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

II. The Action of Sodium Azide on Bacterial Catalase

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Although Keilin and Hartree (1934) and Blaschko (1935) have shown that sodium azide inhibits catalase derived from animal tissue, there is no information about its action on bacterial catalase. It seemed therefore desirable to make a quantitative study of the effects of sodium azide on catalase derived from this source. Since peroxides are metabolic products of many organisms, the inhibition of an enzyme which decomposes these substances might permit their accumulation in bactericidal quantities. The present paper deals with the effect of sodium azide on the catalase activity of bacteria, and an attempt is made to point out the possible significance of this action.

THE INHIBITION OF CATALASE ACTIVITY OF SUSPENSIONS OF WASHED BACTERIA BY SODIUM AZIDE

The first step was to determine the catalase activity of several bacterial species. A volumetric procedure based on the titration of residual hydrogen peroxide by potassium permanganate was found to give excellent results. Germ suspensions were prepared by placing the growths from 24-hour infusion agar slants in M/15 phosphate buffer solution (pH 7.4), centrifuging, and resuspending in buffer solution in order to minimize the effect of peroxidase. It is obvious that this procedure does not permit the determination of the quantitative amount of catalase present in each germ suspension, but is merely an indication of its ability to decompose hydrogen peroxide.

Known amounts, 10–50 ml. of hydrogen peroxide (approximately 0.2 *N*) were added to 1 ml. of germ suspension contained in large tubes, and subsequently incubated at 37°C. for 2 hours. Tests showed this period of time to be sufficient for the completion of the reaction with all of the strains of bacteria studied. Acidulation with 10 per cent sulfuric acid and titration with standard 0.1 *N* potassium permanganate followed. The catalase activity was expressed as ml. of 0.1 *N* hydrogen peroxide decomposed by 1 ml. of germ suspension. The titrations were made in duplicate and the results indicated that the method was accurate to about 0.1 ml. of 0.1 *N* hydrogen peroxide decomposed. The data obtained are presented in table 1.

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Reference to table 1 shows the relative catalase activities of 1 ml. of suspensions of 31 strains of washed bacteria. While the figures do not take into account the numbers of bacteria in the various suspensions, and are therefore not indicative of the catalase activity in terms of dry bacterial weight, they do roughly divide the species into strong, moderate, weak, and negative catalase producers.

Several species of bacteria which produced catalase, as evidenced by the results of this experiment, were selected for further study in order to determine the effect of sodium azide on the enzyme. The technic employed was as follows: germ suspensions were prepared as previously described. Nine-tenths ml. of the suspensions was used, and 0.1 ml. of 0.1 and 0.2 per cent sodium azide was added to make a final concentration of 0.01 and 0.02 per cent of the chemical,

TABLE 1

Catalase activities of suspensions of several species of washed bacteria expressed as ml. of 0.1 N hydrogen peroxide decomposed

ORGANISMS	0.1*N* H_2O_2 DECOMPOSED BY 1 ML. OF GERM SUSPENSION	ORGANISMS	0.1*N* H_2O_2 DECOMPOSED BY 1 ML. OF GERM SUSPENSION
	ml.		*ml.*
S. aureus	110.4	*B. anthracis*	4.3
S. albus	96.3	*B. subtilis*	6.3
S. citreus	98.2	*B. megatherium*	2.6
Pneumococcus I	0.0	*C. diphtheriae*	115.4
Pneumococcus II	0.0	*L. casei*	0.0
Gonococcus	3.3	*L. fulleri*	0.0
Meningococcus	6.2	*P. aeruginosa*	34.9
Streptococcus O90	0.0	*P. fluorescens*	33.5
Streptococcus G7	0.0	*P. vulgaris*	4.4
Streptococcus NY5	0.0	*S. dysenteriae*	5.0
Streptococcus C1	0.0	*E. typhosa*	1.1
Streptococcus C3	0.0	*E. coli*	7.1
Streptococcus SF120	0.0	*C. welchii*	0.0
Streptococcus Y9	0.0	*C. sporogenes*	0.0
Streptococcus K64	0.0	*C. botulinum*	0.0
		C. tetani	0.0

or M/650 and M/325. The bacterial suspensions were allowed to react with sodium azide in these concentrations for 0–48 hours in order to determine the effect of time on the reaction. At definite intervals (0, 3, 6, 12, 24, and 48 hours) 1 ml. of suspension was removed and the catalase activity determined by the method described. Controls were: (1) 0.9 ml. of germ suspension plus 0.1 ml. of buffer solution, (2) 1 ml. of buffer solution, (3) 1 ml. of 0.01 per cent sodium azide, and (4) 1 ml. of 0.02 per cent sodium azide. It was quickly ascertained that sodium azide alone had no effect on the decomposition of hydrogen peroxide, and these controls were discarded from further experiments. Although variations were encountered in day to day titrations, the results indicated a marked inhibition of catalase activity as soon as the germs came in contact with the

chemical. This effect of sodium azide is presented in table 2 which shows its action on suspensions of the strong catalase producer, *Staphylococcus citreus*. The reason for the marked reduction in the catalase activity of the control suspension after 6 hours incubation is obscure.

After the addition of the chemical to suspensions of 11 species of bacteria, 1 ml. was immediately removed and the catalase activity of these samples deter-

TABLE 2

Catalase activity of suspensions of washed cells of S. citreus in the presence of sodium azide

NUMBER OF HOURS	CATALASE ACTIVITY*		
	Control	NaN_3, 0.01 per cent	NaN_3, 0.02 per cent
0	89.3	10.9	7.7
3	89.2	10.6	6.8
6	89.2	10.2	6.7
12	66.5	7.5	3.3
24	66.4	6.3	2.7
48	64.2	3.4	2.1

* Expressed as ml. of 0.1*N* hydrogen peroxide decomposed by 0.9 ml. of germ suspension.

TABLE 3

Catalase activity of suspensions of eleven species of washed bacteria in the presence of sodium azide

ORGANISMS	CATALASE ACTIVITY*					
	Control		NaN_3, 0.01 per cent		NaN_3, 0.02 per cent	
	0 hours	24 hours	0 hours	24 hours	0 hours	24 hours
S. citreus	89.3	66.4	10.9	6.3	7.7	2.7
S. aureus	72.2	67.1	42.5	23.2	40.2	22.5
S. albus	79.0	79.9	21.7	21.4	21.0	18.1
C. diphtheriae	103.7	79.0	21.6	21.4	20.2	10.3
B. subtilis	4.6	4.2	0.5	0.4	0.3	0.2
B. megatherium	2.0	1.9	0.1	0.0	0.0	0.0
P. vulgaris	5.7	5.7	3.6	1.8	2.8	1.9
S. dysenteriae	6.5	6.0	4.2	3.1	4.8	2.6
E. typhosa	0.9	0.7	0.0	0.0	0.0	0.0
E. coli	6.6	6.6	4.2	3.6	3.1	2.2
P. aeruginosa	37.6	41.5	6.9	6.0	3.3	3.2

* See footnote to table 2.

mined. These results are given in table 3, and indicate in all cases that the catalase activity of the suspensions was markedly inhibited by the concentrations of sodium azide employed. With increased time exposure to the chemical, additional inhibition of catalase activity was observed in most instances, but the increase was not striking. These results are also presented in table 3.

Another point of interest is that in general the amount of inhibition caused

by the two concentrations of sodium azide was not markedly different. This leads to the consideration that even 0.01 per cent sodium azide was excessive. This is also indicated by the fact that there was no significant increase in the inhibition of catalase activity when the suspensions of washed cells were allowed to react with sodium azide for greater lengths of time.

This inhibition of bacterial catalase by sodium azide was extremely interesting because of its striking correlation with the ability of the germ to grow in the presence of similar concentrations of the chemical. This may be better illustrated by an examination of the data in table 4.

TABLE 4

Comparison of the effect of sodium azide on growth with catalase activity of bacteria (24 hours, 37°C.)

ORGANISMS	GROWTH IN PRESENCE OF 0.02 PER CENT NaN_3	CATALASE ACTIVITY*	
		Control	0.02 per cent NaN_3
E. coli	−	6.6	2.2
E. typhosa	−	0.7	0.0
P. vulgaris	−	5.7	1.9
S. dysenteriae	−	6.0	2.6
P. aeruginosa	+++	41.5	3.2
S. hemolyticus	+++	0.0	
S. viridans	+++	0.0	
Pneumococcus	+++	0.0	
S. aureus	++	67.1	22.5
S. albus	++	79.9	18.1
S. citreus	−	66.4	2.7
C. diphtheriae	++	79.0	10.3
B. subtilis	−	4.2	0.2
B. megatherium	−	1.9	0.0
Lactobacillus	++	0.0	
C. welchii	+++	0.0	
C. tetani	+++	0.0	
C. botulinum	+++	0.0	
C. novyi	+++	0.0	
C. sporogenes	+++	0.0	

−, no growth; +, poor growth; ++, good growth; +++, control growth.
* See footnote to table 2.

These data show that the most resistant organisms to the action of sodium azide are the streptococci, the anaerobes, and the lactobacilli. These bacteria lack the enzyme catalase. All the germs which produce catalase are susceptible to sodium azide, using growth as a criterion, although this characteristic varies. According to the data given, those germs which are weak or moderate catalase producers are the least resistant to the action of the chemical. Those which are strong catalase producers, with the exception of *S. citreus*, are more resistant. The correlation between the catalase activity of a germ and its susceptibility to the inhibiting action of sodium azide on growth is superior to a relation between the gram reaction of the organism and its resistance to the chemical.

A determination of the viability of washed germ suspensions in sodium azide was desirable to indicate whether or not the bacteria were alive during the period required for a catalase experiment. If the bacterial cells were killed by the sodium azide, the permeability of the cells would probably change, introducing an additional unknown factor into the experiment.

All strains of bacteria used in the catalase studies were found to be viable after exposure to 0.01 and 0.02 per cent sodium azide for 6–24 hours, indicating that even though the catalase activity was strongly inhibited, the organisms were not killed.

REVERSIBILITY OF SODIUM AZIDE ACTION ON THE CATALASE OF SUSPENSIONS OF WASHED BACTERIA

The inhibiting effect of sodium azide on the catalase activity of suspensions of washed bacteria has been established by the previous experiment. The desirability of determining whether or not this action was reversible is apparent. This information would differentiate between actual destruction and mere inhibition by the chemical.

The germ suspensions were prepared in the usual manner employing 24-hour cultures. The organisms used in this experiment were *Bacillus subtilis* and *S. citreus*. The germ suspension in buffer solution was divided into four portions. One portion was used as the control, while sodium azide was added to the other parts to make final concentrations of 0.01, 0.02, and 0.03 per cent. All suspensions were incubated at 37°C. At definite intervals (6, 24, and 48 hours) the tubes were taken out and 2 ml. of each suspension removed. This sample was then divided into two equal parts. The catalase activity of 1 ml. of each suspension was then determined in order to ascertain the amount of inhibition by sodium azide. The other ml. was centrifugated until the supernatant liquid was clear. This was discarded and the sediment suspended in 1 ml. of phosphate buffer solution (pH 7.4) and again centrifuged. In this manner, the bacterial cells were washed three times, and finally the catalase activity of these suspensions was determined. The results of this experiment are presented in table 5.

It is to be noted that while washing reduced the catalase activity of the control suspensions, it also greatly restored the catalase activity of the sodium azide treated organisms. These results confirm previous similar observations that the chemical does not destroy the catalase, but forms a reversible combination. Another point of interest, shown particularly well by *S. citreus*, is the decreased amount of reversibility after additional exposure to the chemical. This may be due to a stronger union between the chemical and bacterial catalase or possibly to some breakdown of this enzyme.

EFFECT OF SODIUM AZIDE ON THE CATALASE ACTIVITY OF ACTIVELY PROLIFERATING CELLS

The experiments on catalase up to this period were performed with resting cells suspended in phosphate buffer solution. It was desirable to see if similar results would be obtained with actively proliferating germs.

For this study the most resistant of the catalase producers were employed, namely *Staphylococcus aureus*, *Staphylococcus albus*, *Pseudomonas aeruginosa* and *Pseudomonas fluorescens*. The germs were cultured on infusion agar containing 0.01 per cent sodium azide, incubated at 37° C for 12, 24, and 48 hour periods, and the growth in each tube suspended in 5 ml. of phosphate buffer solution (pH 7.4). When variations in the amount of growth of a germ on plain infusion agar and on sodium azide infusion agar were found by Hopkins tube determinations, adjustments were made by proper dilution of the control suspensions. In this manner the results were made uniform so that differences in catalase activity could be interpreted as inhibition by sodium azide rather than variation in numbers of bacterial cells. The results are recorded in table 6.

The data obtained are in general comparable with those using resting cells, with the exception of *P. aeruginosa*. Of more importance, perhaps, is the fact

TABLE 5

Reversibility of sodium azide action on the catalase present in suspensions of washed bacteria

ORGANISMS	NUMBER OF HOURS	CATALASE ACTIVITY*							
		Control		0.01 per cent NaN_3		0.02 per cent NaN_3		0.03 per cent NaN_3	
		Before	After	Before	After	Before	After	Before	After
B. subtilis	6	8.3	6.3	1.4	5.5	1.1	5.5	0.9	5.4
	24	6.0	5.7	0.1	5.3	0.0	5.5	0.0	5.5
	6	2.2	1.8	0.4	1.5	0.0	1.6	0.0	1.5
	24	6.1	2.6	0.6	1.4	0.0	1.2	0.0	1.0
S. citreus	6	31.4	22.6	7.6	21.9	5.7	20.7	5.7	19.0
	24	29.0	21.1	6.9	19.9	6.1	16.9	6.2	14.7
	48	28.3	25.1	5.6	8.7	4.5	7.6	2.4	8.1

* See footnote to table 2.

that the experiment indicates no destruction of the catalase-forming mechanism, but probably a combination with preformed enzyme.

From time to time catalase determinations were made on strains of bacteria after serial subculture on 0.01 per cent sodium azide infusion agar. This was undertaken to detect whether or not the organisms became adapted to the presence of the chemical, although from a theoretical point of view this was not expected.

The results indicated rather clearly that although these strains of bacteria (*S. albus*, *S. aureus*, and *P. aeruginosa*) had become adjusted to the chemical judging from the amount of growth, the inhibition in catalase activity was in keeping with that for germs cultured on sodium azide infusion agar for the first time.

The experiments described have dealt primarily with the action of sodium

azide on bacterial cells under various conditions. Two further experiments were proposed in order to point out the possible significance of the inhibiting action of the chemical on catalase.

The first was based on the postulation that the presence of an initial amount of catalase in broth, previous to its inoculation with bacteria, might have some effect on the resistance of the germs to the action of the chemical. The catalase would of course be added since autoclaving destroys the amount originally present. Heavy suspensions of *B. subtilis* and *S. citreus* in phosphate buffer solution were centrifugated and the supernatant fluid tested for catalase activity. This was found to be about half of a similar quantity of whole culture. Dilutions of this supernatant fluid were added to plain broth and to broth containing

TABLE 6

Inhibition of the catalase activity of bacterial cells grown on 0.01 per cent sodium azide infusion agar

ORGANISMS	AGE OF CULTURE	CATALASE ACTIVITY*	
		Control	0.01 per cent NaN_3
	hours		
S. aureus	12	30.9	28.6
	24	39.1	17.6
	48	91.8	31.0
S. albus	12	33.3	9.4
	24	21.4	3.9
	48	92.0	34.4
P. aeruginosa	12	30.4	29.9
	24	39.9	38.0
	48	44.3	37.4
P. fluorescens	12	12.7	9.0
	24	15.9	7.7
	48	39.5	27.2

* See footnote to table 2.

sodium azide, and the tubes inoculated with the homologous germs. The results seemed to show that the initial presence of catalase did not have any effect on the resistance of these organisms to the chemical.

The second experiment followed from the theory that the role of catalase is related to the decomposition of peroxide which accumulates as the result of aerobic respiration. The occurrence of peroxides in appreciable amounts under physiological conditions is yet to be demonstrated. Possibly the only experimental evidence along this line is the indirect data of Altschul *et al.* (1940) who found reduced cytochrome c in combination with hydrogen peroxide in tissue. On the other hand, certain experiments with isolated enzyme systems which react with molecular oxygen produce hydrogen peroxide, and there is the

opinion that there is no good reason for assuming that these reactions would proceed any differently in the cell.

Several technics were investigated but none were sensitive enough to detect peroxide in aerobic bacterial cultures. These were the technics of Fuller and Maxted (1939), of Sevag and Shelburne (1942a) and the simple titration of acidified cultures with weak potassium permanganate. Of the three methods, the first was the most sensitive and it was possible to detect peroxide in some streptococcus and pneumococcus cultures by using this procedure. However, it was not sensitive enough for the desired purpose. We were unable to demonstrate the formation of a toxic amount of hydrogen peroxide in either plain or sodium azide broth cultures.

It is of course possible that one is unable to inhibit catalase selectively with sodium azide without inhibiting the other heme-containing enzymes (Sevag and Shelburne, 1942b), and this might very well account for the close parallelism between the inhibition of catalase and the growth of bacteria in the presence of sodium azide.

SUMMARY AND CONCLUSION

1. Concentrations of sodium azide of 0.01 and 0.02 per cent, corresponding to M/650 and M/325, markedly inhibited the catalase activity of suspensions of washed bacteria.

2. The reversibility of sodium azide inhibition of bacterial catalase was established. The amount of reversibility diminished with increased time exposure to the chemical.

3. The inhibition of catalase by sodium azide in actively proliferating cells was also determined and found to agree closely with the results obtained with resting cells, with the exception of *Pseudomonas aeruginosa*.

4. The introduction of catalase into infusion broth previous to its inoculation with bacteria, did not affect the sensitivity of the bacteria tested to sodium azide.

5. Attempts to demonstrate the presence of peroxide in plain broth and sodium azide broth cultures of bacteria were unsuccessful.

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