STUDIES IN THE BIOLOGICAL FIXATION OF NITROGEN

II. INHIBITION IN *AZOTOBACTER VINELANDII* BY HYPONITROUS ACID

by

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It has been suggested[1,2] that the fixation of nitrogen in *Azotobacter vinelandii* proceeds through the agency of free hydroxyl radicals, *e.g.* according to the scheme:-

$$\underset{\text{(nitrogenase)}}{E_N} + 2OH \longrightarrow E_N(OH)_2 \xrightarrow{N_2} E_N \cdot N_2(OH)_2 \longrightarrow E_N + (N_2(OH)_2)$$

Attention is thus drawn to the possible initial product, which could be hyponitrous acid (HON:NOH) or nitramide ($H_2N \cdot NO_2$). Moreover, hyponitrous acid is stated to be an intermediate in the reduction of nitrate to nitrogen by denitrifying bacteria[3] and in the oxidation of ammonia to nitrate, from which nitrifying organisms derive their energy[4,5]. The present communication records observations on the influence of hyponitrous acid on fixation and on respiration; the role of nitramide is being studied and will be reported on in due course. Fixation has been measured by means of the isotopic method previously described[1]; for the study of respiration the Warburg method was employed[6].

During the course of the investigation, the stability of hyponitrous acid in various media has been determined by measuring the rate of disappearance of the acid from solutions of widely differing concentrations. The same method has been employed to determine whether hyponitrous acid can act as a source of nitrogen for *A. vinelandii*, and the results confirmed by repeated sub-culturing in the presence of the acid in an effort to train the organisms to use this source.

EXPERIMENTAL

I. *Preparation and decomposition of hyponitrous acid*

Sodium nitrite was reduced[7] with sodium amalgam[8] under conditions unfavourable to the formation of hydroxylamine, and the hyponitrite precipitated as the yellow silver salt[5]. Aqueous solutions of the free acid were obtained by grinding the silver salt, in an ice-cold mortar, with the calculated quantity of 0.05 *N* hydrochloric acid and filtering off the silver chloride. Solutions prepared in this way produced no colour with diphenylamine, and no immediate response to starch-potassium iodide reagent, although in the latter test a faint blue colour developed after a few minutes. The intensity of this colour was practically independent of the age of the aqueous solution, so that the initial solution was essentially free from nitrite and did not produce appreciable concentrations of nitrite on standing in the cold. Quantitative tests on 10^{-3} *M* $H_2N_2O_2$ showed that the nitrite present

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increased from 10^{-6} % to $7.6 \cdot 10^{-6}$ % in 48 h. The free acid does not decompose in a manner analogous to that suggested for moderately concentrated solutions of the sodium salt[9], *viz.*

$$3\,H_2N_2O_2 + 2H_2O \longrightarrow 2HNO_2 + 4H_2O + 2N_2$$

but more nearly in accordance with the reaction:

$$H_2N_2O_2 \longrightarrow N_2O + H_2O.$$

In the experiments recorded below, lasting less than six hours, the amount of nitrite formed is insufficient to interfere with fixation.

Analysis of the silver hyponitrite by determination of silver (as AgCl) and of nitrogen (by DUMAS' method) indicated purities of 99.6 % and 98.2 % respectively.

Low concentrations of hyponitrous acid were determined by the following modification of BLOM's test[10]. The test sample was adjusted to pH = 3 by the addition of 2 *N* hydrochloric acid in the amount indicated by a previously-determined neutralisation curve; at this acidity any nitrite present is destroyed. The hyponitrite was oxidised by iodine in the presence of sulphanilic and acetic acids for 10 minutes, after which the excess of iodine was destroyed by *carefully* adding aqueous sodium thiosulphate. α-Naphthylamine was added and the colour allowed to develop for 15 minutes before comparison with a standard nitrite disc in a Nessleriser.

In view of conflicting reports[10,11,12] on the reaction between iodine and hyponitrous acid, a quantitative investigation was made, the results of which showed that the oxidation follows closely the equation

$$5\,H_2N_2O_2 + O_2 \longrightarrow 2\,HNO_2 + 4H_2O + 4\,N_2O,$$

only one-fifth of the hyponitrite nitrogen being recovered as nitrite.

The rates of disappearance of hyponitrous acid from (a) aqueous solution, (b) 0.067 *M* phosphate buffer (pH = 7.3), (c) 0.012 *M* phosphate buffer (pH = 7.3), (d) the sterile medium used for the bacterial cultures[1], and (e) medium inoculated with the bacteria, were determined at 30° C over a wide range of initial concentration. The results are plotted, in Figs. 1, 2, and 3, as $\log_{10} C_t/C_0$ against time (where C_0 and C_t are the concentrations of hyponitrous acid initially and at time t respectively). The plots approximate to straight lines in every case; slight differences in slope are not very significant, except perhaps in the case of the more concentrated phosphate buffer. The reaction has first-order character over a wide concentration range. The constant, λ, in the usual relationship $C_t = C_0 \cdot e^{-\lambda t}$ varies from $7 \cdot 10^{-3}$ to $10 \cdot 10^{-3}$ min^{-1} (except for the high-phosphate buffer, in which the value is $4.1 \cdot 10^{-3}$ min^{-1}) corresponding to a half-life period of about 100 minutes. Fig. 3 shows that the decomposition is not accelerated by the bacteria—indeed rather the reverse.

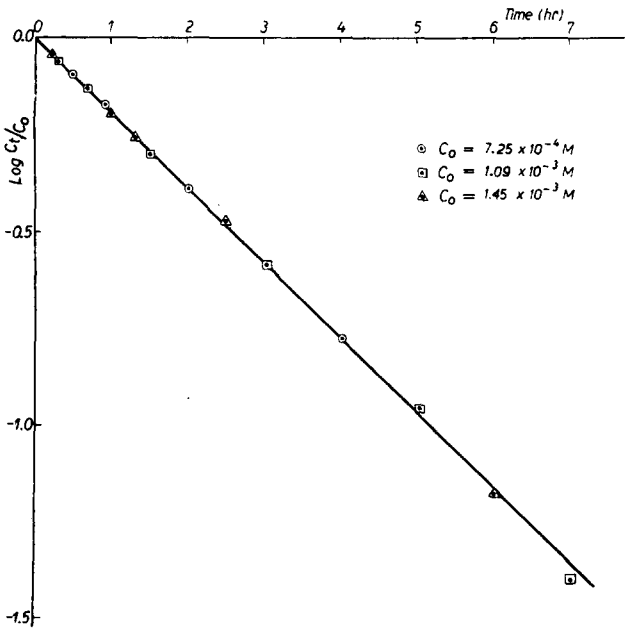

Fig. 1. Rate of disappearance of $H_2N_2O_2$ (aqueous solution)

The bacteria were repeatedly subcultured in the presence of hyponitrous acid, the concentration of the latter at each transfer being 10^{-3} *M*, but in no case was there any

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evidence of adaptation, the rate of disappearance of acid remaining unchanged. It is concluded that *A. vinelandii* does not, and cannot be trained to, use hyponitrous acid as a source of nitrogen.

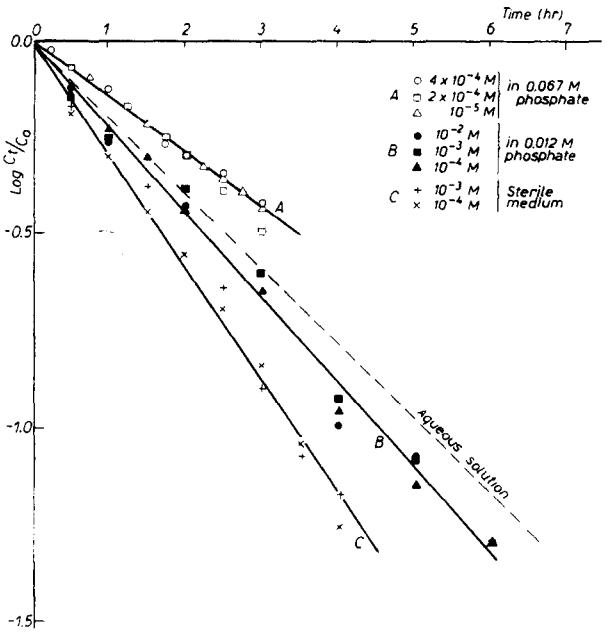

Fig. 2. Rates of disappearance of $H_2N_2O_2$

II. *The isotope experiments*

Identical cultures of *A. vinelandii* containing 10^{10} cells, prepared by subdividing a large culture as previously described[1], were made 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and $10^{-\infty}$ *M* with respect to hyponitrous acid by addition of the appropriate amount of the acid dissolved in sterile medium. An atmosphere of oxygen ($p_{O_2} \sim 0.5$ atmos.), labelled nitrogen ($p_{O_2} \sim 0.04$ atmos.), and argon ($p_A \sim 0.26$ atmos.) was circulated through all the cultures simultaneously and uniformly at a rate of about 100 l per hour. The oxygen and nitrogen were prepared as previously described[1]; argon was obtained from a cylinder, correction for its nitrogen content being made in making up the nitrogen tension. The cultures were kept at 30° C, and circulation continued for 3 hours, during which time the approximate measurement of oxygen uptake indicated that satisfactory aeration and growth occurred. The cells were killed by adding 5 ml of 0.2 *N* sulphuric acid, recovered by centrifugation, and converted to gaseous nitrogen[1] the ^{15}N content of which was measured. Samples of the atmosphere at the beginning and at the end of the experiment were similarly analysed and proved that no leakage of air into the system had occurred. The recovery of nitrogen as ammonia after the Kjeldahl treatment was determined, and found consistently to decrease as inhibition occurred.

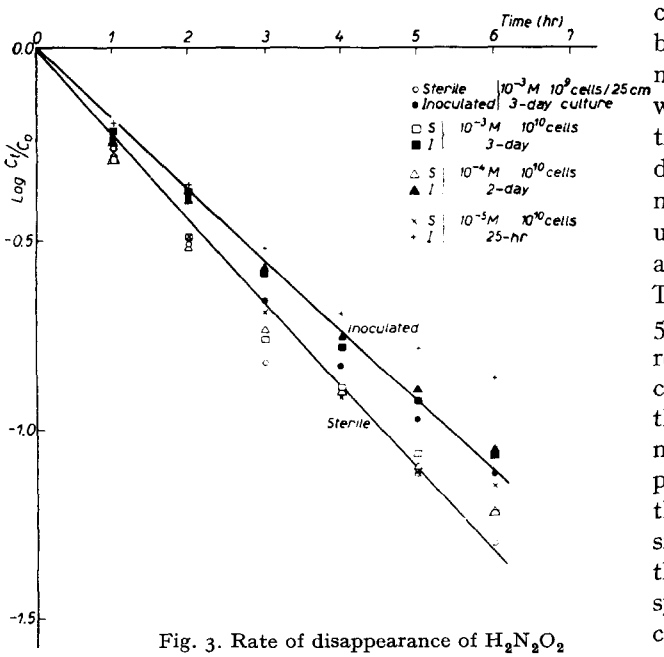

Fig. 3. Rate of disappearance of $H_2N_2O_2$

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The growth constants, k, were calculated from the equation

$$k = \frac{1}{t} \ln \left(\frac{I}{I-E} \right)$$

where t is the time in hours, and I and E the atom % excess over normal of ^{15}N in the circulating atmosphere and in the cells after time t, respectively[1]. Table I records the results of representative experiments; experiment I confirmed qualitatively the results of the respiration studies described below, but the isotopic abundances were too low for accurate quantitative conclusions to be drawn. The oxygen pressure employed, kept deliberately high to avoid oxygen starvation, was probably sufficient to inhibit growth and fixation; in all subsequent experiments, lower oxygen partial pressures were used and satisfactory uptakes of nitrogen observed. The ^{15}N values for air in experiments II and III (0.52 and 0.53 respectively) were taken as valid, since analysis of bacteria exposed to 10^{-2} M $H_2N_2O_2$ and of the original cultures before the run began gave in each case the value 0.53 atom % ^{15}N. The results from Table I are shown graphically in Figs. 4 and 5.

TABLE I

EFFECT OF $H_2N_2O_2$ ON N_2-FIXATION BY *A. vinelandii*

Atmosphere as shown circulated for 3 hours through 10^{10} cells in medium at 30° C.

Expt. No.	*Atmosphere circulated*			*Initial $H_2N_2O_2$ molarity*	*Atom % ^{15}N*			*k (growth constant)*	*$\frac{k}{k \text{ inhibited}}$*
	pN_2	*pO_2*	*pA*		*Ordinary air*	*Circulated atmosphere*	*Cells after 3 hours*		
I	0.04	0.60	0.21	0	0.37	12.80	0.44	0.0020	
				10^{-4}			0.44	0.0020	1.00
				10^{-3}			0.40	0.0007	3.00
				10^{-2}			0.38	0.0003	8.00
II	0.038	0.500	0.262	0	0.52	12.80	0.76	0.0066	
				10^{-5}			0.76	0.0066	1.00
				10^{-4}			0.75	0.0063	1.05
				10^{-3}			0.72	0.0055	1.20
				10^{-2}			0.53	0.0004	16.50
III	0.038	0.500	0.262	0	0.53	12.80	0.75	0.0061	
				10^{-3}			0.73	0.0055	1.11
				$2 \cdot 10^{-3}$			0.68	0.0041	1.49
				10^{-2}			0.53	0.0000	—

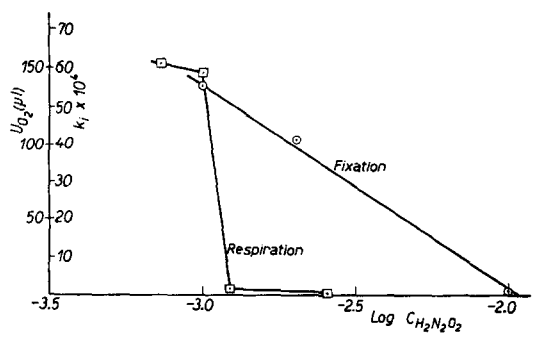

Fig. 4.

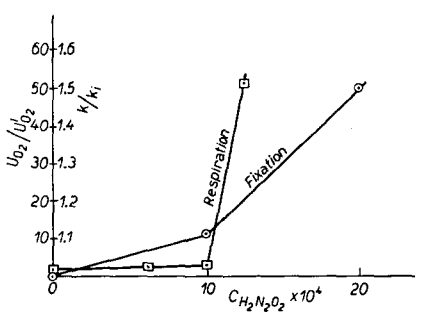

Fig. 5.

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III. *The respiration experiments*

Respiration rates of cultures of *A. vinelandii* were measured, using the WARBURG technique, the medium containing various concentrations of hyponitrous acid. Parallel measurements were made in which the medium contained, in addition to hyponitrous acid, a source of combined nitrogen known to be utilised. Preliminary experiments designed to determine a suitable concentration of this source revealed that relatively low concentrations of AnalaR sodium nitrate or AnalaR ammonium acetate entirely stopped both growth and respiration for a period dependent on the concentration of these salts. Table II summarises the effects observed with ammonium acetate.

TABLE II

EFFECT OF AMMONIUM ACETATE ON GROWTH AND RESPIRATION OF *A. vinelandii*

% w/v of combined N	*Effect*
0.0005	Fixation stopped; respiration unaffected
0.001	Respiration decreased
0.05	Respiration almost stopped
0.10	Respiration stopped for 4 hours
0.20	Respiration stopped indefinitely

Nitrate-nitrogen had less immediate effect, but after a short time produced the same results as the equivalent amount of ammonia-nitrogen. When, after initial cessation, respiration recommenced, addition of more combined nitrogen had no further effect. This phenomenon has been more extensively studied and will form the substance of a later communication.

Table III records typical observations of the inhibition by hyponitrous acid of *A. vinelandii* respiration, and shows also the negligible effect on this inhibition of the presence of a source of combined nitrogen (ammonium acetate).

TABLE III

EFFECT OF $H_2N_2O_2$ ON UPTAKE OF O_2 (IN μl)

Warburg method; air atmosphere; 30° C; $2 \cdot 10^7$ cells of *A. vinelandii* in 2 ml of medium; pH = 7.3; 0.2 ml of 10% KOH in centre wells (+N signifies addition of 50 μg of A.R. ammonium acetate per flask)

Time (min)	*Initial molarity of $H_2N_2O_2 \times 10^3$*								
	0	0.5	0.5 +N	1.0	1.0 +N	1.25	1.5	1.5 +N	2.5
30	30	26	24	22	24	2	2	0	0
60	62	54	50	44	46	3	2	0	0
90	93	82	80	70	72	3	2	0	0
120	126	112	108	98	102	3	6	2	0

DISCUSSION

The results obtained in the respirometric experiments are clear and unequivocal. Hyponitrous acid has no appreciable influence on the process unless its concentration exceeds a value close to 10^{-3} *M*, but above this critical value inhibition is virtually complete. The inhibition is irreversible, since no recovery of respiration rate is observed

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after the passage of time sufficient for the amount of inhibitor present to have fallen well below the critical value (using the data of Figs. 1 to 3 for calculating the concentration drop with time). The inhibition is not specific to nitrogen fixation, since the presence of a small concentration of a source of combined nitrogen has no effect on the cessation of respiration. The isotope experiments, however, show that when the concentration of hyponitrous acid is about 10^{-3} M, fixation is not sharply stopped (as is respiration) although considerable inhibition occurs. Indeed some fixation is observed even at hyponitrous acid concentrations of $2 \cdot 10^{-3}$ M, and is only completely inhibited when concentrations approaching 10^{-2} are present. In view of the irreversibility of the inhibitor with respect to respiration, this finding is all the more striking, and suggests that the fixation process is considerably less sensitive to hyponitrous acid than is respiration. The broad conclusion is, however, that this substance is an irreversible, non-competitive inhibitor of *A. vinelandii*, and is not specific to fixation. This fact, and the inability of the organism to utilise the acid, make it appear most unlikely that it is an important intermediate in the fixation process. It is of interest that ALLEN AND VAN NIEL[13] concluded that neither hyponitrous acid nor nitrous oxide was an intermediate in denitrification.

If the scheme outlined above retains any significance, then the substance of formula $H_2N_2O_2$ cannot be hyponitrous acid. The behaviour of its dehydration product, nitrous oxide (which inhibits reversibly and competitively and is specific to fixation[14]), has still to be explained. While it is true that nitrous oxide is also the dehydration product of nitramide, hydroxylamine nitrite, and ammonium nitrite, it may be that it inhibits by "steric fit" on nitrogenase, as has been suggested[15]. This aspect is being studied as well as the role of nitramide.

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SUMMARY

The rates of disappearance of hyponitrous acid from various aqueous media have been measured, at 30° C, over a wide range of initial concentration. The reaction shows first-order character, the half-life period being about 100 minutes.

The influence of hyponitrous acid on fixation, and on respiration, in *Azotobacter vinelandii* has been determined; the results establish the acid as an irreversible, non-competitive inhibitor, not specific to fixation, although the latter process is somewhat less sensitive to the inhibitor. It is not utilised. Hyponitrous acid is not believed to be a direct, important intermediate in the fixation process.

RÉSUMÉ

La vitesse de disparition de l'acide hyponitreux dans divers milieux aqueux a été déterminée, à 30° C et pour des variations étendues de la concentration initiale. La réaction est du premier ordre, le temps correspondant à 50% de disparition étant de 100 minutes.

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L'influence de l'acide hyponitreux sur la fixation et sur la respiration de *A. vinelandii* ont été déterminées; les résultats obtenus montrent que l'acide est un inhibiteur irréversible, non-compétitif, non spécifique de la fixation, ce dernier phénomène étant d'ailleurs moins influencé par l'inhibiteur. Il n'est pas utilisé. Les auteurs estiment que l'acide hyponitreux n'est pas un intermédiaire direct et important dans la fixation.

ZUSAMMENFASSUNG

Die Geschwindigkeit der Abnahme von Untersalpetriger Säure aus verschiedenen wässrigen Lösungen wurde bei 30° C über einen weiten Bereich der Anfangskonzentration gemessen. Die Abnahme zeigt den Charakter einer Reaktion 1. Ordnung, ihre Halbwertszeit beträgt 100 Min.

Der Einfluss der Untersalpetrigen Säure auf die Fixation und Atmung von *Azotobacter vinelandii* wurde bestimmt. Die Resultate ergaben, dass die Säure ein irreversibeler, nicht-competitiver Inhibitor ist. Er ist nicht spezifisch für die Fixation, obgleich der letztere Prozess weniger empfindlich gegen den Inhibitor ist. Die Säure wird nicht verwendet. Untersalpetrige Säure scheint kein direktes, bedeutendes Zwischenprodukt des Fixationsvorganges zu sein.

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