45 daily = Faeces F, G, H, J, K.

Method of Urine concentration.

The 24-hour specimen of urine was evaporated on a boiling-

water bath to the consistency of a thick syrup. Evaporation was assisted by a fan blowing hot air across the evaporation dish. The residue was extracted with 10% $NaHCO_3$ (about 1/20 volume of original urine), and the extract filtered into a separating funnel. (Since p-aminobenzoic acid is soluble in alkali, it is in this way dissolved out and separated from much of the solid constituents of the urine). The filtrate was acidified with concentrated HCl to a pH of 3 and extracted with about 1 litre of ether in 100-200 cc. quantities. (p-Aminobenzoic acid is extracted by ether from an acid solution, whereas any sulphaguanidine which might have been present in the urine is left behind since it is insoluble in acid-ether). The combined ether extracts were then placed in an evaporating dish and the ether evaporated off, leaving a watery residue. Since this still contained some of the original solid matter of the urine, 250 c.c. hot rectified spirits was added to the residue to dissolve out the p-aminobenzoic and the solution filtered, leaving most of the solid matter behind. The alcoholic extract was now evaporated almost to dryness on a boiling water bath, the residue taken up in a few c.c. of 1% $NaHCO_3$, filtered, the pH adjusted to 7 and the volume made up to 1/50 of original volume of the urine.

Assay of the concentrated urines for p-aminobenzoic acid was carried out as previously described.

Testing of Faeces for Sterility.

1 : 10, 1: 100, 1 : 1000 and 1 : 10,000 dilutions of the faeces were made in sterile normal saline and each dilution tested for the presence of viable bacteria by inoculation into nutrient broth and McConkey lactose-bile salts-neutral red-agar medium.

24-hr. Period.	Urine specimen. (c.c.)	Excretion of p-aminobenzoic acid in micrograms.		Viable bacteria in Faeces.							
				Nutrient Broth.				McConkey's agar.			
		Free.	Acet.	1:10	1:100	1:1000	1:10000	1:10	1:100	1:1000	1:10000
A	1360	+Nil	54	Not tested	Not tested.	Not tested.	Not tested.	Not tested.	Not tested	Not tested.	Not tested.
B	860	+Nil	17	"	"	"	"	"	"	"	"
C	1000	+Nil	40	"	"	"	"	"	"	"	"
Sulphaguanidine course commenced.											
D	1940	+Nil	39	"	"	"	"	"	"	"	"
E	1120	+Nil	5	"	"	"	"	"	"	"	"
F	780	+Nil	3	Heavy growth	Heavy growth	Heavy growth	Heavy growth	Numer- ous col- onies.	Numer- ous colon- ies.	Numerous colonies	Numerous colonies.
G	1400	+Nil	+Nil	"	"	"	"	"	"	"	"
H	680	+Nil	+Nil	"	"	"	"	Few colonies	Nil	Nil	Nil
J	600	+Nil	+Nil	Moder- ate growth.	Slight growth	Slight growth	Nil	"	One colony	"	"
Sulphaguanidine course ended.											
K	780	+Nil	+Nil	Slight growth	Slight growth	Nil	Nil	Few colonies	One colony	Nil	Nil

Footnote. + No p-aminobenzoic acid detectable in 1 cc. of concentrated urine by method used. This indicates that p-aminobenzoic acid content of such urines less than 0.002 micrograms/c.c.

Though the assay method used is only roughly quantitative, as has already been mentioned, and the figures shown for the excretion of p-aminobenzoic acid in experiment 10 can be considered only as approximate, there appears to be no doubt that as the bacteriostatic effect of the sulphaguanidine increases, there is a marked drop in the quantity of p-aminobenzoic acid excreted in the urine. It is, therefore, contended that this experiment clearly shows that the traces of p-aminobenzoic acid excreted in the urine of normal human beings can be considered to originate in the intestine, where it is produced during the growth and multiplication of the intestinal bacterial flora. Experiment 8, in which it is shown that p-aminobenzoic acid can be absorbed from the rectum and pelvic colon, supports this contention.

Relationship of p-aminobenzoic acid and Intestinal Bacterial Synthesis.

Recent work has shown that part of an animal's daily requirements of vitamins - notably of members of the vitamin B complex, namely thiamine, riboflavin, nicotinic acid, inositol, pantothenic acid, biotin and folic acid, and also of vitamin K - is synthesized within that animal's own intestine by its bacterial flora. Deficiency diseases have also resulted from the failure of these bacteria to grow and synthesize vitamins.

These observations taken in conjunction with the facts that p-aminobenzoic acid is an essential metabolite for many bacteria and is actually a growth factor for some bacteria

suggest that p-aminobenzoic acid may exert its major physiological function in higher animals and man by an indirect stimulating action on the intestinal bacterial flora to produce the several vitamins which they have been shown to synthesize, and whose importance in the mammalian organism is well-established. The above demonstration that excreted p-aminobenzoic acid in man is derived from its synthesis by bacteria in the intestine, coupled with its unproven importance as a vitamin in higher animals, lends strong support to this theory.

Martin (1942b) was the first to interpret his own experimental findings and those of others as indicating that p-aminobenzoic acid played its major role in altering the flora of the intestinal tract. Such a theory would also explain the effect of inclusion of sulphonamides in synthetic diets. For example, Mackenzie, Mackenzie and McCollum (1941) and Black, McKibbin and Elvehjem (1941) showed that growth of rats treated with sulphaguanidine was suppressed and that p-aminobenzoic acid reversed this growth-inhibitory effect of sulphaguanidine. On the above hypothesis, sulphaguanidine could be considered to exert its effect mainly on the intestinal bacteria, preventing them synthesizing vitamins and p-aminobenzoic acid by stimulating the growth of these bacteria and providing an essential metabolite for their development, increases their vitamin synthesis, so reversing the sulphaguanidine effect.

In chapter IV attention was drawn to the experimental findings of various investigators which indicated that the

effects of p-aminobenzoic acid, on which its claim to status as a vitamin in human and animal nutrition rests - namely chromotrichial action in man and rats, growth and development of chicks, increased fertility in man and the rat and lactation efficiency in the rat - were adequately explained by its stimulating action on the intestinal bacterial flora, producing various vitamins which are mainly responsible for these effects.

It is, therefore, concluded that the major physiological functions so far attributed to p-aminobenzoic acid are carried out indirectly in man and animals by virtue of its proven role in the metabolism of the micro-organisms which live symbiotically in the intestinal tract of such animals. Whether the physiological actions of p-aminobenzoic acid are entirely indirect through intestinal synthesis of various vitamins, or whether p-aminobenzoic acid has in addition other direct effects, remains, however, unsettled. Certain of its properties and effects suggest that it might also have direct functions, but whether these can be considered physiological is, at best, doubtful.

Direct Effects of p-Aminobenzoic acid on the Mammalian Organism.

(a) Correlation of p-aminobenzoic acid with Sunburn.

Rothman (1926) showed that the p-aminobenzoic acid derivative procaine selectively absorbed the rays of the sun which caused persistent erythema of the skin and pigmentation. Later, Behagel, Rothman and Schultze (1928)

found that the selective absorption of the Dorno rays was caused by p-aminobenzoic acid or its derivatives, namely by compounds with an amino- and a carboxyl-group in para position on the benzene ring, including p-aminobenzoic acid with substituted hydrogen atoms of either or both polar groups. Bird (1942, also found that aqueous solutions of local anaesthetic bases derived from p-amino benzoic acid showed strong absorption of ultraviolet light in the wave-length band 2700-3200 Angstrom units, and concluded that most surface anaesthetics of this type offer interesting possibilities in the preparation of "filters" for the erythema producing rays, i.e. as "sun-screen" preparations. Rothman and Rubin (1942) also prepared a product extremely effective against sunburn by incorporating p-aminobenzoic acid in ointment bases.

The ultraviolet absorption of p-aminobenzoic acid is maximum at 2785 Angstrom units, but at 3000 Angstrom units its absorption is still very great (Rothman and Rubin, 1942), so that the ultraviolet absorption curve of p-aminobenzoic acid completely covers the range of ultraviolet light rays which have a "sunburn action", the maximum effect of which is at 2975 Angstrom units.

Blum (1941, divided the photochemical theory of sunburn action, i.e. the action of the sun's rays on the skin, into two parts:

(1) A substance is present in the photosensitive layers of the skin, the absorption spectrum of which is identical with or similar to the "sunburn action" spectrum;

(2) This substance absorbs the effective Dorno rays, is photochemically altered, and its reaction product causes erythema.

Ansbacher (1944, p.217) points out that p-aminobenzoic acid fulfils these conditions, ie. it is capable or producing erythema when irradiated in saline solution and injected subcutaneously, its absorption spectrum is similar to, or identical with, the "sunburn action" spectrum, and it is reasonably certain that p-aminobenzoic acid is a natural constituent of the skin (although there is no experimental support). He concludes, therefore, that p-aminobenzoic acid or one of its derivatives may be the substance responsible for the reddening and darkening of the skin resulting from exposure to the rays of the sun.

(b) Relationship of p-Aminobenzoic acid to Hormones.

Sieve's (1942) report of some correlation between the actions of p-aminobenzoic acid and the sex hormones has already been reviewed (p.111). He claimed that p-aminobenzoic acid increased fertility in the female, stimulated libido and re-established the menses in cases of amenorrhoea, and caused a great improvement in potency in the male.

Astwood (1943a) found that certain aniline derivatives, including p-aminobenzoic acid and the sulphonamides, inhibited the function of the thyroid gland in young rats when administered in the food or drinking water for a period of 10 days. A daily dose of about 200 mg. p-aminobenzoic

acid/100g. of body weight was required for a full effect. It is possible that these aniline derivatives owe their activity to their structural similarity to tyrosine, and as a working hypothesis it is suggested that these compounds act through a competitive mechanism in the enzyme system responsible for the conversion of di-iodotyrosine to thyroxin. The large dosage required is noteworthy and indicates that this effect is more properly classed as a toxic effect rather than a physiological one.

Thiourea derivatives have a similar effect on thyroid function, and are possibly also specific inhibitors of this same system. From a clinical point of view, however, in the treatment of hyperthyroidisin, thiourea and thiouracil have been shown to be the most promising compounds (Astwood, 1943b).

p-Aminobenzoic acid has also been shown to be capable of inhibiting the destruction of adrenaline (Ansbacher, 1944, p.299), an effect which it shares with other benzoates, some of which in fact are even more active. Here again, therefore, it appears to be unlikely that p-aminobenzoic acid has this function under physiological conditions.

Ansbacher and his co-workers also claimed (Ansbacher, 1944, p.299) that p-aminobenzoic acid potentiated the action of insulin. When injected subcutaneously into mice in a dose of 1/6 unit per 18 g. of body weight, insulin did not reduce the blood sugar to convulsive levels. When, however, p-aminobenzoic acid was given with the insulin,

hypoglycaemic convulsions were produced. p-Aminobenzoic acid itself in doses of 1 to 2 g. per kg. body weight in dogs produced a mild hyperglycaemia, and also depleted the glycogen reserves of the liver.

Martin (1943) suggested that these actions of p-aminobenzoic acid might be motivated via the pituitary and production of thyrotropic hormone, being supported in this view by Mackenzie and Mackenzie (1943) and Astwood, Sullivan Bissell and Tyslowitz (1943), who found that the thyroid-enlarging effect of the sulphonamides and thioureas is probably mediated through the anterior pituitary. Whether these effects of p-aminobenzoic acid are of any significance in the physiology of the normal animal awaits further investigation. In view of the large doses of p-aminobenzoic acid required to demonstrate these actions of p-aminobenzoic acid, the conclusion again appears justifiable that these are not normal functions of this simple primary amine.

SUMMARY.

The quantities of p-aminobenzoic acid reported to occur in various foodstuffs is small. Yeast is by far the richest source, this probably being due to the fact that yeast, like other micro-organisms, synthesizes p-aminobenzoic acid, which is an essential metabolite for its growth and development. This suggests the possibility, overlooked by previous workers, that the negligible amounts of p-aminobenzoic acid present in various animal tissues and fluids, and finally excreted in the urine, is also derived from bacterial growth in the intestine and not from the food. This hypothesis was, therefore, tested by studying the excretion of p-aminobenzoic acid after preventing intestinal bacterial growth by oral doses of sulphaguanidine. The marked drop in the quantity of p-aminobenzoic acid excreted shows that the traces of p-aminobenzoic present in the urine of normal human subjects can be considered to originate in the intestine where it is produced during the growth and multiplication of the bacterial flora. It has already been shown that p-aminobenzoic acid can be absorbed from the large intestine.

These facts, coupled with the unproven role of p-aminobenzoic acid as a vitamin in higher animals and man, and its importance as an essential metabolite for some bacteria and actual growth factor for others, suggest that p-aminobenzoic acid exerts its major physiological functions by an indirect stimulating action on the intestinal bacterial flora to produce the several vitamins which they have been shown to synthesize, and whose importance in the mammalian organism is well established.

Certain of the properties and effects of p-aminobenzoic acid, however, suggest that it might also have direct functions. Thus it has been considered to be responsible for the reddening and darkening of the skin resulting from exposure to the rays of the sun. p-Aminobenzoic acid has also been shown to bear a relationship to endocrine function. It inhibits the function of the thyroid gland and the destruction of adrenaline in the body and potentiates the action of the sex hormones and insulin. However, in view of the large doses of p-aminobenzoic acid required to demonstrate these actions, it is concluded that these are not normal functions of this simple primary amine.

CHAPTER VI.

THE EFFECT OF P-AMINOBENZOIC ACID ON THE RESISTANCE OF ANIMALS TO DISEASE.

During the course of a study on the comparative effects of p-aminobenzoic acid on E. coli and other gram-negative intestinal bacteria of the typhoid, paratyphoid, dysentery, and food-poisoning groups, it was noted that large quantities of p-aminobenzoic acid appeared to inhibit growth of the typhoid and paratyphoid organisms to a larger extent than the others. That p-aminobenzoic acid could itself act as an inhibitor to bacterial growth in concentrations above those which antagonised sulphonamide inhibition is wellknown (Chapter I,pp.18,19,25). However, in view of the possible therapeutic value of p-aminobenzoic acid in typhoid fever, for which no active chemotherapeutic agent is as yet available, it was decided to extend these in vitro findings to experimentally produced typhoid infection in mice. Although the results were disappointing, a curious observation was made — namely that p-aminobenzoic acid administered to mice for some time before inoculating them with B. typhosus apparently diminished their susceptibility to infection. Since untreated typhoid infection in mice is an acute, rapidly-fatal, septicaemia, experiments were also carried out to determine the therapeutic potentialities of p-aminobenzoic acid in chronic infections and guinea-pig tuberculosis was selected for study since here, too, chemotherapy has as yet not made great strides. Tuberculosis in the guinea-pig is a fairly chronic and relatively simple progressive disease, with neither native nor acquired resistance apparently playing any part, so that any differences between

untreated and treated animals could reasonably be attributed to treatment. Such differences, though not dramatic, were easily apparent, and treated animals appeared to be less ill, showed a greater gain in weight and tended to survive longer than did the control animals. Thus again p-aminobenzoic acid, although therapeutically disappointing, appeared to increase the resistance to infection.

Further experiments have shown that p-aminobenzoic acid in large doses apparently neither increases the phagocytic activity of the reticulo-endothelial system nor increases the titre of circulating antibodies produced by injection of an antigen. It therefore seems probable that p-aminobenzoic acid exerts its effect in some way on the natural immunity or "resistance" of the animal.

In the following sections the experimental evidence for the above statements will be detailed and an attempt made to evaluate their significance.

EFFECT OF "LARGE" AMOUNTS OF P-AMINOBENZOIC ACID ON CULTURES OF B. COLI AND VARIOUS PATHOGENIC INTESTINAL BACTERIA.

It has already been shown that a few micrograms of p-aminobenzoic acid is sufficient to promote the growth of E. coli in a purely synthetic basal culture medium to which one thousand times as much sulphapyridine has been added. To test the effect of "large" amounts of p-aminobenzoic acid, it was decided to add 0.01% - 1% quantities to the culture medium. Since the basal medium used in the previous experiments was found to be unable to support the growth of some of the pathogenic intestinal bacteria, McConkey's lactose-bile salts-neutral red-nutrient agar medium was used. This medium was selected since it is one

of the standard selective media for the growth of B. coli, B. typhosus causing typhoid fever, B. paratyphosus A, B and C, all of which may cause paratyphoid fever, B enteritidis and B. typhimurium which cause food-poisoning, B. dysenteriae shiga, B. paradysenteriae flexner and B. paradysenteriae sonne, responsible for bacillary dysentery. The effect of p-aminobenzoic acid on the growth of all these organisms is shown in experiment 11. The tabulated results show clearly that whereas the growth of B. coli is not affected until a 1% concentration of p-aminobenzoic acid is present in the culture medium, the growth of B. typhosus, B. paratyphosus A, and B. paratyphosus B, are already slightly affected by a 0.01% concentration. Further, the growth of B. typhosus is markedly diminished or inhibited by a concentration of 0.1%. The other organisms also show varying degrees of inhibition which is most obvious in the paratyphoid group and least marked in the dysenteric group.

11.4.45.

Experiment 11. EFFECT OF 0.01% - 1% P-AMINOBENZOIC ACID ON B. COLI, TYPHOID, PARATYPHOID, FOOD-POISONING AND DYSENTERY ORGANISMS.

Plates of McConkey media containing 0.01%, 0.1%, 0.5% and 1% of p-aminobenzoic acid were prepared as follows:-

55 g. of Difco McConkey powder consisting of

Bacto-Bile Salts	3 g.
Bacto-lactose	10 g.
Bacto-peptone	20 g.
Sodium Chloride	5 g.
Bacto-Agar	17 g.
Bacto-Neutral Red	0.03 g.

was suspended in cold water, boiled for 1-2 minutes and the required amounts of 2% p-aminobenzoic acid solution in normal sodium hydroxide added. The pH was then adjusted to ±7.3, and the volume made up to 1 litre. The solution was then autoclaved at 15 lbs. pressure for 20 minutes, poured into sterile petri dishes, cooled and allowed to set. To economise on the number of plates required 20-25 tests were conducted on each plate.

Organisms tested.

1. Bacterium coli or Escherichia coli.
2. Bacterium typhosus or Eberthella typhosa.
3. Bacterium paratyphosus A or Salmonella paratyphi.
4. " " B or " schotmülleri.
5. " " C or " hirschfeldii.
6. " enteritidis or " enteritidis Gaertner.
7. " aertrycke or " typhimurium.
8. " shigae or Shigella dysenteriae shiga.
9. " flexneri or " paradysenteriae flexner.
10. " sonnei or " " sonne.

Nutrient broth cultures (incubated for 24 hours at 37°C.) of all the organisms were tested by inoculating one platinum loopful of each culture (un-diluted and diluted 10, 100, 1,000 and 10,000 times with sterile normal saline) on the surfaces of the various

McConkey media. The various dilutions were prepared so as to test out the effect of the different concentrations on p-aminobenzoic acid when small and large numbers of organisms were inoculated. After inoculation the plates were incubated for 48 hours.

The following table shows the results obtained in one of these experiments.

Organism	Dilution	Growth in McConkey Medium plus p-aminobenzoic acid. 0.01%	0.1%	0.5%	1.0%	Growth in McConkey Medium with no p-aminobenzoic acid. Control.
B.coli	Undiluted	Good	Good	Good	Nil	Good
	1:10	"	"	"	"	"
	1:100	"	"	"	"	"
	1:1000	"	"	"	"	"
	1:10000	"	"	"	"	"
B.typhosus	Undiluted	Good	Slight	Slight	Nil	Good
	1:10	"	"	Nil	"	"
	1:100	"	Nil	"	"	"
	1:1000	Slight	"	"	"	"
	1:10000	Slight	"	"	"	"
B.paratyphosus A	Undiluted	Good	Good	Slight	Nil	Good
	1:10	"	Slight	"	"	"
	1:100	"	Nil	Nil	"	"
	1:1000	"	"	"	"	"
	1:10000	Slight	"	"	"	"
B.paratyphosus B	Undiluted	Good	Good	Good	Nil	Good
	1:10	"	"	"	"	"
	1:100	"	Slight	Slight	"	"
	1:1000	"	"	"	"	"
	1:10000	Slight	"	Nil	"	"
B.paratyphosus C	Undiluted	Good	Good	Good	Nil	Good
	1:10	"	Slight	Slight	"	"
	1:100	"	Nil	"	"	"
	1:1000	"	"	"	"	"
	1:10000	"	"	Nil	"	"
B.enteritidis	Undiluted	Good	Good	Good	Nil	Good
	1:10	"	Slight	Slight	"	"
	1:100	"	Nil	"	"	"
	1:1000	"	"	Nil	"	"
	1:10000	"	"	"	"	"
B.aertrycke	Undiluted	Good	Good	Good	Nil	Good
	1:10	"	Slight	"	"	"
	1:100	"	"	Slight	"	"
	1:1000	"	"	"	"	"
	1:10000	"	Nil	Nil	"	"
B.shigae	Undiluted	Good	Good	Good	Nil	Good
	1:10	"	"	"	"	"
	1:100	"	"	"	"	"
	1:1000	"	Slight	Slight	"	"
	1:10000	"	Nil	Nil	"	"
B.flexneri	Undiluted	Good	Good	Good	Nil	Good
	1:10	"	"	"	"	"
	1:100	"	Slight	Slight	"	"
	1:1000	"	"	Nil	"	"
	1:10000	"	"	"	"	"
B.sonnei	Undiluted	Good	Good	Good	Nil	Good
	1:10	"	"	"	"	"
	1:100	"	Slight	"	"	"
	1:1000	"	"	"	"	"
	1:10000	"	"	Slight	"	"

Experiment 12. EFFECT OF 0.1%-1% SULPHAPYRIDINE ON B.COLI, TYPHOID, PARATYPHOID, FOOD-POISONING AND DYSENTERIC ORGANISMS.

Experimental details as in experiment 11. In place of p-amino-benzoic acid, the required amounts of 10% sodium sulphapyridine in distilled water were added.

		Growth in McConkey medium plus sulphapyridine.			Growth in Mc-Conkey medium (Control)
Organism	Dilution.	0.1%	0.5%	1%	
B.coli	Undiluted	Good	Good	Good	Good
	1:10	"	"	"	"
	1:100	"	"	"	"
	1:1000	"	"	"	"
	1:10,000	"	Slight	Slight	"
B.typhosus	Undiluted	Good	Good	Good	Good
	1:10	"	"	"	"
	1:100	"	Slight	Slight	"
	1:1000	"	"	"	"
	1:10,000	"	Nil	"	"
B.para-typhosus A	Undiluted	Good	Good	Good	Good
	1:10	"	Slight	Slight	"
	1:100	"	"	Nil	"
	1:1000	"	Nil	"	"
	1:10,000	Slight	"	"	"
B.para-typhosus B	Undiluted	Good	Good	Good	Good
	1:10	"	"	"	"
	1:100	"	"	"	"
	1:1000	"	"	"	"
	1:10,000	"	"	Slight	"
B.para-typhosus C	Undiluted	Good	Good	Good	Good
	1:10	"	"	"	"
	1:100	"	"	"	"
	1:1000	"	"	"	"
	1:10000	"	Nil	Slight	"
B.enteri-tidis	Undiluted	Good	Good	Good	Good
	1:10	"	"	"	"
	1:100	"	Slight	Slight	"
	1:1000	"	"	"	"
	1:10,000	"	"	"	"
B. aertrycke	Undiluted	Good	Good	Good	Good
	1:10	"	"	"	"
	1:100	"	Slight	Slight	"
	1:1000	"	"	"	"
	1:10,000	"	"	"	"
B.shigae	Undiluted	Good	Good	Nil	Good
	1:10	"	"	Slight	"
	1:100	Slight	Nil	Nil	"
	1:1000	"	"	"	"
	1:10,000	"	"	"	"
B.flexneri	Undiluted	Good	Good	Good	Good
	1:10	"	"	Slight	"
	1:100	Slight	Slight	"	"
	1:1000	"	"	Nil	"
	1:10,000	Nil	Nil	"	"
B.sonnei	Undiluted	Good	Slight	Slight	Good
	1:10	Nil	"	"	"
	1:100	"	Nil	Nil	"
	1:1000	"	"	"	"
	1:10000	"	"	"	"

20-25 g. This was usually found to be 50 million organisms which killed in 24 to 36 hours. If the mouse survived this period, as a rule it recovered rapidly.

EFFECT OF P-AMINOBENZOIC ACID ON EXPERIMENTAL TYPHOID INFECTION IN MICE.

The selective inhibition of the growth of B. typhosus *in vitro* was so well-marked that it appeared worth while to determine whether a similar effect could be obtained in *in vivo* experiments. Experiment 12, which was carried out exactly as was experiment 11, with sulphapyridine added to the medium instead of p-aminobenzoic acid, shows a reverse order of inhibition of the growth of the same organisms. Since this in vitro effect is duplicated by in vivo experiments, sulphonamides having well marked therapeutic value in bacillary dysentery but very little action in typhoid fever, there seemed to be, by analogy, good prospects that large doses of p-aminobenzoic acid might influence typhoid fever. It was decided, therefore, to test its effects in experimentally produced typhoid infection in mice. Following the technique described by Lewin (1938), cultures of a virulent strain of B. typhosus [x] were prepared and the minimum fatal dose determined by intraperitoneal injection into mice of 20-25 g. This was usually found to be 50 million organisms which killed in 24 to 36 hours. If the mouse survived this period, as a rule it recovered rapidly. For injection, a suspension of this number of organisms was made in a volume of 0.25 c.c. by dilution of a 24-hour nutrient broth culture with sterile normal saline. The virulence of the strains was confirmed by demonstrating the presence of high contents of Vi antigen — the strains agglutinating Vi antisera to a titre of 1:800 or higher — and either the absence of

x Made available by Dr. W.Lewin of the S.A.Institute of Medical Research who also very kindly arranged for the mouse injections.

O-antigen agglutination or agglutination only to low titres.

The level of the toxic dose of p-aminobenzoic acid for mice was first determined. Scott and Robbins (1942) observed that the median lethal dose in mice was 2.85 ±0.4 g. per kg. body weight - approximately 50-65 mg. for a 20 g. mouse. When administered orally as the free acid they found that the toxic signs in mice produced by p-aminobenzoic acid consisted of weakness and loss of normal posture, death occurring after several hours. When lethal doses were administered intravenously as the sodium salt, p-aminobenzoic acid produced mild clonic convulsions with death in 5-10 minutes. It was found here that when 10 mg. doses of p-aminobenzoic acid were injected subcutaneously 2-hourly into mice weighing 20-25 g., toxic signs became apparent after 2-3 injections were given, and consisted of hyperexcitability of the muscles of the whole body accompanied by twitching, and followed within a short while by unsteadiness of the gait and finally by actual paralysis, particularly of the hind limbs. When the dose injected was reduced to 5 mg. 2-hourly, no obvious toxicity was apparent. In the following experiments, therefore, this dosage was never exceeded.

The effect of p-aminobenzoic acid when injections were commenced immediately after injection of a lethal dose of B. typhosus was first determined. Since p-aminobenzoic acid is known to be excreted rapidly, the greater part certainly within an 8 hour-period (Chap.III), injections were continued 2- to 4-hourly during the day and until midnight, commencing again the following morning 8 to 9 hours later, and continuing 2-hourly. In experiment 13 these results are reported.

Experiment 13. EFFECT OF P-AMINOBENZOIC ACID ON EXPERIMENTALLY PRODUCED TYPHOID INFECTION IN MICE.

Fifty millions B. typhosus injected intraperitoneally into mice after which animals were divided into two groups. The Test Group received 5 mg. p-aminobenzoic acid (0.25 c.c. of a 2% solution in 1% $NaHCO_3$, pH ±8) subcutaneously at intervals shown. The Control Group received 0.25 c.c. of 1% $NaHCO_3$ only, at the same intervals. The first signs of illness observed were standing-up of fur, diminished activity and huddling-up. Observations continued for 72 hours.

Test Group.

Mouse No.	p-Aminobenzoic acid injected at following intervals (in hours, after infection with B. typhosus.	Incubation (in period. hours)	Survival (in period. hours)
1	½,3,7,16,19,22,26,30,38	-	(Survived 72 hours +)
2	½,3,7,16.	8	19
3	½,3,7,16,19,22.	17	23
4	½,2,4,	4	6
5	½,2,4,6.	6	8
6	½,2,4,6,8,11.	6	12
7	½,2,4,6,8,11,13,21.	8	23
8	½,2,4,6,8,11,13,21.	12	23
9	½,2,4,6,8,11,13,21.	12	23
10	½,2,4,6,8,10.	4	12
11	½,2,4,6,8,10,12.	4	13
12	½,2,4,6,8,10,12.	6	13
13	½,2,4,6,8,10,12,14.	8	22
14	½,2,4,6,8,10,12,14,22.	8	24
15	½,2,4,6,8,10,12,14,22,24,26, 28,30,32,34.	8	Recovered and.. (Survived 72 hours)
16	½,2,4,6.	4	8
17	½,2,4,6,8.	4	10
18	½,2,4,6,8.	4	10
19	½,2,4,6,8.	4	10
20	½,2,4,6,8,10.	4	18
21	½,2,4,6,8,10.	4	18
22	½,2,4,6,8,10.	4	18
23	½,2,4,6,8,10.	4	18
24	½,2,4,6,8,10,18,20.	6	22
25	½,2,4,6,8,10,18,20,22,24,26,28, 30,32,40.	6	41

	Incubation Period.	Survival Period.
Total	155 hours.	394 hours.
Number of mice (n)	24	23
Mean (x)	6.5 hours.	17.1 hours.
Range	4-17	6-41
Standard deviation (σ)	3.2	7.6
Standard error of the mean ($\sigma/\sqrt{n}$)	0.65	1.6
$x \pm 2 (\sigma/\sqrt{n})$	5.2-7.8	13.9-20.3

Survivals - 2 out of 25 = 8%.

Control Group.

Mouse No.	Sodium bicarbonate solution injected at following intervals (in hours) after infection with B. typhosus.	Incubation Period (in hours)	Survival Period (in hours)
1	½,3,7,16.	17	19
2	½,3,7,16,19,22,26,30.	23	32
3	½,2,4,6,8,11,13,21.	9	23
4	½,2,4,6,8,11,13,21.	11	23
5	½,2,4,6,8,11,13,21,23,25.	13	26
6	½,2,4,6,8,10,12,14,22,24,26.	6	28
7	½,2,4,6,8,10,12,14,22,24,26.	24	28
8	½,2,4,6,8,10,12,14,22,24,26, 28,30,32,34.	28 but recovered and survived 72 hours.	
9	½,2,4.	4	6
10	½,2,4,6,8.	4	10
11	½,2,4,6,8.	4	10
12	½,2,4,6,8,10.	6	18
13	½,2,4,6,8,10.	6	18
14	½,2,4,6,8,10,18,20.	6	22
15	½,2,4,6,8,10,18,20.	18	22
16	½,2,4,6,8,10,18,20,22,24.	22	26
17	½,2,4,6,8,10,18,20,22,24,26, 28,30,32,40,42,44.	41	46
18	½,2,4,6,8,10,18,20,22,24,26, 28,30,32,40,42,44,46,48.	Survived 72 hours.	Survived 72 hours.

	Incubation Period.	Survival Period.
Total	242 hours	357 hours.
Number of mice (n)	17	16
Mean (x)	14.2	22.3
Range	4-41	6-46
Standard deviation (σ)	10.3	9.3
Standard error of the mean ($\sigma/\sqrt{n}$)	2.5	2.3
$x \pm 2 (\sigma/\sqrt{n})$	9.2-19.2	17.7-26.9

Survivals 2 out of 18 = 11%.

Twenty-five mice were included in the test group and at the same time a control group of 18 mice were injected 2-hourly with a sodium bicarbonate solution. 8% of the test group and 11% of the control group survived. This difference is not statistically significant and indicates at best that treatment with p-aminobenzoic acid has no beneficial effect. Further analysis of the results show that such treatment may in fact be harmful — although these results must be interpreted with caution as the groups tested are small. The mean period elapsing before signs of illness were first observed was 6.5 ± 0.65 hours in the test group where 24 mice became ill and 14.2 ± 2.5 hours in the control group where 17 mice became ill. The difference between these figures is statistically significant since the means differ by more than twice the standard error (as the calculation $x \pm 2(\sigma/\sqrt{n})$ shows). However when the same analysis is applied to the means of the periods of survivals after infection in the 23 mice of the test group which died (17.1 ± 1.6 hours) and in the 16 mice of the control group which died (22.3 ± 2.3 hours), the difference is not statistically significant although again the treated group fares more badly than does the control group.

Before accepting the obvious conclusion that appears to emerge from experiment 13, namely that the in vivo action of p-aminobenzoic acid in non-toxic doses does not parallel its in vitro effect on B. typhosus, the possibility of modifying factors requires consideration. Lewin (1938) noted that the dose of B. typhosus injected into mice had to be so overwhelming that the mouse would succumb in 24-36 hours, and that

if the mouse survived 48 hours it generally recovered. p-Aminobenzoic acid is rapidly excreted and may not, therefore, be able to reach and maintain a sufficiently high level in the blood and tissue fluids soon enough to counteract the rapid multiplication and thus the rapidly increasing toxic effects of B. typhosus in the mouse. A second possibility also suggests itself. Whereas p-aminobenzoic acid in large concentrations may inhibit growth of B. typhosus, in smaller concentrations it may stimulate it. This fact would explain the adverse therapeutic effect of p-aminobenzoic acid and also indicates how it may actually be harmful.

These possibilities were, therefore, tested experimentally by injecting two groups of mice for 2-3 days with large doses of p-aminobenzoic acid so as to "saturate" their tissues as far as possible, and then infecting both groups with B. typhosus. Thereafter in one group the injections of p-aminobenzoic acid were continued 2-hourly, the other group serving as a control. The results are shown in experiment 14. Twenty-five mice were included in the test group and 15 mice in the control group. The mean period elapsing before signs of illness were observed in the test group where 22 mice became ill was 14.1 ± 1.7 hours, whereas in the control group where only 7 mice became ill this was 15.3 ± 1.6 hours. Though this difference is not significant, and the numbers are too small for accurate statistical evaluation, the control group certainly appears to fare much better than the test group. The mean period of survival of those animals which died in the test group (26.4 ± 2.3 hours, is not significantly different from that of the control group

Experiment 14. EFFECT OF PRELIMINARY INJECTIONS OF P-AMINOBENZOIC ACID ON THE TREATMENT OF TYPHOID INFECTIONS IN MICE.

5 mg. p-aminobenzoic acid (0.25 c.c. of a 2% solution in 1% $NaHCO_3$) injected subcutaneously 2-hourly from 8.30 a.m. to 10.30 p.m. for 2 days and then until 2.30 p.m. on the 3rd day. All mice then injected at 4 p.m. with one lethal dose (50 millions) of B. typhosus intraperitoneally and, thereafter, divided into two groups - the test group continuing to receive p-aminobenzoic acid 2-hourly as shown and the control group receiving no further injections. The first signs of illness and the survival period of the mice in each group were then noted, the observations being continued for 72 hours.

Test Group.

Mouse No.	p-aminobenzoic acid injected at following intervals (in hours after infection with B. typhosus.	Incubation Period (in hours).	Survival Period (in hours)
1	½,3,5,7,16,18,20,22,24,26, 28,30.	17	31
2	½,3,5,7,16,18,20,22,24,26,28, 30,41,44,47,49.	-	Survived 72 hours.
3	½,2,4,6,8,19,21,23.	17	24
4	½,2,4,6,8,19,21,23,25,27,29.	17	3[illegible]
5	½,2,4,6,8,19, 21,23,25,27,29, 31,40.	17	41
6	½,2,4,6,8,19,21,23,25,27,29, 31,40,42,44,48.	6, recovered and survive	72 hours.
7	½,2,4,6,8.	6	18
8	½,2,4,6,8,19,21,23,25.	6	26
9	½,2,4,6,8,19,21,23,25,27,29. 31,40,42,44,48.	-	Survived 72 hours.
10	½,2,4,6.	4	7
11	½,2,4,6.	4	7
12	½,2,4,6.	4	18
13	½,2,4,6,17,19,21.	7	22
14	½,2,4,6,17,19,21.	7	22
15	½,2,4,6,17,19,21,23,25.	18	26
16	½,2,4,6.8,17,19,21,23,25,27, 29,31,40.	18	41
17	½,2,4,6,8,17,19,21,23,25,27, 29,31,40,42,44,48.	26 but recovered and survived 72 hours.	
18	½,2,4,6,8,17,19,21,23,25,27, 29,31,40,42,44,48.	-	Survived 72 hours.

Continued on next page.

Test Group (Continued)

Mouse No.	p-aminobenzoic acid injected at following intervals (in hours, after infection with B. typhosus.	Incubation Period (in hours)	Survival Period (in hours)
19	2,4,6,8,10,12.	12	22
20	2,4,6,8,10,12.	12	22
21	2,4,6,8,10,12.	12	22
22	2,4,6,8,10,12,22,24,26,28, 30.	22	32
23	2,4,6,8,10,12,22,24,26,28, 30.	24	32
24	2,4,6,8,10,12,22,24,26,28, 30,32.	24	36
25	2,4,6,8,10,12,22,24,26,28, 30,32,36.	30	48

	Incubation Period.	Survival Period.
Total	310 hours	527 hours.
Number of mice (n)	22	20
Mean (x)	14.1 hours	26.4 hours
Range	4-30 hours	7-48 hours.
Standard Deviation (σ)	7.8	10.1
Standard error of the mean ($\sigma/\sqrt{n}$)	1.7	2.3
$x \pm 2 (\sigma/\sqrt{n})$	10.7-17.5	21.8-31.0

Survivals 5 out of 25 = 20%.

Control Group.

Mouse No.	Incubation Period (in hours).	Survival Period (in hrs).
1	-	Survived 72 hours
2	Slight illness at 18 hours, recovered, and	" 72 "
3	-	" 72 "
4	18	22
5	-	" 72 "
6	-	" 72 "
7	6	7
8	18	18
9	18	18
10	-	" 72 "
11	18	26
12	-	" 72 "
13	-	" 72 "
14	18	41
15	-	" 72 "

	Incubation Period.	Survival Period.
Total	114 hours	132 hours.
Number of mice (n)	7	6
Mean (x)	16.3 hours	22 hours.
Range	6-18 hours	7-41 hours.
Standard Deviation (σ)	4.1	10.3
Standard error of the mean ($\sigma/\sqrt{n}$)	1.6	4.2
$x \pm 2(\sigma/\sqrt{n})$	13.1-19.5	13.6-30.4

Survivals 9 out of 15 = 60%.

Standard error of the difference between the above two percentages of survival in the test and control groups

$$= \frac{A \times (100 - A)}{N_t} + \frac{B \times (100 - B)}{N_c} = 15.0$$

Where A = Percentage survival in test group.
B = " " " control group
N_t = Number of mice in test group
N_c = " " " control group.

(22 ± 4.2 hours), but here again 20 mice died in the test group as compared to only 6 in the control group.

Since the difference (=40%) between the percentage survivals in the control and test groups is more than twice its standard error (= 15), it is statistically significant despite the relatively small number of mice in the two groups. Campbell (1941) has calculated that if this difference is 2.5 times its standard error the odds are 1 to 80, and if it is 3 times, they are 1 to 369 that a difference as large as this could occur simply by chance.

The significantly greater number of survivals in the control group as compared to the test group of experiment 14 indicates that p-aminobenzoic acid in some way increases the resistance of mice to infection when it is administered for some time before the animals are infected with B. typhosus. When, however, the administration is continued after infection, the beneficial effect is lost and such treated mice with 20% survivors are not significantly different from mice which are not treated at all. Such untreated mice show a survival rate of 14% (Experiments 13 and 15; Control Groups); the difference between the percentage survivals in the two groups (6%) is even less than its standard error (9.4), and, therefore, is certainly not significant. It is suggested that these findings can only mean that p-aminobenzoic acid cannot attain in vivo bacteriostatic concentrations, and that the concentrations that can be reached have a beneficial effect both on the animal organism and the infecting bacillus. When p-aminobenzoic acid is presented to the tissue cells of the mouse (before

infection with B. typhosus, it increases the mouse's resistance to infection, but when infection has already occurred, this effect is completely overshadowed by the much greater stimulating effect of the concentration of p-aminobenzoic acid reached in the blood on the growth of the infecting organism with whose metabolism it has a far more intimate relationship (Chapter I,.

Before attempting to elucidate the nature of the "resistance" built up in the mouse by p-aminobenzoic acid, it was necessary to confirm on larger numbers that preliminary injections of p-aminobenzoic acid do in fact increase the number of survivors, when mice are injected with a lethal dose of B. typhosus. This confirmation is provided by experiment 15, and that a similar beneficial effect can be obtained by oral administration of p-aminobenzoic acid is shown in experiment 16.

Experiment 15. EFFECT OF INJECTIONS OF P-AMINOBENZOIC ACID ON SUBSEQUENTLY INDUCED TYPHOID INFECTION IN MICE.

5 mg. p-aminobenzoic acid (0.25 c.c. of a 2% solution in 1% $NaHCO_3$) was injected subcutaneously at 2-hourly intervals from 8.30 a.m. to 10.30 p.m. for 2 days and then until 10.30 a.m. on the 3rd day, into a test group of 35 mice. A control group of 32 mice was injected at the same time with 0.25 c.c. 1% $NaHCO_3$. All mice were then infected intraperitoneally with one lethal dose of 50 millions B. typhosus. The first signs of illness and the survival period of the mice in each group were noted, the observations being continued for 72 hours.

Continued on next page.

Test Group.

Mouse No.	Incubation Period (in hrs.).	Survival Period (in hrs.
1	8	12
2	10	12
3	10	22
4	12	22
5	12	22
6	12	22
7	12	22
8	22	22
9	22	24
10	22	24
11	22	24
12	22	24
13	22	24
14	22	24
15	22	24
16	22	26
17	22	26
18	22	26
19	22	29
20	22	29
21	-	Survived 72 hours.
22	-	" " "
23	-	" " "
24	-	" " "
25	-	" " "
26	-	" " "
27	-	" " "
28	-	" " "

Continued on next page.

Test Group(Continued).

Mouse No.	Incubation Period (in hrs).	Survival Period (in hrs).
29	-	Survived 72 hours.
30	-	" 72 "
31	-	" 72 "
32	-	" 72 "
33	-	" 72 "
34	-	" 72 "
35	-	" 72 "

	Incubation Period.	Survival Period.
Total	362 hours	460 hours.
Number of mice (n)	20	20
Mean (x)	18.1 hours	23.0 hours.
Range	8-22	12-29
Standard Deviation (σ)	5.4	4.2
Standard error of the mean ($\sigma/\sqrt{n}$)	1.2	0.94
$x \pm 2(\sigma/\sqrt{n})$	15.7-20.5	21.1-24.9

Survivals 15 out of 35 = 43%.

Control Group. Experiment 15.

Mouse No.	Incubation Period (in hours).	Survival Period (in hours).
1	5	10
2	6	10
3	6	11
4	6	11
5	10	12
6	10	12
7	10	21
8	10	21
9	12	21
10	12	21
11	12	21
12	12	21
13	12	21
14	12	21
15	12	21
16	12	21
17	19	21
18	19	21
19	19	21
20	19	21
21	19	21
22	19	21
23	19	21
24	19	21
25	22	24
26	24	48
27	24	48
28	survived 72 hours	survived 72 hours
29	" 72 "	" 72 "
30	" 72 "	" 72 "
31	" 72 "	" 72 "
32	" 72 "	" 72 "

	Incubation Period.	Survival Period.
Total	381 hours	564 hours.
Number of mice (n,	27	27
Mean (x)	14.1	20.9
Range	5-24	10-48
Standard deviation (σ(	5.6	8.7
Standard error of the mean ($\sigma/\sqrt{n}$,	1.1	1.7
$x \pm 2(\sigma/\sqrt{n}$,	11.9-16.3	17.5-24.3

Survivals 5 out of 32 = 16%.

Standard error of the difference between the survival percentages of test and control groups = 10.6

Experiment 16. EFFECT OF ORAL ADMINISTRATION OF P-AMINOBENZOIC ACID ON SUBSEQUENTLY INDUCED TYPHOID INFECTION IN MICE.

Approximately 50 mg. of p-aminobenzoic acid was fed daily to each of a test group of 40 mice by adding 1 part to 100 parts of their food. The average quantity of food consumed per mouse was first determined and found to be ± 5 g. After 2 weeks these mice plus a control group of 40 mice, kept on the same diet without p-aminobenzoic acid and under the same conditions for the same time, were infected intraperitoneally with 1 lethal dose, i.e. 50 millions of B. typhosus.

Test Group.

Mouse No.	Uncubation Period (in hours).	Survival Period (in hours).
1	8	12
2	10	12
3	10	18
4	10	18
5	10	18
6	12	18
7	12	18
8	12	18
9	18	18
10	18	18
11	18	18
12	18	18
13	18	18
14	18	18
15	18	18
16	18	18
17	18	18
18	18	22
19	18	22
20	18	22
21	20	22
22	20	24
23	20	24
24	20	24
25	24	26
26	26	29
27	30	35
28	-	survived 72 hours
29	-	"
30	-	"

Test Group (Continued).

Mouse No.	Incubation Period (in hours).	Survival Period (in hours).
31	-	Survived 72 hours
32	-	"
33	-	"
34	-	"
35	-	"
36	-	"
37	-	"
38	-	"
39	-	"
40	-	"

	Incubation Period.	Survival Period.
Totals	460 hours	544 hours.
No. of mice (n)	27	27
Mean (x)	17.0 hours	20.1 hours
Range	8-30 hours	12-35 hours.
Standard Deviation (σ)	5.2	4.9
Standard errof of the mean ($\sigma/\sqrt{n}$)	1.0	0.94
$x \pm 2(\sigma/\sqrt{n})$	15.0-19.0	18.2-22.0

Survivals 13 out of 40 = 33%.

Control Group.

Mouse No.	Incubation Period. (in hours)	Survival Period (in hours)
1	4	8
2	8	10
3	8	12
4	8	12
5	8	12
6	8	12
7	12	18
8	12	18
9	12	18
10	12	18
11	12	18
12	12	18
13	12	18
14	12	18
15	12	18
16	12	18
17	18	18
18	18	18
19	18	18
20	18	18

Continued on next page.

Control Group (Continued)

Mouse No.	Incubation Period. (in hours).	Survival Period. (in hours).
21	18	18
22	18	18
23	18	18
24	18	18
25	18	18
26	18	18
27	18	18
28	18	18
29	18	18
30	18	18
31	18	18
32	18	24
33	18	24
34	18	24
35	22	26
36	-	Survived 72 hours.
37	-	"
38	-	"
39	-	"
40	-	"

	Incubation Period.	Survival Period.
Totals	510 hours	614 hours.
Number of mice (n)	35	35
Mean (x)	14.6 hours	17.5 hours.
Range	4-30 hours	8-35 hours.
Standard deviation (σ)	4.2	3.9
Standard error of the mean ($\sigma/\sqrt{n}$)	0.71	0.66
$x \pm 2(\sigma/\sqrt{n})$	13.2-16.0	16.2-18.8

Survivals 5 out of 40 = 13%.

Standard error of the difference between the survival percentages of test and control groups = 9.6.

Experiment 15 shows that in the test group of 35 mice 20 or 57% became ill and died, the mean period before onset of symptoms in the mice being 18.1 ± 1.2 hours and the survival period after infection with B. typhosus 23.0 ± 0.94 hours. In the control group of 32 mice, 27 or 84% became ill and died, the mean period before onset of symptoms here being 14.1 ± 1.1 hours and the survival period 20.9 ± 1.7 hours. Although the difference between the incubation periods and the survival periods in the two groups is not statistically significant, again the test group appears to fare better. Moreover, the difference (27%) between the survival percentages of the test and control groups is significant, being more than twice its standard error (10.6%.

Experiment 16 confirms these findings when p-aminobenzoic acid is administered orally (ingested with the food) for a period of two weeks. In the test group here of 40 mice, 27 or 67% became ill and died, the signs of illness being noted after a mean period of 17.0 ± 1.0 hours and death occurring after a mean period of 20.1 ± 0.94 hours. The corresponding figures for the control group of 40 mice in which 35 or 87% became ill and died were 14.6 ± 0.71 hours and 17.5 ± 0.66 hours. Again the test group appears to have benefited from the preliminary treatment with p-aminobenzoic acid, and the difference (20%, between the survival percentages of the test and control groups is significant, the standard error being 9.6.

The results of experiments 14, 15 and 16 are summarised in the following table. The difference between the survival

percentages of all the mice treated with p-aminobenzoic acid before infection with B. typhosus, and that of mice either untreated or treated after infection is 4 times the standard error between these percentages which is double the minimum requirement (or twice the standard error.) for significance.

From these experiments it is concluded that quantities of p-aminobenzoic acid far in excess of those produced under physiological conditions have on the mouse beneficial effects similar to its effects in bacteria.

TABLE SUMMARISING RESULTS OF EXPERIMENTS 14, 15 AND 16. EFFECTS OF PRELIMINARY MEDICATION WITH P-AMINOBENZOIC ACID ON TYPHOID INFECTION IN MICE.

	Preliminary treatment with p-aminobenzoic acid before (but not continued after) infection with B. typhosus.							Preliminary p-aminobenzoic acid treatment continued after infection with B. typhosus.								
Experiment Number.	No. of mice in group.	No. of mice showing illness.	Incubation period. (in hours).	No. of mice dying.	Survival period (in hours).	No. of survivors.	Percentage of survivors.	No. of mice in group.	No. of mice showing illness.	Incubation period (in hours).	No. of mice dying.	Survival period (in hours).	No. of survivors.	Percentage of survivors.	Standard error of the difference between the survival percentages of the two groups.	Significance of the difference between survival percentages of the two groups.
14	15	7	16.3 ±1.6	6	22.0 ±4.2	9	60	25	22	14.1 ±1.7	20	26.4 ±2.3	5	20	15.0	Statistically significant.
	Preliminary treatment with p-aminobenzoic acid before infection with B. typhosus.							No preliminary treatment with p-aminobenzoic acid before infection with B. typhosus.								
15	35	20	18.1 ±1.2	20	23.0 ±0.94	15	43	32	27	14.1 ±1.1	27	20.9 ±1.7	5	16	10.6	Statistically significant.
16	40	27	17.0 ±1.0	27	20.1 ±0.94	13	33	40	35	14.6 ±0.71	35	17.5 ±0.66	5	13	9.6	Statistically significant.
Total	90					37	41.1	97					5	15.5	6.4	Statistically significant.

EFFECT OF P-AMINOBENZOIC ACID ON EXPERIMENTAL TUBERCULOSIS IN GUINEA-PIGS.

In the previous section experimental evidence that p-aminobenzoic acid increases the resistance of mice to infection with typhoid bacilli has been detailed. Since, however, B. typhosus causes in mice a rapidly fatal septicaemia, it was felt that a more chronic disease might provide confirmatory and clearer evidence of this beneficial effect of p-aminobenzoic acid. Tuberculous infection in guinea-pigs was selected for study, since it is generally accepted that B. tuberculosis produces a progressive disease in the guinea-pig with no evidence of any naturally-occurring immunity. Any observable effect of the progress of the disease is, therefore, more likely to be significant.

Two groups of five guinea-pigs were inoculated with 200 millions B. tuberculosis which is about one-fifth the dose required to kill a guinea-pig in 6 weeks. One group was then treated with p-aminobenzoic acid by its addition to the diet, the other group serving as a control. Weights and rectal temperatures were recorded daily and autopsies performed on each animal as soon as possible after death. At the same time, the weights of two other non infected groups of five guinea-pigs kept under the same conditions as the two preceding groups, one with p-aminobenzoic acid in its diet and the other without, were also recorded. The results are summarised in the table and graphs of experiment 17.

Experiment 17. EFFECT OF P-AMINOBENZOIC ACID ON TUBERCULOSIS IN GUINEA-PIGS.

Twenty guinea-pigs, 300-350 g. in weight, were divided into 4 groups, each containing 5 animals. All 4 groups were fed on a bran and oats diet of which they consumed about 25-30 g. daily, plus daily offerings of lettuce leaves. At the outset of the experiment, Groups A and B were inoculated intramuscularly with 200 millions B. tuberculosis from a 14-day culture on Petragnani's and Loewenstein's media. This dose was approximately 1/5 the dose estimated to kill a 300-350 g. guinea-pig in 6 weeks. Groups A and C had 0.4% p-aminobenzoic acid added to their diet so that each guinea-pig in these groups would receive 100-120 mg. p-aminobenzoic acid daily. All animals were weighed daily and their rectal temperatures taken. The rectal temperatures are not detailed since none of the animals showed marked pyrexia nor could the degree of pyrexia be correlated with the apparent progress of the disease.

In Table I, the weekly maximum weights of all the guinea-pigs in the four groups are recorded.

In Table II the weekly maximum rectal temperature of the animals in Groups A and B are recorded. Daily rectal temperatures were not recorded in Groups C and D but the normal rectal temperature of the guinea-pigs was found to be between 100.5° F. and 102.5°F.

In Table III the period of survival and autopsy findings of all the guinea-pigs in Groups A and B are recorded.

In Graph I, the percentage increase in weights of infected animals treated with p-aminobenzoic acid (Group A)

are compared with non-infected animals under the same conditions (Group C).

In Graph II the percentage increase in weights of infected untreated animals (Group B) are compared with non-infected animals under the same conditions (Group D).

TABLE I. WEEKLY MAXIMUM WEIGHTS IN GRAMS. OF GUINEA-PIGS IN GROUPS A.B.C AND D.

	Group A.					Group B.					Group C.					Group D.				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Commencement of Experiment	380	330	325	300	330	360	355	340	310	325	365	345	345	385	330	325	370	345	330	315
1st week	405	350	345	320	360	360	370	360	330	365	390	380	365	410	350	340	405	410	355	315
2nd "	410	340	350	325	360	380	370	360	350	370	440	400	410	450	385	425	435	450	385	350
3rd "	395	360	370	330	390	415	340	345	330	365	445	410	405	460	390	390	460	475	390	390
4th "	410	385	380	330	385	440	375	330	340	355	470	425	420	435	410	415	480	475	405	415
5th "	415	400	405	330	395	440	360	345	340	370	505	435	450	465	445	460	510	525	435	435
6th "	420	415	405	325	390	460	375	350	360	380	550	455	475	500	470	480	525	580	505	470
7th "	410	420	425	340	405	470	375	350	365	385	570	500	495	525	490	490	565	610	495	475
8th "	450	440	445	350	425	Died	400	355	375	390	595	530	520	540	505	520	580	625	495	495
9th "	430	430	450	350	425	-	390	350	365	400	615	550	525	555	515	540	605	650	510	505
10th "	455	440	460	350	425	-	415	350	375	425	680	595	590	600	555	605	655	715	560	555
11th "	465	435	455	350	425	-	410	365	375	450	690	625	610	625	585	580	685	745	580	590
12th "	475	435	470	345	445	-	390	370	355	430	730	670	650	680	620	620	710	785	615	605
13th "	460	440	455	360	425	-	370	390	345	440	705	635	645	645	620	625	700	800	610	600
14th "	500	430	470	370	430	-	355	400	345	450	710	650	645	660	625	620	720	800	620	600
15th "	450	400	440	355	420	-	Died	425	325	435	715	650	665	665	640	640	720	800	630	620
16th "	435	395	465	360	415	-	-	445	325	425	705	675	675	700	650	650	730	830	645	625
17th "	410	390	450	325	395	-	-	435	330	385	Observations not continued.									
18th "	380	385	435	325	340	-	-	395	Died	345										
19th "	Died	375	420	295	Died	-	-	390	-	Died										
20th "	-	355	400	280	-	-	-	370	-	-										
21st "	-	345	395	Died	-	-	-	360	-	-										
22nd "	-	355	400	-	-	-	-	350	-	-										
23rd "	-	355	330	-	-	-	-	355	-	-										
24th "	-	330	Died	-	-	-	-	335	-	-										
25th "	-	Died	-	-	-	-	-	Died	-	-										

TABLE II. WEEKLY MAXIMUM RECTAL TEMPERATURES IN DEGREES FAHRENHEIT OF GUINEA-PIGS IN GROUPS A AND B.

	Group A.					Group B.				
	1	2	3	4	5	1	2	3	4	5
Commencement of Experiment	100.8	101.8	101.4	102.2	101.4	100.6	101.2	101.4	101.6	101.0
1st week	102.2	102.4	102.6	102.8	101.6	102.2	101.6	102.4	101.8	101.6
2nd "	102.8	103	103.4	102.8	102.8	103.2	103	103.2	103.4	103
3rd "	102.2	102.8	103	103.4	102.6	103.4	103.8	103.4	102.6	102.6
4th "	102.6	102.2	103	103	102.6	103.4	102.8	103.4	102.6	102.8
5th "	102.2	102.6	102.4	103	103.2	103.6	102.2	103.2	102.8	102.6
6th "	102.2	102.2	102.4	102.8	102.8	102.6	102.8	102.8	102.8	102.4
7th "	102.4	102.0	102.6	102.8	103	102.8	102.6	103.2	102.8	102.4
8th "	102.2	102.2	101.6	102.8	102	Died	102.2	103	102.6	102.6
9th "	102	102.4	101.8	102.8	102.6	-	102.4	103	102.6	102.6
10th "	102	102.6	103	103.4	102.4	-	102.8	103.6	102.4	102.6
11th "	102.4	102.6	102.8	103	102.2	-	102.8	103	102.6	102.8
12th "	102.4	102.8	102.2	102.6	102.6	-	102.6	103	102.6	102.6
13th "	102.2	103	102.2	103	102.4	-	102	103.4	103.2	103.2
14th "	102.8	103.6	101.8	102.6	102.4	-	102.6	103	103	103.8
15th "	103	103.8	102.8	103.2	103.4	-	Died	102.4	103	103.4
16th "	103.6	103.6	103.4	102.4	103	-	-	102.4	103	103.2
17th "	103	103.8	102.6	102.6	102.6	-	-	103.2	102.4	103
18th "	101.8	103.4	102.6	103.4	101.4	-	-	103.2	Died	102.8
19th "	Died	103.2	102.8	102.6	Died	-	-	103.4	-	Died
20th "	-	103.8	102.4	102.2	-	-	-	102.4	-	-
21st "	-	102.2	101	Died	-	-	-	102.2	-	-
22nd "	-	102.2	101.6	-	-	-	-	102.4	-	-
23rd "	-	101.8	101.2	-	-	-	-	102.2	-	-
24th "	-	102.2 Died	Died	-	-	-	-	101.4 Died	-	-

TABLE III. PERIODS OF SURVIVAL AND AUTOPSY FINDINGS OF GUINEA-PIGS IN GROUPS A AND B.

AUTOPSY FINDINGS.

roup A.	1.	2.	3.	4.	5.
urvival ime af- er in- fection.	122 days	165 days.	154 days	136 days	121 days
ite of njec- ions in eft high.	Small area of caseation; tubercle bacilli present in smear from caseous material.	Very small area of caseation; tubercle bacilli present in smear.	Very small area of caseation; tubercle bacilli present in smear from caseous material.	Small area of caseation; tubercle bacilli present in smear.	Small area of caseation; tubercle bacilli not detected in smear.
ymph land in eft roin.	Enlarged and slightly caseous; tubercle bacilli present in smear.	Slightly enlarged; no caseation. tubercle bacilli present in smear.	Hyperaemic, enlarged, but not caseous; tubercle bacilli present in smear.	Hyperaemic, enlarged, but not caseous; tubercle bacilli not detected in smear.	Enlarged but not caseous; tubercle bacilli not detected in smear.
ymph land n ight roin.	Enlarged, hyperaemic but not caseous; tubercle bacilli not detected in smear.	Slightly enlarged; no caseation. Tubercle bacilli present in smear.	Normal; tubercle bacilli not detected in smear.	Enlarged; no caseation; tubercle bacilli not detected in smear.	Enlarged; no caseation; tubercle bacilli not detected in smear.
iver	Grossly enlarged and studded with miliary tubercles; tubercle bacilli not detected in smear.	Slightly enlarged; studded with fine miliary tubercles; Tubercle bacilli present in smear.	Slightly enlarged and studded with fine miliary tubercles; tubercle bacilli present in smear.	Enlarged; several tubercles scattered throughout; tubercle bacilli not detected in smear.	Enlarged and studded with miliary nodules tubercle bacilli not detected in smear.
pleen.	Enlarged; with several coarse nodules present; tubercle bacilli not detected in smear.	Grossly enlarged with large areas of necrosis; tubercle bacilli present in smear.	Enlarged and studded with nodules; tubercle bacilli not detected in smear.	Enlarged with a few scattered tubercles; tubercle bacilli not detected in smear.	Enlarged with several scattered nodules; tubercle bacilli not detected.

TABLE III (CONTINUED). PERIODS OF SURVIVAL AND AUTOPSY FINDINGS OF GUINEA-PIGS IN GROUPS A AND B.

AUTOPSY FINDINGS.

Group A.	1.	2.	3.	4.	5.
Peritoneal cavity.	No exudate.	Blood-stained exudate.	No exudate.	No exudate.	Clear, straw-coloured exudate.
Para-aortic lymph glands.	Enlarged but not caseous.	Enlarged.	Not enlarged.	Not enlarged.	Enlarged and hyperaemic.
Lungs.	Several fine tubercles scattered throughout both lungs; tubercle bacilli present in smear.	Scattered nodules throughout both lungs; tubercle bacilli present in smear.	A few nodules in both lungs; tubercle bacilli present in smear.	A few nodules in both lungs, but tubercle bacilli not detected in smear.	A few nodules in both lungs; tubercle bacilli present in smear.

Mean survival time after infection = 139.6 days.

TABLE III CONTINUED.

AUTOPSY FINDINGS.

Group B.	1.	2.	3.	4.	5.
Survival time after infection.	46 days	95 days	160 days	117 days	124 days.
Site of injection in left thigh.	Large caseous area in muscle; tubercle bacilli present in smear.	Small caseous areas in muscle; tubercle bacilli present in smear.	Small caseous area in muscle; tubercle bacilli present in smear.	Extensive caseation of muscle; tubercle bacilli present in smear.	Large caseous area in muscle; tubercle bacilli present in smear.
Lymph gland in left groin.	Enlarged and caseous; tubercle bacilli present in smear.	Enlarged and caseous; tubercle bacilli present in smear.	Enlarged and hyperaemic; tubercle bacilli not detected in smear.	Enlarged and caseous; tubercle bacilli present in smear.	Enlarged and caseous; tubercle bacilli present in smear.
Lymph gland in right groin.	Enlarged and hyperaemic; tubercle bacilli not detected in smear.	Enlarged and hyperaemic; tubercle bacilli not detected in smear.	Enlarged and hyperaemic; tubercle bacilli not detected in smear.	Enlarged and hyperaemic; tubercle bacilli not detected in smear.	Enlarged and hyperaemic; tubercle bacilli not detected in smear.
Liver	Grossly enlarged and completely studded with miliary tubercles; tubercle bacilli not detected in smear.	Grossly enlarged and studded with numerous coarse nodules; tubercle bacilli not detected in smear.	Enlarged and studded with large caseous nodules; tubercle bacilli not detected in smear.	Grossly enlarged and studded with caseous nodules; tubercle bacilli present in smear.	Grossly enlarged and studded with nodules; tubercle bacilli not detected in smear.
Spleen.	Enlarged and studded with miliary tubercles; tubercle bacilli not detected in smear.	Grossly enlarged and studded with numerous coarse nodules; tubercle bacilli not detected in smear.	Enlarged with large necrotic areas; tubercle bacilli not detected in smear.	Very grossly enlarged and studded with nodules; tubercle bacilli present in smear.	Grossly enlarged and congested, with numerous nodules; tubercle bacilli not detected in smear.

TABLE III CONTINUED.

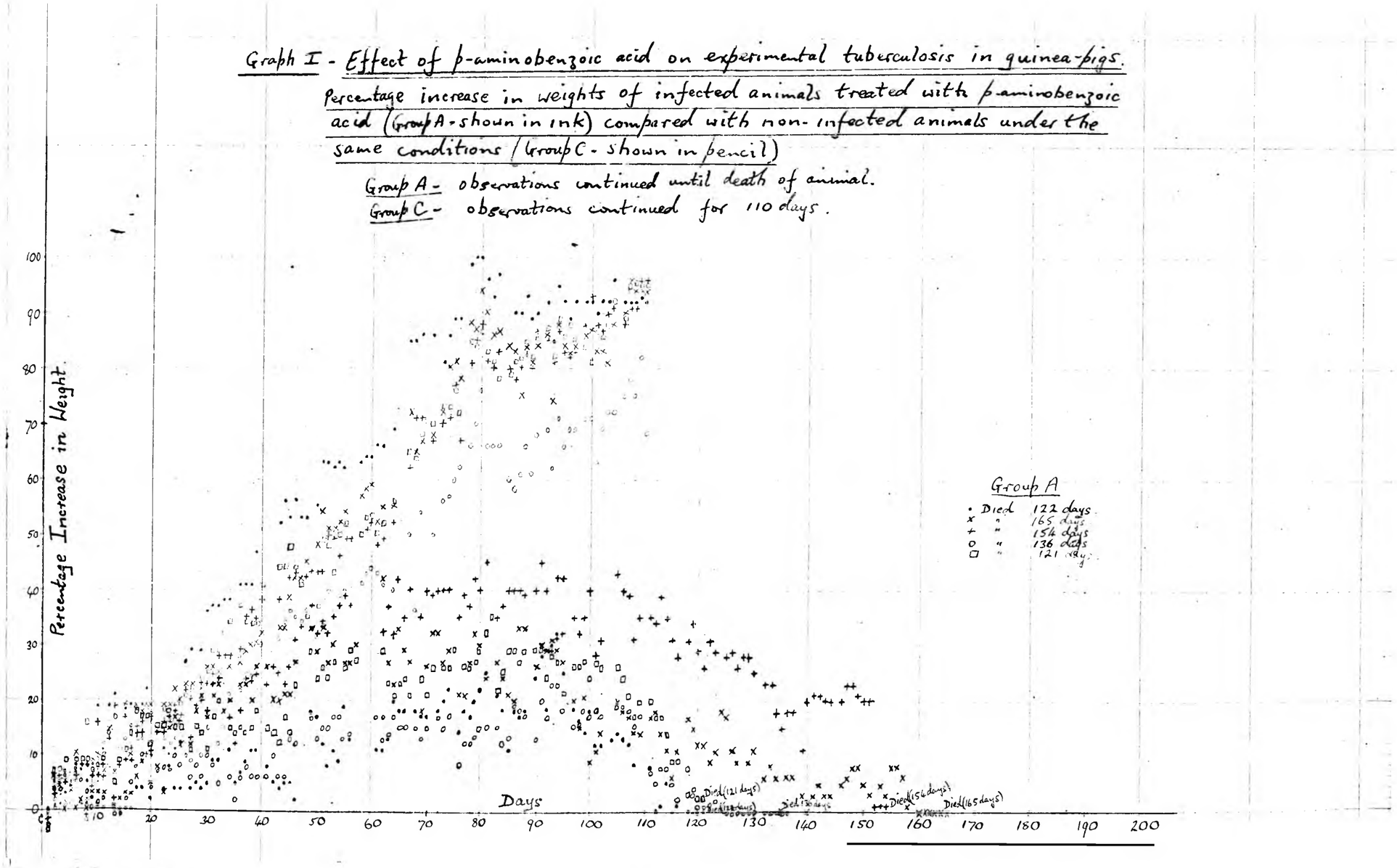
Group B.	1.	2.	3.	4.	5.
Peritoneal cavity.	Excess of blood-stained exudate present; tubercle bacilli not detected in smear.	No exudate.	No exudate.	Excess of blood-stained exudate; tubercle bacilli not detected in smear.	No exudate.
Para-aortic lymph glands.	-	-	Not enlarged	Enlarged and caseous.	Enlarged and caseous.
Lungs.	Numerous tubercles scattered throughout both lungs; tubercle bacilli not detected in smear.	Several tubercles scattered throughout both lungs; tubercle bacilli present in smear.	Numerous tubercle scattered through both lungs. Numerous tubercle bacilli present in smear.	Numerous tubercles scattered throughout both lungs; tubercle bacilli present in smear.	Congested with petechial haemorrhages; tubercle bacilli not detected in smear.

Mean survival time after infection = 108.4 days.

Graph I - Effect of p-aminobenzoic acid on experimental tuberculosis in guinea-pigs.

Percentage increase in weights of infected animals treated with p-aminobenzoic acid (Group A - shown in ink) compared with non-infected animals under the same conditions (Group C - shown in pencil)

Group A - observations continued until death of animal.
Group C - observations continued for 110 days.



guinea-pig, it does produce some resistance in this animal"

It will be seen that treatment of tuberculotic guinea-pigs with p-aminobenzoic acid while not preventing development of the infection prolongs their period of survival (from 108.4 to 139.6 days) and diminishes the severity of the disease since the treated group as a whole shows a greater increase in weight than does the untreated group, although this increase falls considerably short of that of normal guinea-pigs under the same conditions. The data recorded for the two non-tuberculous control groups also indicate that p-aminobenzoic acid per se added to the diet is not responsible for this increase since the weight changes of these two groups are on the whole similar. Nevertheless, at autopsy the recorded findings do not show any significant difference between the treated and untreated groups although the impression was gained that the untreated guinea-pigs showed a greater number of lesions and a more widespread involvement of organs than did the treated guinea-pigs, and this despite the fact that the longer survival period in the treated groups should if anything have permitted more extensive lesions to develop.

On the whole, therefore, the conclusion appears permissible that whereas p-aminobenzoic acid in large doses has no bacteriostatic action on B. tuberculosis in the guinea-pig, it does produce some resistance in this animal after it has been experimentally infected with tuberculosis. These results confirm the previous findings that p-aminobenzoic acid in mice increases their resistance to infection with typhoid bacilli.

The two most probable interpretations of the effect of p-aminobenzoic acid when added to the diet or injected subcutaneously which suggest themselves are:-

(a) the effect may be due to an alteration in the defense mechanism of the host,

(b) the effect may be due to an interference in the in vivo cultural medium of the parasite.

(a) necessitates (i) a study of the development of antibodies and their circulation in the blood stream during administration of p-aminobenzoic acid and

(ii) a study of any stimulating effects of p-aminobenzoic acid on the phagocytic activity of the reticulo-endothelial system.

The possibility of (b, appears to be ruled out by the observation (p.147, that this effect of p-aminobenzoic acid is not seen when its exhibition is continued after infection with B. typhosus. A study, therefore, of antibody formation and reticulo-endothelial activity during feeding with p-aminobenzoic acid was made.

EFFECT OF P-AMINOBENZOIC ACID ON THE ACTIVITY OF THE RETICULO-ENDOTHELIAL SYSTEM.

Yeomans, Snyder, Murray, Zarafonetis and Ecke (1944) investigated the possible therapeutic effect of p-amino benzoic acid in louse-borne typhus fever in man following a report by Snyder, Maier and Anderson (1942) that the mortality of experimental murine typhus in white mice was reduced by the oral administration of the drug. The latter workers found that large amounts of p-aminobenzoic acid were required to demonstrate such an action, which even

occurred, though to a lesser extent, when the oral administration was started one or two days after the inoculation of rickettsiae. Yeomans and his co-workers confirmed this effect in human louse-borne typhus fever and concluded that when treatment was started in the first week of illness, the clinical course of the patients who received p-aminobenzoic acid was much less severe than that of patients not so treated.

Since the multiplication of rickettsiae occurs in the cells of the reticulo-endothelial system, the suggestion was put forward by these workers that p-aminobenzoic acid exerts its effect by stimulating in some way the cells of this system (p.6). In the previous section this possibility was independently suggested by the results obtained when studying the effects of p-aminobenzoic acid on experimental typhoid infection in mice and experimental tuberculosis in guinea-pigs. It was, therefore, decided to test the effect of p-aminobenzoic acid on the activity of the reticulo-endothelial system.

As is well-known, such a study is fraught with great practical difficulties. After a consideration of various techniques discussed by Maher (1944), including the use of thorotrast, which is a stabilised emulsion of thorium dioxide containing 25% by volume, and 19-20% by weight, of thorium dioxide, with from 16-19% of protective colloid, it was decided to use thorium dioxide suspensions and study its deposition in the spleen, both by successive X-ray pictures and by histological examination. After intravenous

injection, thorotrast is deposited in the spleen and liver, and when sufficient has been injected to "block" the reticulo-endothelial system, storage in the spleen appears to be complete within three hours after injection, as observed by X-rays. Further, during the following 72 hours there is no significant shift of the stored material. Since thorotrast was unfortunately not procurable here, various aqueous suspensions of thorium dioxide were prepared by grinding the powder in a morter, and stabilised by the addition of gum acacia. Since 25% suspensions of thorium dioxide were found to be too thick for injection into the femoral vein of the rat, 10% suspensions were finally used.

After being fed for 2 weeks on a diet containing 1% p-aminobenzoic acid, a group of 10 rats was injected with 10% thorium dioxide suspension, 7 rats receiving 0.25 ml., and 3 rats 0.5 ml. At the same time a control group of 10 rats kept under the same conditions as the first group but without p-aminobenzoic acid addition to their diet, were injected with the thorium dioxide suspension in the same proportions. All injections were made into the femoral vein after anaesthetisation of the rat. Larger volumes were not injected since in trial experiments such quantities of the suspension of thorium dioxide were found to produce toxic effects which usually ended fatally. Two hours after injection all the animals were X-rayed * and the plates examined for a concentration of the radio-opaque thorium in the splenic area. Further X-ray plates were taken after 24 hours and again after 48 hours.

The results in all cases were quite negative, neither

* See page 171.

the test nor the control groups showing sufficient absorption of the thorium by the reticulo-endothelial cells of the spleen to be demonstrable radiologically.

Since the possibility existed that insufficient thorium had been injected for radiological examination, all the animals were killed after 48 hours and the spleens removed for sectioning. * The sections were stained with haematoxylin and eosin and examined microscopically by dark-ground illumination. The eosin-stained cells under the dark-ground illumination provided a greenish, almost fluorescent background, against which the highly-refractile, violet-coloured particles of thorium dioxide could easily be observed. Although in all the spleens examined, the amount of thorium dioxide phagocytosed by the reticulo-endothelial cells was considerable, it was approximately the same in both test and control groups.

The only conclusion, therefore, which these observations permit is that p-aminobenzoic acid, as judged by the techniques used, has no effect on the activity of the reticulo-endothelial system. It must be conceded, however, that the relative crudeness of the methods available for investigating the functioning of the reticulo-endothelial system precludes any really accurate assessment of this problem.

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* The radiological investigation was carried out in the Radiology Department of the Johannesburg General Hospital, and the sections of the spleens prepared by the Histology Department, University of the Witwatersrand. To both these departments I wish to express my sincere thanks for invaluable assistance.

Photographs of the X-ray plates and photomicrographs of the splenic sections have not been prepared since no differences were observed between the test and control groups.

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EFFECT OF P-AMINOBENZOIC ACID ON THE DEVELOPMENT OF ANTIBODIES.

It is generally accepted that the reticulo-endothelial system is concerned with humoral immunity no less than with cellular immunity. Recently studies indicating that the lymphocyte elaborates antibodies have also appeared.

With the hope of elucidating the mechanism by which p-aminobenzoic acid increased the resistance of animals to infection, its effect on antibody formation was investigated. This was done by following the development of circulating antibodies after injection of dead typhoid bacilli into two groups of rabbits, one of which was being treated with p-aminobenzoic acid orally, the other group serving as a control. At the same time, total and differential leucocyte counts were done so as to determine whether any apparent changes in the number of lymphocytes occurred. All counts were done at the same time of day.

The results are shown in experiment 18. After two control observations had been made at weekly intervals 0.25 ml. of a killed culture of B. typhosus H9Cl was injected into the test and control groups and the titre of "O" and "H" antibodies determined every week as well as the total numbers of leucocytes and lymphocytes. Each rabbit in the test group received daily by mouth about 400 mg. of p-amino-benzoic acid. At the end of 11 weeks, the experiment was

terminated, since by this time it was apparent that there were no differences between the test and control groups. It must, therefore, be concluded that p-aminobenzoic acid does not exert its effect on resistance to infection by stimulating antibody production, or the number or types of circulating leucocytes.

Experiment 18. EFFECT OF P-AMINOBENZOIC ACID ON THE DEVELOPMENT OF ANTIBODIES.

Two groups, each containing 3 white Angora rabbits were observed for two weeks. At the end of each week, blood was collected from the ear veins, the serum separated and the titre of B. typhosus "H" and "O" antibodies determined by the method of doubling dilutions — starting with a 1:5 dilution of serum to which was added an equal volume of B. typhosus "H" or "O" antigen, so that the first final dilution of serum tested was 1:10. The dilution of serum was then doubled by adding an equal volume of saline so that the final dilution then became 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, 1:1280, 1:2560 until 1:5120 which was the highest dilution tested. For "H" agglutination, the serum-antigen mixture was incubated at 52°C. for 2 hours, and for "O" agglutination at 52°C. for 24 hours. The total white cell counts were done in the usual way. For the differential cell counts, thin blood smears were stained with Leishman's stain and at least 400 cells counted.

Both groups of rabbits were then injected intravenously with 0.25 c.c. of a killed B. typhosus H901 culture which stimulates the production of both "H" and "O" antibodies in the serum. The test group of rabbits were then treated orally with 5 c.c. of 4% p-aminobenzoic acid (= 200 mg.) in 4% sodium bicarbonate twice daily by means of a capillary pipette, and the antibody titres, total and differential white cell counts done at weekly intervals as above. The control group were treated with 5 c.c. 4% sodium bicarbonate twice daily and similarly investigated.

[At end of 5th week rabbit No. 6 was found dead. On the evening previous to death its condition was apparently normal and, at autopsy, no cause for death could be found.]

Titre of "H" Antibodies.

	Test Group.			Control Group.		
Week.	Rabbit 1.	Rabbit 2.	Rabbit 3.	Rabbit 4.	Rabbit 5.	Rabbit 6.
1	< 1:10	< 1:10	< 1:10	< 1:10	< 1:10	< 1:10
2	< 1:10	< 1:10	< 1:10	< 1:10	< 1:10	< 1:10
Injection of 0.25c.c. B. typhosus H901.						
3	1:40	1:160	1:320	1:640	1:160	1:640
4	1:320	1:2560	1:2560	1:2560	1:2560	1:2560
5	1:160	1:1280	1:2560	1:1280	1:2560	1:640
6	1:320	1:640	1:640	1:320	1:320	-
7	1:640	1:1280	1:2560	1:640	1:1280	-
8	1:640	1:1280	1:320	1:1280	1:1280	-
9	1:160	1:640	1:640	1:640	1:640	-
10	1:320	1:640	1:640	1:640	1:640	-
11	1:320	1:1280	1:1280	1:1280	1:2560	-

Titre of "O" Antibodies.

	Test Group.			Control Group.		
Week.	Rabbit 1.	Rabbit 2.	Rabbit 3.	Rabbit 4.	Rabbit 5.	Rabbit 6.
1	1:40	< 1:10	< 1:10	< 1:10	1:40	1:20
2	1:20	< 1:10	< 1:10	< 1:10	1:20	1:20
Injection of 0.25 c.c. B. typhosus H901.						
3	1:640	1:640	1:640	1:640	1:640	1:640
4	1:1280	1:640	1:1280	1:1280	1:1280	1:640
5	1:1280	1:640	1:1280	1:320	1:640	1:320
6	1:1280	1:1280	1:640	1:640	1:640	-
7	1:1280	1:640	1:640	1:640	1:640	-
8	1:640	1:320	1:320	1:320	1:320	-
9	1:1280	1:640	1:640	1:320	1:640	-
10	1:640	1:320	1:320	1:160	1:160	-
11	1:640	1:640	1:640	1:320	1:320	-

Total White Cell Counts/c.mm.

	Test Group.			Control Group.		
Week.	Rabbit 1.	Rabbit 2.	Rabbit 3.	Rabbit 4.	Rabbit 5.	Rabbit 6.
1	10,000	8,800	10,100	12,800	18,500	10,900
2	10,500	5,100	7,800	4,000	6,500	5,200
Injection of 0.25 c.c. B. typhosus H901.						
3	6,600	4,500	7,600	11,000	3,000	5,600
4	7,500	4,100	7,000	7,400	5,500	7,400
5	7,300	3,400	4,900	8,000	5,300	6,900
6	9,400	3,200	5,700	5,500	5,900	-
7	2,800	2,900	6,600	10,500	3,900	-
8	5,100	4,900	6,500	6,100	4,200	-
9	6,500	4,200	6,100	7,200	4,900	-
10	6,400	5,800	12,200	8,600	7,000	-
11	7,100	6,500	7,900	7,600	5,600	-

Percentage and Total Lymphocyte Count/c.mm.

Percentages reported to nearest whole number and Totals to nearest hundred.

	Test Group.			Control Group.		
Week.	Rabbit 1.	Rabbit 2.	Rabbit 3.	Rabbit 4.	Rabbit 5.	Rabbit. 6.
1	48% 4,800	54% 4,700	62% 6,200	46% 5,900	83% 15,400	53% 5,700
2	47% 4,900	50% 2,500	80% 6,200	82% 3,300	71% 4,600	51% 2,700
Injection of 0.25 c.c. B. typhosus H901.						
3	61% 4,000	68% 3,000	62% 4,700	34% 3,700	61% 1,800	81% 4,500
4	44% 3,300	49% 2,000	54% 3,800	47% 3,500	64% 3,500	70% 5,200
5	26% 1,900	54% 1,900	64% 3,100	52% 4,200	56% 2,900	47% 3,300
6	41% 3,800	54% 1,700	54% 3,100	53% 2,900	63% 3,700	-
7	37% 1,000	73% 2,100	54% 3,500	61% 6,400	63% 2,400	-
8	37% 1,900	56% 2,700	73% 4,700	66% 4,000	57% 2,400	-
9	43% 2,800	50% 2,100	71% 4,300	-	57% 2,800	-
10	70% 4,500	69% 4,000	69% 8,400	68% 5,900	55% 3,900	-
11	48% 3,400	29% 1,900	28% 2,200	50% 3,800	68% 3,800	-

The above studies of reticulo-endothelial activity both as regards phagocytosis and the formation of antibodies, therefore, appear to indicate that p-aminobenzoic acid does not exert its effect by the stimulation of a cellular or a humoral immunity. Since it also does not appear to act by reducing the ability of pathogenic bacteria to develop in the tissues or body fluids of the host animal, it must be concluded that at present the mechanism by which p-aminobenzoic acid increases resistance to infection remains unknown.

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SUMMARY.

Large quantities of p-aminobenzoic acid were found to inhibit growth on MacConkey's medium of the typhoid-paratyphoid group of organisms to a larger extent than B. coli and other gram-negative, pathogenic, intestinal bacteria. Since sulphapyridine added to the same medium showed a reverse order of inhibition, affecting the growth of the dysenteric organisms most markedly, and since this drug also has a well-marked therapeutic action in bacillary dysentery, it appeared worth while to determine whether the selective inhibition of the growth of B. typhosus on MacConkey's medium by p-aminobenzoic acid could be duplicated in in vivo experiments.

The therapeutic effect of p-aminobenzoic acid in experimentally produced typhoid infections in mice was, however, found to be disappointing, although it was noted that administration, either orally or by injection, of p-aminobenzoic acid to mice for a few days before infecting them with B. typhosus apparently diminished their susceptibility to infection. From this it has been concluded that quantities of p-aminobenzoic acid far in excess of those present under physiological conditions have beneficial effects on the resistance of the mouse, and that the concentrations which can be reached in vivo without toxic effects have a stimulating effect both on the animal organism and the infecting bacillus. When, therefore, p-aminobenzoic acid is presented to the tissue cells of the mouse before infection with B. typhosus, it increases the mouse's resistance to infection, but when infection has already occurred, this effect is completely overshadowed by

the much greater stimulating effect of p-aminobenzoic acid on the growth of the infecting organism with whose metabolism it has a far more intimate relationship. Similar effects were noted when tuberculous guinea-pigs were treated with p-aminobenzoic acid in large doses, although there were no demonstrable bacteriostatic effects on the tubercle bacillus itself.

This action of p-aminobenzoic acid has been shown to be due in some way to a stimulation of the natural immunity or "resistance" of the animal, and not to an adverse effect on the *in vivo* growth of the infecting bacillus. Attempts to elucidate the mechanism of its action have, however, been unsuccessful, the experiments described indicating that p-aminobenzoic acid does not exert its effect by the stimulation of an increased cellular or humoral immunity, since it apparently neither increases the phagocytic activity of the reticulo-endothelial system nor the titre of antibodies produced by injection of an antigen. The activity of the reticulo-endothelial system was tested by injecting thorium dioxide suspensions intravenously into p-aminobenzoic acid-treated and control rats, and then following its deposition in the spleens by X-ray and histological studies. It must be conceded, however, that the relative crudeness of the methods available for investigating the functioning of the reticulo-endothelial system precludes any really accurate assessment of this problem. The effect of p-aminobenzoic acid on the activity of antibodies was investigated by following the development of circulating antibodies after

injection of dead typhoid bacilli into two groups of rabbits, one of which was being treated with p-aminobenzoic acid orally, the other group serving as a control. Total and differential leucocyte counts were also done so as to study any differences in the number of circulating lymphocytes since these cells have also been shown to elaborate antibodies.

It is, therefore, concluded that p-aminobenzoic acid stimulates the natural immunity or "resistance" of an animal to disease, but that, at present, the mechanism by which it increases resistance to infection remains obscure.

GENERAL SUMMARY AND CONCLUSIONS.

1. In 1940, Fildes focussed attention on p-aminobenzoic acid by suggesting that it was an essential metabolite for bacteria and that it was normally associated with an enzyme system in the bacterial cell. He propounded the hypothesis, since supported by many workers, that the sulphonamide group of drugs owed their bacteriostatic action to their structural similarity to p-aminobenzoic acid which enabled them to displace it from its enzyme and stop an essential line of metabolism in the bacterial cell. Attempts to identify the enzyme system involved in the utilisation of p-aminobenzoic acid have, however, led to inconclusive results. Various other theories have, therefore, been propounded to explain the action of sulphonamides including modifications of Fildes' hypothesis.

2. The estimation of p-aminobenzoic acid in the studies reported in this thesis has been based on its property of antagonising the sulphonamides, and is a modification of the microbiological method published by McLeod in 1940. It was selected since it is more sensitive than the chemical methods and the reagents are readily obtainable. The method of assay consists in comparing the amount of material which is required to neutralise the inhibitory action of sulphapyridine on Escherichia coli growing in a synthetic medium, with that of a standard solution of p-aminobenzoic acid under identical conditions.

3. From a study of the behaviour of various derivatives of p-aminobenzoic acid under the assay conditions used, the finding of other workers is confirmed that the inactive form, in which most of the p-aminobenzoic acid is excreted in the urine, is p-acetyl-aminobenzoic acid. The suggestion is, however, made that under physiological conditions all the p-aminobenzoic acid is conjugated and excreted either as p-acetylaminobenzoic acid or p-aminobenzoyl-glycuronate.

4. In view of the possible significance of p-aminobenzoic acid as a member of the vitamin B complex, a study of its absorption and excretion in man after feeding minute doses was undertaken. Previous studies in the literature have been concerned with the excretion following relatively large doses of p-aminobenzoic acid. The results obtained suggest that the human organism deals with p-aminobenzoic acid as it would a toxic substance and not as a vitamin. When doses up to 25 mg. were fed, it was found that all the p-aminobenzoic acid was conjugated and rapidly excreted, — completely within 16 hours or less — indicating that the normal liver was able to "detoxicate" up to this quantity at one time. Even with minute doses of 1 mg., p-aminobenzoic acid appeared in the urine in the first specimen collected. p-Aminobenzoic acid was found to be rapidly absorbed — certainly within 8 hours — from the intestine, but the absorption was apparently incomplete.

5. Although it has become very difficult to classify the vitamins as a distinct and well-defined group of compounds, the claims of p-aminobenzoic acid to be regarded as a member of the vitamin B complex are reviewed. Ansbacher reported that the absence of p-aminobenzoic acid from the diet, produced achromotrichia in the rat and defective growth in the chick. Other workers have claimed that it improved lactation in the rat and decreased the percentage of still-births. In man it has been claimed to be an anti-gray hair vitamin and also an anti-sterility vitamin. However, many of these reported effects lack confirmation, and observations by different groups of workers are, in fact, actually conflicting. The evidence that p-aminobenzoic acid produces deficiency symptoms — perhaps the most important criterion of a vitamin — must, therefore, be considered inadequate and inconclusive. Further, any action that it appears to have is believed by most workers to be indirect, and on the intestinal micro-organisms rather than on the animal itself. This explanation accords well with the firmly-established effects of p-aminobenzoic acid on micro-organisms.

6. It is noteworthy that as far as its physiological effectiveness in minute amounts is concerned — another characteristic of vitamins — the reported work shows that the quantities required to remove deficiency effects are far greater than those that could be ingested in a normal diet. It appears legitimate, therefore, to conclude that <u>although p-aminobenzoic acid is a growth-factor for bacteria</u>

yeasts and fungi, it does not fulfil the criteria required of a vitamin given in any of the definitions reviewed in this thesis.

7. The negligible quantities of p-aminobenzoic acid occurring in various foods and the fact that yeast, a micro-organism, is its richest source, suggest that the minute amounts of p-aminobenzoic acid present in various animal tissues and fluids, and finally excreted in the urine, are also derived from micro-organisms, viz. bacterial growth in the large intestine, and not from the food ingested. This hypothesis was tested by studying the content of p-aminobenzoic acid in the urine after producing intestinal bacteriostasis with sulphaguanidine. A marked drop in the excretion of p-aminobenzoic acid was shown to occur, indicating that the traces of p-aminobenzoic acid present in the urine of man can be considered to originate in the intestine where it is produced during the growth and multiplication of the bacterial flora. Confirmation of this hypothesis is provided by the observation that p-aminobenzoic acid can in fact be absorbed from the large intestine.

8. The above findings suggest that p-aminobenzoic acid exerts its major physiological function in animals and man indirectly, by stimulating the intestinal bacteria to produce the several vitamins which they have been shown to synthesize, and whose importance on the mammalian organism is well established.

9. In vitro, large quantities of p-aminobenzoic acid were found to inhibit growth of the typhoid-paratyphoid group of bacteria to a greater extent than other gram-negative intestinal organisms. The therapeutic effect of non-toxic doses of p-aminobenzoic acid in experimentally produced typhoid infections in mice was, however, found to be disappointing. Nevertheless, the administration of large doses of p-aminobenzoic acid before infecting the mice, was apparently of benefit. The interpretation put on these experiments is that p-aminobenzoic acid increases the "resistance" of mice to infection, but that this effect is completely overshadowed by the much greater stimulating effect of p-aminobenzoic acid on the growth of the infecting organism with whose metabolism it has a far more intimate relationship. Similar effects were noted in experimentally-produced tuberculosis in guinea-pigs.

10. The mechanism by which p-aminobenzoic acid stimulates the natural immunity or resistance of an animal to disease remains obscure, since it stimulates neither a cellular nor a humoral immunity. The experiments described indicate that p-aminobenzoic acid apparently increases neither the phagocytic activity of the reticulo-endothelial system nor the titre of antibodies produced by injection of an antigen.

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