STUDIES IN THE BIOLOGICAL FIXATION OF NITROGEN

IX. INHIBITION OF FIXATION OF NITROGEN IN *AZOTOBACTER VINELANDII* BY AZIDE AND BY CYANATE

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The similarity in structure between azide ions, cyanate ions, and nitrous oxide molecules has been discussed in relation to the known behaviour of nitrous oxide regarding the nitrogen fixation process, and the need for further knowledge of the influence of these ions on fixation has been emphasised[1]. It has also been established that both ions inhibit respiration in *A. vinelandii*, and that azide also inhibits the phosphorylation process[1]. Azide is reported to affect the anaerobic utilisation of ammonia by *Saccharomyces cerevisiae*[2], and since it is generally agreed that ammonia plays a major part in the fixation process, it is necessary to know whether azide also influences ammonia utilisation (as well as nitrogen fixation) in *A. vinelandii*.

In the course of investigating these questions, some study of the analytical chemistry of azides has been made; the possibility of isotopic exchange of nitrogen between azide ions and nitrogen molecules has also been studied.

EXPERIMENTAL

Bacterial cultures

Cultures for the experiments on fixation were prepared by transferring 1 ml of a 3-day-old shake culture[1] to 50 ml of sterile medium[3] in a 250 ml Kjeldahl flask and shaking for 3 days; about 10^{10} cells, separated from this suspension by centrifugation, were transferred to 400 ml of medium and aerated with moist, sterile air for 20 hours, during which time the culture was stirred magnetically. From this master culture 60-ml portions were transferred to each culture flask[3] for aeration with the experimental atmosphere.

Experimental atmosphere and samples for isotope abundance measurements

The atmosphere, and the nitrogen samples for mass analyses, were prepared according to the methods previously described[3].

Circulating pumps

A new type of circulating pump was used to aerate the cultures with the experimental atmosphere. The head (A, Fig. 1) was made from a solid block of polythene, into which glass inlet and outlet tubes, fitted with valves (B, C) consisting of stainless steel balls bearing on ground glass seatings, were sealed by heating. (These seals were highly satisfactory, and pressures of $\sim 10^{-5}$ mm of Hg could be maintained in the apparatus for many weeks.) The truncated cone (D) was hollowed out of the polythene head. The diaphragm, of 0.125" sulphur-free rubber, was attached to the detachable "button" on the end of the piston by means of a thick circular rubber pad 1.2" in diameter. The whole of the diaphragm and pad was covered with a sheet of vulcanised rubber, affixed with rubber solution, to prevent "belling" of the diaphragm (with consequent loss of pumping rate when pressures below atmospheric were used). The head of the pump was secured to the stand by long brass screws (E), the diaphragm being attached to the polythene by means of a rubber sealing compound. The piston, driven by a constant-speed electric motor through a gear-box and reducing pulleys, had a stroke of 1 cm each side of the central position; this gave

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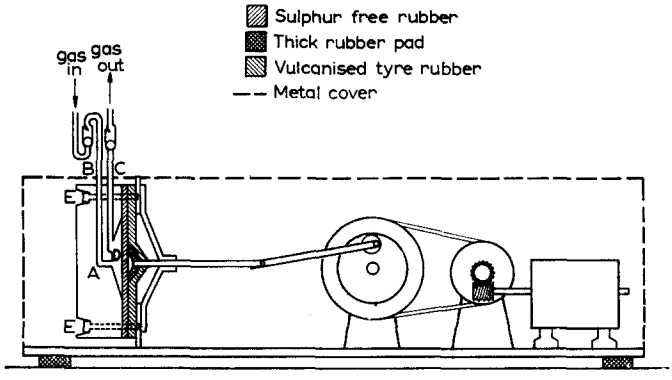

Fig. 1. Diaphragm pump.

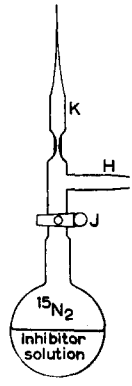

Fig. 2. Isotope exchange apparatus.

a calculated rate of pumping of 390 l/h at 100 strokes per min. The observed pumping rate at atmospheric pressure was very slightly less than this. Bulbs of 500 ml capacity in the gas discharge line served to even out the flow of gas. In the fixation experiments the experimental atmosphere passed into a distributing system designed to ensure that each culture was aerated at the same rate with the same gas mixture.

Ammonia utilisation

To study the influence of azide on ammonia utilisation, a series of cultures in the early logarithmic phase was shaken for the prescribed period, after the addition of the azide and of 0.0005% w/v of nitrogen as $^{15}NH_4Cl$. Oxygen uptake was measured using the Warburg technique in the usual way.

Exchange of nitrogen between N_2 and NaN_3

Isotopically labelled nitrogen was confined over a molar solution of sodium azide in culture medium and the apparatus shaken for 18 h at 30° C (see Fig. 2). A sample of the gas was collected in the previously evacuated and out-gassed tube K, and the abundance of ^{15}N determined mass spectrometrically. No exchange of nitrogen was observed.

Determination of azide

A direct colorimetric method, depending on the red colour produced by the addition of ferric ions to aqueous azide solutions[4,5], was used. The colour persists indefinitely in the presence of an excess of ferric ions. In the concentration range $10^{-3}M$ to $10^{-6}M$ azide, the colour intensity is independent of the presence of Ca^{++}, Mg^{++}, K^+, SO_4^{--}, Cl^-, mannitol, or substances excreted during the growth of *A. vinelandii*, but is affected by PO_4^{---} ions, probably because of the precipitation of ferric phosphate. In view of these findings the procedure adopted was as follows. 9 ml of culture medium containing azide was added to 1 ml of N calcium chloride. The solution was centrifuged for 10 min to remove cells and calcium phosphate, and 9 ml of the supernatant liquid diluted to 10 ml, in a graduated flask, with a standard ($\sim N$) solution of ferric chloride. The extinction coefficient was determined using a Unicam absorptiometer S.P. 400, with a blue filter. Fig. 3 shows the relationship between concentration and extinction coefficient for the range $10^{-3}M$ to $10^{-6}M$ azide. The method is most satisfactory in the range $10^{-3}M$ to $10^{-4}M$, where the error is about $\pm$ 0.5 mg of N as N_3^-. The method was used empirically, new standard solutions being prepared on each occasion to compare with the test solution.

RESULTS

Nitrogen fixation

The results of an experiment using azide as inhibitor are given in Table I. The values of k are calculated from the relationship[3]

$$k = \frac{1}{t} \ln \frac{I}{I-E}$$

where k is a growth rate constant, I and E are the atom % excesses of ^{15}N in the

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experimental atmosphere and in the cells (after time t) respectively. The plot of k_i/k against log $[N_3^-]$ is a straight line (Fig. 4), extrapolation of which to $k_i/k = 1$ indicates that the threshold value, below which azide has no effect, is $10^{-10}M$. The fixation process is therefore extremely sensitive to azide.

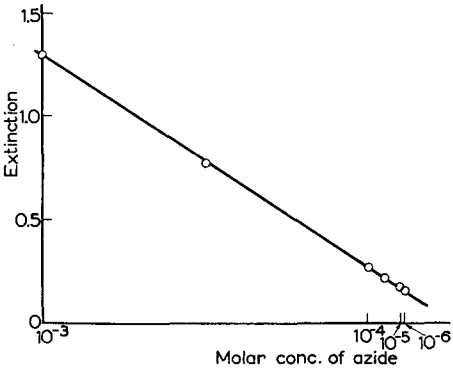

Fig. 3. Colorimetric determination of azide.

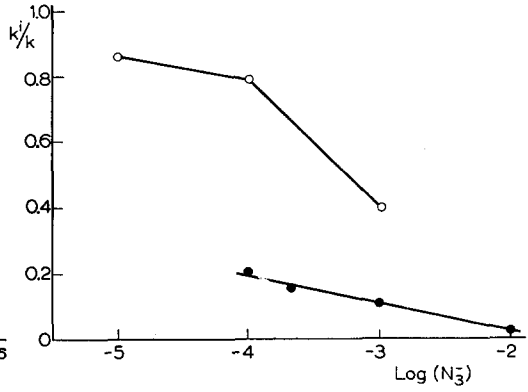

Fig. 4. Influence of azide on nitrogen fixation and uptake of ammonia by *Azotobacter vinelandii*. ○ = $^{15}NH_4$; ● = $^{15}N_2$.

The results of a similar experiment in which cyanate was added to the cultures are given in Table II, and plotted as k_i/k against log $[CNO^-]$ in Fig. 5. From the data, it is clear that cyanate is a very weak inhibitor of fixation; at the lower concentrations employed, a slight stimulation of fixation occurred.

TABLE I

EFFECT OF AZIDE ON FIXATION OF NITROGEN BY *A. vinelandii*

Experimental atmosphere: $p_{N_2} = 0.24$; $p_A = 0.24$; $p_{O_2} = 0.5$ atm. Atom % ^{15}N in atm., before and after = 12.0 ± 0.2. Atom % ^{15}N in cylinder $N_2 = 0.46 \pm 0.02$. Duration of experiment = 4 h. Number of cells per flask = 10^{10}.

Concn. of N_3^- (mol./l)	*Atom % excess of ^{15}N in bacteria*	*k (h^{-1})*	*ki/k*
0	0.816	0.0081	1.00
10^{-2}	0.472	0.00023	0.03
10^{-3}	0.498	0.00092	0.11
$5 \cdot 10^{-4}$	0.512	0.0012	0.15
10^{-4}	0.532	0.0017	0.21

Ammonia utilisation

The data in Table III shows that the uptake of ammonia is not inhibited by azide to the same extent as is the fixation of nitrogen. When the data are plotted as k_i/k against log $[N_3^-]$ (Fig. 4), two lines are obtained; the slope of the one relating to low ammonia concentrations ($< 10^{-4}M$) is apparently identical with that of the line for nitrogen fixation. Thus the constant (A) in the relationship

$$k_i/k = 1 - A \log [N_3^-]$$

has the same value whether moleculat nitrogen or ammonia (in low concentrations)

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TABLE II

INFLUENCE OF CYANATE ON THE FIXATION OF NITROGEN BY *A. vinelandii*

Experimental atmosphere: $p_{N_2} = 0.2$; $p_A = 0.3$; $p_{O_2} = 0.5$ atm. Atom % of ^{15}N in atmosphere = 7.79. Atom % of ^{15}N in cylinder N_2 = 0.44. Duration of experiment = 2 h. Number of cells per flask = 10^{10}.

Concn. of CNO⁻ (mol./l)	*Atom % excess of ^{15}N in cells*	*k (h^{-1})*	*k_i/k*
0	0.58	0.0295	1.00
$6 \cdot 10^{-2}$	0.50	0.0292	0.990
10^{-2}	0.52	0.0293	0.993
10^{-3}	0.59	0.0296	1.003
10^{-4}	0.60	0.0297	1.007

TABLE III

INFLUENCE OF AZIDE ON THE UPTAKE OF AMMONIA BY *A. vinelandii*

Medium: Normal[3] + 0.0005 w/v of N as NH_4Cl. Atom % ^{15}N in NH_4Cl = 30.8. Duration of experiment = 24 h. $T = 30 \pm 3°$ C.

Concn. of N_3^- (mol./l)	*Atom % excess of ^{15}N in cells*	*k (h^{-1})*	*k_i/k*
10^{-5}	18.4	0.039	0.86
10^{-4}	17.4	0.035	0.79
10^{-3}	10.8	0.018	0.40
0	20.3	0.045	1.00

is the substrate. It is interesting to note that at $10^{-4}M$ ammonia, the uptake of inorganic orthophosphate is completely inhibited. A further interesting point is that the uptake of ammonia is inhibited ($\sim$14%) even when the azide concentration is $10^{-5}M$, although both respiration and the uptake of phosphorus are stimulated[1].

The application of the FISHER test[6] in which $\log \frac{k_i}{k - k_i}$ is plotted against $\log [N_3^-]$, to the results for the azide-inhibited fixation and ammonia utilisation cultures gives

TABLE IV

INFLUENCE OF AMMONIA ON AZIDE-INHIBITED RESPIRATION IN *A. vinelandii*

Number of cells = $3.4 \cdot 10^8$/ml. Medium, normal. + A denotes addition of 0.0005% w/v of N as NH_4OAc. Each flask contained 0.8 ml of culture; the side arm, containing 0.1 ml of azide and 0.1 ml of ammonia, was emptied into the flask after 30 min.

Initial concn. of N_3^- (mol./l)	*Time (min)*		*Concn. of N_3^- (mol./l) after addition*	*Time (min)*				*$\frac{U^i_{O_2}}{U^i_{O_2}}$*
	15	*30*		**45*	*60*	*75*	*90*	
0	78	147	0	72	147	247	307	1.00
0	81	149	0 + A	79	157	239	316	1.03
0	72	143	10^{-4}	75	143	221	301	0.95
0	72	145	10^{-4} + A	81	165	251	344	1.21
0	79	148	10^{-3}	71	145	218	295	0.93
0	87	150	10^{-3} + A	77	154	230	314	1.03
0	76	150	10^{-2} + A	52	103	155	201	0.65

* Manometers were re-zeroed before these readings.

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different results. For ammonia, a single straight line is obtained; it appears that the ammonia-utilising system does not involve azide-sensitive enzymes. For nitrogen fixation, however, two straight lines are obtained, and the break in continuity occurs at about $1.5 \cdot 10^{-3} M$; this is sufficiently close to the break in the corresponding graph for azide-inhibited respiration[1] to suggest that the two processes are linked.

Influence of combined nitrogen on the respiration rate of azide-inhibited cultures

The similarity in the inhibition of nitrogen fixation and of ammonia utilisation (at low concentrations) prompted the investigation of the effect of adding ammonia (as ammonium acetate) and nitrate (as potassium nitrate) to cultures of *A. vinelandii* already inhibited by azide. The results are shown in Tables IV (normal medium), V (low (10%) phosphate medium), VI (normal medium, after a lapse of time), for ammonia, and in Table VII for nitrate.

TABLE V

INFLUENCE OF AMMONIA ON AZIDE-INHIBITED RESPIRATION OF *A. vinelandii* IN PHOSPHATE-LOW MEDIA

Oxygen uptake in μl. Number of cells = $1.5 \cdot 10^8$/ml. Medium, 10% phosphate. + A denotes addition of 0.0005% w/v of N as NH OAc.

Concn. of N_3^- (mol./l)	*Time (min)*			
	15	*30*	*45*	*60*
0	86	172	258	341
0 + A	81	172	256	336
10^{-3}	71	145	209	266
10^{-3} + A	78	156	238	303
$3 \cdot 10^{-3}$	50	101	151	196
$3 \cdot 10^{-3}$ + A	66	127	178	232
10^{-2}	33	73	107	137
10^{-2} + A	33	66	101	128

TABLE VI

INFLUENCE OF AMMONIA ON AZIDE-INHIBITED RESPIRATION IN *A. vinelandii* AFTER A LAPSE OF TIME

Oxygen uptake in μl. Number of cells = $3 \cdot 10^8$/ml. Medium, normal. + A denotes addition of 0.0005% w/v of N as NH_4OAc.

Concn. of N_3^- (mol./l)	*Time (min)*			*Time (min)*	
	30	*45*		*30*	*45*
10^{-2} + A	182	266	After lapse of 3 h	80	167
10^{-3} + A	246	341	(manometer	206	332
10^{-4} + A	263	374	re-zeroed)	216	326
0	200	297		238	374

Addition of ammonia immediately revives the respiration rate, and where the azide concentration is below $10^{-3} M$, stimulates oxygen uptake to above the "normal" level. Such marked revival of respiration did not occur when media deficient in phosphate were used, although some recovery was observed. It was also noted that

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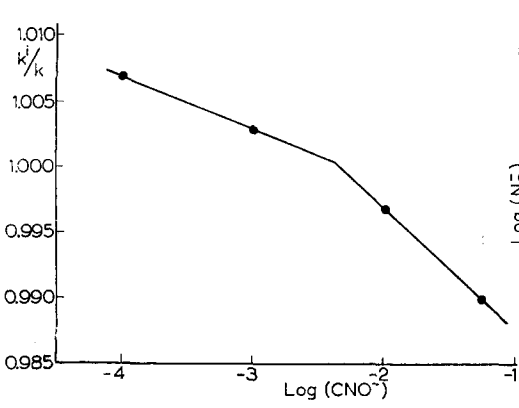

Fig. 5. Influence of cyanate on nitrogen fixation by *Azotobacter vinelandii*.

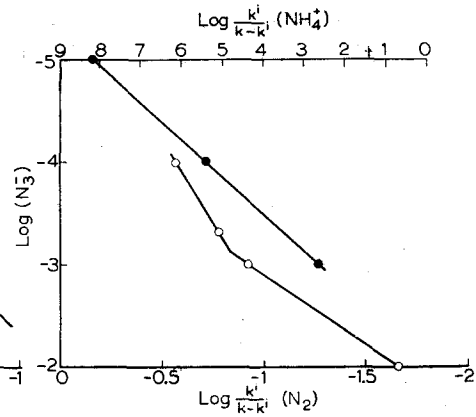

Fig. 6. FISHER test for inhibition of *A. vinelandii* by azide. ● = $^{15}NH_4$; ○ = $^{15}N_2$.

azide inhibition in ammonia-revived cultures recurred after a lapse of time; this was not due to exhaustion of ammonia from the medium, since the cellular nitrogen increased by only 0.0005 mg during this time although the medium contained, initially, ten times this amount of ammonia-nitrogen.

Nitrate has only a minor regenerating effect on azide-inhibited respiration.

Similar experiments with cyanate-inhibited cultures showed that ammonia has no regenerating effect (Table VIII).

TABLE VII

INFLUENCE OF NITRATE ON AZIDE-INHIBITED RESPIRATION IN *A. vinelandii*

Oxygen uptake in μl. Number of cells = $3 \cdot 10^8$/ml. Medium, normal. + K denotes addition of 0.0005 % w/v of N as KNO_3.

Concn. of N_3^- (mol./l)	*Time (min)*	
	30	*45*
10^{-2} + K	131	178
10^{-3} + K	187	228
10^{-4} + K	187	228
0	198	285

TABLE VIII

INFLUENCE OF AMMONIA ON CYANATE-INHIBITED RESPIRATION IN *A. vinelandii*

Oxygen uptake in μl. Number of cells = $6 \cdot 10^8$/ml. Medium, normal. + N denotes addition of 0.0005 % w/v of N as NH_4OAc

Concn. of CNO⁻ (mol./l)	*Time (min)*		
	15	*30*	*45*
$3.8 \cdot 10^{-2}$	10	25	38
$3.8 \cdot 10^{-2}$ + N	11	30	22
$3.8 \cdot 10^{-3}$	106	216	316
$3.8 \cdot 10^{-3}$ + N	121	240	341
0	136	264	399
0 + N	138	269	403

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The above observations are consistent with the specific inhibition of nitrogenase by azide in the range $10^{-2}M$ to $10^{-4}M$, the inhibition of other systems becoming more apparent with the passage of time.

Test for utilisation of azide

If azide inhibits fixation specifically, it is desirable to know whether it can serve as an alternative source of nitrogen, and several authors have suggested that azide can be utilised by certain organisms[7,8,9]. A suitable culture was grown, azide added, and the resulting stand culture incubated at 30° for a given time. The results in Table IX do not suggest that appreciable amounts of azide were taken up; no attempt was made to "train" the bacteria to utilize azide.

TABLE IX

INCREASE IN CELL NITROGEN OF *A. vinelandii* GROWN IN PRESENCE OF AZIDE

Concn. of N_3^- (mol./l)		Duration of expt.	Increase in cell N (mg)
Initial	Final		
10^{-3}	10^{-3}	3 weeks	5
$5 \cdot 10^{-4}$	$5 \cdot 10^{-4}$	2 weeks	4.5
10^{-4}	10^{-4}	3 days	5

$10^{-3}M$ = 10.5 mg of N as N_3^-; $10^{-4}M$ = 1.05 mg of N as N_3^-.

DISCUSSION

An attempt to compare the behaviour of nitrous oxide, azide and cyanate meets with the immediate difficulty that nothing is known about nitrous oxide apart from its effects on the respiratory and the nitrogen-fixing systems. Table X sets out such information as is available, and shows the very great differences in the effects of the

TABLE X

Nature and extent of inhibition	Molar concn. of inhibitor producing effect		
	N_2O	N_3^-	CNO^-
Respiration:			
80 % inhib.	—	$2 \cdot 10^{-2}$	$2.5 \cdot 10^{-2}$
50 %	—	$2.5 \cdot 10^{-3}$ *	10^{-2} *
10 %	$2.1 \cdot 10^{-4}$ *	$4 \cdot 10^{-5}$	$3 \cdot 10^{-3}$
P-utilisation:			
80 %	—	$2.5 \cdot 10^{-4}$	
50 %	—	$7.3 \cdot 10^{-5}$ *	$3 \cdot 10^{-2}$ *
10 %	—	10^{-5}	
N-fixation:			
80 %	—	10^{-4} * (at $p_{N_2} = 0.24$)	—
50 %	—	$1.8 \cdot 10^{-4}$ **	—
10 %	$1.8 \cdot 10^{-4}$ *** (at $p_{N_2} = 0.1$)		10^{-1} (at $p_{N_2} = 0.2$) *

* Reference [1]. ** By extrapolation. *** Reference [10].

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three inhibitors. The obvious conclusion is that compounds having similar bond lengths and electron distributions do not necessarily lead to similar biological effects.

The results of the present investigation show that azide is a specific inhibitor of nitrogen fixation, that it acts reversibly, and that it is not utilised. Considered qualitatively, it is therefore extremely similar to nitrous oxide, and its behaviour therefore supports the suggestion that the latter substance inhibits nitrogen fixation by competing for the active sites on the nitrogenase. When considered quantitatively, however, the results weaken this support since azide is far the more powerful inhibitor; moreover, azide is a well-known powerful general inhibitor of metal-enzyme systems. It is clearly not permissible to deduce the biological behaviour from purely structural and dimensional considerations. This is emphasised by comparing azide with cyanate the latter inhibiting fixation only weakly, being neither specific nor competitive, and being irreversible; the extreme similarity between azide and cyanate, in bond lengths molecular shape, and electron distribution, coupled with their totally different effects on fixation, seems clearly to indicate that these factors are of minor importance only. The inhibition of fixation by cyanate is probably the result of inhibition of respiration.

Assuming that the inhibition of uptake of orthophosphate reflects inhibition of oxidative phosphorylation, it is evident that the mechanism of nitrogen fixation does not involve, in the initial stage, any oxidative phosphorylation. Thus $10^{-4}M$ azide completely inhibits phosphorus utilisation[1], but fixation still continues (though more slowly) in concentrations of azide greater than $10^{-2}M$. The break in the curve for the utilisation of ammonia, at $\sim 10^{-4}M$, suggests that at above this concentration azide inhibits phosphorylating reactions associated with the conversion of ammonia to organic substances. Below this concentration there is a striking similarity in the slope of the curves for ammonia uptake and nitrogen fixation.

It may be pertinent to summarize the information now available concerning the action of azide on *Azotobacter*; Table XI is drawn up from various sources.

Catalase, indophenol oxidase, nitrogenase, and the phosphorus-utilising systems appear to be especially sensitive to azide, but hydrogenase and the respiratory systems are much less so. Since there is some ground for belief that the indophenol oxidase system is linked to the cytochrome system of *Azotobacter*, the response to azide suggests that the cytochrome system may not be of major importance in the respiratory activity of the organism. This is consistent with the suggestion that *Azotobacter* hydrogenase acts in a respiratory capacity[13], to which idea the observed reversibility of hydrogenase lends support[14].

It is remarkable that at $5 \cdot 10^{-3}M$ azide, respiration of *Azotobacter* is inhibited by ~60%, nitrogen fixation by ~95%, and phosphorus uptake completely, yet

TABLE XI

Enzyme system	*Molar concn. of azide producing inhibition*					
	100%	*80%*	*50%*	*40%*	*30%*	*20%*
Nitrogenase	$2 \cdot 10^{-2}$	10^{-4}				
P-utilizing[1]		$2.5 \cdot 10^{-4}$	$7.3 \cdot 10^{-5}$			
Catalase[11]	10^{-3}	10^{-4} (75%)	$5 \cdot 10^{-5}$			
Indophenol oxidase[1]	$5 \cdot 10^{-3}$	$5 \cdot 10^{-5}$ (89%)				
Hydrogenase[12]		10^{-1} (8 %)			10^{-2}	
Respiratory[12]	10^{-1}			$5 \cdot 10^{-3}$		10^{-3}

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multiplication and growth continue. This fact has already been noted for respiration[11], but the almost complete cessation of fixation suggests that azide might be used as an alternative source of nitrogen. No utilisation was, however, observed.

It has been noted that cultures whose respiration is inhibited by azide become stimulated by the addition of ammonia, yet at these azide concentrations inhibition of ammonia utilisation occurs. The question of stimulation is not new, but no satisfactory explanation of its occurrence has been offered. In these laboratories, stimulation of fixation by nitrite, by hydrazine, and by cyanate have been recorded; stimulation of respiration and of phosphorus uptake by azide and by DNP have been noted; and stimulation of hydrogenase activity by low concentrations of azide have been reported[12]. It may well be that closer study of stimulation may throw more light on the nitrogen fixation process than the study of inhibition.

ACKNOWLEDGEMENT

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SUMMARY

The influence of azide and of cyanate on the fixation of nitrogen by *A. vinelandii* has been measured; the effect of azide on the uptake of ammonia has also been determined. It is found that, like nitrous oxide, azide is a specific inhibitor of fixation, it acts reversibly, and it is not utilised; it differs from nitrous oxide in being a much more powerful inhibitor, but may nevertheless act by competing with nitrogen for the active sites on nitrogenase. Cyanate is a very weak inhibitor of fixation, is irreversible, and is neither specific to fixation nor competitive. These results demonstrate that it is not permissible to deduce biological behaviour from purely structural and dimensional considerations.

The significance of these findings is discussed, together with those reported in the previous paper, and the tentative conclusion is drawn that the results support the suggestion that azotobacter hydrogenase may act in a respiratory capacity.

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