

# THE SUSCEPTIBILITY OF PENICILLINASE-PRODUCING BACTERIA TO PENICILLIN

## II. THE EFFECT OF SODIUM AZIDE

CATHERINE C. DIETZ AND AMEDEO BONDI, JR.<sup>1</sup>

*Department of Bacteriology and Immunology, Temple University Medical School,  
Philadelphia, Pennsylvania*

Received for publication March 11, 1948

In a previous publication Bondi and Dietz (1947) reported that various factors could influence the susceptibility of penicillinase-producing bacteria to penicillin. The authors concluded that individual cells of certain organisms that produce this enzyme may be just as susceptible to penicillin as those of organisms that do not produce penicillinase. In a continuation of this study, the effect of sodium azide on the susceptibility of penicillinase-producing organisms has been studied to determine further the mechanisms that influence their susceptibility to this antibiotic.

Woodruff and Foster (1945) studied the effect of various agents on penicillinase activity and found ferrous chloride, iodoacetic acid, and sodium azide to have an inhibitory effect. Two groups of workers, Benedict, Schmidt, and Coghil (1945) and Henry and Housewright (1947), were unable to show an effect by sodium azide on this enzyme. Treffers (1946), in studying the potentiation of penicillin by various agents, concluded that sodium azide, iodoacetic acid, and many other compounds could enhance the action of penicillin without involving the inhibition of penicillinase.

The sodium azide used in this study was prepared from a 1 per cent stock solution in sterile distilled water. This solution was allowed to remain at room temperature overnight and added aseptically to 1 per cent tryptone broth to give the desired concentration. Stock solutions of crystalline potassium penicillin G in sterile saline, 2,000 Oxford units per ml, were kept frozen until used. Fresh penicillin solutions, in the concentrations desired, were prepared from the stock solutions regularly.

The cultures of staphylococci studied were taken either from a stock culture collection or isolated from routine hospital specimens. *Staphylococcus* X-3 "resistant" was obtained by isolation of a resistant variant from the parent culture by passage through broth containing increasing concentrations of penicillin. The latter organism did not gain the ability to produce penicillinase.

Preliminary experiments were run to determine the highest concentration of sodium azide that would not appreciably affect the growth of the organism tested. This was accomplished by adding different concentrations of the chemical to 1 per cent tryptone broth and inoculating sets of such broths with cultures of staphylococci. From the results, summarized in table 1, 0.02 per cent sodium

<sup>1</sup> Present address: Hahnemann Medical College, Philadelphia 2, Pennsylvania.

azide was chosen as the highest concentration that could safely be used without killing the organisms, although some inhibition of growth by this concentration was observed.

In the first series of experiments, the penicillin susceptibilities of 6 strains of staphylococci (3 penicillinase and 3 non-penicillinase-producers) in the presence and absence of sodium azide were determined, as summarized in table 2. This was done by adding different concentrations of penicillin to tubes of 1 per cent tryptone broth with and without sodium azide in final concentrations of 0.02 per cent, 0.01 per cent, and 0.005 per cent. All tubes were inoculated with a drop of

TABLE 1  
*Inhibition of growth of staphylococci by sodium azide*

STAPHYLOCOCCUS	DILUTION OF INOCULUM	GROWTH INHIBITION BY VARYING CONCENTRATIONS OF SODIUM AZIDE					
		None	0.005%	0.01%	0.02%	0.04%	0.08%
H	Undiluted 1/100	4*	4	3	3	2	—
		4	4	3	3	2	—
X-3 "Susceptible"	Undiluted 1/100	4	4	4	4	3	—
		4	3	3	3	—	—
X-3 "Resistant"	Undiluted 1/100	4	3	3	3	—	—
		4	3	2	1	—	—
No. 446†	Undiluted 1/100	4	3	3	3	3	—
		4	3	3	2	1	—
No. 7815†	Undiluted 1/100	4	4	4	4	3	—
		4	4	4	4	2	—
No. 1752†	Undiluted 1/100	4	3	3	3	2	—
		4	3	3	3	1	—

\* 4 = no inhibition of growth; 3, 2, and 1 = varying degrees of inhibition of growth; — = complete inhibition of growth.

† Produce penicillinase.

a 24-hour broth culture of the organism being studied or a 1:100 dilution of that culture. An effect was observed with those staphylococci capable of producing penicillinase that was not seen with those that did not. The former were rendered approximately 15 times more susceptible to penicillin in the presence of 0.02 per cent sodium azide when large inocula were used. The results with staphylococci, incapable of producing this enzyme, are strikingly different. The latter organisms, if affected at all, became at most four times as susceptible as the controls. The resistant variant of *Staphylococcus* X-3 behaved as the rest of the non-penicillinase-producers, despite its resistance to penicillin. The potentiation of penicillin on penicillinase-producing organisms is reduced when the inoculum is small and the concentration of sodium azide is decreased.

From the foregoing experiment, it is concluded that sodium azide had an effect on penicillinase-producing staphylococci over and above that which may be exerted against non-penicillinase-producers. This seemed to indicate some interference with the enzyme by this chemical.

TABLE 2  
*Effect of sodium azide on penicillin sensitivity of staphylococci*

PENICILLINASE PRODUCERS				NON-PENICILLINASE-PRODUCERS			
<i>Staphylococcus</i>	Conc. of sodium azide %	Dilution of inoculum	Inhibiting concentration of penicillin u/ml	<i>Staphylococcus</i>	Conc. of sodium azide %	Dilution of inoculum	Inhibiting concentration of penicillin u/ml
No. 1752	0.02	Undil. 10 <sup>-2</sup>	6.4	H	0.02	Undil. 10 <sup>-2</sup>	0.025
			0.4				0.013
	0.01	Undil. 10 <sup>-2</sup>	102.0 0.4				0.01
No. 7990	0.02	Undil. 10 <sup>-2</sup>	6.4	X-3 "Susceptible"	0.02	Undil. 10 <sup>-2</sup>	0.1
			0.4				X
	0.01	Undil. 10 <sup>-2</sup>	25.6 0.4				0.01
No. 7768	0.02	Undil. 10 <sup>-2</sup>	6.4	X-3 "Resistant"	0.02	Undil. 10 <sup>-2</sup>	12.8
			0.4				X
	0.01	Undil. 10 <sup>-2</sup>	12.8 0.4				0.01
No. 7990	None	Undil. 10 <sup>-2</sup>	102.0	None	None	Undil. 10 <sup>-2</sup>	0.05
			0.8				0.025
	0.01	Undil. 10 <sup>-2</sup>	>102.0 0.8				0.01
No. 7768	None	Undil. 10 <sup>-2</sup>	102.0	None	None	Undil. 10 <sup>-2</sup>	51.2
			0.4				12.8
	0.01	Undil. 10 <sup>-2</sup>	102.0 0.4				0.01

X = no growth in sodium azide control.

To study the mechanism of action of sodium azide, experiments were run to correlate the rate of growth of penicillinase-producing organisms with the rate of destruction of penicillin. To flasks containing 50 ml of 1 per cent tryptone broth were added penicillin to give a final concentration of 0.75 units per ml, and sodium azide to give the concentrations required. All flasks were then inoculated with 0.5 ml of a 24-hour broth culture. Samples were removed at intervals of 0, 1, 2, 4, and 8 hours and heated at 60 C for 30 minutes to stop growth of the organism and to inactivate the penicillinase that might be present. Penicillin assays were

run by the Oxford cup technique, and the percentage of destruction was calculated. Growth was simultaneously determined by measuring optical densities

TABLE 3  
*Relationship of growth to penicillin destruction in the presence of sodium azide*

CONCENTRATION OF NaN <sub>3</sub>	CONCENTRATION OF PENICILLIN u/ml	TIME INTERVAL									
		0 hr		1 hr		2 hr		4 hr		8 hr	
		Pen. des. %	Opt. den.	Pen. des. %	Opt. den.	Pen. des. %	Opt. den.	Pen. des. %	Opt. den.	Pen. des. %	Opt. den.
<i>Staphylococcus 7768</i>											
None	None		0		6		10		52		129
None	0.75	0	4	49	4	100	5	100	9	100	111
0.02	0.75	0	2	0	1	75	1	99	4	100	5
0.01	0.75	0	2	0	5	100	6	100	5	100	54
0.005	0.75	0	2	49	0	100	0	100	9	100	75
<i>Staphylococcus 1752</i>											
None	None		0		7		14		64		143
None	0.75	0	5	47	5	94	5	100	11	100	105
0.02	0.75	0	2	36	1	67	1	100	0	100	3
0.01	0.75	0	3	36	3	76	2	100	3	100	17
0.005	0.75	0	0	47	0	86	0	100	0	100	52

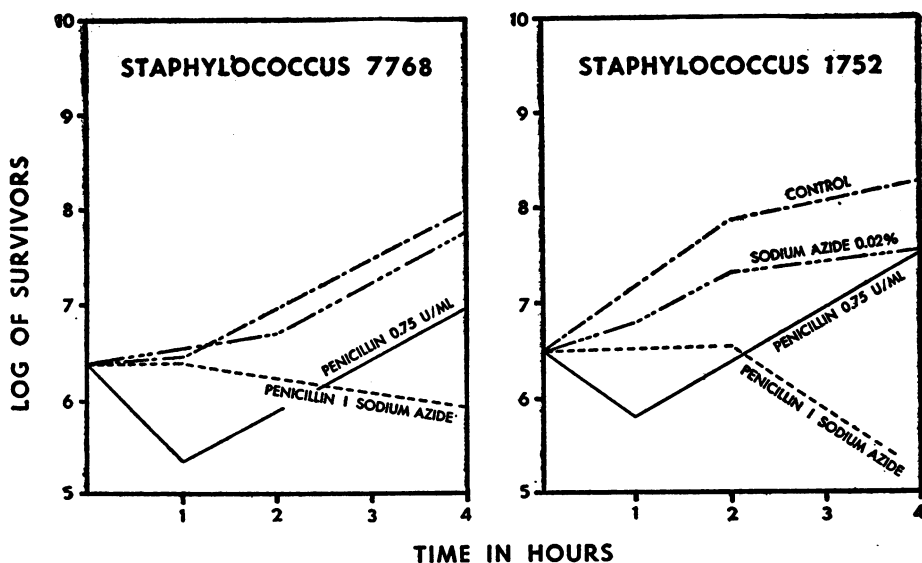


FIG. 1. BACTERICIDAL EFFECTS OF PENICILLIN AND SODIUM AZIDE COMBINED

on a Klett-Summerson photometer. Table 3 summarizes the results obtained with two strains of staphylococci. Similar results were obtained with two other

strains of staphylococci. It was noted that the penicillin in flasks containing no sodium azide, or small concentrations of the chemical, was destroyed more rapidly than the penicillin in flasks containing higher concentrations of sodium azide. Growth occurred after penicillin destruction, and the rate of growth varied with the speed with which penicillin was destroyed. It was also observed that penicillin destruction occurred in the absence of detectable growth. The concentration of penicillin used, 0.75 units per ml, was chosen because it afforded an experiment that could be concluded in one day. The results of a similar experiment, using higher concentrations of the antibiotic, were essentially the same except that the period of time for penicillin destruction and hence for growth to begin is prolonged.

Since turbidimetric readings give evidence only of bacteriostatic action, bacterial counts were made to study the bactericidal activity of penicillin-azide combinations. To flasks of 1 per cent tryptone broth in volumes of 50 ml containing either penicillin, sodium azide, or both, were added 0.5 ml of an undiluted

TABLE 4  
*Effect of NaN<sub>3</sub> on sterile penicillinase of Staphylococcus 7990*

CONCENTRATION OF PENICILLIN u/ml	CONCENTRATION OF NaN <sub>3</sub> %	PENICILLIN DESTROYED, PERCENTAGE				
		½ hr	1 hr	2 hr	3 hr	4 hr
0.75	0	36	61	73	93	100
0.75	0.02	20	58	86	95	100
0.75	0.01	20	63	76	86	100
0.75	0.005	20	60	67	86	100

24-hour broth culture. Bacterial counts were made at the end of 0, 1, 2, 4, and 6 hours on extract agar. These counts, shown in figure 1, demonstrate that sodium azide alone did not appreciably affect growth. Penicillin alone surprisingly caused a drop in count in the first hour of the experiment, but later the number of viable organisms followed a pattern resembling that of the control. A combination of the two agents was followed by an appreciable drop in the total count that persisted for 24 hours.

From the results of these experiments, it is apparent that the destruction of penicillin is delayed by the presence of sodium azide. To determine whether this delay is due to direct interference with penicillin activity or a slowing up of the production of the enzyme the following experiments were run: Sterile penicillinase was prepared by the method of Harper (1943) from *Staphylococcus 7990* and stored in sterile tubes. One-half ml of this material was added to flasks of 1 per cent tryptone broth, containing 0.75 units per ml of penicillin and varying concentrations of sodium azide. Penicillin assays were run at ½, 1, 2, 3, and 4 hours, and the penicillin destroyed was calculated.

From table 4 it is evident that the rate of destruction was the same in flasks containing azide as in the control flasks. Similar results were obtained using penicillinase prepared from *Staphylococcus 1752*. These results indicate that

sodium azide does not interfere with the rate of destruction of penicillin by pre-formed enzyme. Therefore the action is probably due to a slowing down or interference with the production of penicillinase.

#### DISCUSSION

There may be some synergistic or additive effect of combinations of sodium azide and penicillin on all staphylococci studied. Over and above this effect, sodium azide renders penicillinase-producing organisms more susceptible to penicillin. That the action of sodium azide is related to penicillinase is indicated by its greater action on penicillinase producers than on the non-penicillinase-producers that are more susceptible to penicillin and equally sensitive to sodium azide. It is significant to note that *Staphylococcus* X-3, which *in vitro* was made resistant to penicillin but which did not gain the ability to produce penicillinase, was not rendered sensitive in the presence of sodium azide.

Two possible mechanisms for the action of sodium azide seemed apparent at the beginning of this study. Either the action of penicillinase was inhibited or the production of the enzyme by a growing culture was suppressed. The results obtained indicate that penicillin destruction by sterile preparations of the enzyme proceeds at the same rate regardless of the presence of sodium azide in the concentration used. This indicates that the potentiating action of this agent is probably due to a suppression of the production of penicillinase by growing cultures rather than to an inhibition of the enzyme. Although sodium azide is much too toxic to be safely used *in vivo*, it is possible that a compound exerting comparable activity but lower toxicity could be used effectively in conjunction with penicillin to treat infections due to penicillinase-producing staphylococci.

#### SUMMARY

Sodium azide renders penicillinase-producing staphylococci more susceptible to penicillin. The action of this agent is not due to an interference in the activity of penicillinase as indicated by its failure to act in this fashion on sterile preparations of the enzyme. Penicillinase production by a growing culture of *Staphylococcus* is suppressed by sodium azide.

#### REFERENCES

- BENEDICT, R. G., SCHMIDT, W. H., AND COGHILL, R. D. 1945 Penicillin. VII. Penicillinase. *Arch. Biochem.*, **8**, 377-384.
- BONDI, A., AND DIETZ, C. C. 1947 Susceptibility of penicillinase-producing bacteria to penicillin. I. Factors influencing susceptibility, *J. Bact.*, **55**, 843-847.
- HARPER, G. J. 1943 Inhibition of penicillin in routine culture media. *Lancet*, **245**, 569-571.
- HENRY, R. J., AND HOUSEWRIGHT, R. D. 1947 Studies on penicillinase. II. Manometric method of assaying penicillinase and penicillin, kinetics of the penicillin-penicillinase reaction, and the effects of inhibitors on penicillinase. *J. Biol. Chem.*, **167**, 559-571.
- TREFFERS, H. P. 1946 Studies on resistance to antibiotics. I. The action of penicillin on some gram-positive and gram-negative organisms and its potentiation by various inhibitors. *Yale J. Biol. Med.*, **18**, 609-623.
- WOODRUFF, H. B., AND FOSTER, J. W. 1945 Microbiological aspects of penicillin. VII. Bacterial penicillinase. *J. Bact.*, **49**, 7-17.