PARA-AMINOBENZOIC ACID PRODUCTION BY STAPHYLOCOCCI

By WESLEY W. SPINK, M.D., LEMUEL D. WRIGHT, PH.D., JEAN J. VIVINO, AND HELEN R. SKEGGS

(From the Division of Internal Medicine, University of Minnesota Hospitals and Medical School, Minneapolis, and the Medical-Research Division, Sharp and Dohme, Inc., Glenolden, Pennsylvania)

(Received for publication, December 27, 1943)

It is now recognized that several species of bacteria may develop resistance to the antibacterial action of the sulfonamides. Strauss, Dingle, and Finland (1) have shown that pathogenic strains of staphylococci may become resistant *in vitro* by exposing cultures to increasing concentrations of the drugs. Vivino and Spink (2) confirmed these observations, and also pointed out that the development of sulfonamide-resistant staphylococci may take place in the human body. Up to the present time, we have isolated 28 strains of coagulase-positive staphylococci from 24 patients, which showed varying degrees of sulfonamide-resistance. A description of these investigations and their clinical significance will be presented elsewhere (3).

The mechanism whereby bacteria become adapted to grow in the presence of the sulfonamides has been the subject of numerous investigations. The observations of Woods (4) first suggested that para-aminobenzoic acid (PABA) was synthesized by certain species of organisms, and that PABA, an essential bacterial metabolite, competed with the chemically related sulfonamides for a position in a bacterial enzyme system. MacLeod (5) observed that cultures of a sulfonamide-resistant strain of Type I pneumococcus yielded a filtrate which inhibited the antibacterial action of sulfapyridine for *B. coli* to a greater degree than a filtrate prepared from the parent sulfonamide-sensitive strain. Mirick (6) utilized a suspension of a soil bacillus, which could be specifically adapted to oxidize PABA, and found that this suspension rapidly destroyed the diazotizable sulfonamide-inhibiting substance present in the filtrate prepared from a sulfonamide-resistant strain of pneumococcus. More recently Mirick (7) has presented a method for quantitating small amounts of PABA with the aid of specific adapted enzymes of this same soil bacillus. Landy and his associates (8) have investigated with a microbiological assay two sulfonamide-resistant strains of staphylococcus supplied by us, and reported that the sulfonamide inhibitor elaborated by these strains was PABA.

Up to the present time, there has not appeared any confirmatory evidence in support of Landy's conclusion that the antisulfonamide action of staphylococci is related to the synthesis of PABA by the bacterial cells. The purpose of this report is to present the results of observations showing that the resistance

of staphylococci is closely related quantitatively to the production of PABA by the bacteria.

Methods and Materials

Throughout the present study Gladstone's (9) water clear synthetic medium has been utilized in which the presence of sulfonamide inhibitors has been reduced to a minimum. The preparation of this medium is described elsewhere (3). The standard test used for detecting sulfonamide-resistant staphylococci was as follows: Strains to be tested were grown for several generations in the synthetic medium to insure against contamination by any sulfonamide-inhibiting materials. 0.1 cc. of a 10^{-3} dilution of a 24 hour culture in synthetic medium was added to a series of tubes containing 10 cc. of the medium with varying concentrations of sodium sulfathiazole. Under these conditions the total number of bacteria added varied between 40,000 and 180,000. Sodium sulfathiazole was selected because repeated observations with the described standard test had shown this compound to inhibit growth of the staphylococci to a greater degree than either sulfanilamide, or the sodium salts of sulfapyridine, sulfadiazine, and sulfamerazine. After 48 hours of incubation at 37°C., bacterial growth was ascertained and expressed in terms of turbidity with the use of the Evelyn photoelectric colorimeter.

Ten sulfonamide-resistant and 5 non-resistant strains of staphylococci, all coagulase-positive and isolated from patients, were used in an attempt to obtain a diazotizable substance produced by the bacteria. 10 cc. of the synthetic medium was seeded with 0.1 cc. of a 10^{-3} dilution of a 24 hour culture, and then incubated at 37°C. for 96 hours. Supernatants of the cultures were prepared by adding trichloracetic acid to a final concentration of 1.5 per cent and then centrifuging the contents. The colorimetric method of Bratton and Marshall (10) was used to determine the amount of diazotizable material present in the supernatants, utilizing a standard PABA preparation as a control.[1] The intensity of the color was measured with the Evelyn photoelectric colorimeter.

A culture of an aerobic soil bacillus capable of being adapted to oxidize PABA, thus preventing the development of a diazo color, was obtained through the courtesy of Dr. George S. Mirick of the Hospital of The Rockefeller Institute for Medical Research. At the suggestion of Dr. Mirick, the bacillus was grown for approximately 24 hours in 200 cc. of veal infusion broth, pH 7.8, in each of two flasks. One flask contained only the inoculated medium, and the second had 20 mg. per 100 cc. of pure PABA added. When maximum growth was obtained the bacterial cells were washed with phosphate buffer, pH 7.8. The washed cells, concentrated in about 10 cc. of phosphate buffer, were shaken for 30 minutes with the previously described supernatants, a sample of which had given a diazo color reaction. The cells were similarly exposed to a solution containing a known quantity of pure PABA. Immediately thereafter, the diazo test was performed with the treated supernatants. The same procedure was carried out with cells not grown in the presence of PABA.

To test the antisulfonamide action of sulfonamide-resistant and sulfonamide-

[1] Pure PABA was kindly supplied by Dr. D. F. Robertson of Merck & Co., Inc.

sensitive strains of staphylococci, filtrates were prepared from the representative strains by passing 72 hour cultures in synthetic medium through a Seitz filter. The amount of diazotizable substance in each filtrate was quantitated by the diazo reaction. The inhibitory effect of the filtrates upon the bacteriostatic action of sodium sulfathiazole was determined by adding to a series of tubes 1 cc. of the filtrate and 9 cc. of the synthetic medium containing the previously described standard inoculum of a sulfonamide-sensitive strain of staphylococcus. Varying concentrations of sodium sulfathiazole were then added to each of the tubes. A control series of tubes contained varying amounts of pure PABA instead of the filtrates. The amount of PABA used was that amount in solution which gave approximately the same diazo reaction as the bacterial filtrates. The contents of the tubes were incubated for 48 hours at 37°C.

Attempts were made by two of us to identify the diazotizable substance produced by the staphylococci as being PABA by employing the microbiological assay described by Lewis (11). Repeated failures were ascribed to a chemical contamination of the control medium, possibly by PABA or other growth-stimulating substances for the test organism, *Lactobacillus arabinosus* 17-5. More desirable results were obtained in a second laboratory by utilization of the assay method of Landy and Dicken (12). Material was prepared with the synthetic medium at Minneapolis and sent to Glenolden for assay by the other two authors of the present paper. In the first series of observations, filtrates prepared by Berkefeld filtration were assayed. 5 cc. of each sample was autoclaved at 15 pounds pressure for 15 minutes with 5 cc. of 0.2 N NaOH, and then neutralized with 0.2 N HCl. *Acetobacter suboxydans*, as modified by the addition of purines and pyrimidines (13), was employed. The medium and three sulfonamide-resistant strains (605, 611, and 620) were assayed at levels of 0.02 to 0.10 cc. of the original filtrate. The standard curve embraced a range of 0.005 to 0.5 microgram of PABA. Incubation was carried out for 48 hours at 30°C. Turbidity of the cultures, measured with the aid of the Klett-Summerson photoelectric colorimeter, was taken as an index of growth. Two other series of assays were made according to the method of Lewis (11). 1 cc. of each sample was autoclaved with 10 cc. of a 1 N NaOH at 13½ pounds pressure for 30 minutes, and then neutralized with 1 N HCl. The sulfonamide-resistant cultures were assayed at levels of 0.0005 to 0.0025 cc. of the original sample, while the sulfonamide-sensitive strains were assayed at levels of 0.005 to 0.05 cc. The standard curve covered a range from 0.0001 to 0.0010 microgram of PABA. The basal medium was prepared according to the directions of Lewis. A 24 hour culture of *Lactobacillus arabinosus* 17-5, grown in a medium free of PABA, was used as the inoculum. Titration of the acid produced during 72 hours' incubation at 30°C. was used as a measure of the response of the organism to PABA.

RESULTS

Diazotizable Substance Produced by Staphylococci and Its Relationship to Sulfonamide Resistance.—The results of the inhibitory effect of sodium sulfathiazole upon the growth of 15 strains of staphylococci are presented in Table I. It is to be noted that the growth of 5 strains was completely inhibited by a concentration of less than 1 mg. per 100 cc. of sodium sulfathiazole, whereas the inhibition of 10 resistant strains was only accomplished with a concentra-

tion of 200 mg. or more of the sulfonamide per 100 cc. of culture medium. The amount of diazotizable substance found in the supernatants of the cultures of the 15 strains was also compared with the resistance of the organisms to the action of sodium sulfathiazole. An aqueous solution of pure PABA was used as a control in quantitating the diazo reaction. Supernatants of the cultures were used instead of filtrates free of organisms since the end results were the same with both preparations, and supernatants were obtained with greater ease than

TABLE I

Diazotizable Substance in Supernatants of Non-Resistant and Sulfonamide-Resistant Strains of Staphylococci Grown for 96 Hours at 37°C.

Strain No.	Diazotizable substance	Minimal concentration of sodium sulfathiazole completely inhibiting growth
	micrograms of PABA per 100 cc.	*mg. per 100 cc.*
Non-resistant strains		
7	Less than 10	Less than 1
14	" " 10	" " 1
104	" " 10	" " 1
718	" " 10	" " 1
720	" " 10	" " 1
Resistant strains		
604	76	200
605	68	250
606	56	200
610	86	200
611	124	200
612	96	200
613	78	200
614	92	200
615	80	200
616	78	200

were the filtrates. As shown in Table I, the sulfonamide-sensitive strains produced less than 10 micrograms per 100 cc. of diazotizable material, expressed in terms of PABA. On the other hand, the sulfonamide-resistant strains produced from five to slightly over ten times as much of the substance. It should be emphasized that the color reaction cannot be measured accurately with the Bratton-Marshall technique when the concentration is below 10 micrograms per 100 cc. Nevertheless, the results show a marked difference in the amount of diazotizable substances produced by these two groups of staphylococci.

Effect of a Soil Bacillus (Mirick), Specifically Adapted to Oxidize PABA, upon

the Diazotizable Substance.—After the supernatants of the 15 cultures of staphylococci had each been exposed to the soil bacillus, according to the method previously described, no diazo reaction could be developed. Control samples of supernatants similarly treated with the parent strain of Mirick's soil bacillus, which had not been adapted to oxidize PABA, produced the typical diazo color. This is further evidence that the diazotizable material formed by staphylococci is PABA.

Effect of Bacteria-Free Filtrates of Non-Resistant and Sulfonamide-Resistant Strains of Staphylococci upon the Antistaphylococcic Action of Sodium Sulfathiazole.—Filtrates prepared from both sulfonamide-sensitive and sulfonamide-resistant strains of staphylococci inhibited the bacteriostatic action of sodium sulfathiazole for strains whose growth was readily inhibited by the drug.

TABLE II

Antisulfonamide Effect of Filtrates of Non-Resistant and Resistant Strains of Staphylococci, and of PABA, upon Antistaphylococcic Action of Sodium Sulfathiazole

Test material	Concentration of sodium sulfathiazole, *mg. per 100 cc.*								
	0.01	0.1	0.5	1.0	5.0	10.0	25.0	50.0	100.0
Non-resistant strain 14.....	4+	4+	+	0	0	0	0	0	0
Non-resistant strain 14 plus filtrate of non-resistant strain 14.....	4+	4+	4+	4+	+	+	0	0	0
Non-resistant strain 14 plus filtrate of resistant strain 605.....	4+	4+	4+	4+	4+	4+	+	+	+
Non-resistant strain 14 plus 0.27 mg. per 100 cc. PABA.....	4+	4+	4+	4+	4+	4+	4+	+	0

+ = minimum growth. 4+ = maximum growth.

The results of a representative experiment are shown in Table II. The growth of strain 14 was inhibited completely by 1 mg. per 100 cc. of sodium sulfathiazole, while the growth of strain 605 required a concentration of 250 mg. of the sulfonamide. It is of interest to observe that the filtrate of strain 14 inhibited the antistaphylococcic action of the sulfathiazole for this same strain. Reference to Table I shows that this strain produced relatively small amounts of the diazotizable material. The filtrate of the sulfonamide-resistant strain 605 produced a much more marked inhibition of the sulfonamide activity for strain 14. In quantitating the diazo reaction of the filtrate of strain 605, it was calculated that the diazotizable substance represented approximately 0.27 mg. per 100 cc. of PABA. This concentration of PABA acted to inhibit bacteriostasis by sulfathiazole nearly as markedly as did the filtrate of strain 605.

Microbiological Assay of Staphylococcic Filtrates for PABA (Method of Landy and Dicken).—In the first series of assays, the filtrates of two sulfonamide-sensitive strains of staphylococci and three strains resistant to sulfonamide

activity were investigated. As a control, the amount of active material in the synthetic medium was determined. The results are given in Table III. Slight growth stimulation was found to occur in the uninoculated synthetic medium. The 3 sulfonamide-resistant strains produced approximately 30 to 40 times the PABA produced by the 2 sulfonamide-sensitive strains. For comparison, Table III also includes the concentration of sodium sulfathiazole required to inhibit completely the growth of each strain of staphylococcus.

Comparative Microbiological Assay of PABA in Cell-Free Filtrates and in the Medium with Staphylococci Present (Method of Lewis).—Since the first series was carried out with the filtrates of staphylococcic cultures, there existed the possibility that a portion of the PABA had been removed in the process of

TABLE III

PABA Content of Staphylococcic Filtrates (Method of Landy and Dicken), As Compared with the Concentration of Sodium Sulfathiazole Required to Inhibit in Vitro Growth of the Cultures

Sample	PABA	Minimal concentration of sodium sulfathiazole completely inhibiting growth
	micrograms per 100 cc.	*mg. per 100 cc.*
Medium	0.26	
Strain 7*	0.88	Less than 1
" 14*	0.75	" " 1
" 605‡	26	250
" 611‡	32	200
" 620‡	25	200

* Non-resistant to sulfonamide action.

‡ Resistant to sulfonamide action.

filtering the cultures through the Berkefeld candles. Therefore, a second series of assays was made in which the amount of PABA was determined simultaneously in cell-free filtrates and in the medium containing the fully grown cells. These results are presented in Table IV. Both preparations yielded the same amount of PABA. In the light of these findings, there appeared to be no advantage in assaying cell-free filtrates for PABA, and in subsequent determinations, the original medium with the fully grown cells was utilized. It is to be noted that the values of PABA obtained with the method of Lewis were considerably greater than those obtained with the method of Landy and Dicken. In addition, the yield of PABA was much greater with the sulfonamide-resistant strains of staphylococci in comparison with the non-resistant strains.

Comparison of PABA Production by Staphylococci (Method of Lewis) with the Concentration of Sodium Sulfathiazole Required to Inhibit the in Vitro Growth

TABLE IV

Comparative Assay of PABA (Method of Lewis) with Cell-Free Filtrates and with the Original Culture Medium Containing Staphylococci

Strain and material assayed	PABA	Minimal concentration of sodium sulfathiazole completely inhibiting growth
	micrograms per 100 cc.	*mg. per 100 cc.*
7 —cells*	1.7	Less than 1
7 —filtrate*	1.8	" " 1
14 —cells*	1.1	" " 1
14 —filtrate*	1.1	" " 1
7c—cells‡	130	50
7c—filtrate‡	130	50
14c—cells‡	57	75
604— " ‡	200	200
605— " ‡	170	250

* Non-resistant to sulfonamide action.

‡ Resistant to sulfonamide action.

TABLE V

Comparison of PABA Production by Staphylococci (Method of Lewis) with the Concentration of Sodium Sulfathiazole Required to Inhibit in Vitro Growth of the Cultures

Strain	PABA	Minimal concentration of sodium sulfathiazole completely inhibiting growth
	micrograms per 100 cc.	*mg. per 100 cc.*
14*	4.5	Less than 1
104*	3.5	1
715*	6.1	1
716*	4.6	1
717*	5.1	1
724*	7.8	1
727*	3.5	1
14C‡	30	75
116‡	30	20
117‡	33	5
604‡	240	200
605‡	460	250
606‡	30	200
611‡	140	200
616‡	210	200
619‡	220	40
628‡	65	200
629‡	220	10

* Non-resistant to sulfonamide action.

‡ Resistant to sulfonamide action.

of the Cultures.—The third series of assays was accomplished with 18 strains of staphylococci, 7 of which were considered sensitive to the *in vitro* action of sodium sulfathiazole while the remaining 11 were resistant. Lewis' method for assaying PABA was utilized. In the preparation of the samples, the cells were not removed from the culture medium. The comparative results are shown in Table V. It is obvious that the sulfonamide-sensitive strains produced less PABA than the resistant strains. However, the results obtained with the resistant strains were inconstant. In several instances, the production of PABA could not be directly correlated with the *in vitro* resistance of the organisms to sodium sulfathiazole. As examples, strain 606 produced only 0.30 microgram of PABA per cc., and yet a concentration of 200 mg. of sodium sulfathiazole per 100 cc. was required to inhibit completely the growth of this organism. Similar results were obtained with strain 628. On the other hand, strains 604, 611, and 616 produced larger amounts of PABA and required only the same amount of the sulfonamide to inhibit their growth. These inconsistencies will be discussed. It is of interest that the most resistant strain (605) yielded the highest assay of PABA.

DISCUSSION

The foregoing data offer further evidence that the development of sulfonamide-resistant strains of staphylococci is related quantitatively to the production of PABA by this species of bacteria. Nevertheless, this does not rule out the possibility that another mechanism or other mechanisms may be involved in the development of such resistance. The inconstant results which we have obtained with two different methods of assaying PABA merit comment. It is believed that these discrepancies are associated with several sources of error which are inherent in any biological assay, particularly those relating to the assay of PABA. As Mirick (7) has pointed out, there may be substances closely related chemically to PABA which may stimulate growth of the test organism. Furthermore, when substances of unknown composition are supplied to a medium deficient for promoting growth, factors unrelated to the one of specific interest may activate growth. Finally, organisms may adapt themselves to grow in a deficient medium. These alterations in biological activity may be so slight that they cannot be detected readily. The difficulties encountered in assaying PABA in an unknown mixture may be resolved by applying the technique described by Mirick (7) in which a soil bacillus may be specifically adapted to oxidize PABA. According to Mirick, the specific adaptive enzymes of this bacillus can be used to identify as little as 10 micrograms of PABA.

SUMMARY

1. Strains of staphylococci produce diazotizable materials which can be converted to a dye, and the intensity of the color reaction can be quantitated

in the same manner as *p*-aminobenzoic acid. The sulfonamide-resistant strains which we have studied produce more diazotizable substance than non-resistant strains.

2. The development of a color by the diazotizable substance can be inhibited by exposing the substance to a soil bacillus (Mirick) specifically adapted to oxidize PABA.

3. The diazotizable substance produced by staphylococci inhibits the anti-staphylococcic action of sodium sulfathiazole to approximately the same degree as equivalent amounts of pure *p*-aminobenzoic acid.

4. Two microbiological methods for assaying *p*-aminobenzoic acid were employed for quantitating the amount of this material produced by staphylococci. In general, the sulfonamide-resistant strains produced more *p*-aminobenzoic acid than the sulfonamide-sensitive strains. The inconstant results obtained with these biological assays are discussed.

BIBLIOGRAPHY

1. Strauss, E., Dingle, J. H., and Finland, M., *J. Immunol.*, 1941, **42,** 331.
2. Vivino, J. J., and Spink, W. W., *Proc. Soc. Exp. Biol. and Med.*, 1942, **50,** 336.
3. Spink, W. W., and Vivino, J. J., *J. Clin. Inv.*, in press.
4. Woods, D. D., *Brit. J. Exp. Path.*, 1940, **21,** 74.
5. MacLeod, C. M., *J. Exp. Med.*, 1940, **72,** 217.
6. Mirick, G. S., *J. Clin. Inv.*, 1941, **20,** 434.
7. Mirick, G. S., *J. Exp. Med.*, 1943, **78,** 255.
8. Landy, M., Larkum, N. W., Oswald, E. J., and Streightoff, F., *Science*, 1943, **97,** 265.
9. Gladstone, G. P., *Brit. J. Exp. Path.*, 1937, **18,** 322.
10. Bratton, A. C., and Marshall, E. K., Jr., *J. Biol. Chem.*, 1939, **128,** 537.
11. Lewis, J. C., *J. Biol. Chem.*, 1942, **146,** 441.
12. Landy, M., and Dicken, D. M., *J. Biol. Chem.*, 1942, **146,** 109.
13. Landy, M., and Streightoff, F., *Proc. Soc. Exp. Biol. and Med.*, 1943, **52,** 127.