STUDIES ON THE ANTIBACTERIAL ACTION OF THE SULFONAMIDE DRUGS

II. The Possible Relation of Drug Activity to Substances Other Than *p*-Aminobenzoic Acid*

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The relation of *p*-aminobenzoic acid to the mode of action of the sulfonamide drugs has been discussed in the preceding paper (1). During the past year the theory has been advanced that sulfapyridine and sulfathiazole prevent bacterial growth by interfering with the functioning of chemically related coenzyme systems. Fildes (2) first called attention to the chemical relation of sulfapyridine to nicotinic acid and of sulfathiazole to thiamin. West and Coburn (3) also noted the similarity of sulfapyridine and nicotinic acid amide and reported *in vitro* experiments with *Staphylococcus aureus* on the basis of which they suggested that sulfapyridine exerts its bacteriostatic effect by interfering with the formation of cozymase from nicotinamide. Dorfman and his associates (4) observed that sulfapyridine inhibited the respiration of "resting" (5) dysentery bacilli and concluded that it acted by disturbing the respiratory function of the chemically related vitamin, nicotinamide. Using pyridine-3-sulfonic acid and its amide, rather than sulfapyridine, McIlwain (6) showed that sulfonic acid derivatives of nicotinic acid, when added to cultures of *Staphylococcus aureus* in a synthetic medium, inhibited the growth promoted by nicotinic acid, nicotinamide, and cozymase.

The attractiveness of the hypothesis that the group attached to the sulfonamide radical interferes with the functioning of certain vitamins and coenzymes is obvious when one considers the structural similarity of the compounds involved. Sulfapyridine, nicotinic acid, nicotinamide, and cozymase each possess a pyridine nucleus. The thiazole ring common to thiamin and cocarboxylase is contained in the sulfathiazole molecule, and sulfadiazine is similarly related to thiamin and cocarboxylase through its pyrimidine radical. Since nicotinic acid, thiamin, and their corresponding coenzymes are known to play important rôles in bacterial metabolism (7), it seems logical to assume that they may be

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concerned in the bacteriostatic action of the chemically related sulfonamide compounds.

To prove the hypothesis, however, it is necessary first to demonstrate that the antisulfonamide effect of the vitamin or its coenzyme is specific in the sense that it antagonizes only the chemically related sulfonamide drug. For example nicotinic acid and cozymase should inhibit sulfapyridine but not sulfathiazole, sulfadiazine, or sulfanilamide. Thiamin and cocarboxylase, on the other hand, should block the action of sulfathiazole and sulfadiazine but should not influence the action of sulfapyridine or sulfanilamide. The experiments reported in the present paper indicate that the antisulfonamide effect of thiamin, nicotinamide, and their respective coenzymes is in no sense specific and is due to stimulation of bacterial growth rather than to a direct antagonistic action upon the sulfonamide drugs. These observations are discussed in relation to the mode of action of the complex derivatives of sulfanilamide, and a tentative explanation is offered for the variations in bacteriostatic potency exhibited by the different sulfonamide compounds.

Material and Methods

Culture Media.—The medium used in all experiments with *Staphylococcus aureus* was one of known chemical composition described by Gladstone (8). The constituents of the medium and the several fractions, which were sterilized separately by autoclaving or filtering, are listed in Table I. All of the labile fractions (part B) were added separately in the order designated. In the experiments with *B. coli* the medium used was that described in the preceding paper.

Drug, Vitamin, and Coenzyme Solutions.—Solutions of the various vitamins and coenzymes[1] used in the bacteriological experiments were prepared by dissolving each substance either in the basal amino acid fraction of the staphylococcal medium or in the synthetic medium for *B. coli*, depending upon the organism to be used. The vitamins and coenzymes tested, and the final concentrations in which they were employed, are designated below under each experiment. The solutions were sterilized by filtration through Seitz filters. Solutions of sulfanilamide, sulfapyridine, sulfathiazole, thionine, and methylene blue were prepared in a similar manner except that instead of being filtered, the basal medium was brought to a boil just before the drug was added and the solution was then allowed to cool slowly without further heating. Stock solutions of the sulfonamide compounds and of methylene blue were made up in concentrations of 10^{-2} or 10^{-3} molar, whereas thionine was prepared in a saturated solution. The desired concentrations for each experiment were attained by diluting the stock solutions with the appropriate basal medium.

Organisms.—The strain of *Staphylococcus aureus* used was one isolated from a routine throat culture. The organism was transferred from blood agar to 5 cc. of Gladstone's amino acid medium to which nicotinamide and thiamin had been added in final concentrations of 10^{-5} and 10^{-7} molar respectively. Excellent growth was

[1] Cocarboxylase and cozymase were supplied through the courtesy of Merck and Company.

obtained in this synthetic medium, and daily subcultures were made by adding 0.1 cc. of a 24 hour culture to 5 cc. of the medium. The inoculum used in all experi-

TABLE I

Culture Medium for Staphylococcus aureus

A. Basal amino acid fraction	
KH_2PO_4	4.5 gm.
Water	550 ml.
NaOH 1N	26 ml.
S-Aspartic acid	0.20 gm.
S-Valine	0.15 gm.
S-Leucine	0.15 gm.
S-Alanine	0.10 gm.
S-Glutamic acid	0.10 gm.
S-Iso-Leucine	0.10 gm.
S-Phenylalanine	0.10 gm.
S-Lysine-hydrochloride	0.10 gm.
S-Glycine	0.05 gm.
L(−)-Proline	0.05 gm.
L(−)-Oxyproline	0.05 gm.
L(−)-Tyrosine	0.05 gm.
L(+)-Arginine hydrochloride	0.05 gm.

Amino acids dissolved in the buffer solution, made up to 600 ml., adjusted to pH 7.40, tubed in 3 ml. quantities and autoclaved.

B. Labile fractions (added to each tube separately)	
L(−)-Cystine, M/200 in N/10 HCl (Seitz filter)	0.10 ml.
NaOH, N/5 (autoclave)	0.05 ml.
S-Methionine, M/100 (Seitz filter)	0.10 ml.
L(−)-Tryptophane, M/200 (autoclave)	0.05 ml.
Glucose, M/2 (Seitz filter)	0.05 ml.
$MgSO_4 \cdot 7H_2O$, M/60 (autoclave)	0.125 ml.
$Fe(NH_2)_2(SO_4)_2 \cdot 6H_2O$, M/500 in N/50 HCl (Seitz filter)	0.125 ml.
Water (autoclave)	1.4 ml.

C. Vitamin fraction

To make a basal medium for daily subculture and for bacteriostatic experiments, 0.5 cc. of a solution of 10^{-4} M nicotinamide and 10^{-6} M thiamin chloride dissolved in the basal amino acid fraction was added to each tube making final concentrations of 10^{-5} and 10^{-7} respectively.

ments was 0.1 cc. of a 1:10,000 dilution of the daily subculture. Plate counts revealed that the inoculum contained between 10,000 and 20,000 organisms, giving a final bacterial concentration of 2,000 to 4,000 viable organisms per cc. The strain of *B. coli* and the manner in which it was cultured in the synthetic medium have already been fully described in the previous paper.

EXPERIMENTAL

1. Determination of the Concentrations of Thiamin, Cocarboxylase, Nicotinamide, and Cozymase Optimal for the Growth of Staphylococcus aureus

Knight's experiments (9) to determine the quantities of nicotinamide and of thiamin necessary for the optimal growth of the staphylococcus in Gladstone's synthetic medium were repeated, and it was found that 10^{-6} M nicotinamide (or cozymase) and 10^{-8} M thiamin (or cocarboxylase) would insure heavy growth in 42 hours when growth was estimated by noting the degree of clouding in the culture tubes. Growth curves revealed, however, that still higher concentrations of any of these four substances would cause more rapid multiplication of the organism. (Figs. 1 A, 1 B, and 1 C.) Knight's assertion that 10^{-5} M nicotinamide and 10^{-7} M thiamin enabled optimal growth to occur was, therefore, not confirmed in the case of the present strain of staphylococcus. That increasing concentrations of nicotinamide, cozymase, thiamin, and cocarboxylase accelerate the growth rate of the staphylococcus is a fact of primary importance in interpreting the antibacteriostatic effect of these compounds to be described below.

2. Determination of the Minimum Concentrations of Sulfanilamide, Sulfapyridine, Sulfathiazole, Thiamine, and Methylene Blue That Will Prevent the Growth of Staphylococcus aureus

The minimum bacteriostatic concentrations of the various drugs used were determined by adding increasing concentrations of drug to successive tubes and noting the smallest concentration that would inhibit growth completely for 48 hours.[2] This end point was measured with a reasonable degree of accuracy by allowing the concentrations in consecutive tubes to vary only within relatively narrow limits. The concentrations of sulfanilamide in several consecutive tubes were, for example, 6×10^{-4} M, 4×10^{-4} M, 2×10^{-4} M, 1×10^{-4} M, etc. The end points of the sulfonamide drugs were very constant when the same lots of medium and of drug solution were used. New medium and new drug solution, when standardized, showed a maximum shift in drug end point of only one tube. The dye end points were less constant, showing a tendency to shift one or two tubes in successive experiments. To eliminate the significance of any possible variation, the position of the drug or dye end point was checked in all subsequent experiments dealing with bacteriostasis. The minimum bacteriostatic concentrations of the various agents tested are listed in Table II.

[2] Macroscopically detectable growth.

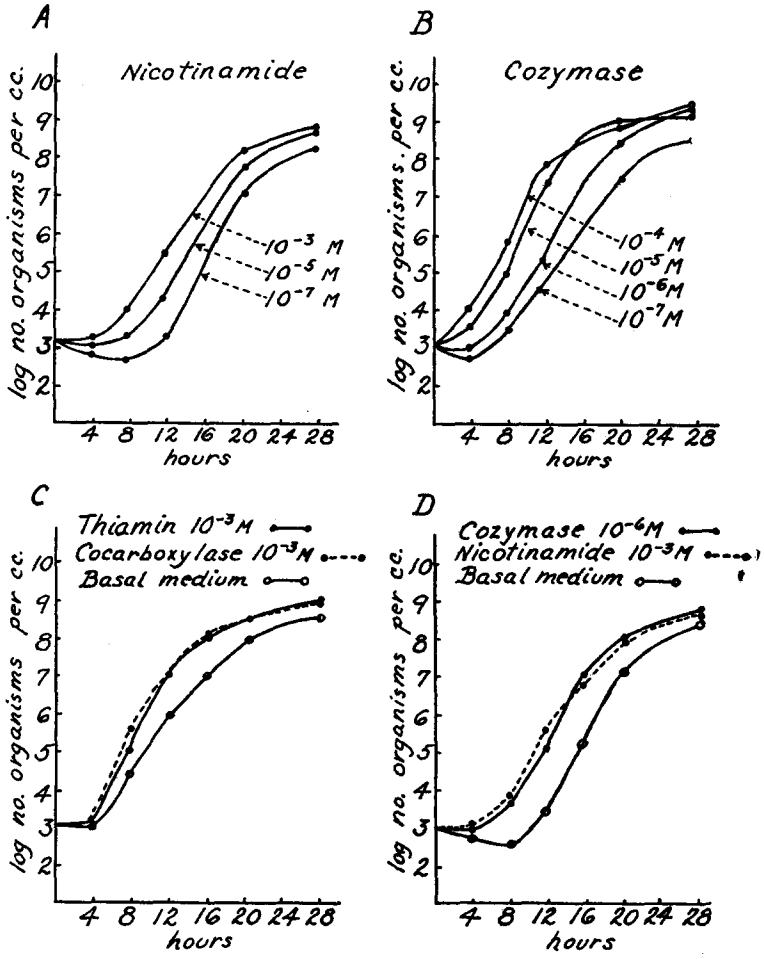


FIG. 1

A. Growth curves of *Staphylococcus aureus* in synthetic medium containing increasing concentrations of nicotinamide.

B. Growth curves of *Staphylococcus aureus* in synthetic medium containing increasing concentrations of cozymase.

C. Growth curves of *Staphylococcus aureus* in synthetic medium containing high concentrations of thiamin and cocarboxylase.

D. Comparison of the growth rates of *Staphylococcus aureus* in 10^{-3} M nicotinamide and in 10^{-6} M cozymase.

3. *The Antisulfonamide Effect of Nicotinamide, Cozymase, Thiamin, and Cocarboxylase*

When *Staphylococcus aureus* was used as the test organism, it was found possible to block[3] the growth-inhibiting effect of sulfanilamide, sulfapyridine, and sulfathiazole with nicotinamide when the latter substance was added to the synthetic medium in a concentration of 2×10^{-3} molar. Lower concentrations of nicotinamide blocked more irregularly and a concentration of 10^{-4} molar nicotinamide had no blocking effect whatsoever. Cozymase blocked the

TABLE II

Minimum Bacteriostatic Concentrations of Sulfonamide Drugs, Methylene Blue, and Thionine As Tested against Staphylococcus aureus in a Synthetic Medium

Sulfanilamide	2×10^{-4} M
Sulfapyridine	8×10^{-6} M
Sulfathiazole	1×10^{-5} M
Methylene blue	1×10^{-6} M (approximate)
Thionine	1:160 dilution of saturated solution (approximate)

TABLE III

Blocking of Bacteriostatic Action of Sulfapyridine and Sulfathiazole by Cozymase

Sulfapyridine			Sulfathiazole		
Drug concentration	Basal medium	Cozymase 10^{-6} M	Drug concentration	Basal medium	Cozymase 10^{-6} M
6×10^{-5} M	+	+	6×10^{-6} M	+	+
8×10^{-5} M	0	+	8×10^{-6} M	0	+
1×10^{-4} M	0	+	1×10^{-5} M	0	+
2×10^{-4} M	0	0	2×10^{-5} M	0	0

+ indicates visible growth of *Staphylococcus aureus* at the end of 48 hours.

growth-inhibiting action of these same drugs far more effectively having an antibacteriostatic effect at concentrations between 10^{-6} and 10^{-7} molar (Table III). Thiamin and cocarboxylase, on the other hand, manifested only a barely detectable antidrug effect even at concentrations as high as 10^{-3} molar. As suggested by the next experiment, this last result may be explained by the relatively slight degree of growth stimulation brought about by thiamin and cocarboxylase in the synthetic medium as compared to that caused by nicotinamide and cozymase.

In cultures of *B. coli* thiamin (10^{-3} M), riboflavin (10^{-5} M), pyridoxine

[3] The term "block" will be used only with reference to the prevention of bacteriostasis.

(10^{-3} M), pantothenic acid (2×10^{-4} M), crystallin biotin (3γ per cc.), crystallin methyl biotin[4] (3γ per cc.), nicotinamide (10^{-3} M) cocarboxylase 10^{-3} M) and cozymase 10^{-4} M) all failed to exert an antisulfonamide effect. All of these compounds also failed to increase the antibacteriostatic action of small amounts of *p*-aminobenzoic acid simultaneously added to the medium.

4. Correlation of the Antibacteriostatic Action of Nicotinamide, Cozymase, Thiamin, and Cocarboxylase with Their Ability to Stimulate Bacterial Growth

Instead of influencing only the chemically related drug sulfapyridine, nicotinamide and cozymase were shown to block the bacteriostatic action of all three sulfonamide compounds. This observation suggested that the blocking effect might be due to stimulation of growth rather than to a direct antagonistic action upon the drug. An attempt was made to correlate the antibacteriostatic effect of nicotinamide and cozymase with their ability to stimulate growth. The rate of growth of the staphylococcus in basal medium was compared to that in medium containing from 10^{-3} to 10^{-6} molar nicotinamide or from 10^{-4} to 10^{-7} molar cozymase. It can be seen from the resulting growth curves (Figs. 1 A and 1 B) that the stimulating effect of cozymase is much greater than that of nicotinamide. As stated above, the limiting blocking concentration of nicotinamide; *i.e.* the lowest concentration that will reverse the inhibition of growth by a sulfonamide drug, is approximately 10^{-3} molar whereas that of cozymase is between 10^{-6} and 10^{-7} molar. If the growth rates of the staphylococcus in 10^{-3} molar nicotinamide and in 10^{-6} molar cozymase are plotted on the same graph (Fig. 1 D), it can be seen that they are approximately the same, indicating a close correlation between the growth promoting properties and the drug blocking effect of the compounds.

Similar experiments were also carried out with *B. coli* grown in the synthetic medium described in the preceding paper. Neither nicotinamide nor cozymase is a growth factor for *B. coli* as each is for *Staphylococcus aureus*, and repeated growth curves showed that cozymase did not stimulate the growth of *B. coli* in the synthetic medium. Failing to stimulate growth, cozymase, even in concentrations of 10^{-4} molar, failed also to block the action of any of the sulfonamide drugs. It appears, therefore, that the antibacteriostatic action of nicotinamide and cozymase is dependent upon acceleration of bacterial growth rather than upon a direct antidrug effect.

5. Lack of Specificity in the Antibacteriostatic Effect of Cozymase

Quantitative experiments were carried out to determine whether or not the blocking effect of cozymase was greater against sulfapyridine to which it is

[4] Obtained through the courtesy of Professor V. du Vigneaud, Cornell University Medical School.

chemically related than against sulfanilamide or sulfathiazole to which it is unrelated. The degree of blocking by cozymase of each of the compounds was found to be approximately the same (Table III).

To determine whether or not the antibacteriostatic action of cozymase bore any specific relation to the sulfonamide group of drugs, cozymase was titrated against thionine and methylene blue. Both of these compounds are dyes which probably achieve their bacteriostatic effect by altering the oxidation-reduction potential of the culture medium (10), a mechanism apparently quite different from that by which the sulfonamide drugs act (1). Cozymase blocked the growth-inhibitory effect of thionine regularly. Less satisfactory results were obtained with methylene blue because of the instability of the bacteriostatic end point of this dye, but the general result was the same as with thionine. These experiments are compatible with the view that the drug-blocking effect of cozymase is unrelated to the chemical structure of the drug involved and merely results from the stimulation of bacterial growth.

6. The Failure of Sulfanilamide, Sulfapyridine, and Sulfathiazole to Affect the in Vitro Action of Cocarboxylase As a Coenzyme

Cocarboxylase is known to function as a coenzyme in the decarboxylation of pyruvate by the enzyme carboxylase contained in yeast. The activity of cocarboxylase may be conveniently estimated by measuring manometrically in the Warburg apparatus the rate of evolution of CO_2 from a carboxylase-cocarboxylase-pyruvate mixture. The method employed in the present studies was that of Lohmann and Schuster (11), the reaction being carried out at a pH of 6.6.[5] The possible inhibitory action of sulfanilamide, sulfapyridine, and sulfathiazole upon the functioning of cocarboxylase was tested by carrying out the reaction in the presence of a final concentration of 10 mg. per cent of drug. Although sulfathiazole, which is chemically related to cocarboxylase, was added in concentrations 50 to 200 times greater than that of the coenzyme, it failed to influence the reaction. Sulfanilamide and sulfapyridine likewise were without effect. In preliminary unpublished experiments with sulfapyridine and the cozymase-apozymase system, Walti also failed to detect any inhibitory effect of the drug upon the action of the coenzyme (12).

DISCUSSION

Direct evidence has been presented in the preceding paper supporting the theory that the sulfonamide drugs prevent bacterial growth by interfering

[5] The dried yeast preparation used as a source of carboxylase and the sodium pyruvate were supplied through the courtesy of Dr. Otto Bessy of the Department of Biological Chemistry, Harvard Medical School. The authors are grateful to Dr. C. L. Gemmel of the Department of Physiology, Johns Hopkins Medical School, for the Warburg apparatus used in these studies.

with the metabolic function of *p*-aminobenzoic acid. According to this theory there are at least two possible explanations for the fact that some of the more common derivatives of sulfanilamide are considerably more potent bacteriostatic agents than the parent drug. First, the greater bacteriostatic powers of the substituted sulfonamide compounds, such as sulfapyridine, sulfathiazole, and sulfadiazine, may be explained by assuming that the radical attached to the sulfonamide group interferes with the metabolism of a second substance essential to the bacterial cell, just as the *p*-amino nucleus of these compounds apparently disturbs the function of *p*-aminobenzoic acid. Such a dual effect on the part of the more complex derivatives of sulfanilamide might well account for their increased bacteriostatic potency. Second, the greater antibacterial powers of the substituted sulfonamide compounds may be explained by assuming that the chemical group attached to the sulfonamide radical enables the compounds to interfere with the metabolism of *p*-aminobenzoic acid more effectively than does the simpler sulfanilamide molecule. According to this second hypothesis the only metabolic function of the bacterial cell interfered with by the sulfonamide compounds is that concerned with the utilization of *p*-aminobenzoic acid, the degree to which this function is disturbed determining the relative bacteriostatic potency of the drug.

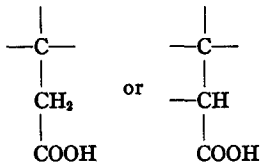
The first of these hypotheses has received support in the publications of Dorfman and his coworkers and of West and Coburn, who have advanced the view that sulfapyridine specifically alters the metabolism of the pyridine-containing coenzyme, cozymase. On purely theoretical grounds the same reasoning might be applied to sulfathiazole and sulfadiazine since both of these compounds are structurally similar to thiamin and its coenzyme, cocarboxylase. The results of the experiments reported in the present paper, however, fail to confirm the view that the chemical group linked to the sulfonamide radical plays a specific rôle in interfering with either the formation or the function of the analogous coenzyme. It has been shown that the antisulfonamide effect of cozymase, emphasized by West and Coburn, is in no sense specific but is due to its action as a growth stimulant rather than to a directly antagonistic action against the drug.[6] Also the *in vitro* activity of the coenzyme, cocarboxylase, was found to be unaffected by the chemically related sulfonamide compound, sulfathiazole, even when the latter was present in concentrations 200 times greater than the coenzyme. Both of these observations cast grave doubt upon the attractive theory that the bacteriostatic effect of sulfapyridine, sulfathiazole, and sulfadiazine is related to their structural

[6] It should be pointed out that *p*-aminobenzoic acid blocks efficiently the sulfonamide drugs in a medium in which it fails to stimulate growth (1). The dissociation of growth stimulation and antidrug effect would appear to be fundamental in determining whether a given chemical compound is specifically related to the mechanism of bacteriostasis.

similarity to nicotinamide, thiamin, and the respective coenzymes. The results likewise fail to substantiate the view that the greater antibacterial power of the more complex sulfonamide drugs is due to a dual effect upon the metabolism of the bacterial cell as compared to the single effect exerted by the simpler sulfanilamide molecule.

The second hypothesis advanced to explain the differences in bacteriostatic potency exhibited by the various common sulfonamide drugs is, on the other hand, entirely compatible with the observations reported in the previous paper. *p*-Aminobenzoic acid was shown to block the bacteriostatic effect of all of the sulfonamide compounds studied, regardless of their chemical structure, and the bacteriostatic potency of each drug was found to be directly proportional to its ability to nullify the blocking effect of *p*-aminobenzoic acid. Both of these observations suggest that all of the sulfonamide drugs studied cause bacteriostasis by interfering with a single metabolic function of the bacterial cell, namely that concerned with *p*-aminobenzoic acid.

Evidence has been presented also that the drugs exert their bacteriostatic effect by competing with *p*-aminobenzoic acid for the enzyme system normally involved in its utilization. If the mechanism of bacteriostasis concerns only the competitive inhibition of this particular enzyme system, it must be assumed that the relative bacteriostatic power of a given sulfonamide drug depends upon its relative ability to disturb the function of this essential system. Such an assumption is not without foundation for analogous variations in competitive inhibition by related chemical compounds are common in enzyme chemistry. For example, Quastel and Wooldridge (13) have shown that various organic acids having in common the structure



inhibit the important respiratory enzyme, succinic dehydrogenase. The authors have attributed the inhibition to the structural similarity of these compounds and the specific substrate, succinic acid, and have postulated that the common chemical configuration causes the inhibitors to become adsorbed on to that part of the enzyme surface which normally adsorbs and activates the substrate. Quastel and Wooldridge have demonstrated in addition that all of the related acids do not cause the same degree of inhibition, the inhibitory effect being quantitatively different in the case of each compound, presumably because of a specific degree of affinity for the enzyme. Thus the present hypothesis advanced to explain the mode of action of the sulfonamide drugs may be considered exactly analogous to the competitive inhibition of succinic dehydrogenase, for the experimental evidence strongly suggests that

sulfanilamide and its derivatives, due to their chemical similarity to *p*-aminobenzoic acid, competitively inhibit the enzyme involved in its utilization and that different sulfonamide drugs inhibit this essential enzyme reaction in different degrees.

To prove conclusively this "unitarian theory" as to the mechanism of sulfonamide bacteriostasis it will be necessary first to identify the enzyme system that utilizes *p*-aminobenzoic acid and secondly to demonstrate that the relative bacteriostatic power of a given sulfonamide drug is directly proportional to its ability to inhibit this particular enzyme system. An attempt is now being made to identify the enzyme (or enzymes) involved in this apparently vulnerable cycle of bacterial metabolism.

SUMMARY

1. In cultures of *Staphylococcus aureus* in a synthetic medium nicotinamide and cozymase were shown to block the bacteriostatic action of chemically unrelated sulfonamide drugs as well as the chemically related compound sulfapyridine. The antibacterial properties of organic dyes totally unrelated to the sulfonamide compounds (methylene blue and thionine) were also nullified by the addition of cozymase to the culture medium.

2. The antagonistic action of the pyridine-containing coenzyme, cozymase, was found, by quantitative study, to be no greater against sulfapyridine than against other structurally dissimilar sulfonamide compounds.

3. The antidrug effects of nicotinamide and cozymase in staphylococcus cultures were observed to be directly proportional to their ability to stimulate the growth of the organism in the synthetic medium. When tested in cultures of *B. coli* in which they failed to accelerate bacterial growth, these same substances failed to influence the bacteriostatic action of the sulfonamide drugs.

4. The *in vitro* action of the coenzyme, cocarboxylase, as measured in the Warburg respirometer, was shown to be unaffected by the chemically related drug, sulfathiazole, even when the latter was present in great excess.

The above observations fail to support the theory that sulfapyridine, sulfathiazole, and sulfadiazine prevent bacterial growth by interfering with the functioning of the chemically related coenzymes, cozymase, and cocarboxylase. The mode of action of sulfanilamide and its more common derivatives is discussed in the light of these observations, and a tentative theory is offered to explain the differences in bacteriostatic potency exhibited by the various sulfonamide compounds.

BIBLIOGRAPHY

1. Wood, W. B., Jr., *J. Exp. Med.*, 1942, **75**, 369.
2. Fildes, P., *Lancet*, 1940, **1**, 955.
3. West R., and Coburn, A. F., *J. Exp. Med.*, **72**, 91, 1940.

4. Dorfman, A., Rice, L., Koser, S. A., and Saunders, F., *Proc. Soc. Exp. Biol. and Med.*, 1940, **45**, 750.
5. Quastel, J. H., and Whetham, M. D., *Biochem. J.*, 1925, **19**, 520.
6. McIlwain, H., *Brit. J. Exp. Path.*, 1940, **21**, 136.
7. Stephenson, M., Bacterial metabolism, London, Longmans, Green and Co., 1939.
8. Gladstone, G. P., *Brit. J. Exp. Path.*, 1939, **20**, 189.
9. Knight, B. C. J. G., *Biochem. J.*, 1937, **31**, 96.
10. Dubos, R., *J. Exp. Med.*, 1929, **49**, 575.
11. Lohmann, K., and Schuster, P., *Biochem. Z.*, 1937, **294**, 188.
12. Walti, A., personal communication.
13. Quastel, J. H., and Wooldridge, W. R., *Biochem. J.*, 1928, **22**, 689.