THE MECHANISM OF RESISTANCE TO SULFONAMIDES

II. ABSENCE OF CORRELATION BETWEEN RESISTANCE AND THE FORMATION OF ARYLMINE BY STAPHYLOCOCCUS AUREUS. NONINTERFERENCE WITH THE UTILIZATION OF GLUCOSE AS A CRITICAL FACTOR IN THE DEVELOPMENT OF RESISTANCE TO SULFONAMIDES

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Woods (1940) reported that 1.2 to 5.8 × 10⁻⁸ M of p-aminobenzoic acid (PABA) added to the medium counteracted the complete inhibition of growth (5-day period) of Streptococcus pyogenes (S. haemolyticus) and Escherichia coli by 3.03 × 10⁻⁸ M of sulfanilamide (SA). That is, one molecule of PABA counteracted the inhibitory action of 25,000 to 5,224 molecules of SA. Using Clostridium acetobutylicum, Rubbo and Gillespie (1940) found that 23,000 molecules of SA were neutralized by one of PABA. Measuring one-half maximal growth (16-hour period) of Staphylococcus aureus, Wyss et al. (1942) reported that one molecule of PABA neutralized 4,660 molecules of SA, and 53 molecules of sulfathiazole (ST). With E. coli, one molecule of PABA neutralized 2,000 molecules of SA, and 27 of ST. Determining the growth (5-day period) of E. coli, Strauss et al. (1941) reported that 0.0001 µg/ml of PABA neutralized 235 µg/ml of SA (a calculated ratio of SA to PABA molecules of 1,870,000), and 1 µg/ml of PABA neutralized 50 to 100 µg/ml of sulfathiazole (a calculated ratio of ST to PABA molecules of 27 to 54). There are in the literature numerous other reports supporting these observations.

At this point it must be emphasized that these observations are made with PABA added to the system from outside and do not therefore supply any evidence regarding the question as to whether or not PABA is synthesized by bacteria under normal physiological conditions. In view of this fact it cannot be claimed that the natural or acquired resistance of bacteria to sulfonamides under normal physiological conditions is due to an antagonism between increased PABA metabolism and sulfonamides unless it can be shown by a definite chemical method that PABA is simultaneously present. However, Landy et al. (1943) and Spink et al. (1944) reported that the development of resistance to sulfonamides in S. aureus (found to be a most striking example in this respect) results in the increased synthesis of PABA. They used a microbiological assay method to demonstrate the presence of PABA in bacterial culture fluids. In other words, the view expressed by Fildes (1940) that there exists an enzyme system responsible for the metabolism of PABA is supported by Landy et al. Since such generalizations have direct bearing on the mechanism of the action of sulfonamides

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and the nature of bacterial metabolism, we undertook a critical study of these reports.

**TABLE 1**

*Noninterference with the utilization of glucose as a critical factor in the development of resistance to sulfonamides*  
(Representative data)

<table>
<thead>
<tr>
<th>SECTION</th>
<th>EXP.</th>
<th>MEDIUM</th>
<th>CONTROL</th>
<th>PER CENT*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Inhibition of growth by</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Tryptophane added</td>
<td>ST</td>
</tr>
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<td></td>
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<td>Tryptophane added</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Tryptophane added</td>
<td>ST</td>
</tr>
<tr>
<td>A</td>
<td>1</td>
<td>Resistant strain</td>
<td>Birth medium alone</td>
<td>35 42 2 12</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Basal medium + glucose</td>
<td>76 88 75 112</td>
<td>2985</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Basal medium + pyruvate</td>
<td>35 100 2 140</td>
<td>2353</td>
</tr>
<tr>
<td>B</td>
<td>1</td>
<td>Susceptible strain</td>
<td>Birth medium alone</td>
<td>45 68 0 0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Basal medium + glucose</td>
<td>84 90 27 35</td>
<td>1560</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Basal medium + pyruvate</td>
<td>27 40 0 0</td>
<td>55</td>
</tr>
<tr>
<td>C</td>
<td>1</td>
<td>Susceptible strain (623)</td>
<td>Birth medium alone</td>
<td>0 27 0 2</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Basal medium + glucose</td>
<td>0 93 0 75</td>
<td>4380</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Basal medium + pyruvate</td>
<td>0 30 0 0</td>
<td>76</td>
</tr>
</tbody>
</table>

* Per cent inhibition of growth was calculated as follows:  
(control growth – growth in sulfonamides) × 100 = per cent inhibition of growth.  
This is the same as: 100 − % control population.

**RESULTS**

1. **Relationship between Arylamine Formation and Resistance to Sulfonamides**

As reported previously (Sevag and Green, 1944a, 1944b, 1944c), the formation of arylamine (PABA?) is not an indispensable process associated with the proc-
cesses of growth of *S. aureus* (see also the preceding article 1). However, under those growth conditions which also involve the formation of arylamine, the amount formed must have a quantitative bearing on the degree of inhibition of growth by sulfonamides if the claims cited above possess a valid experimental basis. With this in mind we have calculated the arylamine measured in the culture fluids as PABA and indicated the molar ratio of SA to PABA, or ST to PABA. These calculations are presented in table 1.

2. Change in Resistance to Sulfonamides of the Resistant Strain upon Continuous Transplantation

The resistance of staphylococci to sulfonamides can be increased by continuous transplantation in increasing concentrations of the drug. Continuous transplantation of the resistant strain in media free from sulfonamides might conceivably affect the resistance of this strain. Accordingly, the sulfonamide-resistant strain was transplanted daily on extract agar, free from glucose and sulfonamides, about 100 times. The resistance of this strain was again tested under conditions similar to those shown in table 1. These results are given in table 2.

**DISCUSSION**

1. Formation of Arylamine (*p*-Aminobenzoic Acid?) by *Staphylococcus aureus*

In the preceding communication the following observations were made: (a) the arylamine formed by strains of *S. aureus* is derived as a by-product of tryptophane metabolism; (b) there is no parallelism between the growth and the amount of arylamine formed; and (c) the growth of the strain of *S. aureus* made resistant to sulfonamides can take place without the formation of arylamine.

The formation of arylamine does not therefore appear to be an indispensable part of the metabolic and growth processes of *S. aureus* when it is rendered highly resistant to sulfonamides. The question as to whether or not this arylamine is partly or wholly *p*-aminobenzoic acid must nevertheless be raised.
2. Quantitative Consideration of the Arylamine Formed, Calculated as p-Aminobenzoic acid, in Relation to Antagonism to Sulfonamides

An analysis of the results given in table 1 shows that arylamine calculated as PABA is several-fold greater than that required to counteract the inhibition of the growth of the resistant strain of *S. aureus* by sulfanilamide (table 1, exp. 2, column 5). A comparison of the results of experiment 2 with 3 (section A, column 5) shows that in the presence of glucose where growth is not inhibited the molar ratio of SA to PABA is 2985. In contrast, in the presence of pyruvate where growth is inhibited 75 per cent, we find a 21 per cent smaller SA to PABA ratio of 2353. These data do not support the assumption that the measurable arylamine is *p*-aminobenzoic acid.

The calculated amount of PABA may, on the other hand, appear to be insufficient to counteract the inhibition by sulfathiazole (table 1, section A, column 6). There should, nevertheless, be a quantitative relationship between the calculated amounts of PABA and the degrees of inhibition. The absence of such relationship is very striking. In this connection a comparison of the per cent of inhibition indicated by experiments 2 and 3 (table 1, section A, column 6) brings out an interesting relationship. In the presence of glucose the molar ST to PABA ratio is 490. A comparison of this ratio with similar ratios, described in the introduction, shows that the amount of calculated PABA in our system is about 10- to 20-fold smaller than that required by the amount of sulfathiazole present; that is, growth should have been completely inhibited. Nevertheless, growth was not inhibited. In contrast, in the presence of pyruvate (section A, exp. 3, column 6) the molar ST to PABA ratio was 22 per cent smaller than in the glucose system, but growth was inhibited 70 per cent. These points cannot be reconciled by assuming that the measured arylamine is related in part or wholly to *p*-aminobenzoic acid.

The results obtained with the susceptible strains of *S. aureus* (table 1, sections B and C) show that the growth of these strains is inhibited under all three conditions of growth (exp. 1, 2, and 3) irrespective of the amount of calculated PABA, or the magnitude of the molar ratio of SA to PABA or ST to PABA. The only striking difference between these and the resistant strain is the absence of the inhibition of growth of the latter strain in the system containing glucose.

In the light of these observations it would seem reasonable to conclude that the presence or absence of inhibition of growth of the resistant strain of *S. aureus* is independent of the amount of arylamine, calculated as PABA. On the other hand, the results discussed below show that the resistant strain has acquired the ability to metabolize glucose in a manner not susceptible to the inhibitory action of sulfonamides.*

* Through the courtesy of Dr. W. W. Spink of Minneapolis, we received 4 sulfonamide-resistant strains of *Staphylococcus aureus* (nos. 604, 605, 611, 616; Spink et al., 1944). These strains, studied under the conditions described above, produced arylamine and behaved toward sulfonamides in a manner comparable to the resistant strain we have used in our experiments. The amount of arylamine produced by these 4 strains, calculated as *p*-aminobenzoic acid, checked the results reported by Spink et al. (1944). These results were approximately the same as those obtained with our sulfonamide-resistant strain.
3. Specialization in Metabolism of Glucose by Staphylococcus as one of the Critical Factors in the Development of Resistance to Sulfonamides

A survey of the results given in table 1 (see also tables 2, 3, and 4, article I) show that the metabolism of glucose is an important factor in increasing the growth of staphylococci several-fold. In the absence of glucose the growth is of smaller magnitude. It is therefore reasonable to believe that the inhibition of glucose metabolism by sulfonamides should also result in the inhibition of growth of staphylococci. Approaching the problem of the development of resistance to sulfonamides from this standpoint, we can readily see (table 1) that in the presence of glucose both sulfanilamide and sulfathiazole fail to inhibit the growth of the resistant strain of S. aureus. This is in contrast to the high degree of inhibition of the growth of the susceptible strains. This phase of the problem, therefore, merits further consideration. In view of the importance of glucose, also of pyruvate and tryptophane, the staphylococci were grown in 1 per cent casein hydrolyzate (basal) medium (see article I): (a) in the absence of added glucose, pyruvate and tryptophane; (b) in the presence of glucose or pyruvate but in the absence of added tryptophane; (c) in the presence of added tryptophane without the addition of glucose or pyruvate; and (d) in the presence of glucose and tryptophane, or pyruvate and tryptophane.

A comparative survey of the results presented in table 1 shows that the resistant strain is resistant to sulfonamides only in the presence of glucose. In the absence of glucose the growth of the resistant Staphylococcus aureus is inhibited. This is in striking contrast to the inhibition of the growth of susceptible strains under identical conditions. These observations indicate that during the development of resistance to sulfonamides S. aureus has acquired the specialized ability of metabolizing glucose in a manner which is not susceptible to the inhibitory action of sulfonamides. It may also be mentioned that, in contrast to the susceptible strains used, the resistant strain utilized pyruvate for growth to a degree equal to that of glucose. However, the growth of the resistant strain in pyruvate was strongly inhibited by sulfonamides. In this connection it is also to be remembered that the growth of the resistant strain in the basal medium (free of glucose or pyruvate) was highly susceptible to the inhibitory action of sulfonamides.

In the light of these observations it would appear that with the development of resistance in a medium containing glucose (as an essential contributor to the growth of staphylococci) there develops a highly specialized sulfonamide-resistant mechanism for the metabolism of glucose. However, this does not guarantee the growth of the resistant strain against the inhibitory action of sulfonamides in the absence of glucose (compare exp. 2 with 1 and 3, section A, table 1). These observations indicate that there may be several alternate pathways for the metabolism of either glucose or amino acids in organisms resistant to sulfonamides.

* Whether or not other resistant or susceptible strains behave similarly requires further experimentation.
4. Alternate Metabolic Pathways in Organisms Resistant to Sulfonamides

(a) Reversibility of Resistance to Sulfonamides. The development of resistance to antibacterial chemotherapeutic and other agents is a well-known phenomenon. A definite answer to the question of the duration or reversibility of acquired and natural resistance to sulfonamides, however, is lacking. The results of our studies presented in table 2 show that the growth of the strain which was highly resistant to sulfonamides in a medium containing glucose (table 1, section A, exp. 2) underwent a marked loss in this respect after 100 or more daily subcultures on extract agar (free of glucose and sulfonamides). This reversal of resistance to sulfonamides occurred without any loss of ability to utilize glucose or produce arylamine. These observations indicate that the resistant strain, on growing repeatedly on extract agar, either “lost” or underwent a change in the sulfonamide-resistant type of glucose metabolism. In this connection the following observations are of interest.

(b) Shifting of Resistance Metabolism from Glucose to Amino Acid Metabolism. As a result of 100 daily subcultures on extract agar (without added glucose, pyruvate, or sulfonamides), the resistant strain of S. aureus appears to have undergone a basic change, or adaptation in sulfonamide-resistant type of metabolism. The results presented in table 2 show that in contrast to the previous strong inhibition of growth by sulfonamides in basal medium, without glucose or pyruvate (table 1, section A, exp. 1), the growth now assumed a high degree of resistance to sulfonamides. Since during the daily subcultures on extract agar the medium did not contain sulfonamides, the acquired resistance does not appear to be a consequence of a new training for the development of resistance to sulfonamides. A critical analysis of the facts available at present would, on the other hand, appear to lead to the conclusion that the “resistance” acquired in a medium containing glucose is an adaptation to the metabolism of amino acids. Since the metabolism of glucose and amino acids is catalyzed by respiratory enzymes, one or more components of this enzyme system are involved in the alternation of the resistance metabolism essential for growth. In this connection a consideration of the effects of preformed or synthesized tryptophane (see below), riboflavin, and panthothenic acid on the metabolism of glucose and tryptophane in counteracting the inhibitory action of sulfonamides appears to be of significance.

5. The Metabolism of Tryptophane in Relation to the Resistance to Sulfonamides

In the conclusion of the preceding article it was suggested that the development of resistance to sulfonamides may result from or involve the increased synthesis of tryptophane. In this connection an analysis of the results presented in table 2 shows that growth in the basal medium (without glucose or pyruvate) in the absence of added tryptophane is inhibited 41 per cent by sulfathiazole. In contrast, the addition of 1 X 10^{-4} M tryptophane reduced the inhibition to 8 per cent. This indicates that the synthesis of tryptophane (essential for growth) from other amino acids, but not the utilization of added tryptophane, is inhibited.
by sulfathiazole. On the other hand, in the presence of glucose, the synthesis of tryptophane is not (or is only weakly) inhibited by sulfanilamide or sulfathiazole. The utilization of added tryptophane, however, is markedly inhibited by both drugs. Evidently, as previously reported (Sevag and Green, 1944c), the interrelation of glucose and amino acid metabolism appears sometimes to determine the inhibition of the synthesis of tryptophane, and at other times the utilization of added tryptophane by sulfonamides. The inhibition of growth in the presence of pyruvate does not appear to be influenced by the synthesis or utilization of preformed tryptophane. Further studies are necessary regarding this point.

CONCLUSIONS AND SUMMARY

The Woods-Fildes concept of the mode of action of sulfonamides, and those concepts which are formulated as extensions or adaptation of theirs, are primarily based on the results obtained with p-aminobenzoic acid added to the systems. Our results show that this PABA concept cannot be extended or applied to conditions of normal bacterial growth, in particular to that of Staphylococcus aureus. There is as yet no adequate, rigorous, chemical or physiological evidence that PABA synthesis occurs under normal conditions of bacterial growth. On the other hand, from the standpoint of the known requirements of a growing cell the inhibition of glucose and amino acid metabolism by sulfonamides should a priori be considered as critical factors. This conclusion is supported by the results previously reported (Sevag et al., 1942, 1943) and by those presented in this paper.

The results presented in this paper show that the development of resistance to sulfonamides is associated with the development of a sulfonamide-resistant type of glucose metabolism. This resistance with respect to glucose metabolism is reversible. The acquired resistance can, however, be adapted by the organisms to carry on a sulfonamide-resistant type of amino acid metabolism. The metabolism of tryptophane synthesized during growth or added to the system appears to exercise a definite role in this respect.

REFERENCES

Sevag, M. G., and Green, M. N. 1944b Lack of correlation between the formation of arylamine (p-a.b.a.?) and resistance of staphylococci to sulfonamides. J. Bact., 47, 451.


