THE MECHANISM OF RESISTANCE TO SULFONAMIDES

I. Factors Controlling the Formation of Arylamine from Tryptophane by Staphylococcus aureus[1]

M. G. SEVAG and MORRIS N. GREEN[2]

Department of Bacteriology, School of Medicine, University of Pennsylvania, Philadelphia 4, Pa.

Received for publication July 19, 1944

Woods (1940) found that *p*-aminobenzoic acid (PABA) in high dilutions antagonized the action of sulfonamide (SA) in a manner similar to a fraction obtained from yeast extract. It was later shown (Rubbo and Gillespie, 1940; Blanchard, 1941) that this fraction from yeast was also PABA. Woods's findings gave rise to a concept (Fildes, 1940) which explains the mode of action of sulfonamides on bacteria by the assumption that PABA is an essential metabolite normally associated with an enzyme. Sulfonamides displace PABA from its enzyme and thereby stop this essential line of metabolism. Landy *et al.* (1943) have concluded that the development of resistance to sulfonamides in *Staphylococcus aureus* results in an increased synthesis of PABA. Using microbiological assay methods, they reported the presence of PABA in the culture fluids of numerous bacteria.

Since the validity of this concept and the dependability of the assay methods have fundamental bearing on the general principles and practice of chemotherapy, we undertook a critical study of the factors controlling the assumed synthesis of "PABA" by strains of *S. aureus*. In view of the experimental data reported previously by us (Sevag and Green, 1944a) and in this paper, there appears to be as yet no evidence that *S. aureus* synthesizes PABA or requires it for growth. It is conceivable, however, that staphylococci may produce minute amounts of PABA. This assumed production of PABA appears to influence neither growth nor resistance to sulfonamides (Sevag and Green, 1944b). On the other hand, it is known that bacteria (Kotake, 1933) produce one or more arylamines, and we have found that staphylococci, likewise, produce arylamines of unknown constitution (Sevag and Green, 1944a). These arylamines on diazotization and coupling with N-(1-naphthyl)-ethylene diamine (Bratton and Marshall, 1939) yield a colored solution of lavender shade similar to those obtained from sulfonamides.

EXPERIMENTAL METHODS

1. Source of the Strains of Staphylococcus aureus

The sulfonamide-resistant and the parent sulfonamide-susceptible strains of *S. aureus* were kindly supplied by Dr. M. Finland of Boston (Strauss, Dingle,

[1] This investigation has been aided by a grant from the Josiah Macy, Jr. Foundation.

[2] George Leib Harrison Fellow and Manfred Wahl Fellow.

and Finland, 1941). As will be seen in the following article (II), the resistant strain was found to be resistant to 0.04 M sulfanilamide and 0.0066 M sulfathiazole. The B-523 strain is toxigenic; it was isolated from a case of osteomyelitis and was supplied through the courtesy of Dr. John Blair (see article III). These strains were supplemented in our work with nine others from various sources.

2. Determination of Arylamine in Culture Fluids

The method used was basically that of Bratton and Marshall (1939). Using the Klett-Summerson photoelectric colorimeter, we were able to measure as little as 0.2 to 0.3 μg/10 ml of arylamine, calculated as PABA. One μg of pure PABA per 10 ml solution gave a reading of 20. Treated in the same manner, one μg of *meta* and *ortho* isomers gave readings, respectively, of 10 and 5. Kynurenine,[3] an amino acid in which the pyrrole ring of tryptophane has ruptured, in equivalent molar concentration gave a reading of 5, identical with that obtained with *o*-aminobenzoic acid. The colors of the dyes produced from these arylamines are indistinguishable with the naked eye.

The values given in the arylamine columns in all of the tables correspond to the colorimetric readings obtained with 3 ml of clear culture fluid obtained by centrifuging the cultures in an angle centrifuge.

In experiments in which arylamine was determined special care was taken to avoid contamination with sulfonamides. The glassware containing sulfonamides was handled separately to prevent the contamination of the stock glassware. That used for the study of arylamine formation was washed in dilute cleaning mixture and rinsed repeatedly with water after routine washing in the laboratory kitchen.

3. Determination of the Growth of Staphylococcus aureus

The cultures were grown in 10 ml of medium at 37 C. After 48 hours of growth 3 ml of the culture were diluted to 10 ml in a standard tube, and the optical density of the suspension was determined with the Klett-Summerson photoelectric colorimeter. A reading of 66 corresponds to 0.14 mg of staphylococcal nitrogen, 1 mg of staphylococci, or approximately 2.5 to 3.5 billion cocci.

EXPERIMENTAL RESULTS AND DISCUSSION

1. Growth of Staphylococcus aureus Independent of Arylamine Formation

If the arylamine found in the culture fluids is PABA and if the latter is an essential metabolite, it would seem reasonable to assume that its formation should run parallel with growth, particularly with the strain made resistant to sulfonamides. That is, the ratio of the amount of arylamine formed to the amount of growth should remain roughly constant. The results presented in table 1 show that there is no parallelism between the amount of arylamine formed and growth. On the contrary, the amount of arylamine formed is less under

[3] We are indebted to Dr. R. W. Jackson of the Eastern Regional Research Laboratory, U. S. Department of Agriculture, Philadelphia, Pa., for the gift of a sample of kynurenine.

optimal, and more under less favorable, conditions of growth. The results presented in tables 2, 3, and 4 show conclusively that the growth of both resistant and susceptible strains of *S. aureus* can take place without the formation of arylamine.

2. Critical Factors Controlling the Formation of Arylamine

(*a*) *Indispensability of Tryptophane.* Tryptophane is an essential growth amino acid for *S. aureus*. There are, however, *nonexacting* strains that are capable of multiplying in the absence of added tryptophane. The nonexacting (Sevag and Green, 1944c, 1944d) strains (both sulfonamide-resistant and -susceptible) are capable of synthesizing tryptophane from other amino acids, yielding a limited amount of growth. In the presence of glucose the synthesis of tryptophane also occurs, thereby resulting in a several-fold increase of growth.

TABLE 1

The interrelation between the concentration of casein hydrolyzate, growth, and arylamine formation by Staphylococcus aureus (R—Finland)

BASAL MEDIUM CONTAINING CASEIN HYDROLYZATE	TURBIDITY READING DUE TO GROWTH	COLORIMETRIC READING DUE TO ARYLAMINE	ARYLAMINE / GROWTH
Per cent			
10.00	174	167	0.96
5.00	134	177	1.36
2.50	114	230	2.02
1.25	107	240	2.24
0.63	91	230	2.53
0.16	56	227	4.06
0.07	43	234	5.44
0.04	40	160	4.00
0.02	36	110	3.06

Basal Medium: M/30 phosphate solution (pH 7.4) of casein hydrolyzate (SMACO, vitamin-free) + 9.0×10^{-4} M of cysteine hydrochloride + glucose 0.5 per cent + 1×10^{-4} M of tryptophane + 2.5×10^{-4} M of $Fe(SO_4) \cdot (NH_4)_2SO_4 \cdot 6H_2O$ + 3.3×10^{-4} M of magnesium sulfate + 1 μg/ml of medium of each of thiamin chloride and nicotinamide.

The *exacting* strain (B-523) is incapable of synthesizing tryptophane from other amino acids, either in the presence or absence of glucose, and it is therefore incapable of growth unless tryptophane is added to the medium.

It can be seen from the results presented in table 2 that (a) the formation of arylamine by the nonexacting strain (R) takes place to a certain extent in the presence of glucose and in the absence of added tryptophane, and (b) the amount of arylamine formed in the presence of added tryptophane (media *B* and *C*) is doubled although there is no increase in growth. In the former case glucose mediates the synthesis of an increased amount of tryptophane, part of which is oxidized to arylamine. In the latter case the amount of arylamine formed is twice as much because of the presence of a greater amount of available tryptophane (this is borne out further by the results presented in table 3). The formation of arylamine by the exacting strain (B-523) requires additional amino acids

such as cysteine, threonine, and isoleucine (table 2, compare sections A with B). This shows that oxidative-reductive reactions involving the metabolism of glucose or tryptophane and other amino acids are responsible for the formation of arylamine as a by-product of tryptophane metabolism.

The indispensability of tryptophane for the formation of arylamine is most clearly shown by the results presented in table 3. It will be seen that in the basal

TABLE 2

Glucose and tryptophane as critical factors in the formation of arylamine(s) during the growth of Staphylococcus aureus

EXP.	MEDIUM	GROWTH FACTORS ADDED TO THE MEDIUM			STAPHYLOCOCCUS AUREUS					
					Resistant (R) strain			Strain B-523		
		$MgSO_4$ 0.01%	Glucose 0.5%	Tryptophane 1×10^{-4} M	Growth	Arylamine	Arylamine / Growth	Growth	Arylamine	Arylamine / Growth
1	*A.* Synthetic medium I*	+	−	−	7	0	0	0	0	0
2	*A.* Synthetic medium I*	+	+	−	32	32	1.00	5	0	0
3	*A.* Synthetic medium I*	+	−	+	11	7	0.63	8	3	0.37
4	*A.* Synthetic medium I*	+	+	+	52	45	0.86	27	2	0.07
5	*A.* Synthetic medium I*	−	+	+	18	7	0.38	24	2	0.08
1	*B.* Synthetic medium II†	+	−	−	11	3	0.27	0	0	0
2	*B.* Synthetic medium II†	+	+	−	96	55	0.57	5	0	0
3	*B.* Synthetic medium II†	+	−	+	22	11	0.50	18	9	0.50
4	*B.* Synthetic medium II†	+	+	+	95	97	1.02	18	37	2.05
5	*B.* Synthetic medium II†	−	+	+	26	30	1.16	14	35	2.50
1	*C.* Casein hydrolyzate (1.3%)	+	−	−	15	0	0	0	0	0
2	*C.* Casein hydrolyzate (1.3%)	+	+	−	105	63	0.60	7	0	0
3	*C.* Casein hydrolyzate (1.3%)	+	−	+	68	26	0.38	13	3	0.23
4	*C.* Casein hydrolyzate (1.3%)	+	+	+	95	114	1.20	40	48	1.20

* *Synthetic Medium I.* Ten ml of the M/30 phosphate (pH 7.4) solution containing 6.6×10^{-4} M of alanine, valine, leucine, glycine, proline, oxyproline, aspartic acid, glutamic acid, 2.5×10^{-4} M of methionine, phenylalanine, tyrosine, hydrochlorides of histidine, lysine, and supplemented with salts and vitamins as indicated in the footnote to table 1.

† *Synthetic Medium II.* This consisted of the synthetic medium I + 3.36×10^{-3} M of threonine + 3.05×10^{-3} M of isoleucine + 9.0×10^{-4} M of cysteine hydrochloride.

medium, free of glucose and preformed tryptophane, the growth of both the non-exacting resistant and susceptible strains takes place without the formation of arylamine. The exacting strain (B-523C) does not grow under these conditions. However, upon the addition of increasing amounts of tryptophane the formation of arylamine is progressively increased for all three strains. In this respect, the resistant strain does not behave differently from the susceptible strains.

(*b*) *Effect of Various Carbohydrates.* The results presented in table 4 show that, in the case of the resistant strain, glucose and pyruvate cause the forma-

TABLE 3

Increased arylamine formation by increasing the concentration of tryptophane in the medium

EXP.	MEDIUM	STAPHYLOCOCCUS AUREUS					
		Resistant		Susceptible		B-523C Highly susceptible	
		Growth	Arylamine formed	Growth	Arylamine formed	Growth	Arylamine formed
		a	b	c	d	e	f
1	Basal medium alone*	44	2	39	0	0	0
2	+ tryptophane 1×10^{-4} M	63	24	42	2	35	2
3	+ tryptophane 25×10^{-4} M	66	75	39	38	39	50
4	+ tryptophane 100×10^{-4} M	66	110	31	80	50	110
5	Basal medium + Glucose	84	72	90	30	65	60
6	+ tryptophane 1×10^{-4} M	69	127	93	40	70	52
7	+ tryptophane 25×10^{-4} M	63	125	69	50	90	55
8	+ tryptophane 100×10^{-4} M	66	170	69	88	85	85

* Basal medium consisted of 1% of casein hydrolyzate and the other ingredients indicated in the footnote to table 1.

TABLE 4

Effect of carbohydrates on arylamine formation

	CARBOHYDRATE (0.5 PER CENT)	STAPHYLOCOCCUS AUREUS					
		Resistant strain			B-523 strain		
		Growth	Arylamine	Arylamine / Growth	Growth	Arylamine	Arylamine / Growth
1	No carbohydrate	51	20	0.39	39	0	0
2	Glucose	120	160	1.33	81	57	0.70
3	*d*-Fructose	114	95	0.83	132	26	0.20
4	Sucrose	127	88	0.69	129	26	0.20
5	*d*-Mannitol	111	75	0.68	144	27	0.19
6	Dulcitol	40	26	0.65	42	0	0
7	Trehalose	127	86	0.68	129	34	0.26
8	*d*-Xylose	51	28	0.55	60	0	0
9	Cellibiose	54	30	0.56	31	0	0
10	Raffinose	60	35	0.58	21	0	0
11	*d*-Mannose	138	52	0.38	144	27	0.19
12	*d*-Sorbitol	51	17	0.33	36	28	0.78
13	Inositol	54	21	0.39	42	2	0.05
14	Rhamnose	48	22	0.46	37	0	0
15	*l*-Arabinose	45	17	0.38	63	0	0
16	*d*-Galactose	144	50	0.38	138	0	0
17	Salicin	48	15	0.31	27	0	0
18	Pyruvate	31	32	1.03	15	0	0
19	Pyruvate	100	140	1.40	30	0	0
20	Pyruvate	60	100	1.66			

The formation of arylamine in carbohydrate-free medium (casein hydrolyzate) in the absence and presence of the following substances showed no change: lactic, succinic, pimelic, citric, and tartaric acids; glycerin and β-glycerophosphate. Neither the growth nor the arylamine formation were influenced by these substances.

tion of the largest amount of arylamine. Pyruvate appears to be the essential link in the metabolism of carbohydrate responsible for the arylamine formation. Judged by the *arylamine:growth* ratio, the effect of other carbohydrates on arylamine formation is greater with *d*-fructose than with sucrose, *d*-mannitol, and trehalose. *d*-Galactose shows no effect whatsoever. This is significant because all these carbohydrates support the growth of staphylococcus to an equal or greater degree than glucose or pyruvate.

Glucose is the most effective carbohydrate for the formation of arylamine by the B-523 strain. In contrast, pyruvate is completely ineffective. Apparently some other carbohydrate intermediate is involved. Most of the other carbohydrates are practically ineffective despite the fact that the growth in the pres-

TABLE 5

Acquisition of the property of increased arylamine formation independent of training of Staphylococcus aureus with sulfonamides

EXPERIMENT ON	STAPHYLOCOCCUS AUREUS RESISTANT STRAIN	BASAL MEDIUM ALONE			
		Tryptophane added			
		None		1×10^{-4} M	
		Growth	Arylamine formed	Growth	Arylamine formed
	Experiments with				
Jan. 25, 1944	(a) 25th daily subculture, no vitamins	35	2	42	12
	(b) + vitamins*	35	8	60	11
Apr. 2, 1944	(a) 93rd daily subculture, no vitamins	45	4	55	15
	(b) + vitamins*			72	29
Apr. 4, 1944	(a) 95th daily subculture	76	18	76	70
				73	47
Apr. 12, 1944	(a) 103rd daily subculture, no vitamins			68	105
	(b) + vitamins*				

* Vitamins: 0.02 μg biotin + 1 μg B_2 + 1 μg B_6 + 0.004 μg folic acid (*Lactobacillus casei* factor) + 1 μg pantothenate per ml of medium.

ence of these carbohydrates is from 60 to 77 per cent greater than in the presence of glucose.

(*c*) *Increased Arylamine Formation without Training with Sulfonamides.* In the presence of a minimal amount of tryptophane (1×10^{-4} M) the amount of arylamine formed by the sulfonamide-resistant strain of *S. aureus* is undoubtedly greater than that formed by the parent sulfonamide-susceptible strain. However, the strain B-523, which has not received any course of training for resistance to sulfonamides, is also capable of producing a large amount of arylamine. It is therefore reasonable to assume that the increased arylamine formation by the resistant strain is unassociated with the phenomenon of acquired resistance to sulfonamides. This is borne out further by the results presented in table 3 and particularly by those in table 5. It will be seen (table 5) that the resistant

strain, which did not produce an appreciable amount of arylamine (in the absence of glucose) in a medium containing 1×10^{-4} M of tryptophane, produced large quantities of arylamine after the ninety-fifth daily subculture on extract agar in the absence of sulfonamides. These acquired characteristics of the organism are markedly susceptible to the combined action of vitamins, because a greater amount of arylamine is formed in their presence than in their absence. This is significant because along with the acquisition of these characteristics the resistant strain lost nearly completely its resistance to sulfonamides in the presence of glucose.

CONCLUSION AND SUMMARY

The arylamine found in the culture fluids of *Staphylococcus aureus* is derived from tryptophane. It may perhaps consist of a mixture of *ortho*-aminobenzoic acid, kynurenine, and other similar oxidation products of tryptophane observed in mammalian (Jackson and Jackson, 1932) and bacterial systems (Kotake, 1933). The chemical characterization of these products is, however, in progress. Any degree of growth of the resistant *S. aureus* can take place without the formation of arylamine. The susceptible strain can also be made to produce large amounts of arylamine without a corresponding change in resistance to sulfonamides. The development of resistance and increased arylamine formation are therefore unassociated processes. On the other hand, a possible link between the development of resistance and increased arylamine formation may arise from the interference of sulfonamides with tryptophane metabolism. The oxidation of tryptophane to arylamine thereby assumes increased proportions. As stated above, the susceptible strains are capable of producing larger amounts of arylamine when the concentration of tryptophane added to the medium is increased (table 3). Conversely, it may be reasoned that the increased arylamine formation, even in the absence of added tryptophane, by the resistant strain is associated with increased tryptophane synthesis. This process may be looked upon as a physiological response to an emergency situation in overcoming the inhibition by sulfonamides. These possibilities are being tested experimentally.

REFERENCES

BLANCHARD, K. C. 1941 The isolation of *p*-aminobenzoic acid from yeast. J. Biol. Chem., **140**, 919–926.

BRATTON, A. C., AND MARSHALL, E. K., JR. 1939 A new coupling component for sulfanilamide determination. J. Biol. Chem., **128**, 537–550.

FILDES, P. 1940 A rational approach to research in chemotherapy. Lancet, **I**, 955–957.

JACKSON, R. W., AND JACKSON, W. T. 1932 The metabolism of tryptophane. III. The availability of kynurenine in supplementing a diet deficient in tryptophane. J. Biol. Chem., **96**, 697–701.

KOTAKE, Y. 1933 Studien über den intermediaren Stoffwechsel des Tryptophans. Z. physiol. Chem., **214**, 1–13.

LANDY, M., LARKUM, N. W., AND OSTWALD, E. J. 1943 Bacterial synthesis of *p*-aminobenzoic acid. Proc. Soc. Exptl. Biol. Med., **52**, 338–341.

LANDY, M., LARKUM, N. W., OSTWALD, E. J., AND STREIGHTOFF, F. 1943 Increased synthesis of *p*-aminobenzoic acid associated with the development of sulfonamide resistance in *Staphylococcus aureus*. Science, **97**, 265–267.

RUBBO, S. D., AND GILLESPIE, J. M. 1940 *p*-Aminobenzoic acid as a bacterial growth factor. Nature, **146**, 838–839.

SEVAG, M. G., AND GREEN, M. N. 1944a Indispensability of tryptophane, and glucose or pyruvate for the formation of arylamines (*para*-aminobenzoic acid (*p*-a.b.a.)?) by *Staphylococcus aureus*. J. Bact., **47**, 450.

SEVAG, M. G., AND GREEN, M. N. 1944b Lack of correlation between the formation of arylamines (*p*-a.b.a.?) and resistance of staphylococci to sulfonamides. J. Bact., **47**, 451.

SEVAG, M. G., AND GREEN, M. N. 1944c The metabolism of tryptophane by *Staphylococcus aureus* as a critical factor in relation to the mode of action of sulfonamides. Am. J. Med. Sci., **207**, 686.

SEVAG, M. G., AND GREEN, M. N. 1944d The mechanism of resistance to sulfonamides. III. Pantothenic acid and tryptophane metabolism: The role of pantothenic acid in the synthesis of tryptophane by *Staphylococcus aureus* and the effect of vitamins on tryptophane in exercising antagonism to sulfonamides. J. Bact., **48**, 631–638.

STRAUSS, E., DINGLE, J. H., AND FINLAND, M. 1941 Studies on the mechanism of sulfonamide bacteriostasis, inhibition and resistance. Experiments with *Staphylococcus aureus*. J. Immunol., **42**, 331–342.

WOODS, D. D. 1940 The relation of *p*-aminobenzoic acid to the mechanism of the action of sulfanilamide. Brit. J. Exptl. Path., **21**, 74–90.