STUDIES OF THE EFFECT OF SODIUM AZIDE ON MICROBIC GROWTH AND RESPIRATION

I. The Action of Sodium Azide on Microbic Growth

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In recent years, publications from this laboratory (Snyder and Lichstein, 1940; Lichstein and Snyder, 1941; Lichstein, 1941) have indicated the value of sodium azide (NaN_3) as an aid in the isolation of streptococci from clinical material containing "spreaders" and other interfering gram-negative organisms. The use of blood-infusion agar containing 0.02 per cent sodium azide for the cultivation of fecal samples holds in abeyance the rich flora of gram-negative bacilli, thus making possible the isolation of streptococci.

The present paper is concerned with a comprehensive study of the action of sodium azide on pure cultures of selected strains of bacteria directed primarily at an understanding of the mechanism of action of the chemical.

THE GROWTH OF SELECTED STRAINS OF BACTERIA ON MEDIA CONTAINING VARIOUS CONCENTRATIONS OF SODIUM AZIDE

In order to ascertain the influence of the chemical on microbic growth, 92 strains of bacteria were selected. These were largely from the stock culture collection of the Hygienic Laboratory. The basic medium for the cultural studies was beef-infusion broth (pH 7.4). Infusion agar was prepared by adding 2 per cent powdered agar to the infusion broth and autoclaving at 121°C. for 20 minutes. Wherever blood was indicated for enrichment or differentiation, 5–10 per cent sterile defibrinated rabbit blood was added with aseptic technic to the infusion agar. The calculated amounts were added to infusion agar and broth, and the solutions sterilized in the autoclave. For the media enriched with blood, the effect of dilution was taken into consideration when the original amount of sodium azide was added. When the blood was incorporated into the medium containing sodium azide there was a darkening due to chemical action, the extent of which depended on the concentration of the chemical.

The amounts of sodium azide generally employed were 0.01, 0.02, and 0.03 per cent, corresponding to M/650, M/325, and M/217 respectively. The sterile media were poured into Petri dishes and allowed to solidify, and subsequently inoculated by streaking the surface with a loopful of an 18–24 hour broth culture of the organism to be tested. Infusion broth containing similar amounts of the chemical was also inoculated.

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All plates and tubes were incubated at 37°C. The strains of meningococci and gonococci were cultured in an atmosphere of 10 per cent carbon dioxide to attain optimum conditions for growth. The results with pure cultures of gram-negative bacteria are presented in tables 1 and 2.

An examination of the data given in these tables indicates that sodium azide exerted a marked bacteriostatic action against gram-negative bacteria as a group. Of the 41 strains tested on infusion agar containing sodium azide, only 24 grew at a concentration of 0.01 per cent, 6 grew at a concentration of 0.02 per cent, while all but *Pseudomonas aeruginosa* failed to grow in the presence of

TABLE 1

Growth of gram-negative bacteria on infusion agar containing various concentrations of sodium azide, 48 hours, 37°C.

ORGANISMS	NUMBER OF STRAINS TESTED	CONCENTRATIONS OF NaN_3 (PER CENT)							
		0.01		0.02		0.03		0.00	
		Number of strains giving growth	Amount of growth	Number of strains giving growth	Amount of growth	Number of strains giving growth	Amount of growth	Number of strains giving growth	Amount of growth
E. coli	3	2	+	0	−	0	−	3	+++
E. typhosa	5	2	++	1	+	0	−	5	+++
A. aerogenes	2	0	−	0	−	0	−	2	+++
P. vulgaris	7	7	+ (NS)	0	−	0	−	7	+++ (S)
S. dysenteriae	3	2	+	0	−	0	−	3	+++
Salmonella	8	5	++	1	+	0	−	8	+++
P. aeruginosa	3	3	+++	3	++	3	+	3	+++
P. fluorescens	1	1	++	1	++	0	−	1	+++
C. violaceum	2	0	−	0	−	0	−	2	+++
*H. ducreyi**	1	1	+	0	−	0	−	1	+++
Flavobacterium	2	0	−	0	−	0	−	2	+++
Gonococcus*	2	1	+	0	−	0	−	2	+++
Meningococcus*	2	0	−	0	−	0	−	2	+++

+, poor growth; ++, good growth; +++, control growth; −, no growth.
(S) spreading. (NS) no spreading.
* Blood agar base used.

0.03 per cent. On several occasions, strains of *P. aeruginosa* freshly isolated from clinical material grew as well on media containing 0.03 per cent sodium azide as on plain infusion agar. The results with broth containing sodium azide were essentially the same, except that *P. aeruginosa* and *Pseudomonas fluorescens*, which were more resistant to the chemical in infusion agar than the other gram-negative organisms, were very susceptible under these conditions.

Fifty-one strains of gram-positive bacteria were similarly tested on the solid media. Blood was added to the agar for the streptococci, pneumococci, and *Corynebacterium diphtheriae*.

It seemed desirable to include several strains of gram-positive obligative anaerobes. In order to obtain the necessary anaerobiosis the method of Buchner (1888) was employed: after inoculation of the slant the cotton plug was flamed and pushed into the tube for about one inch with the aid of sterile forceps. Powdered pyrogallic acid was then placed on the plug, and a few drops of 20 per cent sodium hydroxide added. A rubber stopper was then quickly fitted, and the tube inverted. This method had the advantage of allowing easy observation of the growth from day to day. The data are presented in table 3.

The data given in table 3 show that sodium azide in the concentrations employed had little or no effect on the aerobic growth of streptococci, pneumococci or lactobacilli. The anaerobes were likewise not inhibited. The aerobic sporulating rods, and *Staphylococcus citreus* were very susceptible to the chemical, while *Staphylococcus aureus* and *Staphylococcus albus* were moderately resistant. The hemolysis produced by *Streptococcus viridans* and the pneumococcus was

TABLE 2

Growth of gram-negative bacteria in broth containing various concentrations of sodium azide, 48 hours, 37°C.

ORGANISMS	CONCENTRATIONS OF SODIUM AZIDE (PER CENT)				
	0.01	0.02	0.03	0.05	0.00
P. vulgaris (7)	++	+	−	−	+++
S. dysenteriae (3)	+	+	+	−	+++
E. typhosa (5)	+	+	−	−	+++
E. coli (3)	++	+	+	−	+++
A. aerogenes (2)	+	−	−	−	+++
P. aeruginosa (3)	++	−	−	−	+++
P. fluorescens (1)	+	−	−	−	+++

+, poor growth; ++, good growth; +++, control growth; −, no growth.
( ) number of strains tested.

characterized by a large brownish zone around the colony, as contrasted to the usual small area of greening around the colonies in the absence of the chemical.

The resistance of the streptococci to the concentrations of sodium azide employed was investigated further, particularly in view of the finding presented by Sevag and Shelburne (1942) that the aerobic respiration of certain strains of these organisms are highly susceptible to sodium azide. Eight strains were selected for the study, and broth containing 0.2 per cent glucose was used as the basic medium. To this were added concentrations of sodium azide ranging from 0.01–0.25 per cent. The data obtained, which are presented in table 4, indicate more clearly the marked resistance of some members of this group.

A consideration of the results as presented in tables 1 and 3 shows rather clearly that sodium azide cannot be looked upon as a strictly gram-negative inhibitor although its action against germs having this tinctorial reaction is more striking than with those that are gram-positive. On the other hand, one should not fail to emphasize the susceptibility of several very important gram-positive bacilli such as *Bacillus anthracis* and *Bacillus subtilis* to the chemical.

A conspicuous lack of correlation between the reaction to the gram technic and resistance to sodium azide is noted when a comparison is made of *B. subtilis*

TABLE 3

Growth of gram-positive bacteria on infusion agar containing various concentrations of sodium azide, 48 hours, 37°C.

ORGANISMS	NUMBER OF STRAINS TESTED	CONCENTRATIONS OF NaN_3 (PER CENT)							
		0.01		0.02		0.03		0.00 (control)	
		Number of strains giving growth	Amount of growth	Number of strains giving growth	Amount of growth	Number of strains giving growth	Amount of growth	Number of strains giving growth	Amount of growth
S. hemolyticus	18	16	+++	16	+++	15	+++	18	+++
		2	++	2	++	3	++		
S. viridans	7	7	+++	7	+++	7	+++	7	+++
S. anhemolyticus	3	3	+++	3	+++	3	+++	3	+++
Pneumococcus	3	3	+++	3	+++	3	+++	3	+++
S. aureus	3	3	++	3	++	3	+	3	+++
S. albus	2	2	++	2	+	2	+	2	+++
S. citreus	1	0	−	0	−	0	−	1	+++
C. diphtheriae	2	2	+++	2	++	2	+	2	+++
B. anthracis	1	0	−	0	−	0	−	1	+++
B. subtilis	2	0	−	0	−	0	−	2	+++
B. mesentericus	1	1	++	0	−	0	−	1	+++
B. megatherium	1	0	−	0	−	0	−	1	+++
Lactobacillus	2	2	+++	2	+++	2	++	2	+++
C. welchii	1	1	+++	1	+++	1	+++	1	+++
C. novyi	1	1	+++	1	++	1	++	1	+++
C. sporogenes	1	1	+++	1	+++	1	+++	1	+++
C. tetani	1	1	+++	1	+++	1	+++	1	+++
C. botulinum	1	1	+++	1	+++	1	+++	1	+++

+, poor growth; ++, good growth; +++, control growth; −, no growth.

TABLE 4

Growth of streptococci in 0.2 per cent glucose broth containing various concentrations of sodium azide, 72 hours, 37°C.

STRAIN	CONCENTRATION OF SODIUM AZIDE (PER CENT)					
	0.05	0.10	0.15	0.20	0.25	0.00
C3	+++	++	++	−	−	+++
C1	+++	+++	+++	+++	+++	+++
NY5	++	++	++	−	−	+++
K64	+++	++	++	++	++	+++
O90	++	−	−	−	−	+++
V9	++	−	−	−	−	+++
SF120	+++	+	−	−	−	+++
G7	+++	++	++	++	++	+++

and *Bacillus megatherium*, gram-positive organisms with strong aerobic tendencies, and *Clostridium novyi* and *Clostridium tetani*, obligative anaerobes having

the same response to the gram-technic but distinctly resistant to sodium azide. One of the explanations advanced for the action of sodium azide is that it serves as an inhibitor of aerobic respiration catalyzed by heme-containing respiratory enzymes. On the basis of the data presented in tables 1 and 3, it seemed desirable to establish any variation of its influence on the growth of aerobic facultative anaerobic organisms in an anaerobic environment. Accordingly 12 strains of organisms having this characteristic were cultured on infusion agar containing sodium azide and incubated under anaerobic conditions. In general the results showed that those organisms which were inhibited under aerobic conditions likewise gave no growth under anaerobic conditions. A noteworthy fact is that *S. albus*, *S. aureus*, and *P. aeruginosa* were more sensitive to the chemical in the absence of free oxygen.

According to our present knowledge heme-containing respiratory enzymes are completely inactive under anaerobic conditions. We are therefore unable at present to offer any plausible explanation for the effect of sodium azide on anaerobic growth.

THE EFFECT OF THE AERATION OF BROTH CULTURES OF P. AERUGINOSA ON ITS GROWTH IN THE PRESENCE OF SODIUM AZIDE

On the basis of the results of the previous experiments, the resistance of *P. aeruginosa* to sodium azide presented certain interesting facts which may be summarized as follows:

1. The three strains tested were more resistant than the other gram-negative bacteria studied when cultured on infusion agar containing sodium azide.
2. When cultured in broth, they were very susceptible to the chemical.
3. Under anaerobic conditions sodium azide inhibited their growth.

The most obvious correlation that can be made, on the basis of the above results, is the oxygen tension available to the organisms under the conditions of the experiment. Germs growing on the surface of agar are in close contact with the oxygen of the air, whereas those growing in broth are not. Indeed, the lower portion of a broth culture may be micro-aerophylic or even anaerobic. By definition the germs growing under anaerobic conditions have no contact with free oxygen.

A second point of correlation is the pigment production of the three strains of *P. aeruginosa* employed in these studies. They all produced fluorescine, but their production of pyocyanine varied. This was best observed after chloroform extraction of broth cultures. By this method it was found that cultures of Strain S contained no pyocyanine, while Strain 1 produced a very small amount of the pigment. Cultures of Strain 2 were very rich in pyocyanine. Another important feature is that this pigment can occur in the oxidized or reduced states. It was apparent that when pyocyanine was present in infusion agar, as a result of the growth of Strain 2, it was in the oxidized form and was readily observed diffusing into the agar. When it was formed in broth cultures, it was generally present in the reduced or leuco form, but was readily oxidized to produce the characteristic blue color by simply shaking the culture tubes in order to aerate the medium. Apparently the oxygen tension in broth was lower than

that necessary to keep the pigment in the oxidized state. Since oxygen is required for the production of the pigment it was not present in cultures of *P. aeruginosa* grown under anaerobic conditions.

The observations pertaining to the differences in susceptibility of *P. aeruginosa* to sodium azide under different methods of cultivation, the variations in oxygen tension presented by these methods, and the production of pyocyanine as discussed, suggested the possibility that pyocyanine might have some relation to the resistance of *P. aeruginosa* to the chemical. The work of Friedheim (1931) showed that pyocyanine serves a respiratory function. His data indicate clearly that this pigment is able to increase the respiration of pyocyanine-free strains of *P. aeruginosa* many times. It was also shown that the respiratory action of this pigment was not specific since it increased the oxygen consumption of suspensions of other bacteria and even red blood cells.

From a theoretical point of view, pyocyanine once reduced must be reoxidized in order to satisfy the requirement of reversibility necessary for a respiratory pigment. This reoxidation is supposedly accomplished by atmospheric oxygen. With this as an assumption, the limit of activity of pyocyanine as a respiratory pigment is the availability of oxygen to reoxidize the pigment after it has been reduced by bacterial action.

Because of these observations and certain theoretical considerations an experiment was proposed to note the effect of aeration on broth cultures of *P. aeruginosa*. Two strains were used. One, the S strain, did not produce pyocyanine, and the other, Strain 2, produced a large amount of this pigment.

Ten ml. of infusion broth, with and without the addition of sodium azide, were placed in 50 ml. Erlenmeyer flasks, as well as in 20 ml. culture tubes to serve as controls. The media were inoculated with the germs and incubated at 37°C. Aeration of these flasks during the period of incubation was accomplished in different manners. The large surface area because of the size of the bottom of the flasks gave a simple technic for allowing the oxygen of the air to diffuse into the thin layer of broth. In the second instance the flasks were placed on a mechanical shaking device and the media slowly agitated, while compressed air in a stream of fine bubbles was led into the broth through alundum aeraters[3] in the third group. The results of this experiment are given in table 5.

An examination of the data presented indicates that all three methods of aeration of sodium azide broth cultures of a pyocyanine-producing strain of *P. aeruginosa* increased the growth of the organism to a marked degree. On the other hand, aeration had little or no effect on the proliferation of the strain which produced no pyocyanine. The question arises as to the mechanism of this phenomenon, and two explanations seem to be forthcoming, (1) pyocyanine was able to serve as a respiratory pigment in aerated broth cultures and this respiration was insensitive to the action of the chemical, and (2) aeration caused an increased oxidation or actual removal of metabolic products, and in this manner the influence of the chemical was counteracted.

[3] Cylindrical alundum stones obtained from the Carborundum Co., Niagara Falls, N. Y.

In keeping with the first postulation, pyocyanine can act as a respiratory pigment as demonstrated by Friedheim. As already discussed, on a theoretical basis, the limit of activity of pyocyanine as a respiratory pigment is the availability of oxygen to reoxidize the compound after it has been reduced by bacterial action. The conditions of this experiment provided a liberal supply of air, but there was no way of proving that the pigment was actually responsible for the increased growth of Strain 2 in the presence of sodium azide. Indirectly, this is suggested, since aeration had no effect on the proliferation of Strain S, the

TABLE 5

Effect of aeration on the growth of P. aeruginosa in the presence of sodium azide in broth, 48 hours, 37°C.

TYPE OF AERATION	CONCENTRATION OF SODIUM AZIDE	GROWTH OF STRAIN S*	GROWTH OF STRAIN 2*
	per cent		
Media in culture tubes (surface volume ratio = 0.15)**	0.00	+++	+++
	0.01	++	++
	0.02	−	+
	0.03	−	−
Media in flasks (surface volume ratio = 1.6)**	0.00	+++	+++
	0.01	++	++
	0.02	−	++
	0.03	−	+
Media in flasks (aerated by mechanical shaking)	0.00	+++	+++
	0.01	++	+++
	0.02	−	+++
	0.03	−	++
Media in flasks (aerated by passage of air through media)	0.00	+++	+++
	0.01	++	+++
	0.02	+	+++
	0.03	−	++

+, poor growth; ++, good growth; +++, control growth; −, no growth.

* Strain S produces no pyocyanine.
Strain 2 produces pyocyanine.

** $\frac{\text{surface area in sq. mm.}}{\text{volume in ml.}}$

organism which did not produce any pyocyanine. Before dismissing this theory it would seem desirable to investigate the rôle of pyocyanine more closely, particularly by some more direct and sensitive method if the same were available. The results of gas metabolism studies with this end in view will be presented in a subsequent publication.

The second explanation is possibly somewhat less tangible since one would anticipate any effect resulting from increased oxidation or removal of metabolic products to act alike with both germs and the data indicate otherwise. There

were differences in the compounds elaborated as manifested by the presence of pyocyanine in the Strain 2 cultures, and it is consistent to envision others, but this possibility entails the formation of products which counteract the sodium azide. The improvement of a culture medium by aeration during the growth of an organism is not new. This fact has been demonstrated by Winslow *et al.* (1932). In their work, aeration prolonged the logarithmic phase resulting in approximately ten times more bacteria. The authors suggested as a reason for this phenomenon, that aeration caused increased oxidation of toxic waste products thus providing better conditions for growth.

The results of this experiment have given a better understanding of the differences in resistance of *P. aeruginosa* to sodium azide when cultured in broth and under strict anaerobic conditions. The possible rôle of pyocyanine, and of a liberal supply of oxygen has been indicated. However, the reason for the marked resistance of this organism when cultured on agar containing sodium azide is still obscure, since even if pyocyanine plays an important rôle, only one of the three strains produces it to an appreciable extent.

TABLE 6

Effect of washing the inocula on the growth of P. aeruginosa in the presence of sodium azide, 48 hours, 37°C.

TYPE OF INOCULUM	CONCENTRATION OF SODIUM AZIDE (PER CENT)			
	0.00	0.01	0.02	0.03
Broth culture	+++	+++	+++	+
1:5 dilution of broth culture	+++	+++	+++	+
Washed organisms from a broth culture	+++	+++	+	−
Washed organisms from an agar culture	+++	++	−	−

+, poor growth; ++, good growth; +++, control growth; −, no growth.

THE EFFECT OF WASHING THE INOCULA WITH BUFFER SOLUTIONS ON THE SUBSEQUENT GROWTH OF P. AERUGINOSA IN THE PRESENCE OF SODIUM AZIDE

The resistance of *P. aeruginosa* to sodium azide under certain conditions of cultivation has been described, and some attempt to elucidate this phenomenon presented. However, the previous experiment gave no satisfactory explanation for the marked resistance of this species to the presence of the chemical in agar. In view of this fact, further work was planned in an attempt to clarify this problem. *P. aeruginosa* is able to produce metabolic products which are active against other microorganisms and their products. Upon the assumption that some metabolic product of the germ is able to neutralize the action of sodium azide, washed inocula were employed in this experiment in order to remove such a substance. Germ suspensions of Strain S and Strain 2 were prepared by three successive washings in phosphate buffer solution (pH 7.4).

Eight series of Petri dishes containing plain infusion agar, 0.01, 0.02, and 0.03 per cent sodium azide infusion agar were prepared in the usual manner. Two

series were streaked with material from 24-hour broth cultures of *P. aeruginosa* while two others were streaked with 1:5 dilutions of the same cultures. The remaining four series of plates were inoculated with the washed suspensions of the two strains described above. All Petri dishes were incubated at 37°C., and the results of the experiment are outlined in table 6.

Examination of these data indicates that washing of the inocula resulted in decreased growth of *P. aeruginosa* in the presence of sodium azide. This was not due to dilution of the germ suspension during washing as is evident from the results obtained with the 1:5 dilution of the broth cultures. The experiments were repeated on several occasions with essentially the same results. The results suggest that washing removed some metabolic product of the germs which may have been associated with the resistance of the organisms to the chemical.

SUMMARY AND CONCLUSIONS

1. Sodium azide in concentrations of 0.01 to 0.03 per cent in infusion agar was bacteriostatic for gram-negative bacteria. Of the 41 strains tested, only 3 strains of *Pseudomonas aeruginosa* grew in the presence of 0.03 per cent of the chemical.

2. Streptococci, pneumococci, anaerobes, and lactobacilli were resistant to 0.03 per cent sodium azide. However, the gram-positive spore-forming aerobes, and *Staphylococcus citreus* were very sensitive, while *Staphylococcus albus*, *Staphylococcus aureus*, and *Corynebacterium diphtheriae* were moderately resistant.

3. Results using broth containing sodium azide were essentially the same as those obtained with solid media. An exception was *P. aeruginosa* which was far more sensitive in sodium azide broth.

4. Eight strains of streptococci tested in glucose infusion broth containing sodium azide, proliferated at a concentration of 0.05 per cent; 5 grew at 0.15 per cent; and three grew well in broth containing 0.25 per cent sodium azide.

5. Twelve strains of facultative anaerobes were cultured under anaerobic conditions on infusion agar containing 0.01 to 0.03 per cent sodium azide. The results were essentially the same as those obtained under aerobic conditions, with the exception that *S. albus*, *S. aureus*, and *P. aeruginosa* were more sensitive to the chemical when cultured in the absence of free oxygen.

6. Aeration of sodium azide broth cultures of a pyocyanine-producing strain of *P. aeruginosa* increased the growth of the organism in the presence of the chemical.

7. Using washed inocula, the growth of *P. aeruginosa* in the presence of sodium azide under aerobic conditions was reduced.

REFERENCES

BUCHNER, H. 1888 Eine neue Methode zur Kultur Anaerober Mikroorganismen. Zentr. Bakt. Parasitenk. I. Orig., **4**, 149–151.

FRIEDHEIM, E. A. H. 1931 Pyocyanine, an accessory respiratory enzyme. J. Exptl. Med., **54**, 207–221.

LICHSTEIN, H. C. 1941 The use of sodium azide for the isolation of streptococci. J. Bact., **42**, 293–294.

LICHSTEIN, H. C., AND SNYDER, M. L. 1941 The inhibition of the spreading growth of *Proteus* and other bacteria to permit the isolation of associated streptococci. J. Bact., **42**, 653–664.

SEVAG, M. G., AND SHELBURNE, M. 1942 Cyanide-sensitive bacterial respiratory systems different from the usual cytochrome-cytochrome oxidase system. J. Gen. Physiol., **26**, 1–9.

SNYDER, M. L., AND LICHSTEIN, H. C. 1940 Sodium azide as an inhibiting substance for gram-negative bacteria. J. Infectious Diseases, **67**, 113–115.

WINSLOW, C.-E. A., WALKER, H. H., AND SUTERMEISTER, M. 1932 The influence of aeration and of sodium chloride upon the growth curve of bacteria in various media. J. Bact., **24**, 185–208.