SUMMARY

1. Injections of adrenaline or growth hormone inhibit the anaerobic glycolysis of the rat uterus.

2. In hypophysectomized rats adrenaline inhibits neither the oxygen uptake nor the anaerobic glycolysis of the uterus.

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STUDIES IN THE BIOLOGICAL FIXATION OF NITROGEN

VIII. INHIBITION OF RESPIRATION AND OF PHOSPHORUS UTILISATION IN *AZOTOBACTER VINELANDII* BY AZIDE AND BY CYANATE

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Nitrous oxide is a specific, competitive, reversible inhibitor of nitrogen fixation in *Azotobacter vinelandii*[1,2]. The discrepancy in the results of tests designed to detect utilisation of nitrous oxide[3,4] raises considerable doubt regarding its role as an intermediate in the fixation process. Thus the uptake of labelled nitrogen from labelled nitrous oxide is very slow, and suggests that this substance is not, as has been proposed[5,6], an important intermediate; the amount of labelled nitrogen uptake observed is indeed so small that it might result from adsorption on the cells, or from slight decomposition of the oxide with subsequent fixation of the free nitrogen produced. Moreover, when labelled nitramide was supplied, no increase in cell label was observed[7], although nitramide is notoriously unstable in the medium, nitrous oxide being among its decomposition products[8]. In addition, the preparation of labelled nitrous oxide entirely free from molecular nitrogen has been found extremely difficult[9].

If nitrous oxide be not an intermediate in the fixation process, some other explanation of its competitive inhibition is necessary. It has been suggested that its effect is physical, arising from the similarity in molecular dimensions between nitrous oxide and nitrogen. If this be so, substances having isosteric (and preferably isoelectronic) molecules might behave in a similar manner. Such a substance is carbon

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dioxide, which is known to inhibit *Azotobacter*; a knowledge of the nature of this inhibition would be of great value, but the difficulties of controlling the partial pressure of carbon dioxide in an experimental atmosphere, in the presence of bacterial cultures, are very great except over a narrow range of low pressures (0.5 to 0.25% of CO_2)[10]. There is the further disadvantage that hydration of carbon dioxide is considerable, and is catalysed by HPO_4^- ions.

Azide and cyanate ions are isosteric with nitrous oxide, but although they are isoelectronic with each other, they differ from nitrous oxide in their electronic distribution[12]. These ions are linear, with the dimensions[13,14,15].

$$\left[N \overset{1.15\,A}{\text{———}} N \overset{1.15\,A}{\text{———}} N\right]^-, \qquad \left[C \overset{1.16\,A}{\text{———}} N \overset{1.16\,A}{\text{———}} O\right]^-.$$

Closer similarity with nitrous oxide is shown by hydrazoic acid and methyl azide[16,17]:

$$N \overset{1.12\,\bar{A}}{\text{———}} N \overset{1.19\,A}{\text{———}} O,$$

$$N \overset{1.10\,A}{\text{———}} N \overset{1.24\,A}{\text{———}} N\!<_{CH_3}, \qquad N \overset{1.13\,\bar{A}}{\text{———}} N \overset{1.12\,A}{\text{———}} N\!<_{H}.$$

It would clearly be of interest to measure the effect on the bacteria of either of these, but this is not possible; first, the acid has a relatively high dissociation constant ($1.9 \cdot 10^{-5}$ at 25°); secondly, methyl azide (which has been prepared) is rapidly and completely hydrolysed in the culture medium.

The inference that azide and cyanate ions might behave in a manner comparable to nitrous oxide is nevertheless permissible, provided that:

(i) the ions do not react with the prosthetic groups of the enzymes;

(ii) the ions in solution are not hydrated;

(iii) the field due to the ions does not markedly affect the enzymes.

Both ions are known to react with certain heavy metals (*e.g.* iron, cobalt) to form complex compounds, and azide especially is a well-known enzyme inhibitor[18]. There is no evidence of hydration in the solid state and, on the basis of FAJANS' views on the polarisation of large anions, little would be expected to occur in solution.

In spite of the above provisions, it was resolved to investigate the effect of azide and of cyanate as inhibitors of *Azotobacter*, and to attempt to measure the concentrations at which nitrogenase is affected. An essential preliminary, which forms the basis of the present communication, was to determine the effects of these ions on respiration and on phosphorus utilisation. Azide has long been known to affect biological systems[19], and CLIFTON first showed that it dissociated respiration from synthetic processes[20]. HOTCHKISS observed that at concentrations of azide that stimulated respiration in bacteria, the uptake of inorganic orthophosphate was decreased[21]. It is also known that azide uncouples substrate oxidation from net triphosphate synthesis, both in whole cells and in cell-free preparations[22], and that reduction in the activity of an enzyme may occur simultaneously with stimulation of the synthesis of the enzyme[23]. The effect of cyanate on biological systems has been less extensively studied. There is evidence that the alkaline phosphatase of rabbit serum is inhibited by molar cyanate[24] and that succinic dehydrogenase is inhibited. LEES AND SIMPSON have shown that cyanate reversibly inhibits nitrite

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reduction by resting cells of *Nitrobacter*. No information on the effect of cyanate on nitrogen fixation has been found in the literature.

EXPERIMENTAL

Growth of bacteria

A. vinelandii were grown in Kjeldahl flasks (of 50 to 250 ml capacity), containing 18 to 50 ml of medium[25], shaken at ~500 vibrations per minute by means of a "Microid" shaker, in an incubator at 30 ± 1°. These flasks are admirably suited to the purpose, the long necks guarding against splashing onto the cotton-wool plugs. The method provides excellent aeration and produces uniform cultures with a stationary population of up to $5 \cdot 10^{10}$ cells per ml. No culture prepared in this way, which is now in general use in these laboratories, has been found contaminated.

For respirometric experiments, 1 ml of a 3-day-old culture was transferred to 50 ml of medium and shaken for two days; 1 ml of the resulting culture was added to a further 50 ml of solution and shaken for about 18 h, at the end of which time the population was ~10^8 cells per ml. 0.9 ml of this culture, together with 0.1 ml of the inhibitor dissolved in medium, was used in each Warburg flask for oxygen uptake measurements.

For the experiments on phosphorus uptake, master cultures were prepared in a similar way. 1 ml of the final culture was added to 100 ml of sterile medium, in Kjeldahl flasks, containing the inhibitor; eight flasks were used in each experiment, and were shaken at 30°, samples for analysis being removed at suitable intervals.

Uptake of oxygen was measured by the usual Warburg method[26], synthetic atmospheres being introduced by the method previously described[3].

Phosphorus uptake was deduced from determinations of the orthophosphate content of the supernatant medium, using the method of ALLEN[27]. For the experiments with azide the medium contained 10%, and for cyanate 20%, of the normal phosphate content.

The ratios of uptake of oxygen to uptake of phosphorus were determined by using 3 ml of culture per Warburg flask, with mercury as the manometric liquid, for the measurement of oxygen uptake; the phosphate content of the medium was determined at the beginning and at the end of the experiment.

RESULTS

Effects of azide on respiration

The rates of uptake of oxygen (U_{O_2}) by *A. vinelandii* in the presence of various concentrations of sodium azide are plotted in Fig. 1, from which it is apparent that inhibition occurs at concentrations higher than ~$10^{-5}M$. Below this concentration, however, appreciable stimulation of respiration occurs, the maximum effect being observed at ~$10^{-6}M$ azide, and decreasing with further reduction of azide until the normal rate is restored at ~$10^{-10}M$. When the data are plotted as ratios of inhibited to uninhibited oxygen uptake ($U^i_{O_2}/U_{O_2}$) against the logarithm of azide concentration ($[N_3^-]$), two straight lines, intersecting at $1.5 \cdot 10^{-3}M$, are obtained (Fig. 2). Extrapolation to $U^i_{O_2}/U_{O_2} = 1$ indicates a "threshold" concentration, having no effect on the rate of respiration, of ~$2.5 \cdot 10^{-5}M$.

The data in Table I show the effect of different partial pressures of oxygen on respiration in the presence of azide. The data are in accordance with the relationship

$$U^i_{O_2}/U_{O_2} = 1 - A\,[N_3^-]$$

where A is a constant. Thus oxygen uptake is inhibited competitively, causing a relative decrease in the inhibition by azide. Cyanide[28] and hydroxylamine[29] similarly compete with oxygen.

The plot of $\log \frac{U^i_{O_2}}{U_{O_2} - U^i_{O_2}}$ against $\log [N_3^-]$ (FISHER test[29]) shows a discontinuity at $1.5 \cdot 10^{-3}M$ (see Fig. 3), suggesting that at this concentration either an enzyme

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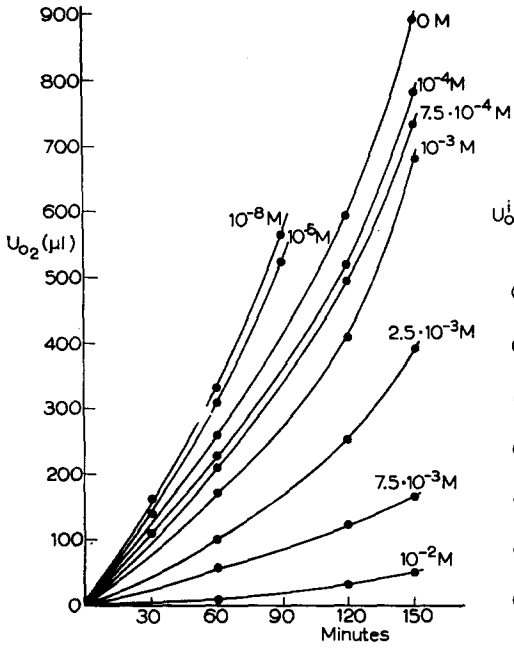

Fig. 1. Oxygen uptake by *Azotobacter vinelandii* in presence of azide.

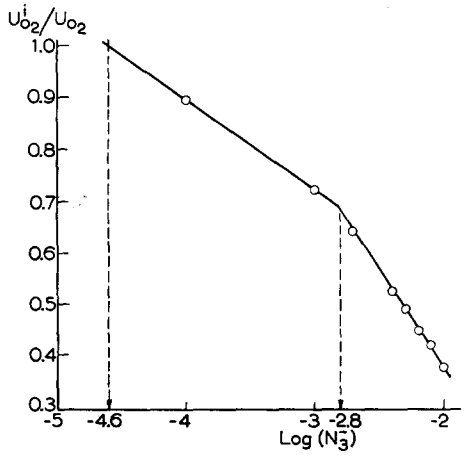

Fig. 2. Inhibition of respiration in *A. vinelandii* by azide.

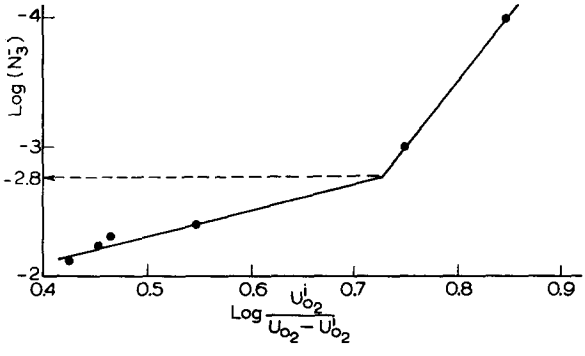

Fig. 3. FISHER test (azide).

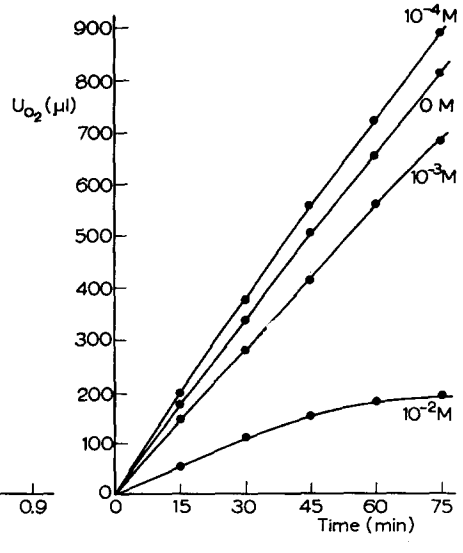

Fig. 4. Oxygen uptake by *A. vinelandii* in presence of cyanate.

system becomes completely inhibited or another enzyme system begins to be affected. In this respect, the behaviour of azide differs from that of hydroxylamine, which appears to concern only a single enzyme system[25].

The effects of azide on respiration are completely reversible; cultures that had

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been inhibited by $10^{-2}M$ azide for 21 days commenced to grow immediately the concentration was reduced to $10^{-4}M$. The effect of the presence of combined nitrogen on the inhibition of *A. vinelandii* by azide and by cyanate has already briefly been reported[30], and will be discussed in a subsequent communication.

Effect of cyanate on respiration

The data shown in Fig. 4 (rates of oxygen uptake at various concentrations of cyanate) indicate that the respiratory system in *A. vinelandii* is less sensitive to cyanate than to azide. A plot of $U^i_{O_2}/U_{O_2}$ against log [CNO^-] (Fig. 5) yields the "threshold" concentration of $\sim 4 \cdot 10^{-4}M$, below which stimulation of respiration occurs, as with azide. In contrast to azide, however, cyanate does not compete with oxygen; Table II shows that the ratio $U^i_{O_2}/U_{O_2}$ is decreased when the partial pressure of oxygen is increased. Cyanate inhibition is irreversible. Cultures inhibited by $10^{-2}M$ and $10^{-3}M$ cyanate for 24 hours are irreversibly poisoned; even cultures stimulated by $10^{-4}M$ cyanate are found to be inhibited four hours later, and are completely inhibited after 24 hours (Table III). The possibility that this delayed inhibition was caused by the conversion of cyanate to a toxic substance, *e.g.* cyanide, in the presence of culture

TABLE I

OXYGEN UPTAKE (μl) BY *A. vinelandii* IN PRESENCE OF AZIDE AND AT DIFFERENT OXYGEN TENSIONS

T = 30.4 ± 0.01° C; population = $9.2 \cdot 10^7$ cell/ml; max. error ± 5 μl.

	Concn. of N_3^- (mol./l)	Time in min			$U^i_{O_2}/U_{O_2}$ (180 min)
		30	60	180	
Air series	10^{-2}	36	68	175	0.44 ± 0.02
	10^{-3}	58	110	323	0.81 ± 0.02
	10^{-4}	50	110	368	0.93 ± 0.025
	0	58	120	398	
$p_{O_2} = 0.5$	10^{-2}	34	52	126	0.52 ± 0.03
	10^{-3}	40	69	198	0.81 ± 0.035
$p_{N_2} = 0.5$	10^{-4}	40	75	231	0.95 ± 0.04
	0	36	69	244	

TABLE II

OXYGEN UPTAKE (μl) BY *A. vinelandii* IN PRESENCE OF CYANATE AND AT DIFFERENT OXYGEN TENSIONS

	Concn. CNO^- (mol./l)	Time in min			$U^i_{O_2}/U_{O_2}$ (180 min)
		60	120	180	
Air series	10^{-2}	68	155	210	0.55
	$5 \cdot 10^{-3}$	66	127	173	0.46
	10^{-3}	69	136	197	0.52
	0	120	254	380	
$p_{O_2} = 0.5$ $p_{N_2} = 0.5$	10^{-2}	40	55	65	0.38
	$5 \cdot 10^{-3}$	37	47	57	0.34
	10^{-3}	38	64	84	0.49
	0	64	114	170	

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TABLE III

SHOWING THE INCREASING INHIBITION OF RESPIRATION IN *A. vinelandii*, BY CYANATE, WITH PASSAGE OF TIME

Oxygen uptake in μl.

Concn. CNO⁻ (mol./l)	*Time in min*						
	15	*30*	*45*	*60*		*255*	*270*
10^{-2}	56	108	150	184		8	14
10^{-3}	163	316	480	620	Manometers	144	213
10^{-4}	182	362	545	706	re-zeroed	148	264
0	160	304	470	615		130	290

TABLE IV

EFFECT ON RESPIRATION OF *A. vinelandii* OF A SOLUTION OF CYANATE 24 HOURS OLD

Oxygen uptake in μl.

Concn. of CNO⁻ (mol./l)	*Time in min*			
	15	*30*	*45*	*60*
10^{-2}	69	110	145	189
0	157	305	460	623

medium, was tested by allowing the inhibitor to stand in sterile medium for 24 hours prior to using it for respiration measurements. Comparison of the data in Table III and Table IV offers no evidence that toxic substances had been formed under these conditions.

Effect of azide on the utilisation of inorganic orthophosphate

The data shown graphically in Fig. 6 indicates that the uptake of inorganic orthophosphate is markedly affected by azide. Thus $10^{-3}M$ azide completely inhibits phosphorus uptake, and $10^{-4}M$ caused partial inhibition, but $10^{-5}M$ results in an

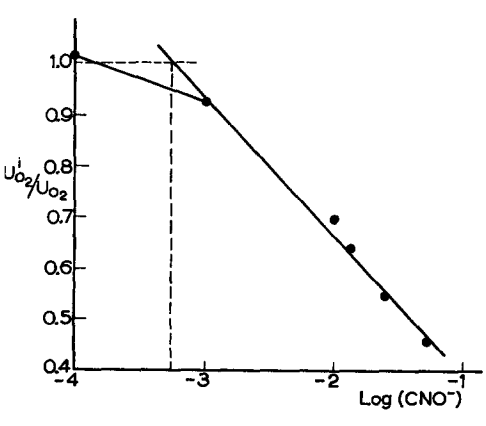

Fig. 5. Inhibition of respiration in *A. vinelandii* by cyanate.

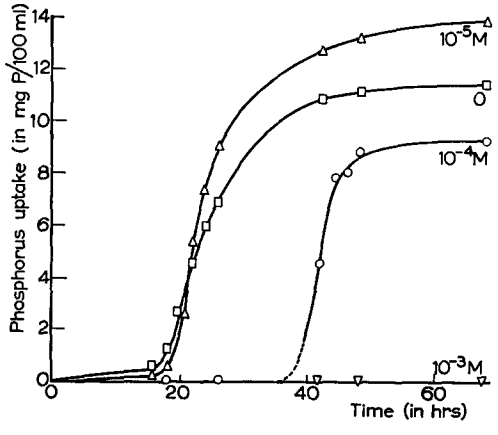

Fig. 6. Influence of azide on utilisation of inorganic orthophosphate by *A. vinelandii*.

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TABLE V

EFFECT OF AZIDE ON THE RATIO OF OXYGEN UPTAKE TO PHOSPHORUS UPTAKE IN *A. vinelandii*

$T = 30.2°$ C; population = $3.7 \cdot 10^8$ cell/ml; time = 3 h.

Concn. of N_3^- *(mol./l)*	U_{O_2} *(µatoms)*	U_P *(µatoms)*	U_{O_2}/U_P
0	193	0.061	3550
10^{-5}	180	0.125	1450
$5 \cdot 10^{-5}$	198	0.250	890
10^{-4}	202	0	∞
$5 \cdot 10^{-4}$	195	0	∞
10^{-3}	178	0	∞
$5 \cdot 10^{-3}$	124	0	∞
10^{-2}	100	0	∞

TABLE VI

EFFECT OF AZIDE ON PHOSPHATE UTILISATION

Orthophosphate uptake in mg/5 ml of medium. Population = $8 \cdot 10^8$ cells/ml. Cultures in 250 ml Kjeldahl flasks shaken at 500 vertical vibrations per min.

Concn. of N_3^- *(mol./l)*	*Time in h*					
	22	*24*	*26*	*42*	*48*	*64*
0	0.045	0.060	0.068	0.108	0.111	0.115
10^{-3}	0	0	0	0	0	0
10^{-4}	0	0	0	0.045	0.088	0.092
10^{-5}	0.053	0.073	0.090	0.126	0.132	0.138

TABLE VII

EFFECT OF AZIDE ON PHOSPHATE UTILISATION

Orthophosphate uptake in mg/5 ml of medium. Population = $9 \cdot 10^8$ cell/ml; cultures in 250 ml conical flasks, shaken horizontally at 100 oscillations per min.

Concn. of N_3^- *(mol./l)*	*Time in h*		
	22	*25*	*42*
0	0.075	0.08	0.146
$2.5 \cdot 10^{-3}$	0	0	0
10^{-3}	0.006	0.015	0.028
10^{-4}	0.011	0.020	0.146
10^{-5}	0.092	0.100	0.160

appreciable stimulation. This conclusion is confirmed by the measurements of the effect of azide on the ratio of oxygen uptake to phosphorus uptake, given in Table V. So far as is known, this is the first reported instance of the stimulation of phosphorus uptake by low concentrations of azide. Attempts to determine the "threshold" concentration above which azide inhibits phosphate utilisation revealed some divergences, depending on the rate and mode of shaking, and on the efficiency of aerating the cultures (see Table VI and VII).

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TABLE VIII

EFFECT OF CYANATE ON THE RATIO OF OXYGEN UPTAKE TO PHOSPHORUS UPTAKE IN *A. vinelandii*

Population = $4.5 \cdot 10^8$ cells/ml; time = 6 h.

Concn. of CNO⁻ (mol./l)	V_{O_2} *(µl)*	V_P *(µatoms)*	V_{O_2}/V_P *(both in µatoms)*
0	4490	0.0090	1380
10^{-4}	4580	0.0090	1410
10^{-3}	4450	0.0090	1370
10^{-2}	3080	0.0090	956
$1.25 \cdot 10^{-2}$	2850	0.0075	1570
$2.5 \cdot 10^{-2}$	2400	0.0060	1660
$5 \cdot 10^{-2}$	2080	0	∞

Effect of cyanate on the utilisation of inorganic orthophosphate

The uptake of orthophosphate by *A. vinelandii* is less sensitive to cyanate than to azide. Inhibition does not occur until concentrations between 10^{-2} and $1.25 \cdot 10^{-2} M$ are supplied, and no stimulation was observed at lower concentrations. Table VIII gives the values of the ratios of U_{O_2}/U_P for cyanate concentrations between $10^{-4} M$ and $2.5 \cdot 10^{-2} M$. When these data are plotted on the same graph as the corresponding data for azide- (and for DNP-) inhibited cultures, the marked differences are clearly demonstrated (Fig. 7).

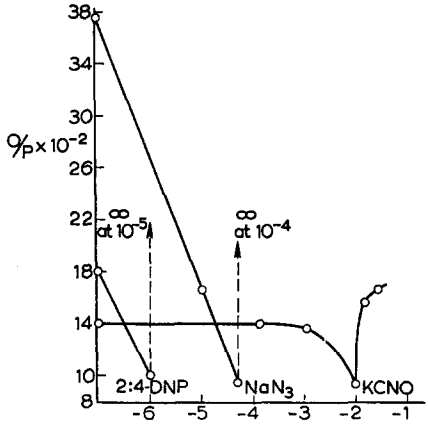

Fig. 7. Influence of inhibitors on ratio of oxygen uptake to phosphate uptake in *A. vinelandii*

SUMMARY OF RESULTS

The significance of the above observations in relation to the problem of nitrogen fixation will be discussed in a subsequent communication in which data on the effect of azide and cyanate on fixation will be presented. It is convenient, however, to summarize the conclusions drawn from the present work.

Both azide and cyanate inhibit respiration and phosphorus uptake in *A. vinelandii*. Azide apparently stimulates the phosphorylating system, even when present at $5 \cdot 10^{-5} M$, but completely inhibits phosphate uptake at $10^{-4} M$. In this range of concentration azide has only a slight effect on respiration; moreover, the inhibition of respiration increases gradually with increasing concentration, and is not complete even at $5 \cdot 10^{-2} M$.

Cyanate, on the other hand, inhibits both respiration and phosphorus uptake to about the same extent, up to $10^{-3} M$, but at higher concentrations the respiratory system becomes more sensitive. Thus at $2.5 \cdot 10^{-2} M$, inhibition of respiration is ~50%, while orthophosphate is inhibited by ~33%.

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These conclusions suggest that inhibition of phosphorylation by cyanate is, at the lower concentrations, a consequence of the inhibition of respiration, but at higher concentrations ($\sim 5 \cdot 10^{-2} M$) phosphorylation becomes the more sensitive process. The behaviour of azide is consistent with the notion that it uncouples the oxidation of substrates from the net triphosphate synthesis, inhibition of respiration occurring only at higher azide concentrations.

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SUMMARY

The effects of azide, and of cyanate, on respiration and on inorganic orthophosphate utilisation, by *Azotobacter vinelandii* have been determined. Both ions inhibit both processes at higher concentrations; both stimulate respiration at very low concentrations, but azide alone markedly stimulates phosphorus uptake when present below $5 \cdot 10^{-5} M$. Azide, but not cyanate, competes with oxygen in inhibiting respiration; azide inhibition is completely reversible, but cyanate inhibits irreversibly. Moreover, cultures supplied with such low concentrations of cyanate that stimulation of respiration occurs are later found to be inhibited. Thus although azide and cyanate are structurally and electronically very similar they exhibit very great differences from each other and also from nitrous oxide in their effects on the bacteria.

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