THE MODE OF ACTION OF SULFONAMIDES*

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* The present review is a critical integration of the material believed to be essential to an understanding of the mode of action of the sulfonamides. In order to bring the treatment of the subject within the scope of a review of this character many supporting references and topics have necessarily been excluded. A much more inclusive annotated bibliographic review will be published in the new series of the Josiah Macy, Jr. Foundation. (The Mode of Action of Sulfonamides, by Richard J. Henry, M.D., 1944.)

The following abbreviations will be used throught this review:

SA = sulfanilamide
HOSA = *p*-hydroxylaminobenzenesulfonamide
SP = sulfapyridine
ST = sulfathiazole
SD = sulfadiazine
PABA = *p*-aminobenzoic acid

A. INTRODUCTION

The field of sulfonamide chemotherapy has exhibited a mushroom growth of tremendous scope and complexity. This has resulted in considerable confusion. It is almost impossible now for an investigator to keep informed of all published works relevant to sulfonamide action. It is therefore timely that an attempt be made to gather in one place the scattered observations, both for the investigator in the field and for one desirous of learning what is known about the action of these drugs, with the object of lessening the confusion and of indicating directions for future investigation.

The primary action of sulfonamides on bacteria is generally believed to be bacteriostatic rather than bactericidal. This must be stated with certain reservations, for the action may become bactericidal if the sulfonamide concentration is sufficiently high or if the presence of any sulfonamide concentration is accompanied by other unfavorable environmental conditions such as poor cultural conditions, adverse temperature, antibodies, toxic proteolytic products, etc. One of the early theories of the therapeutic action of these compounds was that the body defense mechanisms are stimulated. This idea fell into disrepute and is practically discarded (however, see section B2 for further discussion). The question of the possible inactivation of bacterial toxins is still of controversial nature and under investigation. The affinity of sulfonamides for bacterial and other proteins represents a possibility that these compounds are capable also of combining with, and thereby inactivating, toxic proteins. A final answer to this question must await the results of further studies. At present, however, the indisputable fact remains that the ultimate effect of sulfonamides is that of growth-inhibition.[1]

It is the purpose of this review to consider this growth-inhibitory action of the sulfonamides and to attempt a coordination and integration of the available information into a picture of the mode of inhibitory action of sulfonamides which the author regards as the most acceptable.

In 1940, Woods and Fildes made the discovery that *p*-aminobenzoic acid (PABA) is an extremely potent sulfonamide-antagonist. Therefrom arose the theory which has gained almost universal popularity, namely, that a sulfonamide interferes with the utilization of the substrate PABA in an anabolic reaction by competing with the latter for its enzyme. A careful and critical consideration of subsequent investigations reveals that this theory is based on certain assump-

[1] Most of the work leading to these views occurred early in sulfonamide research, and therefore the reader can refer to earlier reviews (160, 193) for complete discussions and references.

tions which have not as yet been proved and, furthermore, that the observations leading to the main arguments presented for its support can be equally well explained on an entirely different basis, and in fact, in certain instances *must* be interpreted on a different basis since the circumstances surrounding these instances exclude the possibility of PABA acting as a substrate. The conclusion is thus ultimately reached that the Woods-Fildes explanation for sulfonamide action cannot be the only possible one compatible with all the known facts regarding sulfonamide action and its antagonism by various substances.

As will be seen, all evidence indicates that sulfonamides achieve their bacteriostatic action by direct inhibition of one or more enzymes; this view enjoys today practically universal support. The two classes of enzymes which must be given consideration are those which catalyze anabolic or catabolic reactions and those which catalyze oxidation-reduction reactions. According to the Woods-Fildes theory the sulfonamide inhibits a hypothetical anabolic enzyme whose substrate is PABA. Others have investigated the effect of sulfonamides on oxidation-reduction enzymes, and there is considerable evidence in favor of the theory that sulfonamides inhibit cell division by a primary inhibition of one or more of these enzymes. It is this theory which is developed in the final section of this review as the one most compatible with all known facts regarding sulfonamide action. It must be forcefully emphasized at this point, however, that there is much work yet to be done before any theory of the mode of action of sulfonamide bacteriostasis can be accepted as final.

B. EMPIRICAL OBSERVATIONS ON THE ACTION OF SULFONAMIDES

The first step in the approach to the problem of a drug's mechanism of action is a consideration of the observations made on its behavior. Therefore, rather than begin with the discussion of these proposed mechanisms, it seems advisable first to acquaint the reader with certain fundamental facts relating to the behavior of the sulfonamides. This condensed account will serve to orient the reader with respect to the problem and will provide a factual background for a more critical reading of what is to follow.

1. The Inhibitory Action of Sulfonamides

The most fundamental fact about sulfonamides is that they are general cell inhibitors. A sulfonamide acts as inhibitor not only towards bacteria, but also towards other cells of practically every variety. This fact is of primary importance since it immediately casts considerable doubt that the inhibitory action of sulfonamides on bacteria is in any way unique, and that the answer to the problem of the mechanism of sulfonamide action can be sought by using bacteria alone. It is apropos, therefore, to recount briefly the various types of cells inhibited by sulfonamides, and thereby justify the use of the term "general cell inhibitor."

a. Bacteria. It is generally accepted today that the basic action of sulfonamides on bacteria *in vitro* and *in vivo* is bacteriostasis. This is not an "all or none" phenomenon; all gradations of decreased bacterial growth rate can be

produced by proper variation of the factors which influence sulfonamide action (discussed in part 3 of this section). Sulfonamides also inhibit other measurable bacterial cell functions; this will be presented in later sections. Because of its essential nature to most of the considerations in this section, it is necessary to anticipate a subject to be discussed at length in a later section (D), and state here that an exceptional feature about sulfonamide inhibition of bacteria is its complete counteraction by certain substances, foremost among which is *p*-aminobenzoic acid (PABA). Particular attention should be paid to references made to PABA and its action, because, according to the most popular theory, PABA is the key to the mechanism of sulfonamide action.

b. Cells other than bacteria; i. Viruses. It has been known for some time that all viruses are not alike and that there are certain groups with group characteristics. The three viruses for which there is adequate evidence indicative of a therapeutic response to sulfonamides (trachoma, lymphogranuloma venereum, and inclusion blenorrhea) and the virus of psittacosis comprise a group of viruses of large particle size which differs from typical viruses in several respects. Several attempts have been made to determine the nature of sulfonamide action on susceptible viruses. Richards *et al.* (220) were unable to demonstrate inclusion bodies and failed to infect baboons with pooled epithelial scrapings following sulfonamide therapy of the infection trachoma, suggesting that the drug caused disappearance of this virus. Holder *et al.* (98) found that contact between a sulfonamide and the virus of lymphogranuloma venereum *in vitro* results in decreased virulence of the virus but no apparent virucidal action, an observation in accordance with observations *in vivo* (112).

Poliomyelitis, choriomeningitis virus infection in mice, pneumonitis virus infection in mice, lymphogranuloma inguinale, canine distemper, and "shipyard eye" or kerato-conjunctivitis have been reported as susceptible to sulfonamide therapy, although most of these claims have been disputed. Many other virus infections which have been investigated, such as smallpox and yellow fever, have invariably been found to be unaffected.

The action of sulfonamides on the susceptible viruses is very interesting inasmuch as with applications of known methods no one has been able thus far to find evidence that viruses have a metabolism of their own. Assuming that viruses are exceedingly minute living organisms, and after making the observation that PABA counteracts the therapeutic effect of SA on mice inoculated intracerebrally with the virus of lymphogranuloma venereum, Findlay (62) extended the Woods-Fildes theory of sulfonamide action (section D1) and suggested that those viruses acted upon by sulfonamide may be those which require PABA (or a substance similar in structure) for their metabolism; for all other viruses PABA would thus not be an essential metabolite. Findlay offered as an alternative hypothesis that in the course of the metabolism of these viruses unaffected by sulfonamide so much PABA is formed by the virus that the chemotherapeutic action of sulfonamide is prevented. So far as is known today, all viruses are associated with living cells; it is possible therefore, that sulfonamide action in this instance might be indirect through an action primarily on the host cell thus rendering it an un-

suitable abode so far as the virus is concerned; however, the work by Holder *et al.* (98) already referred to, indicates that, in the case of lymphogranuloma virus, at least part of the action is directly on the virus itself.

On the other hand, if the virus is a non-cellular entity, it is possible that the action may be direct by interference with its autocatalytic and self-propagative properties. This interference may be in the nature of an adsorption of an inhibitor on the virus itself.

ii. Protozoa. Malaria (Plasmodia):[2] Reports of the effectiveness of sulfonamides on human malaria have been somewhat conflicting. There have been many uncertain or unfavorable and many favorable reports with regard to the therapeutic effectiveness of sulfonamides against human tertian malaria (*Plasmodium vivax*), quartan malaria (*P. malariae*), and estivo-autumnal malaria (*P. falciparum*).

Repeatedly, sulfonamides have been found to be effective against the virulent *P. knowlesi* infection in rhesus monkeys, while they exert no effect on the milder *P. cynomolgi* and *P. inui* infections (33, 34, 35). It appears in general that the sulfonamides are more effective against the more virulent plasmodia.

Certain avian forms of malaria such as *P. praecox* (3) and *P. circumflexum* (186) have been reported as affected by sulfonamides, but *P. lophurae*, *P. cathemerium*, *P. nucleophilum*, *P. relictum*, and *Hemoproteus columbae*, on the other hand, have all been claimed by various workers to be resistant to sulfonamide therapy.[3] In the case of *P. lophurae* infection in ducks, however, there have been several favorable reports, and Marshall *et al.* (188) demonstrated the importance of the blood concentration-time curves in the sulfonamide therapy of this particular infection, showing that maximum effect can be obtained only by keeping the blood sulfonamide level up for a sufficient length of time. It was the opinion of these investigators that the differences in response to sulfonamides of monkey and human malaria on the one hand and avian malaria on the other is at least partly due to differences in the blood concentration-time curves, especially when single oral doses are administered daily. Some of the discrepancies observed in the sulfonamide therapy of different malarial infections undoubtedly are due to species differences in susceptibility to the sulfonamides (34, 188).

PABA antagonizes sulfonamide action on *P. gallinaceum* (182) and on *P. lophurae* (188, 240), but does not antagonize the action of quinine and atabrine, thus indicating that these drugs act on plasmodia through a different mechanism than that of sulfonamides.

Other protozoa: Amebae, paramecia, trichomonads, (80), *Toxoplasma* (229), *Leishmania tropica* (242), and *Entamoeba histolytica* (222) have been reported as inhibited by sulfonamides. Cell division of the flagellate, *Polytomella caeca*, is blocked by SA, this inhibition being antagonized by PABA (163, 164). The flagellate which is inhibited has a volume four times that of the normal average. This is in agreement with numerous analogous observations on bacteria and

[2] For references of the effectiveness of the sulfonamides in malarial infections see the review by Williams (280).

[3] See Marshall (187) for a review of the effect of sulfonamides on avian malarias.

leads to the interesting hypothesis that sulfonamides, within certain limits, inhibit the division of microörganisms rather than growth primarily (164). In the work on *Hydra* (referred to below), however, a similar observation was made but was ascribed to body edema, perhaps brought about by a change in membrane permeability. Both are distinct possibilities but at present the exact cause and nature of this cell volume change is not known.

iii. Other cells. *Halteria*, *Hydra*, *Mesostoma* (flatworm), *Stenostomum* (rotifer), *Dero* (annelid) (59, 148, 149), chick embryo heart tissue culture, bone marrow, wound healing, various yeasts (*Torulospora*, *Torula*, and *Saccharomyces*), various fungi (*Trichophyton gypseum*, *T. purpureum*, *Blastomyces dermatitidis*, *Aspergillus niger*, and *Neurospora crassa*), *Actinomyces hominis* and *A. bovis* (also the clinical infection, actinomycosis), and higher plants (algae, a diatom, *Tradescantia occidentalis*, *Lupinus albus* roots, tomato roots, and *Pisum* roots) have all been reported as inhibited by sulfonamides; the concentrations of sulfonamides required to produce such an effect vary considerably, anywhere from a few mg % to over 100 mg %. PABA counteracts the sulfonamide inhibition of yeast (139) of *T. purpureum* and *T. gypseum* (43, 44), of *Aspergillus niger* (164), of *Neurospora crassa* (268), of algae (30) of a fresh-water diatom (278), of tomato roots (16), and of rootlets of *Pisum* and *Lupinus albus* (183).

Sulfonamide action on plants is of a somewhat unexpected nature. Thus, mitotic irregularities, chromosomal rearrangement, polyploidy resulting in large cells and strange new plant varieties some of which are giants, and transformation into degenerate variants have been observed. Certain of these effects are reminiscent of the action of colchicine. The improbability of any such hereditary mechanism playing a part in the dissociative changes occasionally observed in bacteria under the influence of sulfonamides has been discussed at length by Mellon (192).

Liver tissue, sea urchin eggs, bacterial luminescence, and luminescence of *Cypridina* are also affected by sulfonamides and will be considered in detail in section E8.

Next to the fact that sulfonamides inhibit all these various cell types, the most interesting observation is that in most instances PABA can completely counteract this inhibition. This fact can only strengthen the doubt that the inhibitory action of sulfonamides on bacteria is in any way unique. The tentative conclusion can, therefore, be made that the mechanism of sulfonamide inhibition is fundamentally similar, if not identical, in all cells susceptible to sulfonamides. This certainly would not be unexpected, for it must be remembered that in its gross details the metabolism of bacteria is very similar to that of most other types of cells.

2. The Biphasic Action of Sulfonamides

It is a rather general phenomenon that substances toxic to cells will also stimulate the cells at sub-toxic concentrations, e.g., it is seen with nicotine, narcotics, cyanide, actinomycin, and many others. Thus it is not surprising that low concentrations of sulfonamides stimulate bacterial growth (63, 83, 135, 136).

The primary stimulation of growth by the sulfonamides (before growth-inhibition occurs) observed by many investigators (63, 82) may well be an expression of the same phenomenon. The stimulatory action of sulfonamides is by no means confined to bacteria, for it has been demonstrated in the production of polymorphonuclear leucocytes by bone marrow (63), in phagocytic activity (27), in plant and yeast growth (81, 135, 166), in amplitude of dog heart beat (195) and in the multiplication of *Entamoeba histolytica* (5).

Following the demonstration of the growth-promoting activity of SA in plant and yeast growth, comparable even to indolyl-3-acetic and 1-naphthyl-acetic acids, Grace (81) pointed out that it is difficult to know where to draw the line between substances commonly regarded as inhibitors and those regarded as stimulants. For example, the phytohormone indolyl-3-acetic acid in high concentrations inhibits cell growth in a manner similar to the sulfonamides, and high concentrations of the sulfonamide-antagonist PABA will inhibit bacterial growth very effectively (cf. Dld). According to Grace, low concentrations of the sulfonamides may actually stimulate an infection, their therapeutic effect in such bacterial infections being due to a sufficiently high concentration. This, of course, has a serious clinical implication, namely, the possible danger of underdosage. In certain instances the overall therapeutic effect of these compounds, at ordinary dosage levels, may be a combination of two factors: first, an inhibitory action on the bacteria which are very susceptible to the sulfonamide, the sulfonamide concentration exceeding the range in which stimulation of the bacteria occurs; second, a stimulation of the host's tissues (bone marrow, phagocyte activity, general tissue resistance) which are less susceptible to the sulfonamide whose concentration here lies within the range in which tissue stimulation does occur (the concentrations required to inhibit such tissues *in vitro* have been found usually to be much higher than the levels attained therapeutically *in vivo*). Such a concept receives some support in the preliminary reports of Mellon *et al.* (194) where it is stated that ST, but not SA, increases the oxygen consumption of certain tissues as measured *in vitro* by the Warburg technique. The observed stimulation, however, may have been due to oxidative autolysis of the tissues. In any event, this phenomenon of stimulation in low concentrations and inhibition in high concentrations is not yet understood.

3. Factors Influencing Sulfonamide Action

The intensity of the action of all drugs depends to a great extent on environmental conditions. This is true of cell inhibitors, and sulfonamides are no exception. Much research has been expended upon the effect on sulfonamide activity of certain environmental conditions which are easily varied. Several of the observations made could have been predicted, while one, the effect of pH, has been only recently explained with any degree of satisfaction, and another, the effect that the size of bacterial inoculum has on sulfonamide activity, still defies comprehension. It is important to keep in mind, however, that these studies and their conclusions fit equally well the various hypotheses on how sulfonamides achieve their inhibitory action.

a. Drug concentration; the adsorptive nature of sulfonamide action. Bacterial growth-inhibition by sulfonamides appears to obey the law of mass action, which requires first that the inhibition be reversible, and second, that the inhibition be directly related to inhibitor concentration. This relationship is seen with most cell inhibitors. With respect to sulfonamide inhibition, the first requisite is satisfied by two independent and well-established observations, namely, the inhibition can be reversed by removal of the sulfonamide (157, 169), and the inhibition can be antagonized by PABA and other sulfonamide-antagonists (cf. Section D). PABA-antagonism of sulfonamide inhibition has been shown to obey the law of mass action (cf. D2b) which could not be unless the inhibition itself obeyed the law.[4] The second requisite, that the inhibition be directly related to sulfonamide concentration, has been observed by numerous investigators (27, 82, 126, 154, 193); table 1 gives typical data demonstrating this point. As indicated in some reports (126, 205), this proportionality may not, in every case,

TABLE 1

Showing the degree of bacteriostasis as observed in blood-agar plates containing different amounts of SA and inoculated with a virulent culture of Streptococcus pyogenes

Data from Table IX of Mellon et al. (193)

SA CONCENTRATION		NUMBER OF COLONIES	INHIBITION
M	mg %		
			%
0 (control)		206	
0.00006	1	107	48
0.0006	10	92	55
0.006	100	91	56
0.0075	125	84	60
0.01	167	79	62
0.015	250	59	71
0.03	500	52	75

be strictly linear over a large range of concentrations. If, as assumed by some (Section D), sulfonamides affect more loci in the cell as their concentration is increased, one would expect to observe occasionally such discontinuities in relationship.

A rather interesting question is whether sulfonamide action takes place at the cell surface or within the cell. There is the definite possibility that bacterial metabolism in general takes place predominantly at the surface (2);[5] if this were

[4] In view of the competition which exists between sulfonamides and PABA it may almost be assumed that PABA action is also a result of reversible combination. Other sulfonamide-antagonists (Section D) apparently do not act competitively, but this by no means precludes their combining by a reversible combination.

[5] The term "cell surface" as related to bacteria is ambiguous since it has been demonstrated by staining techniques that surrounding the inner protoplasm of the cell there is the protoplasmic membrane, outside of which is the cell-wall (125). Studies with the electron microscope (203, 204) have confirmed the existence of a solid cell-wall distinct from

true, it would almost be assumed that sulfonamide action occurs at the surface, since there is little doubt that this action is primarily one of inhibition of some metabolic function. Feinstone *et al.* (56, 57) observed that strong adsorption or diffusion through the cell is not a criterion of drug activity, and claimed to have obtained evidence with the aid of the electron microscope that the drugs do enter the cell. This, of course, would not rule out the possibility that the inhibition produced is due to enzyme inhibition at the cell surface; it would depend on the location of the functional enzymes which are interfered with. Electrokinetic experiments have revealed that SA and PABA behave alike at the bacterial surface, thus suggesting that their action is at the surface (18).

The combination of sulfonamide with whatever component of the cell it inhibits may be regarded as an adsorption. The term "adsorption" is variously understood today. As used throughout this review it is meant to imply an interaction at a surface, for instance at the surface of an enzyme molecule. Such interaction between two molecules may be mediated through electronic van der Waals attraction, attraction of electric dipoles or multipoles, Coulomb attraction,

TABLE 2

Effect of varying the number of organisms inoculated on the per cent of the control population; data from Table 2 of Libby (155)

ORGANISM AND DRUG TESTED	% OF CONTROL POPULATION WITH AN INOCULUM PER 10 ML OF:		
	50 million	25 million	12.5 million
Type II pneumococcus:			
Sulfapyridine	61	51	44
N^4-sulfanilyl sulfanilamide	54	44	38
Paratyphoid A:			
Sulfapyridine	77	57	46
Streptococcus C493:			
Sulfanilamide	68	57	34

hydrogen bond formation, or primary chemical valence. It is impossible at present to say which form of interaction applies to sulfonamide action, but as long as the interaction is reversible the law of mass action must apply equally well to each. This allows one in theoretical considerations to treat them as one and the same.

b. Size of inoculum. In the presence of a constant amount of sulfonamide, the inhibition of bacterial growth is inversely related to the number of organisms present (20, 27, 126, 154, 162, 177, 260); or, in other words, as the size of inoculum is increased a greater amount of sulfonamide is required to produce the same inhibition (see table 2 for a typical set of data). Apparently in some cases inoculum size has little effect (17, 181), but these have certainly been in the

the inner fluid or potentially fluid protoplasm. It is impossible at present to say at which locus, protoplast, the protoplasmic membrane, or the cell-wall, metabolic reactions would be most likely to occur.

minority. Nitti *et al.* (211) claimed that the importance of inoculum size varies with the organism, being of less importance with meningococci and gonococci than with streptococci and pneumococci, and of no significance whatsoever in growth-inhibition of the mold, *Aspergillus niger*.

If the ratio of volume of medium to volume of bacteria were not so high in such experiments one would suspect that, where this inverse relationship is observed, reduction of concentration resulting from adsorption of the sulfonamide from the medium by the bacteria was a factor. Several checks on this possibility have revealed either no measurable, or only a very minute decrease in sulfonamide concentration after contact with bacteria (126, 177). It would appear, therefore, that for the production of a certain level of inhibition of bacterial growth, there is, in most instances, a trend in the direction of a definite number of molecules of sulfonamide being present per bacterium. This direct relationship is not seen between cell suspensions and various other inhibitors such as narcotics, nor would it be expected. The relationship of amount of an enzyme per cell to the inhibitor concentration is the determining factor in such a heterogeneous system. This relationship does not change by varying the number of organisms in a given system. The observed inverse relationship between sulfonamide effect and inoculum size must therefore be due to some unknown variable.

Since certain bacteria have been shown to produce sulfonamide-antagonists, which in the case of some organisms can be found in the medium, it would at first seem quite logical that this should be the answer to the problem. The larger the inoculum the greater the amount of antagonists produced, and thus the greater the amount of sulfonamide required. This very promising explanation was apparently shattered when no antagonists were found in the medium of *Escherichia coli* and of pneumococcus cultures, although in both instances the inverse relationship between sulfonamide concentration and inoculum size existed (126, 162). Furthermore, if production of sulfonamide-antagonist were responsible for this phenomenon, and if the antagonist produced by bacteria is PABA, as believed by some, the inverse relationship should not be observed with HOSA as inhibitor, since PABA does not reverse HOSA action (section C); however, the inverse relationship does exist (20). Here is an empirical observation which appears to be inconsistent with general chemical principles and at present defies explanation. The paradox awaits clarification.

c. Composition of the medium; in vitro vs. in vivo. Occasionally throughout this review the sulfonamide concentrations[6] used in various investigations will be cited, presumably to provide some basis for comparison of results. Actually, however, such comparisons in most instances are of little significance. The reasons for this statement will become apparent after reading the section on sulfonamide-antagonists (D). Suffice it to say at this point that the extreme variability in the amounts of sulfonamide-antagonists in various media alone makes comparison of results *in vitro* practically impossible (154, 179, 180, 262,

[6] Throughout this review, for the convenience of the reader, all concentrations will be expressed two ways, in molarity (M) and in milligrams per cent (mg %).

263, 265). With the little that is known of the effects of various components of culture media on bacterial metabolism, and in view of the extreme variability of media employed in sulfonamide research, it seems utterly hopeless to attempt to evaluate the results of every investigator in the light of the medium employed.

A good example of the role played by the medium is the observation by MacLeod and Mirick (180, 181) that properly prepared fresh calf-liver infusion has no sulfonamide-counteracting action, and to produce the same results in plain broth and peptone broth, 20 and 40 times, respectively, as much sulfonamide is required. Another fact which complicates the situation is that if the medium is not optimal for growth, sulfonamide action is more pronounced (179, 259). Dilution of cultures with water, physiological saline solution, or buffer solutions either kills or injures some cells (181). This probably explains the observation that increasing the sodium chloride concentration of the medium results in increased sulfonamide action (160, 193). This is presumably another example of supplementation of adverse influences on the bacterial cell.

Since sulfonamide-antagonists have been found in every body tissue so far examined, it would be expected that there would be some lack of correlation between sulfonamide activity *in vitro* and *in vivo*. White *et al.* (277) found that, although no sulfonamide is active *in vivo* unless it is active *in vitro* or can be decomposed to a compound which would be active *in vitro*, compounds can be active *in vitro* while inactive *in vivo*. This lack of complete correlation between activity *in vivo* and *in vitro* has been observed by others.

d. pH. Varying the environmental pH has a very definite effect on sulfonamide activity. This was first noted by those interested in sulfonamide therapy of urinary tract infections, an instance where the pH can be controlled within certain limits. Investigation showed that sulfonamide activity in urine is increased as the pH is raised, e.g., from the range 5.5–6 to 7.5–7.8 (93). These investigations of sulfonamide activity at various urine acidities were made primarily in quest of an answer to an important clinical question, and not to ascertain the nature of the effect of pH on sulfonamide activity. Subsequent work designed specifically to give such information seemed at first to indicate two things: first, that it is the anion which is the active agent in a sulfonamide solution (which is of course compatible with the earlier observations that sulfonamide activity increases with urine pH) (75, 235), and second, that ion for ion all the sulfonamides are approximately equal in activity (75). Thus, the activity of a sulfonamide at any pH would be governed only by its acidic dissociation at that pH.

Cowles (37) and Brueckner (24), after making careful comparisons of bacteriostatic sulfonamide concentrations with ionization curves, found that an amendment would have to be made to the simple "ionic" theory. They observed that, in general, sulfonamide activity is at a maximum when the pKa of a sulfonamide is close to the pH of the culture medium, and decreases progressively as the pKa values depart in either direction from this pH. The maximum activity is, therefore, at the pH where 50 per cent is in the ionized form and 50 per cent in the non-ionized form. This suggests that only the non-ionized form can enter the

cell and, once in, only the ionized form is active; several examples are known where the intact organic molecule is better able to penetrate the cell membrane than the ions. As the pH departs in either direction from the pKa value, sulfonamide activity decreases. As Brueckner recognized, the mathematical approach he and Cowles made to the problem of sulfonamide activity is empirical.

Up to this point, all attempts at explaining the relative activities of sulfonamides were based on correlating in some way the activities with the acid dissociations of the compounds. There is no disagreement that pH does affect sulfonamide activity; but the "ionic" hypothesis met certain difficulties. Further analysis of the data used in its support revealed that the "ionic" interpretation was unjustified (128). Furthermore, such facts as the activity of compounds incapable of ionization, e.g., SG, the increase in activity of undissociable sulfonamide molecules with an increase in pH, and the decrease in activity of sulfonamide anions with an increase in pH (232) cannot be explained on such a basis. This would indicate that possibly the pH and the acid dissociation of the compound are not the only factors involved, and in fact may be only secondary to some more basic variable.

Bell and Roblin (12), aware of the inadequacy of the simple "ionic" theories, and seeking an approach to the problem which would utilize some fundamental physical property related both to structure and activity, observed that the more negative the SO_2 group of a sulfonamide, the greater the activity. They pointed out that the more negative the SO_2 group, the more closely it resembles the COO^- group of PABA at pH 7, for at this pH the carboxyl group of PABA is over 99 per cent ionized and consequently carries a negative charge. Since the SO_2 group of a sulfonamide in the ionized form is much more negative than in the non-ionized form, the former should be much more active than the latter. This theory therefore allows for an activity of undissociated sulfonamides. They disagreed with the contention (75) that ions of different sulfonamides are equally active, since the experimental data indicate that the ions of stronger acids are less potent. Since the electron-attracting power of the R group (on the N attached to the SO_2 group) is proportional to the acid strength, it follows that the greater the acidity, the less negative the SO_2 group of the ionic and molecular forms, and the less the activity of both. Up to a certain point this decrease in activity with increasing acid strength is more than compensated for by an increasing proportion of the active ions. Beyond this point, an increase in acidity is not paralleled by a proportionate increase in ions; therefore, the dominating effect is now the decreasing negative character of the SO_2 group which is accompanied by a decreasing activity. The activity of the undissociated forms should show a continuous increase as acid strength decreases. Thus, when the compounds become such weak acids that the effect of the highly active ions is negligible, the curve relating pKa to activity should pass through a minimum and then increase as acid strength decreases. On the basis of these theoretical and experimental considerations, Bell and Roblin believed that the optimum of N^1-substituted SA derivatives has been reached, insofar as inherent bacteriostatic activity is concerned.

Kumler and Daniels (134) have recently presented theoretical considerations indicating that a fundamental factor for activity is the contribution of the resonating form of the compound with a coplanar amino group. The negative character of the SO_2 group is thus a concomitant factor associated with the resonating form. Observations at variance with pre-existing theories are reconciled by this new theory. Compounds which appeared to be exceptions to the Bell-Roblin theory (sulfanilylurea, sulfanilylguanidine, sulfanilamide-1,2,4-triazole), and those which do not fall within the scope of their theory (sulfones, ring N-methyl and N-methylsulfapyridine and sulfathiazole compounds), can be adequately accounted for on the basis of resonance. Furthermore, it is seen that, so far as activity is concerned, whether the most active species of the compound is the anion, cation, or neutral molecule is an incidental property. Kumler and Daniels agree with Cowles and Brueckner in their explanation for the maximum in the activity *vs.* pKa curve for N^1-mono-substituted sulfanilamides, that the undissociated molecules get to the site of action more easily but that once there it is the ion that is more active. They point out, however, that the optimum ratio of ions to undissociated molecules need not be 50/50, but could be almost any ratio, depending on the relative rates of the two separate reactions.

It might be expected that pH would also influence the sulfonamide-antagonistic activity of PABA. If either the undissociated or the ionic form of PABA is more active than the other, then the pH should affect PABA action. Lwoff *et al.* (164) reported that the antagonistic activity of PABA parallels its dissociation curve, being maximum at its isoelectric point, in the case of SA-inhibition of the flagellate *Polytomella*. It was considered that the undissociated form of PABA penetrates more readily into the cell than the ion. These authors claimed that PABA-activity varies little or not all with pH with respect to *E. coli* or *Aspergillus niger*. If this were true, it might be interpreted to mean that, in these latter two instances, it is not necessary for the PABA to gain entrance to the cell in order to exert its action.

Brueckner (24), however, found that when pH was varied in cultures of *Staphylococcus aureus*, changes occurred in the molar ratio, sulfonamide/PABA, which did not correlate with changes in drug activity. This indicated that PABA activity might also be changing with pH variation. Experiments demonstrated that PABA does not increase in activity as its ionic concentration increases; it became decreasingly effective as the pH rose from 6 to 9, the range in which PABA rapidly becomes more than half dissociated. Thus, it is seen that the molar ratio of sulfonamide/PABA at varying pH levels cannot be interpreted exclusively in terms of sulfonamide activity. If the sulfonamide loses potency with increasing pH at the same rate as PABA, then no change in molar ratio should occur as the pH is increased. This was found to be the case with SD and ST at pH levels of 7 and above. If, on the other hand, the sulfonamide becomes increasingly active as the pH rises, as SA does, then changes in the molar ratio should occur; this was observed experimentally.

Fisher *et al.* (68) found that apparently only the non-ionized form of PABA is active as a growth inhibitor in fertilized sea urchin eggs. This may mean that, here again, only the non-ionized form can penetrate the cell, although once

in it may be the ion which is active (in this instance as an inhibitor rather than as an antagonist). This appears to be true in fertilized sea urchin eggs for the inhibitory actions of local anesthetic bases which have PABA or benzoic acid as their base (132). The effect of pH on the fungistatic activity of PABA and benzoic acid, and on the inhibitory action of benzoic acid on yeast fermentation and bacterial growth, also point to such a conclusion (39, 97).

In any event, it seems that this problem of pH-effect on PABA activity and that of other sulfonamide-antagonists requires clarification. Inasmuch as most culture media contain sulfonamide-antagonists and the organisms themselves seem capable of producing them (cf. D2c), the evaluation of investigations on the relationship between pH and sulfonamide activity cannot be on a sound basis without further knowledge. The problem is by no means simple; changes in pH might also affect membrane permeability, the enzymes, etc.; then, too, there is the effect of proteins, salts, etc. on the ionization and neutralization of the sulfonamide, of PABA, etc.

e. Temperature. The bacteriostatic and bactericidal action *in vitro* of the sulfonamides is definitely increased by an increase in temperature (7, 150, 151, 276). Several investigators have compared the action at approximately 37°C (human body temperature) and at several degrees higher. It was found that the increase in temperature *per se* is very definitely not the sole cause of the observed effects. This, of course, is of clinical interest, as will be seen below. In some instances, a particular concentration which is bacteriostatic at 37°C becomes bactericidal at slightly higher temperatures. The most quantitative experiments were those on streptococci reported by White (1939) who found that at 30°C SA concentrations less than 0.058 M (1000 mg %) are inactive, at 36°C concentrations less than 0.0058 M (100 mg %) are inactive, and at 39°C concentrations of 0.00058 M (10 mg %) or less are bactericidal. About 100 times as much sulfonamide was required at 37°C as at 39°C to produce the same effect. As the temperature is increased above 37°C, the ability of PABA to antagonize sulfonamide action decreases markedly (150, 151).

That increased temperature results in increased sulfonamide activity is also apparent *in vivo*. This has been observed in gonococcal infection of the chick's chorioallantoic membrane (8), in pneumococcal septicemia of rabbits (193), in human gonococcal infection where artificial fever is employed in conjunction with drug therapy (7, 8), and in infected wounds (85).

The facts indicate (section B3b) that the reaction in which the sulfonamides are involved is of an adsorptive nature. Most adsorptions are strongly exothermic, and it may be said that as a general principle such an absorption decreases with increasing temperature. Many adsorptions, however, apparently are not so simple because they do not follow this rule; e.g., they may increase with temperature up to a certain point and then decline. "Activation" energy and other factors are thought to be the cause of such phenomena, but as yet they are by no means completely understood (2). Studies of the effect of temperature on inhibitions (of an adsorptive nature) of enzyme systems are even more difficult to interpret since, besides the various factors involved in the ad-

sorption process *per se*, the effect of temperature on enzyme activity and probably other factors are to be considered. An example of the effect of temperature on enzyme inhibition is seen in the work on sulfonamide inhibition of bacterial luminescence (106, 107). Depending on the circumstances, a rise in temperature can increase or decrease the inhibition.

f. Age of culture; length of experiments. Though it may be that, qualitatively, the action of sulfonamides on bacteria is the same regardless of the age of the culture, it would be expected that the action would vary quantitatively with age. Critical data on this particular problem are very scanty and conflicting. Long and Bliss (160) claimed that the effect of SA is less on young cultures than on old ones. On the other hand, MacLeod and Mirick (18) reported that, as is seen with various unfavorable agents such as bacteriophage, water, etc., older cells are more resistant to sulfonamides. The ages of the cultures used by various investigators have differed considerably, and it seems probable that in many of the quantitative differences observed, this factor has played an important part. The previous history of the bacteria, i.e., their previous environment and growth conditions, may also be important.

The lengths of the time allowed to elapse before experimental observations are made also have varied tremendously, extending from fractions of an hour up to almost a week. It is a hopeless task to attempt to keep an actively growing bacterial culture constant in every respect over any but the shortest period of time. Though the concentration of sulfonamide does not change, other factors which influence its action change considerably with time: the pH changes, sulfonamide-antagonists are produced by the bacteria, bacterial substrates are used up, poisonous products accumulate, the bacteria age. Even if the bacterial growth is completely inhibited a certain amount of metabolism persists, and there is the possibility that the culture may "escape." This "escape" may be brought about by either a sufficient number of cells autolyzing and releasing substances which reverse the sulfonamide effect (103) or an acquisition of drug-fastness (103, 237). In instances where bacterial growth is only retarded, the bacteria may within a relatively short time resume a growth rate equal to the control. This may be partially due to development of resistance; but this would be unnecessary for, as soon as the number of bacteria reached a certain range, the amount of sulfonamide present would no longer be inhibitory (cf. B3b). It can be calculated from the data of Sevag and Shelburne (247) that sulfonamide effect may change from hour to hour even in the early hours of an experiment.

It is, therefore quite possible that, by varying the lengths of experiments, qualitative as well as quantitative differences in results may be obtained. Such results would lead to logical but spurious conclusions. A good example of this has been emphasized by Lewis and Snyder (154) who were faced with the paradox of organisms growing better with SA than without it. It was discovered, however, that the results were due to a continued reproduction near a maximum over a longer period than in plain broth. The SA was actually depressing.

g. Sulfonamide structure. It is not within the scope of this review to consider in any great detail the tremendous mass of literature dealing with the effect of

chemical structure on sulfonamide activity (212). The theoretical approaches to this problem supplied by Bell and Roblin and by Kumler and Daniels have already been presented. A few of the more important empirical principles, however, may be mentioned.

After it was firmly established that the activity *in vivo* of Prontosil and similar compounds is due to their breakdown to SA, investigations were planned to determine whether the *p*-aminobenzene-sulfonamide nucleus is the essential basis for all sulfonamide activity.

That the *p*-amino group is not necessary for activity was illustrated by various findings. Compounds in which the *p*-amino group is oxidized show activity, in many instances considerably greater than that of SA. Nitrogen itself does not appear to be required in all cases for activity, e.g., 4,4′-diacetyldioxyphenylsulfone is an effective agent (152). With few exceptions, however, all active compounds contain a nitro or amino group in *para*-position to the sulfur. Kumler and Daniels (134) are of the opinion that the evidence indicates the amino group to be the functional part of the molecule for activity. The generally accepted fact that no sulfonamides with a substituted *p*-amino group are active, and the general chemical inactivity of the SO_2 group as compared with the very reactive amino group support this conclusion.

Nor is the SO_2 group essential as is evidenced by the activity both *in vivo* and *in vitro* of *p*-nitrobenzoic acid (120), and the activities *in vitro* of *p*-aminothiophenol and 4,4′-diaminodibenzene disulfide, both of which are antagonized by PABA (84). It has been suggested that the SO_2 part of the molecule may be responsible for certain of the toxic reactions caused by sulfonamides (224, 281). The nature of the sulfonic end of the benzene ring may determine the diffusibility into or affinity for bacterial cells (252); its effect may also be indirect, through its influence (including that of R substituents) on the *p*-amino group (84, 134).

Inhibition of carbonic anhydrase, on the other hand, depends on an unsubstituted SO_2 group but not a *p*-amino group (184).

A rather interesting series of investigations has revealed the fact that the sulfur of active compounds can be replaced by other elements (arsenic, carbon, phosphorus, selenium, tellurium) and retain activity which, in some cases at least, is even increased (84, 225). Collier (36) is of the opinion that, in such cases where substitution of the sulfur results in increased activity, the polarity of the molecule has been increased, thus intensifying the reactivity of the active group (presumably the *p*-amino group).

Since there is no longer any doubt about the fact that PABA is able to reverse true SA-like activity both *in vivo* and *in vitro*, we now have a qualitative biological test (the specificity of which may be questioned, however), whereby it can be determined whether or not a substance exerts its activity by the same mechanism as SA, SP, SD, etc. It is quite possible that many of the compounds referred to above which are unlike SA in basic structure, although possessing definite anti-bacterial activity, act by a mechanism dissimilar from that of SA. Accordingly, a reëvaluation of the activities of all these various compounds must be made. Thus, Green and Bielschowsky (84) found that with but one

exception, replacing the sulfur by selenium or tellurium gives very active compounds which, however, are not antagnoized by PABA. From this it would appear that the NH_2⟨⟩S nucleus may be basic for true SA-like activity after all (84). Kuhn *et al.* (133), however, observed PABA antagonism of 4,4′-diamino-benzophenone, 4,4′-diamino-diphenylsulfone, phosphanilic acid and carbopyridin; this indicates that, using PABA reversal as a criterion, the sulfur is not indispensable for true SA-like action.

This particular problem is not only important from the standpoint of furthering the understanding of the mode of action of SA and related compounds, but also because it opens up the definite possibility of new therapeutic anti-bacterial agents which are more active, not being counteracted by sulfonamide-antagonists. Presence of the latter is frequently the cause of therapeutic inefficiency with SA, SD, ST, etc.

4. Delay vs. no Delay in Sulfonamide Action

A point of some importance has been the almost universal observation that sulfonamide action *in vitro* and *in vivo* is delayed approximately from 2 to 6 hours after primary contact between sulfonamide and organism (17, 157, 162, 177, 205). Probably its greatest importance lies in the interpretations based on it. Thus, those who championed the theory that SA has to be transformed into an active substance before any effect occurs, claimed that the delay in action corresponds to the time of sulfonamide activation. In fact, it was the belief of some that the oxidative processes of bacterial multiplication were required for the conversion. As will be seen in section C, it seems fairly certain now that this idea must be abandoned. Then many of those who believe that sulfonamides interfere with bacterial nutrition have expressed the opinion that the slow development of bacteriostasis parallels the gradual depletion of bacterial stores of essential metabolites whose formation has been inhibited by sulfonamide action. It must be noted that such an interpretation presupposes that such action is an inhibition of the formation rather than the utilization of the essential substance, the latter being the foundation of the original proposal of Woods and Fildes (cf. section D1c).

A strong argument against the latter thesis is that in certain instances such a great delay in action is not seen (126, 163, 247), e.g., inhibition has been observed as early as 15 minutes following contact with sulfonamide.

Those instances in which the delay is relatively short (say of the order of 10 to 40 minutes) can be readily explained on the basis of the adsorptive nature of sulfonamide inhibition (section B3a). Adsorptive processes, unlike interactions between most molecularly dispersed reactants, are often slow to reach equilibrium. This has been tendered as a possible reason for the delay of sulfonamide action when observed (27, 205). Cowles (37) reported that he was able to show that it takes time for sulfonamide to enter bacteria and for equilibrium to be reached. Since distribution of the drugs varied, it was suggested that the rate of diffusion of the sulfonamides may be a contributory factor. Of course, as already stated, it is not known whether actual penetration of the cell

is necessary for bacteriostatic action, but a certain lapse of time would be required for adsorption equilibrium to be reached even at the cell surface. This may be a matter of a few to many minutes, probably varying with conditions. Sulfonamide inhibition of luminescence in luminescent bacteria and of the luciferin-luciferase system of *Cypridina*, however, is practically immediate, and this inhibition is in the nature of a reversible adsorption (108, 111). Similarly, inhibition of carbonic anhydrase occurs within two minutes, and this inhibition is reversible (283).

Boroff *et al.* (17) observed a delay in the antagonistic action of sulfonamide-counteracting substances as well as that seen in sulfonamide action. This is in agreement with the interpretation of the delay in action being due to slow adsorption. The variability in delay observed may be due to the presence in some instances of substances tending to interfere with adsorption of the drug. Organisms previously grown in sulfonamide-containing medium are rapidly inhibited when transferred to fresh sulfonamide medium (27). This can be taken to mean that sulfonamide adsorption had occurred in the original culture. The one observation which is somewhat difficult to fit into this interpretation is that when bacteria are exposed to sulfonamide at a low temperature not permitting growth and are then brought to a temperature which initiates cell multiplication, a delay in sulfonamide action is seen (126, 260). Similarly, the delay in action is longer at 27°C than 37°C (260). Thus it must be admitted that the reason why in some instances inhibition is greatly delayed, while in other instances it is but slightly delayed, is not apparent. The answer to this question would seem to be worth seeking as it should provide further insight into the mode of action of these compounds.

5. *Specificity of Sulfonamide Action*

a. One drug on different bacteria. When the chemotherapeutic success of Prontosil in streptococcal infection was first reported, it was supposed for a short time that this sulfonamide was specific for the streptococcus. It was soon discovered that other bacterial infections are also susceptible to Prontosil. Today, with the thousands of derivatives which have been prepared, it is seen that any one drug, which possesses any activity at all, varies tremendously in effectiveness on different bacteria, although probably no organism is completely insensitive (84, 212).

A very important question to the therapeutist is whether the relative efficiency of the various sulfonamides is constant with all bacterial infections. It has been the general impression of many investigators, and clinicians as well, that certain sulfonamides are more or less specific for certain infections. A well-known example of this is the belief, which has been put into practice in clinics all over the country, that SA is preferable to its newer derivatives in the treatment of streptococcal infections. However, there is actually very little critical clinical supporting evidence, and some reports have even tended to discount this viewpoint (64, 114).

An answer to this question was sought in analysis *in vitro*, which showed that the various commonly used sulfonamides are non-specific for numerous

bacteria (75, 85, 287), this non-specificity extending even to the tubercle bacillus (70). Green and Parkin (85) also claimed that the sulfonamides are not specific for different organisms, as judged from the local action in infected wounds as well as from studies *in vitro*. The first careful approach to this problem *in vivo* (not wound infections) was made by Marshall *et al.* (189). Thirty-three typical SA derivatives were compared on streptococcal infection in mice, the comparative therapeutic effect being based on blood concentrations giving the same response. On a weight (mg %) basis, no derivative was significantly more active than SA; on a molar basis, SP was more active. On the other hand, all derivatives but one were more active than SA against pneumococcal infection. SP, ST and SD were also much more active against infection by *E. coli* than SA. It was logically concluded therefore that there is a definite specificity of sulfonamides on bacterial infections in mice.

There seems, therefore, to be a great discrepancy between results obtained *in vitro* and those obtained *in vivo*. The data published by Marshall *et al.* (189) reveal that some sulfonamides worked better *in vitro* than *in vivo*, and *vice versa* (no *in vitro* data were published on SP, SD, or ST, however). A rather attractive explanation is that the various sulfonamides are not specific for certain bacteria as borne out by the *in vitro* studies, but that certain sulfonamide-antagonists present *in vivo* are capable of greater antagonism towards the action of one particular sulfonamide on one bacterium than on another. In support of such a hypothesis is the finding that serum *in vitro* antagonizes the inhibitory action of SA and ST on pneumococci and staphylococci but not on streptococci (246). This of course would explain why SA *in vivo* may be specific for streptococcal infection. Because of its great clinical importance, this problem of sulfonamide specificity deserves more thorough investigation.

b. Different drugs on one bacterium. It is beyond the scope of this review to consider in detail the relative activities of the many sulfonamides which have been prepared, and the effects which various substituents, side chains, etc., have on activity. Some phases of this problem have already been touched on. It may be stated, however, that the main question at present is whether the change in activity associated with a change in sulfonamide structure is due to a change in basic mechanism of inhibition, or merely due to an enhancement or suppression of the same mechanism brought about by an altered adsorption coefficient or acid dissociation constant. Various theories attempting a correlation of sulfonamide activity with certain physical characteristics of the molecule have already been discussed (B3d). Further evidence, *pro* and *con*, for each of these two possibilities will receive some consideration in subsequent sections; it seems safest to conclude in favor of neither at the moment.

6. "Sulfonamide-fastness"

a. Natural resistance. One of the least understood of the phenomena of sulfonamide action is the extreme variability in susceptibility of various bacteria to the action of the compounds *in vitro* as well as *in vivo*. Even various strains of one species vary greatly (145). It has been shown, however, that all bacteria are more or less susceptible to sulfonamides (82, 84).

Attempts to explain this variance have been made on the basis of the anticatalase theory

of sulfonamide action and the Woods-Fildes theory; these will be discussed later and will be found to be inadequate. Other possibilities which must be considered include differences in the ability of the sulfonamide to penetrate the cell (if this is necessary) and differences in enzyme patterns. For example, Felsenfeld (58) studied various mannitol-fermenting strains of *Shigella sonnei* and found that colony forms with the greater fermentative activity were the more resistant to sulfonamides as well as to adverse physical conditions such as heat and drying.

Part of the general success of sulfonamide-therapy in the treatment of a particular infection probably depends on the capacity of the organism involved to stimulate antibody formation, although differences in antigenicity of strains are not primarily responsible for the variation in effectiveness of sulfonamide therapy (236).

b. Acquired resistance. Quite a large volume of literature has appeared reporting the acquisition by bacteria of increased resistance to the sulfonamides, both *in vitro* (82, 121, 145, 237, 243, 262, 263) and *in vivo* (76, 274).[7] All of the organisms examined for this aptitude have exhibited it; they include pneumococci, staphylococci, streptococci, meningococci, diphtheria bacilli, *Shigella spp.*, *Vibrio comma*, *E. coli*, gonococci, *Brucella abortus*, and *Hemophilus parainfluenzae*. In fact, it is the belief of some investigators that all organisms susceptible to the bacteriostatic action of the sulfonamides are capable of developing resistance to them (121, 262). Organisms have been trained to grow in concentrations even up to 0.018M (300 mg %) SA; this is, of course, with relatively small inocula. It must be emphasized, however, that no organism has ever been made totally resistant.

Because of its high clinical importance, the phenomenon of sulfonamide-fastness has received considerable attention. However, inasmuch as many of the issues involved are still in the controversial stage, only those regarded as essential to an evaluation of the theories proposed for the mode of action of sulfonamides will be considered here.

An extremely important feature of acquired "sulfonamide-fastness", especially from the clinical viewpoint, is that when an organism is made fast to one sulfonamide it is also resistant to the others (121, 145, 243). There have been cases reported in which acquired resistance is apparently not carried over from one sulfonamide to the others, but it is suggested by Kirby and Rantz (121) that such discrepancies are due to technical differences in the experiments, e.g., resistance to a sulfonamide may be missed if too high a concentration is used in the test. The latter authors have shown that the degree of resistance developed is correlated with the bacteriostatic potency of the sulfonamide; organisms made resistant to certain bacteriostatic concentrations of one sulfonamide are equally resistant to equivalent bacteriostatic concentrations of the others. This of course would seem to indicate, as Kirby and Rantz pointed out, that the acquisition of "fastness" represents an interaction between the bacteria and the one structural unit common to all the sulfonamides, namely, the *p*-aminobenzene nucleus, and, furthermore, that this interaction involves the same enzyme system as that concerned with PABA antagonism. Actual contact between the sulfonamide and the enzyme system involved, in fact inhibition of the same, appears to be prerequisite for the development of resistance, because PABA completely protects bacteria from such a change (262, 263).

The development of resistance in a strain is a gradual process (121, 237), developing most quickly to the least effective compound, and the more easily the less the original sensitivity of the organism (243).[8] Gonococcal colonies, for example, which develop after plating heavy suspensions on inhibitory concentrations of sulfonamide-agar are 5 to 10 or 50 to 100 times as resistant as the parent strain; and on further plating, occasional variants are obtained 1000 times as resistant as the original, thus approximating a logarithmic acquisition of resistance (145).

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[7] Acquisition of increased resistance to sulfonamides may not be confined to bacteria, for there is evidence that the flagellate *Polytomella* (164), *Endamoeba histolytica* (222), and macrophages (101) can develop increased tolerance.

[8] Resistance, as used here, refers to bacteriostatic concentrations of the sulfonamides.

The biochemistry, and especially enzymology, of bacteriology are still in their infancy; and it is difficult to get any insight into the problem at hand, namely, the reason why certain bacteria are greatly affected by certain environmental conditions while others are comparatively resistant, and what mechanisms the susceptible organisms have at their disposal to adapt themselves to unfavorable circumstances. The phenomenon of acquired resistance to a therapeutic agent is by no means new or confined to the sulfonamides, for it has been known for some time that the spirochete of syphilis can become "fast" to arsenicals, the pneumococcus to ethyl-hydrocuprein, and trypanosomes to various triphenylmethane dyes. Various possibilities have been proposed as to the mechanism of development of sulfonamide-fastness, and each has certain points in its favor. It may be that this phenomenon is more apparent than real, and that it merely represents a "weeding out" of the more susceptible bacteria, a true example of selection or survival of the fittest (181, 261). The fact that sulfonamide-fastness in a particular strain is a gradually developing process would be in agreement with such a hypothesis. That variability in sulfonamide susceptibility within a bacterial strain exists is now definitely established (238). Certainly, such a scatter of susceptibility to environmental influence in a presumably homogeneous population is a well-known phenomenon. Cases of bacterial adaptation without multiplication are known (48, 261) and it would, therefore, be of great interest to know if resting bacteria can develop sulfonamide-resistance. Such an approach may answer the question of the extent to which this phenomenon is a result of selection.

Schmidt and Sesler (236) utilized the other available method of approach to this problem, namely, a quantitative study of the sensitivity of individual organisms (pneumococci) composing a sensitive strain and the changes in sensitivity occurring during the strain's acquisition of resistance. It was found that the pneumococci present after the first exposure to SP were significantly more resistant than any organism in the original sensitive strain, and, within certain limits, organisms of increased resistance were formed on each additional exposure to sulfonamide. This indicates that resistant organisms are formed as a result of some action of the sulfonamide on the organism. These investigators emphasized that the individual pneumococci of a sensitive or resistant strain vary somewhat in their sensitivity, but that these differences are relatively small and not to be confused with the larger differences in sensitivity existing between the sensitive and highly resistant strains. They felt that although "breeding out" does not appear to be the main factor in the acquisition of increased sulfonamide-resistance, it has not been ruled out completely and that it probably does play a part. The question is whether or not the acquisition of drug-fastness is one of selection of hereditary variants which are specifically induced by the presence of the sulfonamide. Sulfonamides produce evolutionary changes in plants (B1b), and it may be that some type of hereditary mechanism is in operation also in bacteria.

As will be discussed in section D1h, it has been proposed that the varied sulfonamide sensitivity of organisms in general is directly related to their ability to synthesize PABA (or sulfonamide-antagonist), or to the rate of its release from the organisms into the medium. However, the available data are too confusing to warrant any blanket subscription; this is an aspect of the general problem which must be analyzed more thoroughly. Certainly it is possible that this may be the answer to the question with some bacteria at least; but there is no *a priori* reason why the mechanism of acquiring resistance to a drug must be the same in every instance.

One very plausible hypothesis takes cognizance of the extreme adaptability which is inherent in bacterial organisms (48, 113, 181, 261). The bacteria may develop insensitiveness to sulfonamides by adjusting their metabolic reactions in such a way as to render unnecessary for growth that particular reaction (or reactions) which is ordinarily inhibited by sulfonamides and the functional integrity of which is ordinarily essential for growth (237). This change may involve either the utilization of a new substrate (which may be synthesized by the cell) in a reaction unsusceptible, or less so, to sulfonamide action, and which can replace the one whose metabolism is blocked in susceptible cells, or the development of new intermediate metabolic pathways as shunts which bypass the susceptible reac-

tion. Such possibilities apply whether sulfonamide action is due to inhibition of anabolic reactions or inhibition of oxidative enzyme systems. Karström (113) has described variants with new enzymatic properties due to "adaptive" enzymes whose development and specificity are guided by the chemical structure of the substrate. These "adaptive" enzymes, however, are not permanent since they are lost when the specific conditions responsible for their development are removed; acquired sulfonamide-resistance, on the other hand, is usually retained long after the sulfonamide is removed (121, 180, 237).

A critical examination of the various enzyme systems of bacteria before and after development of sulfonamide-resistance should contribute much to the solution of this important problem.

7. *Synergism with Specific Antibodies and Bacteriophage*

There have been numerous reports of synergistic or potentiating action between sulfonamides and specific antibodies, both *in vivo* and *in vitro*. That their similar effect is attained through dissimilar mechanisms is borne out by three facts: first, PABA has no antagonistic effect on antibody action (264); second, acquisition of sulfonamide-fastness does not alter the susceptibility of bacteria to antisera (180); third, a bacterial strain may be resistant to serum and sensitive to sulfonamide, resistant to sulfonamide and sensitive to serum, sensitive to both, or resistant to both (19).

Sulfonamides also synergize and increase the lytic activity of bacteriophage (290) and lysozyme (207), in contrast to most antiseptics which inhibit or destroy bacteriophage. Though it is possible that some direct connection may exist between the action of sulfonamide and the action of antibodies or phage which would account for their tendency to summate their actions, it seems more probable that their combined effects represent a summation of unrelated influences unfavorable to the bacterial cell, i.e., they do not compete for the same receptor group in (or on) the cell.

Summary: To recapitulate briefly what has been given in some detail: sulfonamides inhibit not only the growth of bacteria but also the growth or other functions, or both, of numerous other cells; sulfonamide action, like the action of many other toxic substances, is usually biphasic; the activity is directly related to the sulfonamide concentration and to the temperature, inversely related to the inoculum size, influenced greatly by the structure of the sulfonamide itself and influenced by changes in pH. It varies not only from bacterial species to species, but even from strain to strain; and under certain conditions the relative activities of various sulfonamides can vary from bacterial organism to organism; bacteria can be trained to resist sulfonamides to a surprising extent; sulfonamide inhibition is synergized by antibodies and bacteriophage. This summarizes what is known about the relationships existing between the sulfonamide, the cell, and the environment. It does not tell us how the sulfonamides effect their inhibitory action.

C. ASSUMPTIONS THAT THE ACTION OF SULFONAMIDES IS DUE TO THEIR TRANSFORMATION INTO CHEMOTHERAPEUTICALLY ACTIVE FORMS

With this section begins consideration of the mechanism of sulfonamide action. Actually, the first part of this is not concerned with the mode of action, but with the question of whether or not the sulfonamide as such is the active agent. The idea that sulfonamides must first be oxidized before attaining activity is the foundation of two theories of the mode of action of sulfonamides, and therefore, must be described before considering the theories.

The material included in this section could perhaps have been dispensed with briefly in the introduction along with the other early proposals which have been outmoded, since it is generally conceded today that the amino-sulfonamide needs undergo no transformation to be bacteriostatic, it being the active form *per se*. The evidence for this is so conclusive that no lengthy, documented consideration will be given here. Because of the possible bearing on certain unsolved problems (sulfonamide toxicity, potentiation by oxidizing compounds, etc.), the author believes it worthwhile to outline the highlights of the earlier theories of sulfonamide action based on the assumption that a sulfonamide must first undergo some oxidative transformation before becoming active.

1. Oxidation of p-aminobenzenesulfonamide (SA) to p-hydroxylaminobenzenesulfonamide (HOSA)

Early in sulfonamide research the question arose as to whether *p*-aminobenzenesulfonamide (SA), as such, is the active agent. Mayer and Oechslin in 1937 put forth the hypothesis that SA is oxidized in the animal body and *in vitro* to *p*-hydroxylaminobenzenesulfonamide (HOSA), the substance responsible for the chemotherapeutic and bacteriostatic property. The finding of methemoglobin in the blood of animals and patients receiving SA, and in blood cultures containing SA, had suggested the formation of an oxidized product of the drug which was responsible for the oxidation of hemoglobin. SA, *per se*, is not an oxidant and is, therefore, incapable of such oxidative action, whereas certain oxidation products (exact nature unknown) of SA have been shown to be capable of such action.

The action of sulfonamide-antagonists, which will be considered in the next section, is difficult to fit into this hypothesis. Perhaps the greatest single piece of evidence against it is that PABA is unable to antagonize the action of SA oxidation products *in vitro* (83, 84, 223). Since it is well established that PABA can completely antagonize the therapeutic action of SA, it follows that only bacteriostatic substances which are antagonized by PABA have a true SA-like mode of action.

It is true that HOSA *in vitro* is more active than SA with some bacteria. HOSA *in vivo*, however, has proved to be no more active than SA, undoubtedly because of the great instability of HOSA, which is reduced to SA in blood very promptly. Proponents of this theory were of the opinion that HOSA is formed from SA slowly in the immediate vicinity of the bacterium, perhaps by the tissue cells of the host as well as by the bacterium itself, and probably with the aid of ferrous iron catalysis. Although it is conceded today that this is not the mechanism of sulfonamide action *in vitro* or *in vivo*, the fact, that oxidation products of sulfonamides, in certain cases at least, are much more active than their reduced forms, may offer certain therapeutic possibilities. Their instability precludes their use in any circumstances except where their oxidized state can be maintained by imposed oxidizing conditions, e.g. in local wound therapy.[9]

[9] Neter (206, 208) observed that azochloramide potentiates the action of sulfonamides *in vitro*, whereas other chemotherapeutic substances such as optochin, merthiolate, and actinomycin do not even exhibit synergy (206). Schmelkes and Wyss (234) confirmed this azochloramide potentiation and, ruling out the possibilities that it might be due to a chemical reaction between the agents resulting in the formation of a more toxic compound on the grounds of the molecular ratio of the agents employed, and that it might be a result of a lowering of the sulfonamide threshold of the bacteria on the basis that the other compounds used by Neter produce no such effect, offered as a possibility the inactivation of sulfonamide-antagonists. Azochloramide and other chlorine compounds were found to inactivate PABA and peptone reversal of SA action on *E. coli*.

In the case of azochloramide potentiation, it is possible that the antagonist PABA is chlorinated and thereby loses its power of antagonism, for Wyss *et al.* (288) have found that 2-chloro-PABA and 3-chloro-PABA do not antagonize SA action.

In the light of some recent findings, another possible explanation of sulfonamide potenti-

2. *Anti-catalase Theory*

Based on the assumption of HOSA formation from SA *in vivo* and *in vitro* the "anti-catalase" theory was formulated, according to which, the therapeutic or bacteriostatic effect of SA is brought about indirectly by the following chain of events: *a*, SA is oxidized by the bacteria to HOSA; *b*, the latter inhibits catalase;[10] *c*, hydrogen peroxide resulting from bacterial metabolism, normally decomposed by catalase, now accumulates; *d*, when sufficient peroxide accumulates the bacteria are destroyed or their growth is hindered.

As stated, the biological evidence against the anti-catalase concept of the therapeutic action of sulfonamides is rather conclusive. Among the arguments which refute it are: *a*, Some bacteria are sulfonamide-sensitive in the absence of catalase; *b*, Type 3 strains of hemolytic streptococci which produce no detectable peroxide are susceptible to sulfonamide; *c*, certain peroxide-resistant organisms are sulfonamide-sensitive; *d*, certain bacteria are as sensitive to SA *in vitro* as to HOSA; *e*, PABA cannot antagonize the action of HOSA; *f*,

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ation by azochloramide is conceivable, namely, that the potentiation is due to formation of HOSA or some other oxidation product of SA (this, of course, assumes that SA does not ordinarily undergo any oxidative change into an "active" agent). In the first place, Goldberger (80), in rather extensive experiments both *in vitro*, and *in vivo* by local application in infected wounds, found that oxidizing agents in general (Lugol's solution, azochloramide, dichloramine-T, hydrogen peroxide, zinc peroxide, potassium permanganate, etc.) potentiated sulfonamide action, while substances such as merthiolate and mercurochrome, did not. This potentiation was also shown in the effect on other unicellular organisms such as protozoa, spermatozoa, and certain fungi. Neter (209) and Crile (38) have confirmed Goldberger's observation that azochloramide enhances the activity of sulfonamide in localized infections. Other previous reports had claimed that hydrogen peroxide increases the effectiveness of local sulfonamide therapy. In the second place, as already stated, PABA is unable to inhibit the action of oxidation products of SA, for instance, HOSA. This would explain the observation that azochloramide "inactivates" PABA antagonism of SA activity. As already stated, the bacteriostatic action of such oxidation products is many-fold that of SA, at least on certain bacteria (84).

It is somewhat difficult to determine which hypothesis is correct. On the one hand, if the sulfonamide-antagonist is PABA it might be expected that this substance would be oxidized before any sulfonamide present. As a matter of fact, it has even been proposed that at least some of the sulfonamide-antagonistic action of PABA is a result of the latter being more easily oxidized than the sulfonamide (this assumes that the active drug agent is an oxidized product of the sulfonamide) (191). As will be seen in the next section, however, oxidized derivatives of PABA have been found to counteract sulfonamide action. Furthermore azochloramide destroys the reversing power of peptone, and there is evidence that the antagonistic activity of peptone is not due to its PABA content (section D). Acting on the suggestion that azochloramide may potentiate sulfonamide by oxidizing the latter, Sevag (244) studied a simple mixture of the two substances in the Warburg respirometer and observed no evidence of an oxidation. This, however, was in the absence of any iron or living material, and it is known, for example, that peroxide with ferrous iron catalysis is a rapid oxidant of SA (252). It may be that neither proposal is correct; but now that media devoid of sulfonamide-antagonists are available it may be that this problem can be solved.

[10] Hydroxylamine and oximes in general are known to inhibit catalase; actual inhibition of this enzyme by HOSA has also been adequately demonstrated. SA itself has relatively little anti-catalase activity (25, 36, 72, 184). This hypothesis was not based on an oxidative action of the oxidation derivatives of SA as was the original idea of Mayer and that of Shaffer and others, (this latter was concerned with the poising of the redox potential and will be considered presently), but rather on an anti-enzymatic action analogous to the inactive complex formation of carboxy-hemoglobin.

sulfonamides act in the absence of conditions necessary for the production of peroxide: e.g., certain anaerobes are susceptible *in vitro* and *in vivo*, and certain facultative organisms are susceptible under anaerobic conditions *in vitro*; *g*, HOSA can completely inhibit the aerobic respiration of streptococci and pneumococci (248). When respiration is completely inhibited there can be no formation of peroxide; a catalase inhibition under such conditions could not allow the accumulation of toxic amounts of a substance which is not being produced in the first place.

Thus one can conclude that while certain toxic and side effects of the sulfonamides may be caused by small amounts of oxidation products formed *in vivo* (102), and that under certain conditions with some organisms catalase inhibition may play a part in bacteriostasis, the principal mode of sulfonamide action both *in vivo* and *in vitro* is by some mechanism other than inhibition of catalase. It is true that the oxidation products exert an inhibition of bacterial growth *in vitro* but apparently this is by a different mechanism than the inhibition produced by the reduced compounds. This is further supported by the finding that *m*-nitrobenzenesulfonamide is as active as the *p*-nitro compound (223), a relationship certainly not existing (except under certain conditions to be discussed later) between the amino isomers. This mechanism would not necessarily have to be connected with catalase in any way.

In line with the discovery that PABA is unable to antagonize the action of sulfonamide oxidation products and the possibility that some toxic reactions *in vivo* may be due to such compounds, it has been shown that PABA is unable to inhibit sulfonamide rashes and fevers or acute toxic effects (167, 266). This, however, must not be regarded as too significant, because the actual mechanisms of sulfonamide toxic reactions are not yet understood; it has been recognized for some time that some of the toxic reactions are an allergic response.

3. The Poise of Oxidation-reduction Potential as Responsible for Sulfonamide Action

Once the hypothesis appeared that the active agent of sulfonamide action is some oxidation product of the parent compound, it was quite natural that interest should be aroused in the changes of oxidation-reduction potentials taking place in cultures during sulfonamide action. Investigation of such changes suggested that sulfonamides (in an oxidized form) might poise the E_h of of bacterial cultures at a level too high to permit bacterial multiplication.

Those who believed that sulfonamide oxidation products are the active inhibiting agents either were of the opinion that these products poised the potential at a high level because of their oxidizing power, or believed that the increased potential was a result of peroxide accumulation in the culture. Roblin and Bell (221) concluded from their experiments, however, that the high potentials obtained *in vitro* are due to the oxidizing agents employed in the determination, rather than to any SA oxidation products. This, of course, would not rule out the possibility that, in the absence of added oxidizing agents, sulfonamide bacteriostasis is accompanied by a high culture potential. However, the observation that the electrode potential of cultures in the state of sulfonamide bacteriostasis remains high can be argued from both sides. On the one hand, it can be claimed (as above) that the presence of oxidizing substances in the culture prevents the metabolic reactions required for growth. But on the other hand, it is possible that the metabolic reactions are hindered primarily, thus preventing the decrease in potential observed during growth and which probably is a natural result of bacterial metabolism (94). The fact that the active sulfona-

mide is now known to be not in an oxidized state definitely indicates that the latter possibility would explain the high potentials if actually present during sulfonamide action.

D. SULFONAMIDE-ANTAGONISTS[11]

It was called to the reader's attention very near the beginning of this review that one of the most unusual facts about sulfonamide action is that certain substances can completely counteract it, and frequent reference to this phenomenon has already been made. The phenomenon of drug antagonism was not wholly unknown previously but this observation was so clear cut, and it came at a time when everyone was so anxious to get at the secret of sulfonamide action, that immediately it stirred the whole sulfonamide field to the point that nearly all the research in the field in the last few years has been devoted to the study of sulfonamide-antagonists. As a result of these investigations several theories of sulfonamide action have evolved, and in order to understand and critically view these theories, it is necessary to present in some detail what is known about sulfonamide-antagonists and their actions. The theories themselves will be developed and discussed as an integral part of this presentation.

1. Para-aminobenzoic Acid (PABA)

The sulfonamide-antagonists can be conveniently divided into two groups: antagonists of known composition, and antagonists of unknown composition which for the main part are mixtures. One of the theories which arose as a result of the study of antagonists is the Woods-Fildes theory, which, since the time of its appearance in 1940, has enjoyed practically universal subscription by the investigators in the field. The Woods-Fildes theory gained this support because it correlates very neatly some of the major observations of sulfonamide action. Since its appearance, many observations have been found compatible with the theory and these have served to make its general acceptance more nearly complete. However, several other important observations made in this same period of time have found no place in this theory and to a great extent have therefore been ignored. It is timely that a critical examination of this theory be made. The Woods-Fildes theory, as well as one other to be discussed, is based on the action of the antagonist, PABA. Before attempting an interpre-

[11] Apparently it is becoming customary to call these substances "sulfonamide inhibitors". It is suggested that to avert confusion the term "inhibitor" be retained in its original connotation, that of the inhibitor of a cell function or reaction, and that such a term as "antagonist" be applied to substances which prevent the action of inhibitors.

Sulfonamide-antagonists have been called "anti-sulfonamides" by some, their action being called "anti-sulfonamide action". In the field of immunology the prefix "anti-" before a substance connotes that an "anti-substance" has been produced as a response to an antigen and can combine with that antigen. It is best that the term "anti-sulfonamide" be reserved for such use.

"Reversal of sulfonamide action" has also been frequently referred to in the literature. This as used has seldom meant that sulfonamide action existed which was subsequently reversed, rather in most instances it has meant that the sulfonamide action was prevented from ever developing. Although this is a point which is presumably only of importance as a matter of terminology, the use of the term "reversal" has been avoided in this review.

tation of the mechanism of this antagonism, it seems proper to consider all that is known about PABA and its properties.

a. Distribution and isolation. The first experimental indication that there may be a fundamental SA-antagonist appeared in the work of Woods (284) in which it was shown that yeast extracts contain a sulfonamide-counteracting factor whose chemical properties suggest a close chemical relationship to SA itself. PABA was found to have high activity in sulfonamide-antagonism, and it was suggested that the sulfonamide-antagonist in yeast might be PABA. Rubbo and Gillespie (226) believed they had obtained the benzoyl derivative of PABA from yeast. Isolation and chemical characterization of PABA from yeast was reported by Blanchard in 1941 and subsequently by others (14, 174, 228)[12]. PABA was obtained both in a free and in a combined form. The latter, Blanchard suggested, may be the sulfonamide-antagonist which Loomis *et al.* (161) had obtained from yeast, and may be the substance from which PABA is derived when yeast is autolyzed. The exact nature of this combined form is obscure, the substance obtained by Loomis *et al.* (161) was insoluble in ether, but it may be a peptide (14); non-diazotizable, and not inactivated by acetylation, whereas the physical and chemical properties of PABA are exactly the opposite. Green and Bielschowsky (83) also found evidence of an ether-insoluble SA-antagonist in their bacterial extracts.

Part of the antagonist obtainable from bacteria (83) and plasma (174), and practically all contained in normal human urine (179) is in a conjugated, inactive form which becomes active only following hydrolysis. It appears that the antagonist is conjugated in the body and excreted in the urine in this inactive form. These antagonists may be PABA but they have not been characterized. It may be interesting to note in this connection that PABA fed to man or animal is excreted partly as its acetyl derivative and perhaps as a glucuronate (92).

Sulfonamide-antagonists have been found in many diverse places, but as will be seen, whether or not they are PABA has not been definitely established except in yeast. It has also been suggested that, in all organisms other than bacteria in which PABA counteracts sulfonamide inhibitory activity, the PABA plays a part in the normal metabolism (probably as an essential metabolite) of the particular sulfonamide-susceptible organism. There is no experimental evidence for this postulate; the possible "wide distribution" of PABA in the plant and animal kingdoms is still an open question.

b. As Sulfonamide-antagonist. Woods and Fildes (284, 285) first demonstrated PABA antagonism of sulfonamide inhibition of bacterial growth. This antagonism to the action of all the sulfonamides on bacteria *in vitro* has been confirmed by many workers under very wide experimental conditions (75, 83,

[12] Landy and Dicken (140), Lewis (153), and Mitchell *et al.* (200) found that of all substances asayed for PABA by their microbiological methods yeast is the richest source. Substances assayed and found to contain PABA included liver, spinach, oat seeds, mushrooms, meat extract, urine, blood, and peptone. These microbiological assay methods depend on the growth-factor specificity of PABA for *Acetobacter suboxydans*, *Lactobacillus arabinosus*, and a strain of *Neurospora crassa*, respectively.

91, 179, 227, 286); in fact, to date no single report denies the counteraction of sulfonamide action on bacteria by PABA. PABA antagonism is also manifest *in vivo*. In mice, PABA has been shown to antagonize SA, SP and ST in hemolytic streptococcal infection (218), to antagonize SA, SP and ST in pneumococcal infection (167), SD in meningococcal infection (218, 270), and ST in infection by *Klebsiella sp.* (205).

PABA has been found to antagonize sulfonamide action in experimental malaria (182, 188, 240); in experimental lymphogranuloma venereum virus infection (62); on a fresh water diatom (278) and algae (30) (autotrophic plants in comparison to pathogenic bacteria which are heterotrophic); on *Pisum* seedlings (279); on *Pisum* and *Lupinus* rootlets (183); on tomato roots (16); on a dermatophyte (43); on *Neurospora crassa* (268); on *Aspergillus niger* and the flagellate *Polytomella caeca* (164); on yeast (139); on luminescence in growing cultures of luminescent bacteria (105); on pigment formation by *Pseudomonas aeruginosa* (271). The antagonistic activity of PABA varies considerably with different organisms.

The relationship existing between sulfonamide and the antagonist PABA is a competitive one as has been shown by Wyss (286) and by Wood (281) using *E. coli*. In the presence of PABA the growth-stimulating concentration of SA is increased (136), and, over a wide range of concentrations, the ratio of sulfonamide to the amount of PABA required for antagonism is more or less constant (84, 173, 205, 227, 269, 281, 284, 287); these two observations are to be expected from the mass law relationship and, therefore, they offer further support for such a relationship. It has been found that one mole of PABA will counteract approximately 1,000 to 26,000 or more moles of SA (43, 70, 75, 163, 226, 281, 284, 287). There have been reported instances in which this ratio is nearer unity (133, 169, 246, 286). Much of the responsibility for this extreme variability in the ratio probably lies in the variable amounts of sulfonamide-antagonists already present in practically all culture media. The other sulfonamides under the same experimental conditions require relatively more PABA to counteract their action (70, 75, 84, 115, 205, 246, 281, 284, 287). Analysis of the quantitative data shows that the potency of each sulfonamide is directly proportional to its ability to counteract the anti-bacteriostatic action of PABA, in other words, the amount of PABA required to counteract bacteriostasis is approximately the same for the minimal bacteriostatic concentration of each sulfonamide (75, 128, 281, 284). This is to be expected from the law of mass action (124). As has been pointed out by several investigators (173, 227, 281), such a large ratio as one mole of PABA to 1,000–26,000 moles of SA does not rule out competitive inhibition between the sulfonamide and PABA; it could be that the relative affinities are widely separated. These high molar ratios, however, are figured from data obtained in a pH range where practically all the SA activity is a function of the SA-ion, and if the ratio of SA-ions to PABA is used to represent the actual competitors in the reaction, values much nearer unity are obtained (75).

Other substances closely related to PABA, procaine for example, also show this phenomenon of sulfonamide-antagonism. According to Woods (284) the

action of procaine is slightly delayed, and it may be, therefore, that hydrolysis of the ester is necessary before it becomes active (179, 228, 284). Similarly *p*-nitrobenzoic acid, *p*-hydroxylaminobenzoic acid, *p*-aminobenzamide, *p*-aminobenzaldehyde, and *p*-nitrobenzaldehyde may owe their antagonistic action to their conversion to PABA (196, 218, 227, 228, 284). Not only procaine, but other local anesthetics derived from PABA counteract sulfonamide action *in vivo* and *in vitro*, whereas others not derived from PABA are devoid of activity (115), and as Krahl points out (130) it would be very difficult to prove or disprove that the PABA derivatives owe their activity to hydrolysis to PABA. Johnson *et al.* (107) obtained evidence indicating that the inhibitory action of procaine on bacterial luminescence is not due to its hydrolysis to PABA; inhibition by procaine was counteracted by increased pressure, whereas pressure had little or no effect on the inhibition caused by PABA. It is of interest to note that *p*-nitrobenzoate in sufficient concentration is itself capable of growth-inhibition which is reversed by the further addition of a small amount of PABA (196). This certainly indicates that the *p*-nitrobenzoate molecule does not have to be reduced in order to have an affinity for the same locus at which sulfonamide inhibition occurs.

Recently, Auhagen (4) found that *p*-aminobenzoyl-*l*-glutamic acid is 8 to 10 times more active than an equimolar concentration of PABA in counteracting *in vitro* the SA-inhibition of *Streptobacterium plantarum*. The *p*-aminobenzoyl derivatives of *d*-glutamic acid, *l*-aspartic acid, *l*-leucine, *d*-leucine, glycine, and glycyl-glycine were inactive. The antagonistic activity of *p*-aminobenzoyl-*l*-glutamic acid obviously cannot be a result of transformation to PABA.

Comparison of properties of the isomers of PABA and SA. The structural similarity between the active sulfonamides and the antagonist PABA has been stressed as a fundamental basis for their mutual competition for an enzyme surface (12, 60, 91, 173, 227, 281, 284). This raises the question of the activity of the *ortho-* and *meta*-isomers of PABA. Rubbo and Gillespie (226, 227) found that PABA is approximately 10,000 times more active than either its *ortho-* or *meta*-isomer as a growth factor for *Clostridium acetobutylicum*, and suggested that the very small activity of the isomers may be due to PABA impurities in the preparations. With respect to sulfonamide-antagonistic activity, however, PABA was only 5 times as active as the same preparation of *meta*-isomer. This throws considerable doubt on the suggestion that the activity is due to PABA as an impurity; and it would seem, therefore, to indicate that the isomer can counteract sulfonamide action, though less actively than the *para* compound. Using concentrations required for counteraction of sulfonamide as a criterion of activity, others have reported that the *ortho-* and *meta*-isomers are antagonistic, although their activity in this respect is much less than that of PABA (284). There have been a few reports of failure to demonstrate any sulfonamide-counteracting activity of the isomers; in some of these reports, the concentrations used are not stated, and in the others the concentrations used were below those at which others have observed the antagonistic activity. The *ortho*-isomer has been shown to antagonize the therapeutic action of SP on mice infected with

pneumococcus or *Streptococcus pyogenes*, though much less so than PABA (218). It is significant, however, that in these experiments the same amounts of the two isomers were administered.

The isomers have also been reported as unable to replace PABA as growth factor for *Acetobacter suboxydans* (140), and the fungus *Neurospora crassa* (268). Lewis (153) reported the *ortho*- and *meta*-isomers to have 0.00005 and 0.009 per cent, respectively, of the growth-stimulating activity of PABA for *Lactobacillus arabinosus*, and expressed the belief that even this relatively weak activity may be due to PABA-impurity in the compounds. The *ortho*-isomer, however, is capable of replacing tryptophan as a growth essential for *L. arabinosus* and *L. casei*, but not for various other lactobacilli (254). This activity is not inhibited by orthanilamide in a concentration 10,000 times that of the *o*-aminobenzoic acid. This fact is insufficient to rule out the possibility of a competition due to structural similarity, since higher concentrations of orthanilamide might have produced an inhibition.

Although definite proof in the form of critical quantitative data is lacking, it would seem probable that the isomers of PABA do possess properties similar to PABA, and that the differences in activities (as growth-factor and sulfonamide-antagonist) of the isomers are due to differences in adsorptive affinities for the same locus.

Then, too, there is the question whether the isomers of SA possess any true SA-like activity. Early reports claimed them to be inactive, but with one exception failed to state the concentrations employed; Nitti *et al.* (210) found them to be inactive in the concentration 0.006 M (100 mg %). That inactivity of the isomers could not be a result of a lack of adequate blood concentrations, nor to a failure of the compounds to adsorb on, or diffuse through, the bacterial cell was shown by Feinstone *et al.* (56, 57). In electrokinetic experiments Bradbury and Jordan (18) observed that metanilamide behaves like aniline at the bacterial surface, not like SA or PABA. These authors believed that the activity of the *para*-compounds is due to polar resonance which, of course, is impossible with the *meta*-isomer, but they were at a loss to account for the inactivity of orthanilamide, since this compound is capable of resonance. Kumler and Daniels (134) explain this apparent discrepancy by assuming a hydrogen bond to exist between the amino and the sulfone groups; the amino group is therefore not free, a structural prerequisite for activity (cf. B3g).

Although it is generally accepted today that the isomers of SA are inactive, actually there is very little critical experimental evidence to support this viewpoint. Wyss *et al.* (289) obtained greater respiratory inhibition of several bacteria with orthanilamide than with either the *meta*-compound or SA, the latter two inhibiting to the same extent. These experiments were carried out in a synthetic medium devoid of sulfonamide-antagonists. Sevag *et al.* (245) have found that orthanilamide exerts a greater inhibition on the carboxylase activity of *E. coli* than SA in the same concentration, metanilamide a lesser inhibition. The important observation, however, was that peptone, serum albumin, and globin at any particular concentration antagonize the inhibitions by the *ortho*-

and *meta*-isomers to a very much greater extent than that by SA. The possible explanation of this will be discussed later, but suffice it to point out here that this observation could very well explain the inactivity of these compounds *in vivo* where sulfonamide-antagonists are ever present, and *in vitro* when media containing antagonists are employed. It may be, therefore, that although the isomers are of much less or perhaps no value therapeutically, they have been too hastily regarded as having no fundamental action akin to SA. Then too, the mere fact that they are inactive at the concentrations in which SA exerts its effect does not rule out the possibility of their acting similarly at higher concentrations.

Actually, there is a question whether structural dissimilarity does rule out competition for the same enzyme. McIlwain (171) noted the lack of specificity between certain bacterial inhibitors and their antagonists of corresponding structure. The phenomenon of adsorption in general does not necessarily imply that two substances adsorbing onto the same surface must be structurally related, and so long as the adsorption of each substance is reversible there will be a competition according to their relative affinities. For example, narcotics of entirely different molecular configuration can inhibit the same enzyme. Thus, Möller and Schwartz (202) report that PABA can counteract the inhibitory action of germanin, neostibosan, arsphenamine, and neo-arsphenamine (compounds devoid of any structural similarity to PABA) on *Streptobacterium plantarum*.

c. As essential metabolite.[13] Woods and Fildes (60, 284, 285) proposed the theory that sulfonamides function by interfering with an essential metabolite, and thus inhibit growth,—the essential metabolite being *p*-aminobenzoic acid. They further proposed that such inhibition requires an inhibitor closely related to the essential metabolite so that it can fit the same enzyme, but sufficiently unrelated to be an inadequate substitute for the essential metabolite. The Woods-Fildes theory thus was based on the existence of a competition between the sulfonamide and PABA for an enzyme surface, and as already seen, this competition has been amply confirmed.

Cases of competitive inhibition of enzyme reactions by substances related to substrates or products are well known (89): succinic dehydrogenase by malonic acid; lipase by acetophenone and other non-polar compounds containing a carbonyl group; lactate dehydrogenase by α-hydroxybutyric acid, glyceric acid, mandelic acid, hydroxymalonic acid, glyoxylic acid and oxalic acid; invertase by β-glucose, α- and β-fructose, β-*l*-arabinose, and α- and β-galactose.

Recent investigation has revealed other anti-bacterial agents which are related to growth essentials in the manner that SA is related to PABA and whose mutual specific effects may be readily explained in terms of competitive inhibition. Bacterial growth inhibited by the addition of certain sulfonic acids or their amides can be restored by adding corresponding carboxylic acids or their deriva-

[13] An essential metabolite is a food substance essential to the organism but which the organism may be capable of synthesizing. A growth factor, on the other hand, is not only essential but must be supplied as such for the organism since the latter is unable to synthesize it.

tives (170). It is considered that the carboxylic compounds play an essential role in growth reactions which are interfered with at enzyme surfaces by the similarly constituted sulfonic compounds. The growth-inhibition produced by α-amino sulfonic acids can be reversed with α-amino carboxylic acids (171). When bacteria are made independent of added amino-carboxylic acids by training, the α-aminosulfonic acids lose their inhibitory power.

Sulfonic acid analogs of pantothenic acid (e.g., pantoyltaurine) are bacterial growth-inhibitors, their action being negated by concomitant addition of pantothenic acid (9, 253). Pantothenic acid is a growth factor for all organisms which are found susceptible to pantoyltaurine (175). Bacterial inhibition by pyridine-3-sulfonamide is unaffected by PABA or pantothenic acid, but is antagonized by nicotinamide (and nicotinic acid); inhibition by pantoyltaurine is antagonized by pantothenic acid but not by nicotinamide or PABA (173, 175). Pyridine-3-sulfonic acid inhibition is also completely antagonized by thiazolecarboxylic acid, coenzyme I, and "iron ion" (201). Pantothenic acid antagonizes pantoyltaurine's chemotherapeutic activity *in vivo* as well as its inhibitory action *in vitro* (176). The antagonism of pantoyltaurine by pantothenic acid and the antagonism of pyridine-3-sulfonamide by nicotinic acid are both competitive, since as with PABA and sulfonamide, a constant ratio exists over a wide range between the inhibitor and the amount of antagonist required for counteraction (170, 175, 176). Snell *et al.* (225) report that long-continued administration of pantoyltaurine to mice and rats produces evidence of pantothenic acid deficiency, thus indicating that this compound may interfere specifically with pantothenic acid metabolism in animals as well as in bacteria.

Indoleacrylic acid inhibits bacterial growth, and this action can be counteracted by the addition of even a trace of tryptophan (61). As Fildes stated (61), this latter observation, plus the fact that, in this instance there is complete absence of a competitive relationship between inhibitor and inhibitor-antagonist, suggests that the action of this inhibitor is more likely to be concerned with the formation of tryptophan rather than its use. There should be a quantitative relationship between indoleacrylic acid and some precursor of tryptophan.

This, as Fildes (61) emphasized, calls attention to a rather important point, namely, if sulfonamide action were a case of inhibiting PABA-synthesis, it would not be expected that the inhibitor should have a quantitative relation with the product PABA but with a precursor, and furthermore, the addition of PABA just sufficient for the needs of the organism should cause growth in spite of the presence of any amount of inhibitor, which of course is contrary to all observations. Others also have concluded that sulfonamide action is inhibition of the utilization rather than the synthesis of PABA (84, 163).

In view of the fact that PABA has been isolated from yeast, the effect of sulfonamides and its relation to PABA in this organism is of especial interest. Landy and Dicken (139) carried out a rather thorough investigation of the effect of sulfonamides (SA, SP, SG, and ST) on *Saccharomyces cerevisiae* in synthetic media. All of the compounds were found to inhibit yeast growth completely in concentrations ranging from 10 to 25 mg %. This inhibition was completely

counteracted by PABA, and it is of interest to note that the molar ratio: sulfonamide/PABA in this antagonism compares very well with that reported for bacteria. It was discovered that the supernatant of 16-hour yeast cultures contains a substance which neutralizes the inhibition by ST. In view of these facts, Landy and Dicken believed it quite probable that the mechanism of sulfonamide action on yeast is similar, if not identical with that on bacteria, and they suggested that PABA may be important in yeast metabolism as Woods and Fildes' "essential metabolite."

It is extremely difficult to interpret experiments involving the use of drug-antagonists, though this is one of the most promising approaches to the solution of the problem of drug action. There is danger in assuming prematurely that antagonistic agents characterized by the effects on growth are the compounds normally involved in the inhibited reactions. Moreover, the metabolism of different bacteria, even of different strains of some species, differs considerably, thus greatly complicating the picture. The fact that complex media may contain various antagonists cannot be ignored, and indicates that much may have to be learned from the use of such antagonists in synthetic media of known composition. When bacteria are put in such artificial media they may be far removed from their optimal environment. Carrying over results thus obtained to the interpretation of drug action on the bacteria in the host must be done with caution. The evidence so far is confusing, and to make order out of chaos is not easy. It would appear, on the one hand, that the mechanism of sulfonamide action and its antagonism is far from simple. On the other hand, the fact that the potency of each sulfonamide is proportional to its ability to nullify the effect of PABA-antagonism, plus the fact that PABA counteracts all sulfonamides, suggest that bacteriostasis is produced by interference with a single metabolic function of the cell. In order to prove that PABA is connected with such a metabolic function in the role of an essential metabolite, it would be necessary to show that PABA is actually an essential metabolite, identify the enzyme system with which it is associated, and then demonstrate that the effect of sulfonamide is directly proportional to the inhibition of this enzyme system.

As will be seen in the next part of this section, certain bacteria require PABA for their growth. Certain investigators have expressed the belief that the requirement of PABA as a growth factor by these few bacteria indicates rather definitely that PABA plays a definite role (e.g., as an essential metabolite) in the metabolism of other bacteria in which PABA is not a growth factor. This supposition of course is used as an argument for the Woods-Fildes theory which presumes such a role for PABA in the cell. It must be remembered, however, that those bacteria which require PABA for growth are saprophytes and as such are not as exacting in their nutritional requirements as the pathogens. It is a curious fact that, to date, PABA has been claimed to be essential for the growth of only one of the pathogenic bacteria (diphtheria bacilli, cf. part d). Even in this instance, however, the pathogenicity of the particular strain used was not recorded. Hence, there is no justification for the generalization that PABA has a metabolic role in all bacteria merely because it is a growth-factor for some of

them. It must be admitted, however, that some of the bacterial products which will be mentioned subsequently may well be PABA, although if this were true it would not necessarily indicate that PABA is an essential metabolite. Landy *et al.* (141), assuming that the sulfonamide-counteracting substance they found to be produced by all pathogens assayed is PABA, expressed the opinion that it is unlikely that so many unrelated species of bacteria would all make this compound unless it is an essential metabolite. In instances where PABA is an essential metabolite but not a growth factor, it would be very difficult to prove this until more is known about the particular enzyme system involved.

As will be seen in the next section, PABA antagonizes the sulfonamide inhibition of carboxylase, an instance in which PABA can scarcely be claimed to be playing the part of an essential metabolite. This is even more true of PABA-antagonism of sulfonamide inhibition of starch digestion by diastase and of methylene blue adsorption onto charcoal (54). Thus, that sulfonamide inhibition is a result of displacement of PABA, functioning as an essential substrate, cannot be the only possible explanation compatible with the known facts. Other interpretations will be presented in section F1.

d. As a growth factor.[14] Up to the time of Woods' and Fildes' publications (60, 284), PABA had been isolated from neither bacteria nor yeast, nor had it been proved to be a growth factor or essential metabolite. Shortly after these publications appeared, Rubbo and Gillespie (226) reported PABA to be a growth factor, as well as a SA-counteracting factor, for *Clostridium acetobutylicum.* Lampen and Peterson (137) and Park and Wood (216) were unable to reproduce Rubbo and Gillespie's experiments on PABA as a growth factor unless biotin was also present, and the latter authors expressed the belief that the substances (perhaps glucose) used by Rubbo and Gillespie contained biotin.

PABA has also been shown to be a growth factor for *Acetobacter suboxydans* (138, 140, 142) and probably for *Lactobacillus arabinosus* (100, 153). In the latter instance, lactic acid production is stimulated by PABA. It has also been claimed that PABA is a growth factor for *Corynebacterium diphtheriae* type *gravis* (29).

PABA concentrations in and above the range 1.5×10^{-5} M (0.2 mg %) to 0.01 M (140 mg %) have been reported by various investigators as growth-inhibitory (4, 84, 91, 226, 227, 248, 284). It has been noted (248) that the supposed growth function and the SA-counteracting action of PABA is thus restricted to a "zone of limited concentration", which is unusual for known bacterial growth factors. It would seem, however, that this consideration *per se*

[14] Recent work by Eyster (55) reveals that growth substances can achieve their effects indirectly as well as directly. Eyster shows that auxins are growth substances because they bring about the release of certain enzymes such as diastase from an adsorptive combination with protein colloidal substances, in which form they are relatively inactive. A rather analogous situation may exist in the case of thiamin stimulation of cocarboxylase (diphosphothiamine) activity in sea urchin egg extracts (131). It is thought that the thiamin might produce this effect by displacing the cocarboxylase from a combination with catalytically inactive protein, thus allowing the cocarboxylase to combine with the catalytically active protein.

does not constitute a conclusive argument against the possibility of PABA being an intermediary metabolite, since practically any substance will inhibit cell metabolism beyond some concentration level.

As stated previously, the fact that PABA is a growth factor for some bacteria has been used as evidence for the Woods-Fildes theory. Let us now examine this evidence further. Bacteria susceptible to sulfonamides may be arbitrarily divided into two groups: one for which PABA is a growth factor, and the other for which PABA is not a growth factor. The assumption is made that the only difference between the two groups, insofar as the relationship of PABA to the metabolism is concerned, is that, in the one, sufficient PABA can be manufactured by the cell for its needs. Accordingly, when both types of bacteria are inhibited by a sulfonamide, the antagonism offered by the concomitant addition of PABA would be the same in both cases, i.e., when the sulfonamide inhibits the bacterium it does so by displacing PABA which is essential for growth; whether the bacterium is capable of manufacturing PABA thus has no direct relationship to the mechanism of sulfonamide inhibition. This assumption makes the inhibition of bacterial growth resulting from withholding PABA from a bacterium for which it is a growth factor analogous to sulfonamide inhibition of bacterial growth, no matter whether the bacterium inhibited by the sulfonamide requires PABA for growth or not. This is supported by the report of Wyss *et al.* (288) that the SA-counteracting activity of PABA parallels its growth-factor activity both for *C. acetobutylicum* and for the "aminobenzoicless" mutant of *Neurospora crassa*.[15] Actually, however, there is indication of a real danger in making such an assumption. That there may be dissociation between these two phenomena (growth-factor activity and sulfonamide-antagonistic activity) is suggested by the fact that although *p*-aminophenylacetic acid is ten times more active than PABA as a growth factor for *C. acetobutylicum*, it possesses no SA-counteracting activity with the same organism (228). Nor does *p*-aminophenylacetic acid have any sulfonamide-antagonistic activity for other organisms (163).

[15] Tatum and Beadle (268) were able to produce an X-ray induced mutant of *Neurospora crassa* which is characterized by the loss of ability to synthesize PABA. A single gene is involved in this mutation, the gene apparently controlling an essential step in the synthesis of PABA. Growth when PABA is supplied is indistinguishable from that of the normal strain, but the mutant is unable to grow on synthetic media devoid of PABA (or the less active substances acetyl-*p*-aminobenzoic acid, *p*-nitrobenzoic acid, aniline and a few others). A concentration of 0.006 M (100 mg %) SA inhibits the growth of both strains to the same extent and in both cases this inhibition is counteracted by PABA. Since the quantitative effects of SA were the same on the two strains it was concluded that PABA utilization is interfered with rather than its synthesis. Results also indicated that the synthesis of PABA in the normal strain does not involve the introduction of an amino group into a preformed benzene ring, the nitrogen or amino group probably being incorporated before the ring is formed. The similarity in the action of SA and PABA in this particular instance seems especially significant inasmuch as it has been demonstrated that PABA (also pyridoxin and pantothenic acid) is involved in the respiratory mechanism of *Neurospora* (79). When mutants are used which require one of these substances for growth, the respiration of the mutants in media containing adequate substrate, but deficient in the particular "growth-factor," is increased upon the addition of the factor.

e. As vitamin.[16] A vitamin is a substance which the animal body requires for normal growth or other normal function, and which the organism itself cannot synthesize. Thus, fundamentally, the terms "growth factor" as applied to bacterial nutrition and "vitamin" as applied to animal nutrition are analogous. The suggestion that PABA occupies an integral position in bacterial nutrition, and the belief of some investigators that PABA is widespread in the animal and vegetable kingdoms make the question of its effect on the animal organism of considerable interest.

PABA has been tentatively added as a member of the vitamin B complex on the basis of its being essential to reproduction and lactation in rats, and growth-promoting in chicks. Subsequent studies have revealed real discrepancies in such an interpretation of the role of PABA in animal nutrition. Recent evidence suggests that PABA is not a growth factor for chicks, but that its action may be indirect by stimulating intestinal bacteria to produce certain essential factor(s) (22).

It is not safe at present to conclude that PABA *per se* behaves in any instance as a vitamin. Although this by no means excludes the possibility that PABA is present in the animal organism, it removes for the present at least one source of support for the idea.

f. Its relation to the development and prevention of resistance to sulfonamides. In the discussion on the acquisition of sulfonamide-resistance (B6) it was stated that one of the explanations offered for this phenomenon is that the bacteria increase their production of PABA, so that a correspondingly larger amount of sulfonamide is required for inhibition (82, 83, 84, 143, 163). This is a logical extension of the Woods-Fildes theory. The establishment of its validity depends on a direct demonstration of an increased production of PABA in the sulfonamide-resistant strain as compared to its parent strain, and furthermore, that this increased production is proportional to the amount of resistance developed.

There have been several reports that the acquisition of resistance is accompanied by an increased production of sulfonamide-antagonist (82, 83, 179, 198). This antagonist was believed to be PABA either on the mere fact that the substance was a sulfonamide-antagonist, or on the fact that the substance was destroyed by a soil bacillus trained to oxidize PABA (198),[17] or on the basis of certain physical and chemical properties of the substance, such as solubility and diazotization (143, 198), or on the basis of microbiological assays (143, 228). The observation that *S. aureus* when grown in increasing amounts of sulfonamides produces a yellow pigment believed to be a PABA derivative has also been considered as evidence that sulfonamide-resistance is a result of increased PABA production (258).

Green and Bielschowsky (83) were among those who observed that resistant strain washings have a greater sulfonamide-antagonistic action than those of the parent; but they found no strict correlation between sensitivity and yield of sulfonamide-counteracting factor, and thus came to the conclusion that a complete explanation of acquired (and natural) resistance cannot be put solely on such a basis. Landy *et al.* (143) found that sulfonamide-resistant strains of

[16] See György (88) for a review of this subject.

[17] The antagonist also specifically activated the PABA-oxidizing enzymes of the soil bacilli grown in its presence. Inasmuch as growth took place during this process, it would appear that PABA could not be an essential metabolite for these bacteria; yet this particular organism is sulfonamide-sensitive (199).

S. aureus produce greater amounts of PABA than do their parent strains (supernatant assayed by the microbiological test of Landy and Dicken, and by chemical methods), and that the amount synthesized by the resistant strains appears sufficient to account for their resistance. On the other hand, resistant strains of *E. coli*, *Vibrio comma*, *Shigella dysenteriae*, and pneumococcus failed to synthesize greater amounts of PABA than did their parent, non-resistant strains. The possibility of other, unknown sulfonamide-counteracting substances being produced in greater quantity by the resistant strains was not ruled out in this investigation.

The results have been inconsistent. In the first place, although in many instances there has been an increased production of sulfonamide-antagonist accompanying the development of resistance, the methods used for identifying the substance as PABA can be questioned. (This particular criticism will be given in greater detail subsequently.) Moreover, it is not even established that there is a strict parallelism between the production of any sulfonamide-antagonist and resistance, perhaps because the mechanism of acquiring resistance to sulfonamide is not identical in every case. Thus, the prerequisites stated at the beginning have not been satisfied. Although it must be admitted that the possibility has not been ruled out that sulfonamide-resistance in some instances is a result of an increased production of sulfonamide-antagonist (perhaps PABA), it must be concluded at present that this cannot be the mechanism in every case, and that it has not even been definitely shown to be the mechanism in any case.

Actual contact between the sulfonamide and the enzyme system involved, resulting in inhibition of the same, appears to be essential for the development of resistance (121, 262). Thus, PABA completely prevents the bacteria from developing such resistance; methionine, nucleic acid, and peptone, on the other hand, delay acquisition of resistance but do not prevent it (262, 263). This cannot be used as supporting evidence for the mechanism for the evolution of sulfonamide-fastness discussed above. It seems reasonably certain that antagonists achieve their action by causing removal in some way of the sulfonamide from its site of action. Since it is logical to assume that resistance develops as a protective response on the part of the organism to the stimulus of the toxic substance sulfonamide, it follows that, unless the toxic state exists, resistance will not develop.

g. As "catalyst" related to the synthesis of substances such as methionine. In the Woods-Fildes theory PABA is considered to be a substrate. Kohn and

[18] Although these publications of Kohn and Harris will be referred to several times, it seems best to mention at this point that the pH fell in the course of their experiments, in some instances as low as 4.7 (126). In view of the established fact that sulfonamide activity varies considerably with pH, and the definite possibility that the activity of sulfonamide-antagonists may also be affected; quantitative results and their interpretation in these publications can be seriously questioned. The theory proposed, however, should receive consideration here inasmuch as it was partly based on previously established facts, and furthermore, because the essence of the theory, that sulfonamide inhibition is primarily on the synthesis of the essential food substance, has received verbal support elsewhere (241, 281).

Harris (91, 126)[18] formulated a new theory which transferred PABA from the role of a substrate to that of a catalyst. The essential facts underlying this interpretation are the following: First, as already discussed, many workers have reported that a latent period exists before sulfonamide action manifests itself *in vitro*. Second, methionine is able to counteract sulfonamide action, but only at low concentrations of sulfonamides. Third, ethionine, norvaline, and norleucine inhibit bacterial growth and synergize SA-action, possibly by competing with and displacing methionine in the cell, since addition of methionine (or peptone), but not PABA, abolishes these inhibitions. It is as if these compounds endeavor to take the place of methionine in a reaction normally involving methionine but are an inadequate substitute for the methionine; thus the reaction ceases. The fact that PABA does not affect inhibition by ethionine was regarded as placing the methionine antagonism as a reaction secondary to primary reactions involving PABA.

From this evidence, Kohn and Harris schematized sulfonamide 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 catalyzed by PABA (termed *primary reactions*). When the stores of X fall below a critical concentration, growth rate decreases. PABA always remains effective as an antagonist because the primary reactions now have available PABA and all the secondary reactions which follow are restored. It is the primary reaction involving methionine synthesis which is inhibited by low concentrations of sulfonamides, since this inhibition can be antagonized by PABA as well as methionine. As the drug concentration is increased more primary reactions become inhibited, and the synthesis of the other X components, in turn, is inhibited.

Two objections can be raised against this theory: first, as already stated, the delay in sulfonamide action is not always observed; second, as pointed out by Sevag *et al.* (246), there is no known example where an excess of enzyme produces an inhibition, and it is well-established that PABA acts as an inhibitor in concentrations above those which antagonize sulfonamide activity. PABA antagonism has been observed in sulfonamide-inhibited systems where its action would be difficult to conceive as catalytic (e.g., the carboxylase and charcoal systems already referred to). Before accepting such a complicated explanation for PABA antagonism which, besides having the above objections to it, could apply to only one phase of sulfonamide-antagonism, it seems best to attempt to find a simpler explanation which would apply to all instances of this antagonism.

h. As detoxicant. Among the various capabilities of the relatively simple compound PABA, other than that of sulfonamide-antagonism, is its ability as a detoxicant. Although unable to counteract the trypanocidal action of pentavalent arsenicals in infected mice, it does protect the animals against toxic doses of the drugs (231). A similar protection is afforded mice against the toxicity of the trypanocidal drug Stibosan (sodium *m*-chloro-*p*-acetylaminophenyl sti-

bonate), a pentavalent antimony compound (280). Here again there is no interference with the drug's trypanocidal potency *in vivo*.[19]

Whether or not the action of PABA in these instances is in any way related to PABA-antagonism of sulfonamide action is not yet known. Actually, the phenomenon of antagonism of sulfonamide inhibition of cell growth may be regarded as a detoxication, and it may be that both detoxications are an expression of some fundamental property of the PABA molecule.

Summary: PABA has been isolated from yeast and characterized; it antagonizes sulfonamide inhibition of bacteria *in vitro* and *in vivo*, and of many other cells, on a competitive basis with the sulfonamide; it behaves as a growth factor for several non-pathogenic bacteria; it has not been shown conclusively that it acts as a vitamin in any case; it acts as a detoxicant for certain antimony and arsenic compounds; it is regarded by the Woods-Fildes theory as an essential metabolite which is displaced from its enzyme by sulfonamide, a proposal for which there has never been any direct evidence; there is no consistent relationship between sulfonamide-antagonist production by bacteria and their susceptibility to sulfonamides; PABA antagonizes sulfonamide inhibitions of systems in which it cannot possibly be an essential metabolite; it has also been proposed as a catalyst in the cell, a theory which can be seriously questioned. A critical analysis of these facts will be deferred to the final section, but it may be stated here that the only definite conclusion that can be made at present is that PABA by some type of adsorptive phenomenon counteracts sulfonamide inhibition.

2. Other Sulfonamide-Antagonists of Known Composition

a. Methionine. The action of methionine is very interesting and has received considerable attention. Methionine has been found to antagonize the action *in vitro* of SA, SP, ST, SD and SG, and, with but a few exceptions, under conditions in which no growth-stimulation is observed with the methionine alone (15, 91, 95, 126, 262). The *l*(−) form is about ten times as effective an antagonist as the *d*(+) form (91). Strauss *et al.* (262), working with *E. coli*, found that methionine can reverse SG and SA, though it is less active than PABA. No reversal of bacteriostatic concentrations of SP, ST, and SD was observed, thus suggesting that these compounds may act at some loci in the cell other than those affected by SA and SG. Other experimental results have indicated this (91, 128, 263).

Harris and Kohn (91) found that methionine is effective only against low concentrations of sulfonamides, and, as with PABA, higher concentrations are required to exert antagonism on sulfonamides other than SA. On the other hand, observations (15, 91) indicate that unlike the condition existing with PABA, there seems to be no constant ratio between the amount of SA present and the amount of methionine required to neutralize it.

[19] SA itself is reported to exert a definite protective action against liver necrosis from acute carbon tetrachloride poisoning in rats (71). A sparing effect of SP on the intoxication of the leucopoietic tissue by benzene has been demonstrated in rabbits (168). PABA failed to inhibit the leukotoxic action of benzene.

Bliss and Long (15) reported that methionine concentrations of 1 per cent or more have an inhibitory action on *E. coli*. They also made the interesting observation that SA is able to neutralize this methionine inhibition to a certain extent. It was their experience that the range of SA concentrations over which methionine is capable of antagonism is very narrow, although the range of effective methionine concentrations is very extensive. The former observation might account for the failure to obtain methionine antagonism of sulfonamide action in some instances.

The metabolism of methionine in bacteria is almost as obscure as that of PABA. Bacterial growth does not occur if the NH_3 of the basal medium is replaced by methionine (91). It is not oxidized, decarboxylated, deaminated, or hydrolyzed by washed suspensions of *E. coli*. Therefore, the role of methionine in the metabolism of *E. coli* is neither to supply nitrogen nor energy for growth. Kohn and Harris (127), however, recently obtained a culture of *E. coli* requiring methionine as a growth factor by growing the bacilli in an amino acid-purine mixture containing SA. Cultivation in SA alone, or in methionine alone, did not alter the methionine requirement of the organism. The apparent paradox was explained by suggesting that SA-resistance developed in methionine-free medium involves adaptive metabolic pathways which protect methionine synthesis, whereas, in media containing methionine, such adjustments are not necessary.

Although there is no doubt that methionine can antagonize sulfonamide inhibition, there is no conclusive indication as to its mechanism of antagonism. Bliss and Long (15) suggest, without evidence, that methionine, and perhaps arginine and lysine (which show some sulfonamide-antagonistic activity), are not themselves anti-bacteriostatic, but rather are precursors of a substance which has such activity, and is an essential metabolite whose production is hindered by sulfonamide. The explanation offered for methionine antagonism by Kohn and Harris has already been discussed.

b. Amino acids, purines, urethane, etc. Various amino acids and purines have been shown to counteract sulfonamides under certain conditions: arginine and lysine (15); glutamic acid, glutamine and casamino acids[20] (95, 179, 205); aminoids[21] (205); glycine, serine, allothreonine, guanine and xanthine in a medium containing methionine, their individual affects being additive (128).

As stated above, guanine and xanthine in the presence of methionine antagonize SA inhibition, but in the absence of methionine, guanine and xanthine increase SA action, although having no effect on growth in basal medium, with or without methionine (128). Hypoxanthine and adenine potentiate SA inhibition, with or without methionine; in the absence of SA these 6-purines are without effect on growth. Thus, the ability to potentiate methionine's antagonism of SA inhibition apparently is dependent on substitution at positions 2 and 6 of the purine nucleus; potentiation of SA inhibition, on the other hand, seems associated with substitution in position 6 alone. When SA action is completely counteracted by PABA, all these purines are without effect. Further-

[20] Acid hydrolysate of casein.

[21] Trade-name of biuret-free material used in culture media.

more, bacteria (*E. coli*) made resistant to SA possess a changed response to the purines; growth is inhibited by hypoxanthine and adenine but not by xanthine or guanine; SA addition does not alter this effect, but PABA or methionine abolishes it completely. All of these observations suggest that metabolic relations for SA, methionine and purines exist which are as yet unknown.

Snell and Mitchell (256) found that, although inactive alone, in the presence of suboptimal amounts of PABA, the purines adenine, guanine, xanthine, and hypoxanthine further antagonize sulfonamide action on *Lactobacillus arabinosus* and *L. pentosus;* methionine showed no antagonistic action under similar conditions[22]; with *L. pentosus* and *L. casei*, on the other hand, antagonism is affected by the purines (also by methionine in the case of *L. casei*) without PABA, provided the medium contains biotin concentrate. The nature of the substance present in biotin concentrate which effects purine activity is unknown. These various lactobacilli, as well as others, are stimulated in their growth by these purines and PABA. In contrast to the lactobacilli, the growth of *Acetobacter suboxydans* is not affected by the purines adenine, guanine, and xanthine in the absence of PABA, but these purines act as growth accessories in that they increase the response to PABA, thereby suggesting a relationship between purines and PABA both for growth and for sulfonamide-antagonism (144). These results with adenine and hypoxanthine are conflicting, but it must be emphasized that different bacteria were used in each case. That the difference found is a result of the fact that different bacteria were used, is borne out by evidence obtained by Kohn and Harris (128). Adenine, but not guanine, has been found to annul the chemotherapeutic efficiency of SA in hemolytic streptococcal infection in mice (190).

Glucose has been reported to counteract sulfonamide action, under certain conditions, in concentrations (0.6 to 2.6 per cent) at which the glucose alone does not stimulate bacterial growth (95).

Lamanna and Shapiro (136) found that mercuric chloride can counteract SA bacteriostasis and *vice versa*, neither result being dependent on the growth-stimulating capacity of low concentrations of the antagonist. Mixtures of both substances in stimulatory concentrations proved to be inhibitory. These investigators were of the opinion that such results probably arise because the two substances stimulate or inhibit separate enzymes whose reaction rates are in some way interdependent.

A very important group of substances which has been shown to antagonize sulfonamide action are those primarily related to cell oxidative metabolism, e.g. coenzymes and nicotinic acid. They have commanded considerable recent attention, but since they are more concerned with respiratory functions, they will be considered in the succeeding section.

Urethane, a general cell inhibitor, has been found to antagonize SA inhibition

[22] In this report it was claimed that methionine failed to exert any additional sulfonamide-antagonistic activity under these specific conditions; however, since the medium employed contained casein hydrolysate it may well be that optimal amounts of methionine were already present.

of growth and luminescence in growing cultures of luminescent bacteria (105). Counteraction of sulfonamide inhibition of the growth of other bacteria has also been observed (173). As with methionine, it is reported that the antagonism by urethane of sulfonamide inhibition is not competitive, the molar ratio: urethane /SA varying approximately between 1 and 100 (173). The mechanism of this antagonism by urethane *in vitro* has recently been explained satisfactorily by Johnson *et al.* (109) on the basis that the sulfonamide and urethane combine reversibly with each other to form an inactive complex. This will be discussed in greater detail at the end of this section. Other substances found by Johnson *et al.* to antagonize SA inhibition of bacterial luminescence include ethyl alcohol, butyl alcohol, chloroform, ether, acetone, glycocoll, arginine, and xanthine.

The substances of known composition which are reported to antagonize sulfonamide inhibition thus include PABA and its derivatives, methionine, certain purines, certain amino acids, glucose, mercuric chloride, coenzymes, nicotinic acid, and urethane.

3. Sulfonamide-Antagonists of Unknown Composition

a. Proteins (serum, etc.) Meat extract and infusion, blood, plasma, serum, various exudates and transudates, albumin, gelatin, casein, fibrin, edestin, and sterile nutrient broth interfere with the activity of sulfonamides, *in vitro*, though to a less extent than peptone (17, 77, 96, 179). The products of protein digestion apparently have greater power of antagonizing sulfonamide than the parent protein (87).

The mechanism of antagonism of these substances is not definitely known. Boroff *et al.* (17) were of the opinion that the antagonistic activity of such material could not be explained purely on the basis of growth-promotion. These various substances have never been directly assayed chemically for PABA; and, pertinent to the question of whether protein antagonism is due to its PABA content, it is highly significant that no aryl amine has ever been demonstrated in protein. It is doubtful whether protein gives the bacterium mechanical protection from sulfonamide, for other substances such as gum, starch, and saponin, which should protect in a similar manner, have no such action (77).

One series of investigations, however, does give a clue to the mechanism whereby protein material antagonizes sulfonamide inhibition. Early cataphoretic studies indicated that Prontosil, but not SA, is adsorbed onto serum albumin and bacterial protein but not to globulin (239, 257). Kimmig and Weselmann (119), also using the cataphoresis technique, confirmed the negative results with globulin, but found all the sulfonamides studied, which included Albucid, Neo-Uliron and SP, to be adsorbed onto serum albumin. That the combination is an adsorption was indicated by the finding that dissociation could be produced by the addition of animal charcoal. By means of ultra-filtration, it was shown that the sulfonamides are held to the proteins by a pressure exceeding eight atmospheres. Davis and Wood (40, 41), using an entirely different experimental approach to the problem, confirmed the latter findings. Their conclusions were based on dialysis experiments which indicated that sulfonamides are ad-

sorbed onto albumin but not globulin. In normal plasma 20 per cent of the SA present is bound, 40 per cent of SP, 55 per cent of SD, and 75 per cent of ST. Experiments with *E. coli* in synthetic medium, with and without albumin added, suggested that the concentration of unbound sulfonamide determines the level of bacteriostatic activity, the bound sulfonamide being apparently inactive (40). The data indicated that it is the anion of the sulfonamide which becomes bound, and, if true, the binding tendency should be proportional to the dissociation constant and to the bacteriostatic activity of the sulfonamide. McClintock and Goodale (169) placed SA in albumin solutions undergoing digestion with trypsin and demonstrated that a combination takes place between the ring amino group of the SA and the albumin, and also the early hydrolytic products of the albumin. This conjugate was bacteriostatically inactive.

Sevag *et al.* (245, 246, 250), after demonstrating antagonism by peptone, serum albumin, and globin of sulfonamide inhibition of bacterial growth and respiration, of catalase activity, and of carboxylase activity, suggest that antagonism in such instances may be due to the ability of these substances to favor the dissociation of the sulfonamide from the inhibited enzyme's surface by the formation of an inactive sulfonamide-protein complex, i.e., the peptone or protein has greater attracting or adsorptive power for the sulfonamide than the enzyme or enzymes which the sulfonamide would inhibit in their absence (cf. 128). Johnson *et al.* (109), in their recent experiments on antagonism of SA inhibition of bacterial luminescence, obtained evidence that serum and peptone owe at least part of their antagonistic activity to their SA-combining power. Whether or not formation of an inactive complex can completely account for the antagonistic activity of these various materials cannot at present be answered; it seems fairly certain, however, that it is at least part of the story.

b. Peptone. The first material discovered to have sulfonamide-antagonistic properties was peptone (15, 91, 157, 177, 205).[23] Lockwood and Lynch (159) showed that this phenomenon is general, peptone being antagonistic to SA, SP, and ST in the case of pneumococci, staphylococci, colon bacilli and hemolytic streptococci. According to Lockwood (157), bacteria under the influence of sulfonamide are unable to break down complex protein but are still able to utilize for growth simple protein-split products, such as peptone.

Lockwood's proposal has been tested by various methods and found to be untenable. Long and Bliss (160) tested a known proteolytic streptococcus to see whether it was more or less affected by SA in peptone-free horse and rabbit sera than a non-proteolytic strain. The two strains grew equally well in the presence or absence of SA in broth. In serum, the proteolytic strain grew more rapidly but the difference between the growths of the two strains was not altered in any way by SA. Furthermore, a direct estimation of the activity of streptococcal proteolytic enzyme has revealed no change in activity under the action of SA (77). Abderhalden (1) found that the sulfonamide derivatives Uliron,

[23] No distinction will be made between peptone, neopeptone, or proteose-peptone though in some instances there may be a difference in their action, qualitatively (160) as well as quantitatively (153).

Albucid, SP, ST, and Prontosil, in concentrations of 20 and 200 mg% have no influence on the action, *in vitro*, of pepsin, trypsin, and serum di- and polypeptidases.

After the discovery of the great sulfonamide-antagonistic powers of PABA it was perhaps natural to think that the counteracting power of peptone (and other antagonists of organic origin) may be due to its PABA content, and claims to this effect were made (174). These claims were based on non-specific chemical assays or on microbiological assays.

Absence of PABA in Peptone. There have been several indirect indications that peptone's antagonistic activity cannot be due to its PABA content (91, 126), but the final word in such a question depends on whether or not PABA can be positively detected in peptone. Eckert (49), using a modified Marshall method for the determination of PABA, found that peptone broths sometimes gave a slight color reaction which he ascribed to the presence of tryptophan. Kohn and Harris (128), following Blanchard's (14) technique and the analysis of Bratton and Marshall, found the PABA content (bound and unbound) of various peptones to be very small. Evidence was obtained by Kohn and Harris which led to the following interpretation of peptone antagonism: The active substances in peptones can be divided into two groups, the first composed of the four amino acids methionine, glycine, serine and allothreonine, and the second made up of the purines xanthine and guanine, which are only antagonistic to sulfonamide action when in the presence of methionine and which potentiate sulfonamide action in its absence. The purines do not affect the competition between PABA and the sulfonamides, this being interpreted by Kohn and Harris to mean that purines are secondary antagonists and potentiators. Possibly the small amount of PABA found in peptone plays a part. The summation of all these factors accounts for almost all the antagonism against SA. Against the heterocyclic sulfonamides, however, this group accounts for nearly all the antagonism only when growth-inhibition is less than approximately 65 per cent. Above this, another factor of great power, whose nature is unknown, is active. It is water-soluble, not a known, naturally occurring amino acid or PABA. It was obtained from pancreas, which seems to be the best source, but is neither insulin nor other protein. The fact that this substance is concerned only with antagonism of the heterocyclic derivatives of SA suggests that these derivatives either act at more than one locus in the cell, or form an inactive complex with some component of peptone and pancreas. The methods used in this investigation ruled out any antagonism due to growth-stimulation.

Peptone is both a growth-stimulator and sulfonamide-antagonist and, as already discussed, it has been suggested that the sulfonamide-antagonism is a result of the growth-stimulation. Further evidence against the "growth-stimulation" hypothesis, appears in the work of Sevag *et al.* (246) who found that peptone counteracts the SA inhibition of respiration when there is no growth. When peptone is added to a culture, growth and respiration increase in parallel with each other. It was observed that the greater the increase, the greater the inhibition by sulfonamide. If sulfonamide-antagonistic action were due to non-

specific growth-stimulation, the opposite would be expected. The experimental findings are in agreement with the idea that to a great extent the respiratory increase is coupled with growth, and that this part of the respiration is sulfonamide-sensitive; thus, it would be expected that the greater the increase in respiration (and growth) the more there is to be inhibited. As already stated, counteraction of sulfonamide inhibition of carboxylase activity in *E. coli* and *S. aureus* by peptone suggested (245, 246) that peptone and bacterial enzyme protein compete for the inhibitor sulfonamide, and as a result of this competition, sulfonamide action is counteracted. It is known that peptone forms reversible complexes with various substances. For example, tannic acid combines reversibly with invertase, precipitating it and thus inhibiting its activity (213); and this inhibition is counteracted by egg albumin or peptone which displace the tannic acid by combining with it. Analogously, protamine and the α- and β-globulins from human plasma inhibit bacteria, their inhibition being counteracted completely by human plasma (244). Since human plasma *per se* is not inhibitory, and in fact is capable of counteracting anti-bacterial substances, it is evident that the α- and β-globulins are neutralized in the plasma by proteins.

c. Necrotic tissues and abscesses. It was an early, clinically important, observation that in the presence of abscesses and tissue necrosis the sulfonamides are helpless (7, 120, 158). Attempts to find sulfonamide-antagonist in pus have been conflicting: MacLeod (179) claimed invariable success, and Fox (74) reported persistent failure. There is, however, a wealth of indisputed clinical and experimental evidence that tissue breakdown products contain sulfonamide-antagonists. It is apparent, for example, that whether or not sulfonamides can produce bacteriostasis in the presence of these products depends on the concentration of sulfonamide which can be obtained in that area. Sulfonamides have given good results in pyogenic infections where they can be applied locally in high concentration. On the other hand, the presence of large amounts of sulfonamide-antagonist in tissue breakdown products, as compared to the relatively small amounts in serum, explains the marked difference in therapeutic response between rapidly spreading infections, such as erysipelas or pneumonia, and localized purulent foci that can only be reached by the ordinary therapeutic blood levels which are too low to cope with the antagonists present in the purulent foci. Dead bacterial cells also have a sulfonamide-antagonistic effect (157).

It is not known whereby, or even what, substances present in tissue necrosis and abscesses antagonize sulfonamide-action; certainly PABA has not yet been identified in such loci. In fact, it seems improbable that the antagonistic activity in these loci is due to PABA. The sulfonamide-antagonistic action of PABA is limited to a zone of concentration with definite limits (Dld), and if the failure of sulfonamides in the presence of tissue necrosis were due to PABA, it would seem to be somewhat of a coincidence that, in every instance, the PABA concentration should lie within the particular concentration range capable of antagonizing the concentration of sulfonamide drug present. Such a relationship would not be required if a substance such as peptone were responsible for the sulfonamide-antagonism, for peptone concentration can be greatly increased above its antagonistic level without disappearance of the antagonism (D3b).

d. Tissue Extracts. Sulfonamide-antagonist has been found in various normal animal tissues (including muscle, liver, kidney, pancreas, and spleen), turnip, enzymatic casein hydrolysate (not acid or alkaline hydrolysate), transplanted rat sarcomas, and some higher fungi (including the common mushroom) (83, 179, 284). The antagonists obtained from these sources are to a greater or lesser degree in a conjugated form. Some of the properties of these naturally occurring antagonists (e.g., ether solubility) are similar to those of PABA

but some of the antagonists have been found to differ chemically in several respects from PABA (179).

Various body fluids and tissue extracts have been analyzed for PABA content by Lewis (153) and Landy and Dicken (140) by microbiological assay methods. All body fluids and extracts (urine, blood, liver, etc.) thus tested were found to contain PABA, appreciable amounts of which in many instances were in an inactive form, being activated by alkaline hydrolysis. Using the variant of *Neurospora crassa* unable to synthesize PABA (section D1a) in a biological assay for PABA, Bonner (16) found tomato roots to contain PABA or a substance with similar action. Using the same assay method, Mitchell *et al.* (200) assayed beef liver, spinach, oat seeds, mushrooms and fresh yeast for PABA and concluded from their results that the assay procedure of Landy and Dicken responds to only a fraction of the total amount of PABA obtainable after acid or alkaline hydrolysis. It was also found that enzymatic hydrolysis or autolysis is not always sufficient to lead to the maximum effect. Separation of partially purified liver fractions by a "chromatogen technique" has also indicated the possible presence of PABA in this tissue (29).

Using a modified Marshall method for detection of PABA, Eckert (49) was unable to obtain a positive reaction with blood filtrates from normal animals. Kisch and Strauss (122), on the other hand, report that normal blood and urine contain small amounts of chromogenic material which upon diazotization gives the color reaction of PABA. Teply *et al.* (269) found a counteracting acid-labile factor(s) present in liver extracts and grass juice which was distinct chemically and physically from PABA and which had properties similar to those reported for folic acid preparations. It is seen, therefore, that although sulfonamide-antagonists undeniably exist in various tissues, and although some of these may be PABA, this compound has not unquestionably been identified with any of them.

e. Bacterial products. Certain bacteria have been shown to give off or contain sulfonamide-antagonist (179); in a few instances, failure to find antagonist in bacteria has been reported (179). There seems to be no doubt, however, that antagonists are produced by certain bacteria at least. The question of most importance is their identity, and it has already been dealt with in connection with the discussions relative to sulfonamide-fastness (D1f). One important investigation which was not mentioned, however, is that of Landy *et al.* (141). Culture filtrates and hydrolyzed whole cultures were assayed for PABA by the method of Landy and Dicken (140) which depends on the specificity of the need of *Acetobacter suboxydans* for PABA as a growth factor. Using this criterion, all organisms studied elaborated PABA to a greater or lesser degree. The organisms included strains of the genera *Staphylococcus*, *Streptococcus*, *Bacillus*, *Brucella*, *Corynebacterium*, *Eberthella*, *Escherichia*, *Clostridium*, *Klebsiella*, *Mycobacterium Proteus*, *Salmonella*, and *Shigella*.

As to whether these sulfonamide-antagonistic bacterial products are PABA it can only be repeated that, although some of them may be PABA, so far only tests have been used, the specificity of which can be questioned. It has been demonstrated rather definitely that at least some of these antagonists act by a non-specific growth-stimulation (82, 83).

Summary: A great number of substances with a great diversity of source antagonize sulfonamide inhibition. The first question to be answered is whether their antagonistic activity is due to their PABA content. This obviously cannot 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 owe their activity to their PABA content. This assumption still persists largely today. The assays which have been employed to determine the presence of PABA in these substances fall into three groups as follows: First is the "sulfonamide-antagonistic" assay. This is obviously non-specific;

no more proof of this is needed than to look at methionine and urethane. Second, physical and chemical properties such as ether-solubility and diazotizability. Solubility is obviously non-specific and diazotizability merely indicates a primary arylamine. The danger of assuming such diazotizable substances to be PABA has been emphasized by reports that bacteria can produce diazotizable aromatic amines which apparently are not PABA. Miller (197) found that filtrates of both susceptible and resistant streptococcal cultures, although containing a primary aromatic amine as shown by diazotization and coupling with dimethyl-α-naphthylamine, had no antagonistic effect on sulfonamide action. Fox (73) claimed to have found a diazotizable aromatic amine formed by bacteria during sulfonamide bacteriostasis; but it was not PABA, its formation being prevented by PABA antagonism of the sulfonamide action. In the third group fall the microbiological tests. Although Landy and Dicken (140) showed that of fourteen related compounds none possessed more than one-tenth the growth-factor activity of PABA for *Acetobacter suboxydans*, one can still question the specificity of the test. Mirick (199) has recently pointed out several possible sources of error in such bioassays. Furthermore, there is evidence that sulfonamide-antagonism and growth-factor activity cannot be assumed to be associated phenomena (D1d). Thus, there is no absolutely conclusive proof that PABA is present in these substances. This is of especial significance in the case of bacterial products, because demonstration of PABA production by bacteria is vital to the Woods-Fildes theory. Chemical or physical analysis of a universally recognized specific nature must be performed on all these various substances to decide the issue. So far, such analysis has been applied to yeast, in which PABA was found, and to peptone, in which PABA was found only in insignificant quantities.

As to how sulfonamide-antagonists act, sufficient data are at hand to warrant the conclusion that interference with sulfonamide action can be divided into at least three distinct categories. In the first, the antagonism is by some specific interference with sulfonamide action. PABA certainly is an example of this type of antagonist. Methionine and mercuric chloride also appear to be representatives of this group. The observation that amino acids containing aromatic groups (tryptophan, tyrosine, and phenylalanine) inhibit SP adsorption onto activated carbon particles suggests that part of the antagonistic activity of amino acids may be due to a similar action at the loci in the cell where sulfonamide adsorbs (146). Antagonism by specific interference will be discussed further in the last section. In the second category, antagonism is unspecific by growth-stimulation, i.e., growth-stimulation by an action on the cell unrelated to the mechanism of sulfonamide inhibition. A prerequisite for this phenomenon is sub-optimal growth to begin with. It is known that sulfonamide action is greater the poorer the nutritional environment of the organisms (77, 83, 193). On the other hand, it must be emphasized that the mere possession of growth-stimulatory properties does not mean that a substance will antagonize sulfonamide action (91, 160, 165, 181, 205). In fact, one compound, asparagine, has been found to enhance sulfonamide activity in spite of the fact that in the

absence of sulfonamide it is a growth-stimulant (233). There is no doubt that the two phenomena, growth-stimulation and specific interference, are not associated. This was first shown by Lynch and Lockwood (165) who demonstrated that though some substances such as peptone, act both as growth-stimulants and as a sulfonamide-antagonists, there are other substances which exert either growth-stimulation or sulfonamide-antagonism alone, PABA being a representative of the latter group. That PABA does not stimulate growth (except when a growth factor) in concentrations which counteract sulfonamide action (or in any concentration for that matter) has been adequately confirmed (136, 196, 248). Further evidence for this dissociation appeared when it was found (91, 126) that, out of an extensive list of amino acids tried, only methionine and PABA exerted sulfonamide-counteracting action, although many of them stimulated growth more than methionine in the absence of sulfonamide. As has been seen, PABA does not belong to this group, nor can any considerable part of the activity of methionine or peptone be considered as belonging here. Certain nutrient substances such as glucose and certain amino acids, however, undoubtedly owe at least part of their antagonistic activity to a non-specific growth-stimulatory action. In the third group, antagonism results from the formation of an inactive complex between sulfonamide and antagonist. That such direct interaction may account for PABA-antagonism of sulfonamide action has received but little consideration until recently, because of the very large molar ratios of PABA/SA observed in antagonism; and it was argued that the molar concentration of PABA required for antagonism should be of the same order as that of the sulfonamide if antagonism were a result of an interaction between the sulfonamide and PABA. But as seen in section B3d, the ratio of "active" sulfonamide to PABA is near unity. Direct procedures have never been used to determine if PABA does combine with sulfonamides, but the recently reported results of Johnson *et al.* (109) are fairly conclusive on this point. These investigators showed that when SA and PABA inhibit bacterial luminescence they do so with certain definite group characteristics. When both inhibitors are present simultaneously, there is no combination between PABA and SA. In contrast to such combinations of inhibitors in which both inhibitors are of the same type (space does not permit a description of the characteristics of each type inhibitor according to Johnson *et al.*), combinations of different type inhibitors can result in antagonism or synergism, depending on temperature and concentration conditions. Such instances of antagonism are a result of the formation of an inactive complex by a reversible adsorption, perhaps through hydrogen bonds. This apparently is the mechanism of sulfonamide-antagonism by urethane, and also probably in part of the antagonism by various other substances. Adsorption between protein and sulfonamide has been adequately demonstrated by various techniques, and it has been suggested that this, to a major extent at least, is the mechanism of antagonism by proteins and protein degradation products, such as peptone.

There is, therefore, antagonism by specific interference, by non-specific growth-stimulation, and by inactive complex formation. Henceforth it should

be useful to ascertain in which of these categories a given antagonist belongs. Two things, however, must be emphasized: first, one antagonist might fall into two or even all three categories, and second, other mechanisms of antagonism are conceivable and must not be lost sight of. The various possible ways in which an antagonist could antagonize inhibitor action are reviewed in the last section.

E. EFFECTS ON RESPIRATORY MECHANISMS

The fundamental action of sulfonamides is inhibition of cell multiplication. This is an *effect* of sulfonamide action, and preceding sections have dealt with observations made on this effect and resultant theories as to its *cause*. We now proceed to a consideration of phenomena believed to be more closely related to the cause. One of the functions of all cells is respiration, and this can be measured with relative ease and accuracy. This section deals with the observations and their interpretations of the effects of sulfonamides on the respiratory mechanisms of bacteria and other cells.

1. General Considerations

It is common knowledge that the energy for cell division and growth, as well as for maintenance, and for synthesis of any essential metabolite, etc., must come from respiratory oxidative processes. These oxidative-reductive respiratory processes are catalyzed by the dehydrogenases, the flavoproteins, the cytochromes, cytochrome oxidase, etc. If any inhibitor blocks the activity of any one enzyme in a respiratory chain the overall activity of the chain is decreased to the same extent, provided, of course, that there is no shunt or by-pass available through which the uninhibited components of the chain can continue to function. Furthermore, if these energy providing respiratory reactions are inhibited, cell division and growth are also inhibited. Sulfonamides could conceivably play the part of a respiratory enzyme inhibitor and thereby bring about their bacteriostatic effect. Several investigators have tested this hypothesis, and several have made experiments to determine which step if any in the chain of respiratory enzymes is inhibited by sulfonamides. Any theory of sulfonamide action based on inhibition of respiratory processes hinges on whether or not an inhibition of these processes can be observed experimentally, e.g., a decrease in aerobic oxygen consumption or anaerobic CO_2 production during sulfonamide action.

2. Inhibition of Bacterial Respiration

Barron and Jacobs (10) demonstrated a slight inhibiting effect by low concentrations (0.01 M; 173 mg %)[24] of SA on the oxygen consumption of heavy

[24] It will be noted that this concentration as well as many others quoted in this section are very high as compared to the concentrations usually used in such experiments as referred to in previous sections (in the range of a few to about 50 mg%). The reader will recall that an inverse relationship exists between inoculum size and concentration of sulfonamide required for inhibition (section B3b); these high concentrations are therefore

saline suspensions of resting streptococci and Friedländer's bacilli in the Warburg apparatus, but no effect on *E. coli* and gonococci. Chu and Hastings (31), using higher concentrations of SA (ca. 0.04 M; 692 mg %), found inhibition of respiration in several experiments with suspensions of washed pneumococci; similarly resting cultures of gonococci and meningococci, although poor respirers, were also inhibited; in the case of *Streptococcus pyogenes* the respiration was so small that experimentation proved to be unsatisfactory. The inhibitions of respiration reported by these two groups of workers fell into the range of 5 to 50 per cent.

Respiratory inhibition of resting and actively dividing dysentery bacilli by SA, ST and SP has been demonstrated by Dorfman and coworkers (46, 47). Ely (50) obtained approximately 50 per cent inhibition of oxygen consumption of resting *E. coli* in synthetic medium and in rabbit serum by 0.06 M (1000 mg %) and 0.05 M (830 mg %), respectively. Kohn and Harris (126) also reported that the oxygen consumption of suspensions of *E. coli* in phosphate buffer + $MgSO_4$ + NaCl was not affected. In saline-glucose medium, at lower sulfonamide concentrations, it was claimed that the oxygen consumption per bacterium did not fall appreciably, and in higher concentrations there was a decrease in oxygen consumption, but it lagged behind the fall in growth rate. The authors concluded that sulfonamides do not have a direct influence on respiratory enzyme action; but calculations made from their published data show that there was inhibition of respiration in all but one instance (see table 3).[25] Unfortunately, a correlation between inhibition of respiration and inhibition of growth cannot be made, because growth rates were determined from the rate of oxygen consumption rather than from direct bacterial counts.

3. Correlation of Respiratory Inhibition with Growth-Inhibition

As can be seen from the reports cited above, it was not possible on the basis of these studies to learn whether or not the inhibition of growth by sulfonamides was the result of the inhibition of the bacterial respiratory enzymes. A more complete set of experiments carried out by Sevag and Shelburne (247, 248) to determine the effect of SA on respiration and growth of *S. pyogenes* and pneumococcus Type 1 showed a definite relationship between these two effects. After measuring simultaneously at various time intervals the increase in the number

considered low in view of the large inocula used in such experiments. Justification for using such large inocula and concentrations will be given in part 7 of this section.

[25] It has been known for some time (215) that *E. coli* produces hydrogen gas, which of course renders unreliable experiments based on manometric measurement of oxygen absorbed or carbon dioxide produced unless this is taken into consideration and accounted for. Sevag and Jane Henry (246) have recently observed that this production of hydrogen from glucose by *E. coli* is inhibited by sulfonamides (0.04 M; 690 mg % SA) and that this inhibition does not necessarily parallel the inhibition of oxygen consumption. The above-mentioned reports apparently overlook this phenomenon of hydrogen production; accordingly all such experiments with *E. coli* will have to be reinvestigated. At the higher inhibitions obtained, however, there is little doubt that oxygen consumption was being inhibited.

of streptococci (and the mg of streptococcal nitrogen) and respiration in the presence and absence of SA (0.04 M; 690 mg %), they concluded that the

TABLE 3

Calculation of inhibition of respiration per cell of E. coli in a medium supporting growth; data for calculations obtained from Table 2 of Kohn and Harris (126)

SULFONAMIDE CONCENTRATION		CMM. O_2 CONSUMED PER HOUR PER 10^7 CELLS (FROM PLATE COUNT)	% INHIBITION OF O_2 CONSUMPTION PER CELL
	0	2.7	
	10^{-4} M	2.6	4
	10^{-3} M	1.7	37
SA	0	2.2	
	10^{-4} M	1.9	14
	10^{-3} M	1.4	36
	0	2.3	
	3×10^{-6} M	2.2	4
	3×10^{-5} M	1.0	57
SP	0	1.9	
	3×10^{-6} M	1.8	5
	3×10^{-5} M	1.1	42
	0	2.5	
	10^{-6} M	2.4	4
	10^{-5} M	1.7	32
ST	0	1.8	
	10^{-6} M	1.9	*
	10^{-5} M	1.7	6

* Apparently there was stimulation rather than inhibition in this instance.

inhibition of both the aerobic and the anaerobic respiration results in proportional inhibition of growth. After recalculation (figure 1) of the data (for *S. pyogenes*) reported by these investigators on a per cell or mg N basis[26] it is apparent that

[26] In certain instances, respiratory data have not been reported on a per cell basis. No conclusions concerning the presence or absence of respiratory inhibition in the individual cells can be drawn unless this is done, for a change during the experiment in the number of cells present must affect the base line from which the inhibition is judged. As a matter of fact, in certain instances, data expressed even on a per cell basis may be misleading as a result of changes in cell size during the course of the experiment or of different cell sizes under different experimental conditions. In such instances the data should be expressed on a mg N (Q_{O_2}) basis (78). A method of measuring growth in bacterial suspensions which is gaining in popularity, namely the photometric method, does not allow for variations in cell size or in non-parallelism between turbidity and viable cell account. These factors should always be checked. It is mandatory that the investigator be certain that his method of growth measurement is providing values sufficiently accurate for the interpretations based on them.

Several other works (90, 234), in reporting experiments of sulfonamide action on growth and respiration of bacteria, gave no indication of whether the inhibition of respiration as reported was on a per cell (or Q_{O_2}, etc.) basis; in some instances, data which the reader

in actively growing cultures, sulfonamides produce a definite inhibition of respiration coincident with inhibition of cell multiplication. Approximately 65 per cent inhibition of aerobic respiration or approximately 45 per cent inhibition of anaerobic respiration results in (or accompanies) complete bacteriostasis. The inhibition of both aerobic and anaerobic growth paralleled the inhibition of respiration. Low concentrations of PABA were capable of completely counteracting the growth-inhibition by SA. It was shown (247) that whether or not the cultures exhibited active growth depended on the nature of the

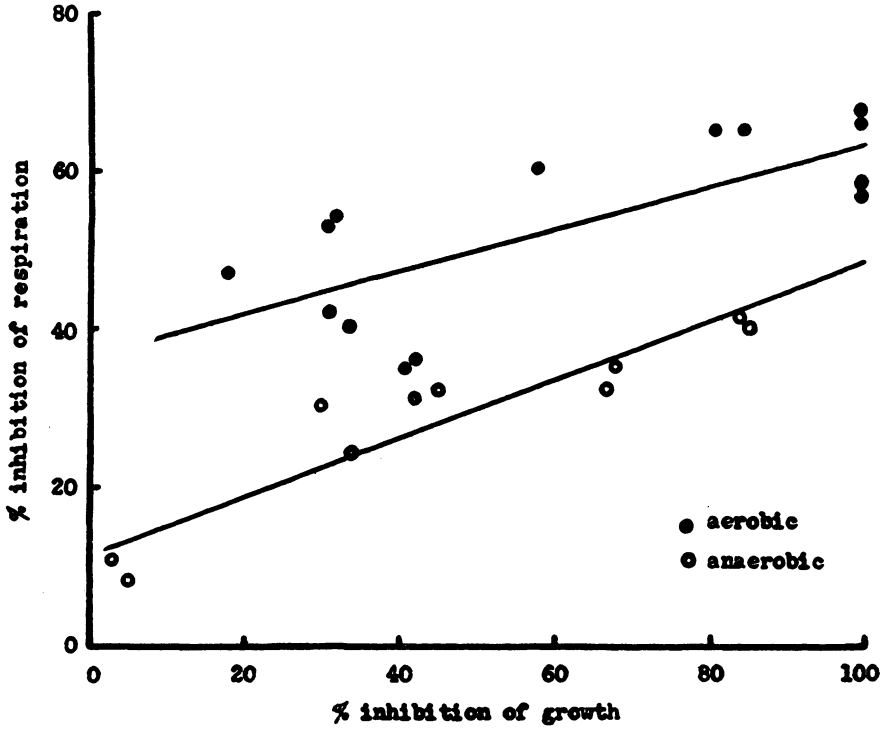

FIG. 1. CORRELATION OF INHIBITION OF AEROBIC AND ANAEROBIC GROWTH WITH INHIBITION OF AEROBIC AND ANAEROBIC RESPIRATION OF STREPTOCOCCUS PYOGENES; CALCULATED FROM DATA IN TABLES 3 AND 4 OF SEVAG AND SHELBURNE (247)

medium. Suboptimal media permitted fairly active respiration of *S. pyogenes* but no measurable growth. The respiration of the bacteria in such a state was

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would require to determine this for himself were either never obtained or not published. Apparently these workers were going on the assumption that the oxygen consumption per cell would not be decreased, and were using oxygen consumption measurements as an indirect method of determining growth rate—a method which has been used in following normal bacterial growth and which has been proposed as a method of evaluation of germicides (23). It is suggested that, inasmuch as several have reported definite inhibition of bacterial cell respiration, such an assumption cannot be made.

SA-sensitive, but less so than that of bacteria in active growth phase. The fact that the added respiration concomitant with growth is relatively more sulfonamide-sensitive indicates strongly that it is intimately associated with bacterial growth and multiplication.[27]

Clifton and Loewinger (32) claimed to have found that SA markedly inhibits the oxygen consumption of washed suspensions of *E. coli* in the presence of various substrates (these investigators were aware of the fact that *E. coli* produces hydrogen). However, in this publication the calculations of respiratory inhibition were not made on a per cell (or similar unit) basis, nor were data given permitting such a calculation for aerobic respiration. Thus a conclusion with regard to the existence of an inhibition of oxygen consumption is not permitted here. Sufficient data were, however, given for an analysis on this basis under anaerobic conditions. The analysis shows that SA (0.00063 M; 11 mg %) inhibits CO_2 production coincident with inhibition of division, both inhibitions being antagonized by PABA.

Thus it is seen that there is good evidence that sulfonamide inhibition of bacterial multiplication is directly related to the inhibition of respiratory mechanisms, either aerobic or anaerobic.

4. Relation Between the Structure of Sulfonamides and Coenzymes

Various authors have drawn attention to the similarity between the structures of various sulfonamides and coenzymes of respiratory systems (60).[28] Moreover, there is a considerable body of evidence, to be considered below, indicating some relation between sulfonamide action and the normal function of coenzymes.

With these as a background, Sevag and coworkers (248, 251) enunciated explicitly the idea that the chemotherapeutic substances which have structural similarity to the whole or part of the coenzyme molecules may combine specifically with the specific proteins (of the dehydrogenase-containing coenzyme I or II, or of flavoproteins functioning as dehydrogenases, or of other enzymes such as carboxylase, etc.) of the respiratory enzymes. This might result in displacement of the coenzyme by the drug or the formation of an inactive "drug-protein-coenzyme" complex. In such cases, as where drugs are active but do not possess structural similarity with the known coenzymes, mutual affinity

[27] A similar phenomenon has also been observed in the SA-inhibition of bacterial luminescence, i.e., the inhibition is generally less when the metabolism is low (109).

Sevag and Shelburne (247) also showed that HOSA, *p*-aminobenzenesulfonhydroxamide, benzenesulfonhydroxamide, benzhydroxamic acid and hydroxylamine inhibit both the aerobic and anaerobic respiration of hemolytic streptococci. Sevag *et al.* (250) demonstrated that HOSA (also hydroxylamine) is capable of inhibiting non-heme type of enzyme systems as well as the heme type. It must be remembered, however, that there is evidence (section C) that oxidized sulfonamides exert their action by a mechanism differing, in at least some respects, from that of the reduced sulfonamides.

[28] For the role of coenzymes, nicotinic acid, thiamin, etc. in bacterial metabolism see Stephenson (261). Dickens (42) called attention to the similarity of the inhibitors pyridine, quinoline and acridine compounds to the active pyridine group of coenzymes and suggested that they may displace the coenzymes by preferential adsorption on the protein carrier, forming an inactive complex.

between the drug and the specific enzyme proteins was considered as a definite possibility. Such an affinity is certainly not without precedent, as will be referred to presently.

Accordingly, SP would be a potential competitor of coenzymes I and II (di- and tri-phosphopyridine nucleotides: DPN and TPN), and ST (likewise SD) of cocarboxylase. These coenzymes are present in the respiratory systems of most living cells.

Experiments having to do with the inhibition of respiration by sulfonamides have been reported by Dorfman and coworkers (46, 47). Here the inhibition of respiration in the presence of nicotinic acid and its derivatives was studied, dysentery bacilli being the organisms used. They concluded that there is strong indication that SP and SD action is related to the metabolic role of nicotinamide (the pyridine component of the coenzymes). The addition of SP (0.0012 M, 30 mg %) after incubating dysentery bacilli with nicotinamide resulted in respiratory inhibition varying from 8 to 15 per cent. When, however, SP was added before nicotinamide, inhibition of 80 to 95 per cent was obtained. Respiration stimulated by a certain preparation of DPN was inhibited by SP in a manner qualitatively similar to the inhibition of the respiration stimulated by nicotinamide. Much the same results were obtained with ST, the similarity in activity being regarded as not surprising in view of reports of isosterism of the pyridine and thiazole rings (127). ST was also found to inhibit anaerobically the nicotinamide-stimulated fermentation, a result to be expected if ST inhibits reactions involving the nicotinamide-containing coenzymes. SA inhibited aerobic respiration approximately 15 per cent but had no effect on nicotinamide-stimulated respiration; thus, there was no evidence that the small inhibition by SA is related to the nicotinamide-containing coenzymes.

Growth-inhibitions (46) were found to parallel the respiratory inhibitions, with one peculiar exception: PABA completely counteracted ST inhibition of growth but exerted no antagonistic action on ST inhibition of nicotinamide-stimulated respiration. Thus the inhibition of nicotinamide-stimulated respiration seems to be independent of PABA, although it was also discovered that the amount of PABA required to counteract the inhibition produced by a given amount of ST is inversely proportional to the nicotinamide concentration present. Dorfman and coworkers reasoned that if the thesis is correct, that the inhibition of nicotinamide-stimulated respiration is dependent on the ring linked to the sulfonamide group rather than on the SA part of the molecule, then acetylation of SP should not abolish the respiratory inhibition although it should markedly decrease the bacteriostatic activity of the compound. This was found to be the case. Thus, here again is evidence that SP and ST act differently than SA, possibly by acting at metabolic loci in addition to those affected by SA. The interpretation was made that SP and ST, but not SA, compete with nicotinamide or related compounds for enzymes essential for the oxidation of lactate and glucose (or some intermediate derived therefrom).

The respiratory inhibition effected by SP and ST was found to be competitive since it was dependent on the relative concentrations of sulfonamide and respira-

tory stimulator. Furthermore, the inhibition was reversible since the respiration and growth could be restored by washing the drug out of the cell; this, of course, is evidence for the theory that the sulfonamides inhibit the respiration by forming a dissociable enzyme complex.

These results are not incompatible with those of Sevag and coworkers and Clifton and Loewinger, although it is somewhat difficult at present to bring them entirely into alignment, the approach to the problem in the two instances being different. In the experiments of Dorfman *et al.* it was difficult to determine the relative importance of respiratory inhibition in stopping growth, although it was shown that nicotinamide (or DPN) can partially antagonize growth-inhibition at certain concentrations of SP or ST.

5. Inhibition of the Dehydrogenases

The dehydrogenases are oxidative-reductive enzymes composed of a protein component and a coenzyme (DPN or TPN). They function by "dehydrogenating" (and thereby oxidizing) substrates. Under normal conditions the reduced dehydrogenase is then oxidized by the oxidative-reductive enzyme next in line, which is in turn oxidized by the next enzyme in line, and so on. In the presence of oxygen, the ultimate hydrogen acceptor in this chain is oxygen. Methods are available whereby a direct measurement of dehydrogenase activity can be made. These consist of adding, in the absence of oxygen, a dye (e.g., methylene blue) which can be reduced by reduced dehydrogenase, and which changes color on reduction. By following the color change the rate of dye reduction can be determined.

Several studies have been reported in which bacterial dehydrogenase activity and the effect of sulfonamides thereon have been measured. MacLeod (178) determined dehydrogenase activity in pneumococci by methylene blue reduction in the presence of various substrates, and found that, whereas the glucose dehydrogenase was not affected by SP in a concentration of 0.0005 M (12.5 mg %), the dehydrogenase activity for glycerol, lactate, and pyruvate was suppressed. Sevag *et al.* (246), on the other hand, have repeatedly found that both the aerobic oxygen consumption and the anaerobic fermentation of *S. pyogenes*, *Staphylococcus aureus*, pneumococcus, and *E. coli* in glucose are strongly inhibited by sulfonamide. Dorfman and Koser (46) also obtained inhibition of the aerobic and anaerobic metabolism of glucose with SP and ST (but not SA) in the dysentery bacillus. Clifton and Loewinger (32) have confirmed both these observations in *E. coli* with glucose as substrate and SA as inhibitor, namely, inhibition of aerobic and anaerobic respiration but no appreciable effect on the dehydrogenase activity using methylene blue as hydrogen acceptor. This was interpreted to indicate that the inhibition must occur after the initial activation of the substrate, presumably on a hydrogen-carrier system somewhere between the original dehydrogenase system and the final hydrogen-acceptor. This may be dependent on the relative affinities of SA and methylene blue for the susceptible enzyme.

SA in a concentration of 0.002 M (33 mg %) does not inhibit the dehydrogenation of *d*-alanine by *d*-amino acid dehydrogenase to pyruvic acid (21).

Fox (72) has determined manometrically the effects of sulfonamides on isolated enzyme systems of *E. coli*. It was found that, aerobically, the oxidation of lactate alone is markedly inhibited, the oxidation of glucose, succinate, fumarate, malate, and pyruvate being unaffected. Under anaerobic conditions, only pyruvate dismutation is depressed; lactic acid dehydrogenase and glucose fermentation are not inhibited. This sulfonamide inhibition of the lactate system aerobically and the pyruvate-lactate system anaerobically was found to be approximately proportional to the bacteriostatic action in growing cultures with equivalent sulfonamide concentrations. In view of the fact previously mentioned, that *E. coli* produces hydrogen, these experiments by Fox would have to be repeated if this was not taken into consideration (no experimental details were given in the brief report). Bucca (25), working with gonococci, has recently reported that SA inhibits lactic acid dehydrogenase but not glyceric acid dehydrogenase. It must also be emphasized that in experiments such as referred to above in which the Thunberg technique of methylene blue reduction is employed to measure the rate of dehydrogenase activity, if a large inoculum is used in the test a correspondingly large amount of sulfonamide must be used before any inhibition is to be expected, because of the relationship existing between inoculum size and amount of sulfonamide required to produce a certain inhibition of overall respiration and bacterial multiplication (section E7). In general, these results support the theory that during sulfonamide inhibition certain respiratory enzymes are being inhibited.

6. Inhibition of the Respiratory Enzyme Carboxylase[29]

Apropos the suggestion that the sulfonamides interfere with cellular components with similar structure, Sevag *et al.* (251) found that ST (0.0014 M; 35 mg % and 0.0055 M; 137 mg %) and other derivatives containing the thiazole ring exercised decidedly greater specific inhibiting effect on the carboxylases of *S. aureus* and *E. coli* than SP, SD, or SA. It is interesting to note that the specific inhibitory effect of ST on the carboxylases of *S. aureus* and *E. coli* in comparison to SA, SP, and SD is in accord with experimental results, *in vivo* and *in vitro*, of other investigators. Rammelkamp and Jewell (219) found that ST, whether added directly to the blood or administered orally, was superior to other sulfonamides in increasing the blood's bactericidal action against *S. aureus*. Similarly, ST was found superior against growth *in vitro* of *S. aureus* and *E. coli* (262). But it must be emphasized that there is no *a priori* reason why the sulfonamides should give the same results at the same concentrations, even if they all acted by an identical mechanism. The important question here is whether the ratio of effectiveness of the compounds is the same on organisms rich in carboxylase (e.g., staphylococci) as it is on organisms containing no carboxylase (e.g., pneumococcus).

Wood and Austrian (282) reported that the action of carboxylase *in vitro* is

[29] The enzyme carboxylase contains the thiazole ring and catalyzes the decarboxylation of pyruvic acid.

unaffected by a 0.0004 M (10 mg %) solution of ST, a concentration 50 to 200 times the concentration of coenzyme. This failure was no doubt due to the low concentrations of sulfonamide used for the amount of coenzyme present. Sevag and Shelburne (246), using 100 mg of air-dried brewer's yeast which was washed with alkaline phosphate to eliminate cocarboxylase while the specific protein of carboxylase remained as a source of specific protein carrier for carboxylase activity, found that approximately 2700 times more ST (by weight) than cocarboxylase was required for about 65 per cent inhibition of the latter's activity. The inhibition was observed to decrease as the amounts of cocarboxylase were increased. This is interpreted as showing a competition between ST and cocarboxylase for carboxylase protein. In such a system, thiamin exerted no antagonistic action, either by itself or in conjunction with coenzyme. Pyridoxin (vitamin B_6) failed to show any effect on ST inhibition of anaerobic respiration of *E. coli*, *S. aureus*, or brewer's yeast; thiamin had no counteracting effect on ST inhibition of *S. aureus*. ST inhibition of anaerobic respiration of *S. aureus* was antagonized by cocarboxylase at pH 6.2, which is the optimal pH for carboxylase activity, whereas at pH 7.16 no antagonism was obtained either in this organism or in *E. coli*. Evidently pH is an important factor here.

The inhibiting effect on yeast carboxylase was non-differentiable among SA, SP, SD, 2-aminopyrimidine, ST, sulfamethylthiazole, 2-sulfanilamido-5-ethyl-4-thiazolone, 2-aminothiazole; the exception was sulfamethyldiazine which was completely ineffective on the carboxylases of the organisms studied (251). The authors stated that though this supports the hypothesis that sulfonamide affinity may in part be related to structural similarity between components of the drug and the corresponding respiratory coenzymes, carboxylase could not be found in pneumococci, an organism whose growth is strongly inhibited by ST, so that ST must inhibit here by some other mechanism. At their face value these observations do not conclusively show that ST is a specific inhibitor of carboxylase in every instance. As already seen, there have been rather frequent indications that sulfonamides inhibit more than one enzyme.

The nature of the inhibition of the carboxylase system by ST appears to be an adsorption of the compound on the enzyme system in some way rather than on the substrate (246). Experiments were carried out as follows: in one case, ST (0.00414 M; 103 mg %) was allowed to be in contact with *E. coli* before pyruvic acid (0.05 M) was added, and after addition of the latter there was 34 per cent inhibition of anaerobic CO_2 production. In the second case, conditions were reversed: ST was in solution with the pyruvic acid, and the inhibition of the bacterial respiration after their addition was only 3 per cent. This indicates that pyruvic acid (the substrate) has a greater affinity for *E. coli* than ST when exposed to the bacteria at the same time. Of course, with the passing of time, the inhibitions observed in the two instances should approach one another as equilibrium is established.

A most important observation made by Sevag *et al.* (245, 246) is that PABA counteracts the inhibitory action of ST on the carboxylase activity of *E. coli* and *S. aureus*. PABA in sufficiently high concentrations is itself capable of inhibiting

the carboxylase activity of *E. coli* (246).[30] The combined actions of ST and PABA, in concentrations which are inhibitory separately, are not additive. This may be interpreted in two ways. First, the PABA may antagonize the ST action, while the inhibition produced by the former persists[31]; this may well be, since the sulfonamide-counteracting action of PABA and its inhibitory action in high concentrations might be dissociated phenomena. Second, the separate inhibitions may be of such a nature that they are not synergistic. It is to be remembered, however, that if such is the case the actions of both substances must be on the same enzyme, since the carboxylase system is composed of only one rather than a chain of enzymes (the various possible ways in which a substance could interfere with such an enzyme will be discussed in F 2). It is of interest to note that the specific inhibition of the carboxylase system by acetaldehyde is not antagonized by PABA (244).

Two things in particular evolve from the various results just described which seem to be of extreme significance with regard to the mode of antagonism of sulfonamide action by PABA. In the first place, the antagonism of the sulfonamide inhibition of the carboxylase enzyme system by PABA scarcely can be explained on the basis of the latter's functioning as an essential metabolite or substrate. Secondly, in the experiments of Sevag *et al.* with live bacteria, the experimental conditions were such that growth could not take place, yet antagonism of respiratory inhibition was obtainable. Since the antagonism of the inhibition of respiration of sulfonamides in the presence of PABA takes place in the absence of growth, it is reasonable to conclude that the counteraction of the growth-inhibiting effect of sulfonamides with PABA occurs through the pathway of respiratory enzymes.

7. Certain Proposed Criticisms and Objections

Kempner (116) stated, "the fact that the inhibitory action of the sulfonamides on pneumococcus and staphylococcus growth is unaltered, whether the bacteria gain their energy by oxidation or by anaerobic frementation shows, just as does the anaerobic *p*-aminobenzoic acid effect on sulfonamide-pneumococcus cultures, that the sulfonamides do not act by way of inhibiting bacterial oxidation or fermentation." This interpretation does not seem to be warranted. Fermentations or anaerobic respiratory processes are oxidative even though oxygen *per se* is not involved, and it is well known that many substances are capable of inhibiting anaerobic oxidative processes (50, 214). As a matter of fact, it has been shown that in yeast, which, like many bacteria, is capable of both aerobic and anaerobic growth, growth is inhibited by narcotics to the same extent whether under aerobic or anaerobic conditions (69). Such a finding merely intimates that the inhibitor is acting at a point in a respiratory chain which is common to both aerobic and anaerobic oxidation.

[30] Inhibition of bacterial growth by high concentrations of PABA has been referred to in the previous section. Sevag finds that these concentrations also inhibit bacterial respiration, and that serum counteracts both the respiratory and growth inhibitions.

[31] This interpretation has also been suggested in certain instances of PABA antagonism of sulfonamide inhibition of bacterial growth (288).

The inverse relationship existing between inoculum size and the sulfonamide concentration required for growth-inhibition has also been found to apply to respiratory inhibition (247). It has been the custom of several workers, including Ely (51) and Sevag and Shelburne (247, 248), to use very large inocula of bacteria (over one billion organisms per ml) in respiration experiments in order to obtain more reliable respiratory values. In order to produce inhibition of growth and respiration in such large inocula, sulfonamide concentrations 50 to 100 times the average therapeutic blood level were required. In the light of the observed inverse relationship, and the fact that the inhibition obtained under these conditions is completely reversible (both by PABA and by removal of adsorbed sulfonamide), there seems to be no reason why such inhibition should be different from that obtained with lower sulfonamide concentrations on fewer organisms. Wyss *et al.* (289) criticized this practice of using large sulfonamide concentrations, and claimed that their experiments with such concentrations indicated that results so obtained should not be regarded as an expression of typical sulfonamide activity. What they claimed for their experiments is probably true, for they used very small inocula (less than one-thousandth of the bacterial concentrations used by Ely, and Sevag and Shelburne) with the high sulfonamide concentrations.

Some workers have failed to obtain a respiratory inhibition of growing cultures of *Brucella melitensis*, hemolytic staphylococcus, and *E. coli* (86, 117, 118). The reason for these failures is not apparent. It should be remembered, however, that bacteria are notoriously susceptible to many factors and vary accordingly, and that experimental procedure in such experiments is as yet far from standardized. The answer must lie in future work and is of the utmost importance. Of the three criticisms of the "Inhibition of Respiration" theory which have been offered, this is the only one which cannot be satisfactorily rebutted. However, in view of the numerous and consistent reports of sulfonamide inhibition of bacterial respiration and the other considerations presented in this section, these few instances of failure to observe respiratory inhibition are not believed to constitute a serious obstacle. It must be remembered that oxygen is only one of the possible hydrogen acceptors in certain cellular oxidative reactions. There are many respiratory reactions in which acceptors other than oxygen participate. Therefore, the mere fact that no inhibition of oxygen consumption is observed under certain conditions is not in itself a disproof of an effect upon respiration.

8. Sulfonamide Inhibition of Oxidative Metabolism in Cells other than Bacteria

Plasmodia. SA markedly inhibits the oxygen consumption of *Plasmodium knowlesi* (a plasmodium producing a malarial infection in monkeys which is susceptible to sulfonamide therapy), but has no apparent effect on the anaerobic CO_2 production (34). The oxygen consumption of *P. cathemerium* is also inhibited by SA and ST (273).

Liver and Muscle. Chu and Hastings (31) found that SA concentrations corresponding to ordinary therapeutic levels (ca. 0.00075 M, 13 mg %) have no effect on, or slightly increase the oxygen consumption of rat liver and diaphragm. Higher concentrations (0.0075 M, 130 mg % and 0.038 M, 650 mg %) gave definite respiratory inhibition. This inhibition is antagonized by methylene blue

(194), indicating that the dye can function as a carrier at a point in a respiratory chain which is inhibited by the sulfonamide. Laves (147) also reported sulfonamide inhibition of aerobic respiration of diaphragm muscle, heart muscle brei, and liver brei. The inhibition of muscle respiration was antagonized by addition of coenzyme. That dehydrogenase activity is interfered with was demonstrated by the Thunberg technique. Laves stressed that, since only about 40 per cent of diaphragm muscle respiration goes *via* the cytochrome system, it is significant that sulfonamide can produce a 50 to more than 80 per cent inhibition of its aerobic respiration.

Bioluminescence. Bioluminescence in luminous bacteria and in the small Crustacean *Cypridina* is a result of the oxidation-reduction reaction of the luciferin-luciferase system, in which luciferin is the substrate and luciferase the enzyme. Johnson and Moore (111) found that SA in concentrations of approximately 0.006 M (100 mg %) readily inhibits bacterial luminescence (*Achromobacterium fischeri*, *Photobacterium phosphoreum*, *Vibrio phosphorescens*, and others were used in the following studies) in a manner resembling that of narcotics in general. The inhibition of luminescence appeared at a slightly lower concentration than growth-inhibition. PABA over a wide range of concentrations had no appreciable antagonistic effect on SA inhibition, and in fact, in high concentrations, added to it. These results were obtained with mature or washed cell suspensions. Later, however, PABA was found to antagonize SA inhibition of growth and light production in growing cultures of luminous bacteria (105, 107). The success of PABA in antagonizing sulfonamide action on actively growing cultures as compared to its failure with resting cells may be of some significance.

Johnson *et al.* (106, 110) showed that SA falls in a group of narcotics with the barbiturates, chloral hydrate, and PABA, which decrease the light intensity of luminous bacteria apparently by a chemical or adsorptive combination with an enzyme, since the inhibition is irreversible by pressure, although reversible by removing the inhibitor. Approximately one molecule of SA combines with a molecule of enzyme.

In an intensive study of the relationships between the SA inhibition of bacterial luminescence and temperature, it was noted that the heat of reaction for the combination of PABA with the enzyme luciferase appears to be approximately 4,000 calories higher than that for SA. This led to the expectation that PABA would combine about 1,000 times more readily than SA with the enzyme; actually, however, more PABA was required to produce an inhibition than SA. Thus, either the calculated heats of reaction are not real or PABA loses much more entropy in the process of adsorption than SA, which would be explained if the ionized form only of PABA and the undissociated molecules of SA are adsorbed (a situation apparently not true with sulfonamide inhibition of bacterial growth, at least). Concerning PABA antagonism of SA inhibition of bacterial growth, it was suggested that, if PABA, in its combination with the bacterial growth enzyme as normal substrate, has a heat of reaction greater than that of the SA combination, and the entropy change is approximately the same, it is quite understandable why only a small concentration of PABA is required for the an-

tagonism; for approximately each 1,300 calories difference, PABA should combine ten times more readily than SA. In luminescence, however, even if PABA combined with the luciferase preferentially in the presence of SA, it was reasoned that the SA inhibition would not be antagonized, since PABA cannot take the place of the normal substrate, luciferin, in the reaction. The result that would be expected, and which in fact was observed, of the combined action of PABA and SA would be an increased inhibition. This line of reasoning is based on the assumption that PABA is the normal metabolite of the bacterial growth enzyme inhibited.

SA (0.003 M; 50 mg %), ST (0.0006 M; 15 mg %), SP (0.001 M; 25 mg %), PABA (0.005–0.05 M; 70–700 mg %), and urethane (0.52 M; 2200 mg %) each independently and reversibly decrease the velocity constant of luminescence of the purified luciferin-luciferase system of *Cypridina* without decreasing the total light (108). The results showed quite satisfactorily that, in this system at least, the action of inhibitor is on the enzyme (luciferase), not on the substrate (luciferin), and in addition that a competitive action between substrate and inhibitor is not involved. The separate inhibitions by SA and PABA were partially additive when the two were present simultaneously. No PABA sulfonamide-antagonistic effect was seen at the concentrations used.

Sea Urchin (Arbacia) Eggs: That SA inhibits oxygen consumption and cell division of sea urchin eggs in a manner practically indistinguishable from typical narcotics was shown by Fisher and Henry (67) and Fisher *et al.* (68). Because of the important bearing that this work has on the "Inhibition of Respiration" theory of the mode of action of sulfonamides, and because of the development of these ideas in section F, it seems apropos that this work be presented in some detail:

Eggs of *Arbacia punctulata*, an echinoderm, are ideal to work with from many standpoints, not the least of which are their self-sufficiency in nutrition and the fact that cell division can be initiated at will by fertilization. Analysis of the action of narcotics (urethane and chloral hydrate) on respiration and cell division in the fertilized eggs and on respiration of the unfertilized eggs led to the interpretation that in the unfertilized resting egg one respiratory system is functioning, and that upon initiation of division by fertilization a second respiratory system is added in parallel to supply the energy for cell division (the respiration of the sea urchin egg increases upon fertilization). It was proposed that when a substance interferes with the latter system cell division is inhibited, and that narcotics inhibit this system considerably before the other,—thus accounting for the relatively little effect of narcotics on respiration as compared to cell division. The other respiratory chain, the one which is present in the inactive unfertilized egg, supplies the energy utilized in maintaining the cells in a basal state. SA, in the range of concentrations from 0.0005 M (9 mg %) to 0.04 M (690 mg %), was found to inhibit the respiration and division of fertilized eggs, but had no effect on unfertilized egg respiration (except in the very highest concentration possible, 0.04 M). This suggested that SA was, in the concentrations used, inhibiting practically specifically the respiratory system or chain (termed the *activity system*) fur-

nishing the energy for cell division. Further confirmation of this was obtained by employing combinations of inhibitors. Azide had no effect on respiration in the unfertilized egg and produced a maximum inhibition of fertilized egg respiration of about 50 per cent. On the possibility that azide might be inhibiting the activity system specifically, azide-SA and azide-narcotic combinations were applied to fertilized eggs. In both cases it was found that, over the range of concentrations of SA and narcotic which presumably inhibit the activity system when used individually, there was no additive inhibition when azide was used in combination at a concentration sufficient to produce the maximal effect of azide alone. From these two separate lines of evidence, it was concluded that SA action in this instance is indistinguishable from the action of typical narcotics. Thus, it becomes evident that it is not inhibition of the overall total respiration of a cell which is significant in inhibition of cell division, but rather the inhibition of that fraction which is specifically concerned with providing the energy for cell division.

PABA over a wide range of concentrations exhibited no sulfonamide-counteracting effect; in fact, in higher concentrations, it inhibited cell division and had a tendency toward additive inhibition with SA (68).

Since, in its gross details, the respiratory metabolism of bacteria is very similar to that of most other types of cells, the results cited above must be assumed to provide evidence in favor of the hypothesis that growth-inhibition is a result of inhibition of respiratory processes.

9. Neutralization by Respiratory Enzyme Factors of the Growth-Inhibition Caused by Structurally Related Inhibitors

The antagonizing effects of the group of substances primarily related to the respiratory systems on sulfonamide growth-inhibition will now be considered. It is perhaps unfortunate, that in all the experiments to be discussed below respiratory measurements were not made in parallel with the observations on growth. Due to this fact, the following experiments on bacterial growth as a basis for correlation with the theory of respiratory enzyme inhibition should be considered with some degree of reservation.

Staphylococcus aureus requires nicotinic acid or nicotinamide for the synthesis of coenzyme; preformed coenzyme also satisfies this requirement for growth.[32] There have been reports that coenzyme I but not nicotinic acid is able to antagonize sulfonamide inhibition, and this has been interpreted to mean that the sulfonamide is preventing the synthesis of the coenzyme from nicotinic acid (259, 282). In these experiments nicotinamide was not tested, so that it is impossible to tell whether there was inhibition of coenzyme synthesis from nicotinamide or whether there was inhibition of nicotinic acid conversion to its amide (46). The

[32] Experiments on the effects of nicotinic acid on sulfonamide action may be much more complicated than is at first apparent. Kligler *et al.* (123) have recently found that, when nicotinic acid is lacking in a medium otherwise suitable for growth, the addition of glucose inhibits the growth of organisms (e.g., *Proteus*, dysentery bacilli, staphylococci) which are able to ferment this sugar in the presence of nicotinic acid.

latter is a definite possibility, since there is evidence that pyridine-3-sulfonic acid inhibits this conversion (170). Furthermore, the results obtained by Dorfman and Koser (indicating that the respiration of dysentery bacilli stimulated by DPN is inhibited in the same manner as the respiration stimulated by nicotinamide) do not support the idea that only DPN-synthesis is interfered with. This can also be interpreted as indicating that sulfonamides are blocking the dehydrogenase activity of these bacteria.[33] Some investigators have failed to obtain antagonism by coenzyme with this organism (263). The explanation for these conflictions in results is not apparent, but it might be pointed out that coenzyme preparations obtained from natural sources may be contaminated with other substances.

There is other evidence that sulfonamide does not inhibit coenzyme synthesis. Axelrod (269) reports inability to find any effect of SP on the synthesis of coenzyme I from nicotinic acid by red blood cells *in vitro*. Nicotinic acid, nicotinamide, and coenzyme I have been reported by Teply *et al.* (269) as antagonistic for SP inhibition of the growth of *Lactobacillus arabinosus*. Teply *et al.* concluded that their data presented no evidence that SP inhibits coenzyme I synthesis, and that it seems more probable that the function of the coenzyme is interfered with. In these experiments, however, apparently no allowance was made for the stimulation of growth by the antagonists in the absence of SP.

The crux of the question whether or not the sulfonamides with side groups specifically inhibit coenzymes containing an analogous group, as already outlined, depends on whether or not the inhibition by the structurally unrelated compounds can be antagonized by the addition of the particular coenzyme or enzyme component to the same extent as those compounds which are related. It has been shown by Wood and Austrian (282), using *S. aureus*, that nicotinamide and coenzyme I antagonize equally the action of unrelated compounds and the related SP. The antibacterial actions of methylene blue and thionine are also antagonized by coenzyme I.[34] Moreover, the antagonizing effects of nicotinamide and coenzyme are directly proportional to their ability to stimulate growth in synthetic media. In the case of *E. coli*, where these compounds do not enhance growth, they fail to counteract sulfonamide action (95, 128, 262, 282). Thiamin, riboflavin, pyridoxin, pantothenic acid, adenylic acid, ascorbic acid, inositol, choline and biotin exhibit no antagonism toward sulfonamide action on the vari-

[33] Mann and Quastel (185) demonstrated the presence of a hydrolytic enzyme, coenzyme I-nucleotidase, in fresh brain tissue responsible for the breakdown of the coenzyme, and which can be completely inhibited by high concentrations of nicotinamide. The stimulating effect of nicotinamide on lactic dehydrogenase activity of tissues in the presence of the nucleotidase is not a result of synthesis of nicotinamide to the coenzyme, but due to a competition of nicotinamide with the coenzyme for the nucleotidase. If a similar situation exists in bacteria, the interpretation of experiments using nicotinic acid, nicotinamide and coenzyme as sulfonamide-antagonistic factors is rendered even more difficult.

[34] It is known that these dyes serve as acceptors for dehydrogenase systems. These inhibitory effects must arise from the higher concentrations used. The counteraction of their inhibitory action by coenzyme I may indicate that it has greater affinity for its specific protein than these dyes possess. These facts also indicate that dehydrogenase activity of these bacteria must be restored before any growth processes can take place.

ous organisms which have been studied (95, 128, 259, 263). The above facts would seem to indicate that acceleration of growth by these vitamins, etc., are not determinant factors in antagonizing the sulfonamide action. Of distinct interest, however is the ability of thiamin to antagonize sulfonamide inhibition of fungus growth (147) and the development of paresis in pigeons on a sulfonamide-containing diet (52). Sulfonamides are reported as capable of producing (presumably directly) a peripheral neuritis (13), and it is well known that thiamin deficiency can also cause this.

McIlwain (170) has emphasized that in so complex a process as the utilization of nicotinic acid, part of which at least is for synthesis of pyridine coenzymes, many reactions must exist. Accordingly, unless the action of inhibitors is confined to one reaction, or several reactions are inhibited in the same manner, it is to be expected that several types of inhibition will be observed. He studied the inhibitors, pyridine sulfonic acid and its amide, and the antagonistic effects of nicotinic acid, its amide, and coenzyme I on *Staphylococcus sp.*, *Proteus sp.* and *E. coli*. Pyridine sulfonic acid inhibited the growth of *Proteus sp.* when nicotinic acid was the growth-promoter, but not when nicotinamide was used. As pointed out, this would suggest that the conversion of nicotinic acid to its amide is blocked. But growth promoted by coenzyme I was very strongly inhibited by pyridine sulfonic acid. It was considered as evident, therefore, that the fate of nicotinamide is not solely coenzyme I or its derivatives. Furthermore, it would appear that part of the block at least is at the degradation of the coenzyme to simpler units used in some synthesis (as already stated, nicotinamide itself may prevent such a degradation). Staphylococcus growth presented a similar picture: pyridine sulfonamide inhibited nicotinamide-promoted growth even to a greater extent than that promoted by the acid. These inhibitions were competitive, whereas the inhibition of coenzyme-promoted growth was not strictly competitive, and might represent superposition of competitive and non-competitive types of inhibition. The inhibition produced by the sulfonic acid was always of this latter type. Though this is a perfect example of how complicated inhibitor action may be, it is relatively clear that these inhibitors are specifically affecting the utilization of nicotinic acid or its derivatives.

There have been several reports on experiments *in vivo* which are very interesting and would seem to have an important bearing on this general question. Adenine sulfate[35] has been found to counteract the therapeutic action of SA, SD, SP, and ST on hemolytic streptococcal infection in mice (190). It was suggested (without experimental data) that these sulfonamides interfere with the normal utilization of adenine in these cases. Raiziss *et al.* (218) were able to obtain only a slight antagonism of SA and SP action on pneumococcal and hemolytic streptococcal infection in mice by nicotinic acid and vitamin C, the latter being given

[35] Adenine is a component of coenzymes I and II, and flavin adenine dinucleotide, in other words a component of dehydrogenases and flavoprotein enzymes. It is also a component of nucleoproteins of the living cells. These facts no doubt account for its being not only a possible essential nutrient for certain bacteria, but also offer a basis for specific affinity for bacteria, and therefore potential competition with inhibitors which exhibit affinities for the above adenine-containing enzymes of bacteria.

in the same amount as that of PABA which counteracted the sulfonamide action completely. This does not, therefore, preclude complete antagonism of sulfonamide action by nicotinic acid, or by vitamin C for that matter, at titrated dosage levels.

At present there is insufficient indisputable evidence to conclude more than that, in some bacteria, sulfonamides interfere with nicotinic acid metabolism. But again we are met with the paradox that in no single case of sulfonamide growth-inhibition has PABA been reported to fail in completely counteracting the process.

10. Resemblance of Sulfonamide Action to That of Indifferent Inhibitors

The sulfonamides, in their action, resemble indifferent inhibitors as exemplified by narcotics—the term narcotic being used in the sense that a cell function (growth and division in the case of bacteria) is being inhibited. If sulfonamides exercise their inhibitory effect by adsorbing onto specific proteins of respiratory enzymes (dehydrogenases) then their action is that most widely ascribed today to typical narcotics (50).[36] Narcotics have been observed to exert an inhibition of bacterial respiration (69, 275).[37]

The action of narcotics on cells is identical with that of sulfonamides; they both

[36] The present status of the mechanism of narcosis has been recently summarized by Fisher (65).

[37] This hypothesis of narcotic-like action raises a very interesting question: if sulfonamides bring about their therapeutic effect *in vivo* by narcotizing bacteria, why is not the animal host narcotized? Apparently the bacteria are susceptible to sulfonamide at a much lower concentration than the tissues of the host—a relation not existing with our ordinary narcotics and anesthetics. It is a well known observation that in order to produce a specific drug effect on different cells, widely different concentrations of the drug are often required. Zeller (291) found, for example, that the affinity of SA for the enzyme cholinesterase varies considerably with the source of the enzyme, even from organ to organ.

As a matter of fact there is some evidence suggesting that the host's tissues are in a state of basal anesthesia while on sulfonamide therapy, e.g., the effects of narcotics and anesthetics are potentiated by sulfonamide therapy (26). That sulfonamide therapy produces a slight sedative action in which alertness and judgment are impaired is a well-known clinical fact (160). For example, aviators are being grounded while receiving such treatment. Moderate doses of SA activate first and later depress the central nervous system of mice in the manner of a partial narcosis (267). In acute poisoning by the sulfonamides depression of the central nervous system is probably the most spectacular and consistent of the effects seen (156). That sulfonamides show a certain predilection for cells of the central (and peripheral) nervous system has also been indicated in studies showing degenerative cellular changes as a result of toxic doses of the drugs (13). Further evidence of the central nervous system depressant action of sulfonamides is the observation that SA, SP, and SD are capable of depressing the electrical activity of the monkey brain as measured by the electroencephalogram; ST, on the other hand, produces excitation accompanied or followed by convulsions (104).

It must be admitted, however, that some or all of such toxic reactions of sulfonamides *in vivo* may be produced by a mechanism totally unrelated to that of bacterial growth-inhibition. Thus, phenylsulfonamide, which has no *p*-amino group, is several times as toxic to mice as SA and brings about a similar picture of intoxication; the toxicity of acetyl sulfonamide is also similar (224).

are general cell inhibitors. As already seen, there have been several investigations (bioluminescence and sea urchin egg studies) in which the resemblance between sulfonamides and narcotics has been noted.

Summary: Sulfonamides inhibit the aerobic and anaerobic respiration of bacteria and other cells, whether in a resting state or actively dividing. When sulfonamides inhibit actively dividing cells, the inhibition of growth is directly related to the inhibition of respiration, indicating that the respiratory inhibition is responsible for the growth-inhibition. The identities of the inhibited respiratory enzyme or enzymes responsible for the growth-inhibition are not definitely known, but it has been shown that sulfonamides inhibit certain dehydrogenases and carboxylase, and it seems fairly certain that it is inhibition of this coenzyme-protein type of enzyme which secondarily results in growth-inhibition.

F. THE MECHANISM OF THE SULFONAMIDE INHIBITION OF CELL DIVISION

At present, there seem to be only two incontestable fundamental facts regarding the action of sulfonamides on bacteria, namely, sulfonamides inhibit bacterial multiplication, and certain substances antagonize this inhibition. We consider now some of the mechanisms which could possibly account for these two phenomena. The most widely considered hypothesis to date for the inhibition (that of Woods and Fildes) involves the antagonism of inhibition by PABA and in fact has been made to depend on the antagonism by PABA. It is therefore appropriate in considering the mechanism to commence by considering pertinent facts with regard to this antagonism.

1. Possible Ways in which PABA and Other Antagonists Could Counteract Sulfonamide Action

In recapitulating the following possibilities of antagonist action it is not pretended that the list is exhaustive. This is practically a virgin field in enzymology, and it is quite probable that mechanisms of inhibitor-antagonism are existent which are as yet unknown or unthought of.

a. Antagonists might act catalytically in promoting removal of sulfonamide from the cell. This was considered as a possibility for PABA antagonism by Woods (284). With what is known today regarding PABA antagonism this possibility seems remote. There is no evidence that any sulfonamide-antagonist works in this manner.

b. Preferential oxidation of sulfonamide-antagonists. This assumes that sulfonamides in order to be active must first be oxidized and that the antagonists are more easily oxidized than the sulfonamides thus preventing their activation. The primary assumption here has been definitely proved to be fallacious (section C); sulfonamides do not undergo an oxidation in order to become active.

c. Antagonist as a catalyst. This was proposed for PABA by Kohn and Harris (section Dlg); according to this idea, sulfonamides compete with PABA in the reactions which the latter catalyzes. The antagonistic action of other substances on a basis of their being catalysts would be difficult to conceive; according to the scheme of Kohn and Harris these substances, such as methionine, occupy posi-

tions in reactions which are secondary to that catalyzed by PABA. The recent evidence, indicating the possible presence of PABA or a similar substance in bacteria, would seem to make this a possibility, but certain actions of PABA such as the antagonism of sulfonamide dehydrogenase inhibition cannot be so explained; this, along with other criticisms presented in section Dlg, restricts the acceptance of this viewpoint.

d. Direct interaction between sulfonamide and antagonist, forming an inactive complex. This cannot account for the counteracting action of PABA or methionine, but other substances, including urethane, ethyl alcohol, butyl alcohol, chloroform, ether, acetone, glucose, urea, peptone, albumin, bacterial protein, glycocoll, arginine, and hypoxanthine, have been shown by one method or another to combine more or less with sulfonamide (section D). These substances are of two categories, namely those which do and those which do not take part directly in metabolism. Apparently this mechanism can account for the antagonism by urethane, but whether or not the antagonism displayed by such substances as protein, peptone, and certain nutrient substrates is solely a result of inactive complex formation cannot be said. In such instances, it is quite possible that more than one mechanism is in effect.

e. Unspecific growth-stimulation. PABA, methionine, and peptone are among those antagonists whose activity cannot be explained on such a basis (section D). Amino acids and glucose undoubtedly antagonize sulfonamide inhibition by this mechanism. Under appropriate conditions, other individual substances acting as nutritive or oxidizable substrates will undoubtedly be shown to antagonize sulfonamide inhibition.

f. The Antagonist as an essential metabolite, competing with the sulfonamide for the enzyme. This was the role ascribed to PABA by Woods and Fildes (section D). The evidence for and against this proposal has already been presented, but in view of the importance of establishing whether this proposal is adequate, let us collect and examine this evidence in detail.

i. For: The relation between PABA and sulfonamide is a competitive one.

Against: Eyster's work (54) on charcoal adsorption and diastase activity results in strong doubt that the antagonism of sulfonamide by PABA is peculiarly significant, i.e., there is really no reason to think that PABA is a special molecule in the cell from the mere fact that a competition exists. Similarly, PABA antagonizes the sulfonamide inhibition of respiration (section E) and the inhibitions of the enzyme luciferase, an oxidative-reductive enzyme (section E8), and carboxylase (section E6). Thus, sulfonamide inhibits systems in which PABA is not used and the inhibition is still removed by PABA.

ii. For: Structural similarity between sulfonamide and PABA.

Against: Sulfonamide inhibition is antagonized by substances without structural similarity (section D). Peptone and amino acids containing aromatic groups (tryptophan, tyrosine, and phenylalanine) also inhibit SP adsorption on activated charcoal (146).

iii. For: PABA is a growth factor for some organisms.

Against: Only a growth factor in a few cells, which with one possible exception

(diphtheria bacilli, the pathogenicity of which was not reported; section D10) are non-pathogens, and these are less exacting in their growth requirements than the pathogens. Microbiological assays and sulfonamide-antagonistic assays, the specificity of which may be questioned, have indicated that PABA is widely distributed in and is a product of living cells, including bacteria. These assays should be repeated by exact chemical methods. The growth-factor activity and sulfonamide-antagonistic activity of PABA cannot be assumed to be associated phenomena, since at least one instance is known where a substance even more active than PABA in growth-factor activity is completely devoid of sulfonamide-antagonistic activity (section Dld). In instances where PABA is a growth-factor, it should be used up during sulfonamide-antagonistic action, thus allowing inhibition eventually to reappear. This has never been reported.

iv. For: The probable presence of PABA in all cells; PABA antagonizes sulfonamide inhibition of almost all synthesizing cells which have been tried.

Against: Same objections as under *iii.*

v. For: The fact that other growth substances antagonize inhibitions by certain analogs.

Against: Same as under *ii.* There is no doubt that some inhibitor analogs compete with substrates, but mere similarity in structure does not necessarily indicate such a relationship.

vi. For: Stimulation of PABA production caused by the presence of sulfonamide.

Against: The data are conflicting (section Dle).

vii. Against: Inhibition of other enzyme systems:[38] Respiratory systems (coincident with inhibition of growth, and also in the absence of growth, aerobic and anaerobic); respiration of bacteria, the sea urchin egg, muscle, liver, etc. (section E); carboxylase (section E6), carbonic anhydrase (section B3g); cytochrome oxidase (25), succinoxidase, luciferase (section E8), tyrosinase (11), cholinesterase (291), sucrase (53), amylase (53), diastase (54), protective proteinases (1). The Woods-Fildes idea is untenable for the sea urchin egg since PABA in this case does not antagonize sulfonamide inhibition.

There is thus good reason to accept the idea that PABA normally enters as an intermediate or end product in the metabolism of many diverse types of cell, including bacteria (final judgment on this must be reserved until PABA is definitely identified in these cells by conclusive chemical methods). However, there is clearly much evidence to show that this relation to normal metabolism is not necessary in order that PABA shall antagonize an inhibition by sulfonamide, i.e. PABA will antagonize sulfonamide inhibitions of enzymes whether or not PABA has any effect of its own on the enzyme system. As the question of PABA as an essential metabolite does not need to enter in these cases, it may be very seriously

[38] The following enzymes have been reported as not being inhibited by sulfonamides: catalase, peroxidase (section C); cytochrome oxidase, polyphenol oxidase, xanthine oxidase, uricase, urease (184); glucose dehydrogenase (Thunberg method), glyceric acid dehydrogenase (Thunberg method), *d*-amino acid dehydrogenase (section E5); pepsin, trypsin, serum di- and poly-peptidase (section D2a); phosphatase (53).

questioned whether such a consideration need be introduced in any case. This point is further stressed by the fact that antagonism occurs with many compounds other than PABA.

It would therefore appear, in the light of the additional data brought to light since the initial observation by Woods and Fildes, that there is very little basis for the presumption that sulfonamides inhibit growth by interfering with PABA metabolism specifically, or for that matter specifically with the metabolism of any analogous compound.

This consideration of course does not rule out the idea of sulfonamide specifically interfering with PABA metabolism. However, to be consistent one must apply similar consideration to methionine, peptone, protein, etc. In other words, the specific relation to PABA cannot be accepted unless at the same time a specific relation to these various other substances, which can be found in cells normally, is likewise accepted. The fact that the effect of PABA on sulfonamide inhibition is competitive cannot be used to support the idea that sulfonamide inhibits specifically a mechanism concerned with PABA metabolism, since, in the case of charcoal, diastase, etc., the antagonism between sulfonamide and PABA is competitive although sulfonamide is inhibiting a system which has no relation whatsoever to PABA. The fact, that methionine antagonism of the sulfonamide effect is not competitive (section D2a) while the PABA antagonism is competitive, does not of course indicate that the two antagonisms are not by the same mechanism. For it has long been recognized that inhibition of an enzyme may be competitive or non-competitive (89), and presumably therefore the effect of an antagonist on an inhibitor may also be competitive or non-competitive.

It is thus found that the Woods-Fildes theory, although consistent with certain observations, is not adequate for all observations.

g. Mutually exclusive action. As a corollary to the hypothesis that the action of sulfonamides is due primarily to an interference with the respiratory systems of cells (which will be considered in detail subsequently) and the observation that PABA antagonizes SA inhibition of respiration, it is necessary to imagine as Sevag and Shelburne (248) point out, that PABA in some way favors the removal of the SA from the catalytic system involved without itself interfering in the operation of that system.

Sevag *et al.* (246) quote the following two examples in which an enzyme is protected from one inhibitor by another inhibitor: Hopkins *et al.* (99) found malonate and succinate to prevent the inactivation of succinoxidase by oxidized glutathionine. Protection by succinate could be relatively easily understood because it is the substrate for the enzyme, and it could be that the inhibitor in this case is competing with the substrate for the same activation center on the enzyme, but malonate itself is an inhibitor for the enzyme. Potter and Dubois (217) have recently shown in addition that succinate antagonizes various sulfhydryl inhibitors such as quinone and *p*-phenylenediamine, cysteine and cystine, and that malonate, while producing a definite inhibition itself, prevents any additive inhibition by quinone or *p*-phenylenediamine. They interpret the latter phenomenon as one of inhibitors whose actions are "mutually exclusive,"

although each of the two types of inhibitor combines with the enzyme at a different locus. Thus malonate (and also succinate) shields the enzyme from sulfhydryl reactants; apparently the presence of the former makes it impossible for the latter to approach its combining site on the enzyme.

Dixon and Keilin (45) demonstrated the complete protection afforded xanthine oxidase aerobically and anaerobically from cyanide by uric acid, adenine, guanine and hypoxanthine. Neither uric acid nor guanine had any effect on the enzyme in the absence of cyanide. The protection afforded this enzyme by hypoxanthine was at first thought to be an example of a substrate protecting its enzyme against an inhibitor by not allowing the latter's approach. This explanation proved to be inadequate, for neither hypoxanthine nor the hydrogen-acceptor alone could confer protection, complete protection being obtainable only when both were present, i.e., when the oxidation of hypoxanthine was actually proceeding. The other substances gave protection without the presence of the hydrogen-acceptor.

The "mutually exclusive" action as originally described above was proposed for an instance in which the substance providing the shielding action was producing an inhibition *per se* through its very shielding action. It seems quite plausible that such a shielding may occur in some instances in which an inhibition does not result from the shield, i.e., adsorption of the substance giving the protection does not inhibit the enzyme activity (at that concentration at least).

Some such mechansim must be the explanation for the observations made by Eyster (54) on the charcoal model and on the digestion of starch by diastase. In the charcoal model, PABA counteracted SA inhibition of the adsorption of methylene blue, this antagonism being definitely shown as competitive. When the PABA concentration was increased sufficiently an additive inhibition was seen. In the case of diastase activity, PABA antagonized SA inhibition and *vice versa*, thus emphasizing the fact that a balance exists between the two substances.

There are many examples in cell physiology where two substances, separately toxic, completely or in part nullify each other's effects when mixed in proper balance, e.g., copper and calcium ions. Of extreme interest and pertinence is the finding by Valko and Dubois (272) that relatively harmless surface-active cations can completely antagonize the antibacterial action of highly toxic cations. These authors explain the phenomenon by what they call "ionic exchange", i.e., the harmless cations compete successfully with the toxic cations for the same spaces (presumably the carboxylic groups of the protein material). Duponol was found to antagonize the antibacterial action of acriflavin and, as pointed out by Valko and Dubois, it is unnecessary to assume that sodium dodecyl sulfate can act as an essential metabolite, an explanation favored by McIlwain (172) for the antagonistic action of tryptic casein, nucleic acid, and yeast extract for acriflavin. These observations offer a close parallel to those of Eyster on the charcoal model. Again, however, we face the question: since the toxic cation is toxic presumably because of an adsorption onto some specific locus, why is not the nontoxic cation toxic, when and if it combines at the same locus? This cannot be answered as

yet with any great degree of satisfaction, but some such mechanism as already described may well be in operation.

Other examples of "mutual exclusion" apropos the problem with which we are chiefly concerned, namely, antagonism of sulfonamide inhibition, have been reported:

SA antagonizes the bacteriostatic effect of mercuric ion and *vice versa* (136).

PABA counteracts SA inhibition of light production in growing cultures of luminous bacteria (E8).

PABA antagonizes ST inhibition of carboxylase (245). With this particular enzyme system, concentrations of PABA which alone inhibit do not produce an additive inhibition when added to inhibitory concentrations of ST; thus here the ST and PABA inhibitions are "mutually exclusive" in the same way as those reported for the two types of inhibitors of succinoxidase.

PABA is capable of shielding bacteria from inhibitors other than sulfonamides, among them being germanin, neostibosan, arsphenamine, and neoarsphenamine (202).

It seems necessary to point out, however, that in certain instances the antagonism or shielding action of PABA may not be in any way related to its action as an inhibitor. Thus, it may be that its antagonistic action takes place in both low and high concentrations, but at the higher concentrations PABA acts also as an inhibitor by some unrelated mechanism.

The exact mechanism of these antagonisms is yet to be learned, but there is ample evidence that antagonism of toxic substances can occur under circumstances indicating conclusively that the antagonism is a physical, surface phenomenon. Such an interpretation must be made of PABA's antagonism in several instances, and thus far there has appeared no definite evidence why this should not apply in every instance.

2. Possible Ways in Which an Inhibitor can Stop Cell Division

At the present time there seems to be no disagreement that sulfonamides inhibit certain enzymes, and that this is undoubtedly the primary mechanism of their action on cells. Let us next examine the various mechanisms whereby an inhibitor can interfere with cell multiplication and see which one agrees best with what is known about sulfonamide action.

a. Inhibition of one or more anabolic reactions which supply material (protoplasm) for increased cell mass. This may or may not be a factor in cell division. For example, in the early divisions of fertilized ova there is frequently no increase in protoplasm, but rather a repeated subdivision of that already present to daughter cells which become increasingly smaller with each division. On the other hand, it has been observed that certain cell inhibitors inhibit division while the cell mass itself increases up to a certain point. Interference with anabolic reactions which form enzymes responsible for other anabolic reactions must also be considered.

Inhibition of an anabolic reaction supplying material for increased cell mass may be brought about in several ways:

i. Direct interference with the enzyme. This forms the basis of the Woods-Fildes theory of sulfonamide action. The considerations regarding this theory have already been presented. While there seems little doubt that sulfonamides interfere with enzymes, there is actually no definite evidence that the enzyme inhibited is specially concerned with PABA as substrate.

ii. Direct interference with the substrate. In contrast to SA and PABA which interfere with the enzyme luciferase, azide inhibits *Cypridina* luminescence by combining with the substrate, luciferin (28, 108). This is merely cited as an example of this type of inhibition. There is no indication that this mechanism could account for inhibition by sulfonamide.

iii. Interference with the infusion of raw materials or the effusion of waste products. Changes in membrane permeability brought about by sulfonamide action could interfere with the infusion of necessary substrates or the effusion of waste products. An increased concentration of such products may produce an inhibition by any of the other mechanisms outlined. Reaction products which are normally substrates for other reactions may accumulate to the point where they, because of the reversible nature of the enzyme reaction which formed them, cause the reaction to slow down and eventually come to equilibrium. Such an accumulation would be due to an inhibition of the enzyme system which utilizes these products as substrates. It must be remembered again, however, that it is an opinion held by many that the great majority at least of bacterial metabolic reactions take place at the cell surface.

iv. Prevention of formation of substrate. This obviously would be a secondary effect, the primary inhibition being on the reaction producing the substrate; this latter inhibition could be of any type.

v. Inhibition of the formation of the enzyme itself. Very little is known concerning enzyme formation, but it is conceivable that a sulfonamide could inhibit this formation in some way. Decreased enzyme concentration would result in decreased reaction rate unless an excess of the enzyme existed. The enzyme concentration would decrease with time, depending on the rate of its disintegration. This could explain the delay in sulfonamide action observed by many. There is some evidence that in certain instances sulfonamides hinder the synthesis of respiratory coenzyme (section E9).

b. Inhibition of one or more oxidative enzymes concerned with supplying energy for the production of increased cell mass or cell division or both. The possible validity of such a mechanism hinges upon whether or not the respiratory processes are inhibited during inhibitor action; and, as was seen in the case of sulfonamides, the observations on bacteria with relation to this mechanism have, with but a few exceptions, shown such an inhibition. The evidence in favor of the hypothesis has indicated that the enzyme systems affected are the dehydrogenases (protein-coenzyme systems). This is interesting, since it is these same enzymes which narcotics inhibit.

In interpreting results from respiratory experiments it must be remembered that a substance conceivably could interfere with dehydrogenase action in any one of several ways. On the basis of what is known about these enzymes (50)

one may list the possible modes of action of a dehydrogenase inhibitor as shown[39] in fig. 2.

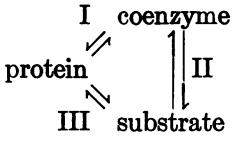

Fig. 2. Bonds connecting the three components of the enzyme-substrate complex denote reversible combinations. The enzyme is capable of functioning only when its two components, the coenzyme and protein, are associated.

i. at locus I, by
  a. adsorption onto the protein,—competition with coenzyme for the protein.
  b. adsorption onto the coenzyme,—competition with the protein for the coenzyme.

ii. at locus II, by
  a. adsorption onto the coenzyme,—competition with the substrate for the coenzyme.
  b. adsorption onto the substrate,—competition with the coenzyme for the substrate.

iii. at locus III, by
  a. adsorption onto the substrate,—so that the protein is no longer specific for the substrate or unable to function with the substrate (would be competitive).
  b. adsorption onto the protein,—the protein is no longer functional.

It is conceivable that a substance may so change the oxidation-reduction potential of an oxidative enzyme system that it can no longer function in its normal capacity. This has been proposed for the action of azide on cytochrome oxidase (6). There are two possible ways in which this could be brought about. First, the substance added, if an oxidation-reduction system itself, could poise the enzyme system at a non-functional level depending on the relative amounts of the two systems present. Since, as concluded in section C, the sulfonamides do not become oxidized before or during their bacteriostatic action, this could not occur. Second, the substance could by combination with the enzyme in any of the ways outlined above cause a shift in the oxidation-reduction potential of the system. Thus we see that this latter proposal is fundamentally not an alternative mode of interfering with oxidative enzymes from those already presented.

The above approach at best is extremely hypothetical but serves its purpose if only to illustrate the complexity of the situation and the caution which must be exercised in interpreting certain types of experiments. The coenzymes are non-specific for a variety of substrates (it is the protein component which confers the specificity on the protein-coenzyme complex) (214). Further complications

[39] Many enzymes other than respiratory enzymes are similarly composed of a protein component and a coenzyme; with such enzymes this consideration would also apply.

arise from the fact that, in the case of bacteria, enzyme (protein) saturation with coenzyme has frequently been found to be rather low and dependent to a great extent on the nature of the medium (78).

Laves (147), after demonstrating inhibition of aerobic and anaerobic respiration of liver brei and heart-muscle brei (section E8), supported the theory, which he apparently accredits to K. Mulli, that sulfonamides inhibit intracellular oxidation processes. Inhibition of enzyme activity was considered as probably resulting from either a direct poisonous action on the coenzyme, or what corresponds to mechanism *i*, *a* above. Sevag *et al.* (section E4) have also proposed what is essentially mechanism *i*, *a* for the inhibition of dehydrogenase activity by sulfonamides in bacteria. They have also suggested the possibility of the formation of an inactive "drug-protein-enzyme" complex which corresponds to *iii*, *b*. Similarly, Dorfman and Koser (46) suggested mechanism *i*, *a* for the SP and ST inhibition of the respiratory enzyme containing nicotinamide. That sulfonamides have a definite affinity for protein, which in magnitude is directly proportional to its bacteriostatic activity, is well-established (section D3a).

The mechanisms schematized so categorically above must not be taken too literally. Adsorption of a substance on the protein surface, say as in *iii*, *b*, may indirectly affect the relationship existing at locus (I) by change of electrical charge, polarity, steric hindrance, etc.

Sulfonamides stop cell growth and so do numerous other inhibitors, such as narcotics, cyanide, azide, etc. But of these, the action is more like that of the so-called "indifferent inhibitor" typified by the narcotics, dyes, etc. The effects of sulfonamides on bacteria and other cells are reversible for a time (section B3a) as are the effects of many indifferent inhibitors. Most typical of the indifferent inhibitors are the narcotics, and, as seen in section E10, there is much evidence to suggest similarities between the effects of narcotics and sulfonamides in forms higher than bacteria.

Concerning the similarity of sulfonamides to narcotics, it is typical of narcotics to inhibit function (e.g., cell division) completely with relatively little effect on respiration, i.e., inhibition of division increases with respiratory inhibition, but the former increases at a faster rate so that by the time division is completely blocked only a relatively small part of the overall normal respiration of the cell is inhibited. This of course does not mean that as the sulfonamide concentration is increased beyond that just required to stop division completely, respiratory inhibition will not also increase. As we have seen (section E), Sevag and others find inhibition of oxygen consumption by sulfonamides but, as with narcotics, it is relatively less than the inhibition of cell division (at or below the concentration of sulfonamide which just completely stops multiplication). Whence it is very likely that sulfonamides are blocking the energy production requisite for cell multiplication.

The complete picture of sulfonamide effect on oxygen consumption must account for the large oxygen consumption still left when cell division is completely stopped, i.e., the question arises as to what mechanism will account for

complete inhibition of cell division and very little inhibition of oxygen consumption or carbon dioxide production if these are significant at all in the action of sulfonamides. The suggestion proposed for the action of narcotics on various cells (65, 67, 69) would seem to apply here, and, in fact, is perhaps most clearly illustrated by the action of narcotics and SA on sea urchin eggs (see section E8). From this work it is evident that it is not inhibition of the overall total respiration of a cell which is significant in inhibition of cell division, but rather the inhibition of that fraction which is specifically concerned with providing the energy for cell division. Sulfonamides and narcotics inhibit this fraction more or less specifically, thus accounting for the complete inhibition of cell division and a relatively small inhibition of total respiration.

One must conclude, therefore, that an inhibition can be antagonized without the antagonist necessarily being a normal component of the reacting system in question. This fact destroys the force of the suggestion for the mechanism of sulfonamide action that, since PABA antagonizes sulfonamide action competitively, and since PABA is a growth factor for some cells, sulfonamides act by interfering with the metabolism of PABA.

Seeking now a more satisfactory explanation, and taking account of the newer investigations, there seems to be much reason for grouping the sulfonamides with such so-called indifferent inhibitors as the narcotics. In this connection, it has already been suggested that normal cell division depends upon the normal function of an unknown, but *specific fraction* of the total oxidative reactions of the cell, and that the indifferent inhibitors affect cell division by inhibiting that specific set of reactions. In keeping with this general consideration, Sevag and coworkers have claimed, and a recalculation of their data establishes, that oxygen consumption in bacteria is in fact inhibited by concentrations of sulfonamide which stop growth. In the sea urchin egg, the only case which has thus far been adequately investigated, and one which is more suitable for general investigation than are bacteria, it seems very clear that SA stops cell division by interfering with a specific fraction of the total oxidative metabolism of those cells. Accepting this view, it is probably necessary to couple with it the supposition that sulfonamide-antagonists exclude the sulfonamides from the cell catalyst or catalysts without themselves interfering with the action of those catalysts.

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