STUDIES IN THE BIOLOGICAL FIXATION OF NITROGEN

X. DISAPPEARANCE OF HYDRAZINE FROM CULTURES OF *A. VINELANDII*

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SUMMARY

The effect of hydrazine on the growth of *Azotobacter vinelandii* has been studied. It was found that small inocula fail to multiply in media containing hydrazine and that addition of hydrazine to proliferating cultures arrests growth. Both inhibitory effects are reversible.

The rate of hydrazine disappearance from cultures of *A. vinelandii* depends directly on the density of the culture and is independent of initial concentration. The disappearance is due to the formation of a non-toxic, acid labile, hydrazine compound which accumulates in the medium surrounding the cells. Two coloured reaction products with picryl chloride, believed to be the picryl derivatives of this hydrazine compound, and a small amount of pyruvic acid were also isolated from such culture supernatants.

INTRODUCTION

One of the most important aspects of the problem of biological nitrogen fixation is the identification of the intermediate compounds and reactions through which molecular nitrogen is transformed into ammonia and thence into organic nitrogen compounds. If a reaction scheme is envisaged, in which all the individual steps are reductive, then hydrazine must be an intermediate, thus,

$$N_2 \xrightarrow{2H} \underset{\text{"diimide"}}{[NH = NH]} \xrightarrow{2H} \underset{\text{hydrazine}}{NH_2{-}NH_2} \xrightarrow{2H} 2NH_3$$

This theory led to investigations of the effects of hydrazine on *Azotobacter*[1-3] and other fixers[4]. BURK AND HORNER[1] have shown that it inhibits growth in *A. vinelandii* and that it cannot be utilised as a source of nitrogen by the organism. However, AZIM[2,5] found that cultures of *Azotobacter* caused hydrazine to disappear from the surrounding medium at rates greater than could be accounted for by aerial oxidation. Recently BACH[3] using heavy suspensions of resting cells observed a similar interaction.

This investigation is a re-examination of the phenomenon of "disappearance" and the changes brought about in *A. vinelandii* cultures by the addition of small amounts of hydrazine.

EXPERIMENTAL

Analytical

Hydrazine: Two methods were used to estimate hydrazine in the supernatant culture liquids. The azine method is based on the formation of a yellow reaction product with *p*-dimethylaminobenzaldehyde in aqueous alcoholic solutions under acid conditions[6]. The procedure used was that of WALT AND CHRISP[7]. Optical densities were measured on a Hilger "Spekker" photoelectric absorptiometer using Bright Spectrum Filter 601 and 1-cm cells. Beer's law is obeyed over the range 0 to 2.0 p.p.m., slight deviations becoming apparent at higher concentrations. The picryl chloride method is based on the reaction of hydrazine with picryl chloride to give hexanitrohydrazobenzene[8] which in weakly alkaline solutions assumes an intense violet colour. CHERKESSOV AND KUL'BERG[9] and RILEY[10] used this reaction to develop colorimetric methods but neither of these two methods was suitable in our case; the first makes use of a heterogeneous system in which the reagent is present in a fine suspension, whilst the second utilises non-aqueous solvents which cause the salts present in the bacteriological medium to precipitate out. The following procedure was adopted.

Reagent: 0.2 % w/v ethanolic picryl chloride. Freshly prepared solutions from pure material were colourless.

Buffer: Phosphate buffer of pH 7.28. Phosphate = 1 *M* (KH_2PO_4 = 3.81 %; K_2HPO_4 = 12.54 %).

Procedure: 4 ml of the test solution and 1 ml of buffer were placed in a 10-ml graduated flask. 5 ml of the reagent were added slowly and with shaking. The contraction in volume was made up with distilled water and the colour intensity measured after 5 min. It is important that the reagent is added to the buffer and not the other way round, as sudden mixing causes the picryl chloride to come out of solution as an oil. The pH of the solution, measured with a glass electrode, is 8.35 to 8.37, *i.e.* about 1 pH unit higher than that of the aqueous buffer. Under these conditions the colour develops within 5 and is stable for a further 25 min. Optical densities were measured on a Unicam S.P. 300 G.P. absorptiometer using a green filter.

Total hydrazine: Total hydrazine (*i.e.* free plus acid labile) was determined by the azine method after hydrolysing the sample in 3 *N* hydrochloric acid at 100° for 15 min. The hydrolysate was then diluted with distilled water until it was 1 *M* with respect to acid, the concentration required for the azine method.

Pyruvic acid: The pyruvic acid which accumulates in the supernatant culture liquids during hydrazine disappearance was estimated as 2,4-dinitrophenylhydrazone, according to the general procedures for the determination of α-keto acids[11,12]. In these methods ethyl acetate is commonly used to extract the hydrazones from the acid reaction mixture; this treatment also extracts appreciable amounts of the reagent which is present in excess. It was found that the subsequent extraction of the ethyl acetate layer with dilute aqueous sodium carbonate produced a reddish brown artifact which passed into the aqueous phase and interfered seriously with the analytical procedure. Washing of the aqueous alkali layer with ethyl acetate removed this interfering coloration; it also removed some of the pyruvic acid hydrazone, giving rise to irregular calibration curves. Table I below shows the overall recovery of pyruvate when using ethyl acetate (washed carbonate layer), ether and chloroform. Clearly, chloroform gives the best results.

TABLE I

Efficiency of the extraction methods					
Potassium pyruvate in mg	*Theoretical equivalent in PDH* mg/ml*	*O.D.***	*Measured O.D.*	*Per cent recovery*	*Extraction method*
3.69	7.84	1.144	0.682	59.6	Ethyl acetate (washed)
2.76	5.88	0.859	0.542	63.0	
1.84	3.92	0.573	0.381	66.5	
0.92	1.96	0.286	0.207	72.3	
3.00	6.38	0.930	0.720	77.5	Ether
2.25	4.78	0.700	0.428	61.1	
1.50	3.19	0.465	0.328	70.5	
0.75	1.59	0.231	0.195	84.5	
2.42	5.14	0.750	0.653	87.1	Chloroform
1.81	3.85	0.562	0.490	87.3	
1.21	2.57	0.375	0.326	86.9	
0.605	1.28	0.187	0.164	87.7	

* PDH, 2,4-dinitrophenyl hydrazone of pyruvic acid.
** Estimated from calibration curves using synthetic PDH repeatedly crystallised from ethyl-acetate (*trans* isomer only).

When estimating pyruvate in culture liquids by this method it was found that traces of other coloured compounds were also formed. The pyruvic acid hydrazone was separated from these by chromatographing in butanol–ammonia and eluting from the paper the slower spot of the two isomeric pyruvic 2,4-dinitrophenyl hydrazones (*trans* isomer[13]). The recovery in this step, determined with synthetic 2,4-dinitrophenyl hydrazone of pyruvic acid, repeatedly crystallised from ethyl acetate (pure *trans* isomer by chromatography), was 81 %.

Hence, the overall recovery of the method, including both the extraction and chromatographic steps, is $87 \times 81 = 71$ %. The concentration of pyruvic acid in the supernatant liquids was estimated from the amount of the *trans* isomer of the 2,4-dinitrophenyl hydrazone of pyruvic acid which was extracted and isolated by the above procedure, on the basis of a 71 % overall recovery.

Cell nitrogen: The amount of nitrogen contained in the cells was determined by the Kjeldahl method applied on a semi-micro scale. The cells contained in a known volume of culture were separated by centrifugation, suspended in sterile medium, re-centrifuged and then digested with sulphuric acid using mercuric oxide as catalyst. The ammonia formed was steam distilled over alkali, collected in dilute boric acid and titrated with hydrochloric acid.

Bacteriological

The strain of *A. vinelandii* used was the same as employed by previous workers in this laboratory[2,14–16] and was originally derived from the stock culture at the Technische Hoogeschool, Delft.

Medium: The organism was grown in a medium similar to the one used previously in this laboratory[2,14–16] the only difference being the addition of a small amount of citric acid (0.110 g/l) in order to increase the amount of iron in solution[17]. The slight

precipitate formed during the preparation of the medium was removed by filtration. The pH of this medium was 7.08 as opposed to 7.26 to 7.28 for the same medium containing no citric acid.

Hydrazine containing medium was prepared by adding, under aseptic conditions, small volumes of hydrazine sulphate previously sterilised by autoclaving. Potentiometric titration of the medium showed that hydrazine sulphate behaved like a monobasic acid; consequently, an equivalent amount of sterile sodium hydroxide solution was also added in order to neutralise the effects of hydrazine sulphate on the pH of the medium.

Cell counts: The number of cells per unit volume of culture was determined in a standard Hawksley Thoma counting chamber.

Cultures: In order to provide efficient aeration, the cultures were grown in Kjeldahl flasks shaken on a microid shaker at 30°. The rate of shaking was such as to maintain a completely broken surface on the culture with the liquid splashing up to the first third of the neck of the flask. Flasks of capacity 250 and 50 ml and containing 100 and 20 ml of medium respectively, were used for growth experiments. Under these conditions of shaking, the cells remained entirely dispersed and no pellicles were formed.

Kinetic experiments

Preparation of cultures: In these experiments hydrazine was added to the culture in its logarithmic phase of growth. Inocula were prepared by inoculating sterile medium (20 ml) in a Kjeldahl flask (50 ml capacity) from an agar slope and shaking at 30° for 24 h. 1 ml of this culture was then transferred to another Kjeldahl flask containing 20 ml medium and shaken for another 24 h. 1 ml of this culture (population 1 to $2 \cdot 10^9$ cells/ml) was then transferred to 100 ml of sterile medium in a 250-ml Kjeldahl flask and shaken at 30°. After 17–22 h, depending on the strength of the culture desired, a small volume of sterile hydrazine sulphate solution (1 % of total) and the equivalent amount of sterile sodium hydroxide solution were added, and shaking was continued at 30°.

Disappearance of hydrazine: The rate of hydrazine disappearance was determined by withdrawing samples at time intervals, centrifuging and estimating the hydrazine content of the clear supernatant by the azine or picryl chloride method. When using the azine method the sample was made 1 *M* in hydrochloric acid immediately on withdrawing from the culture flask in order to avoid any losses due to aerial oxidation of the hydrazine. When using the picryl chloride method the sample was made 0.015 *M* in acetic acid. This reduced the pH to about 4.7, which is sufficiently acid to prevent the aerial oxidation of hydrazine. This small amount of acid did not affect the final pH of the coloured solution or the intensity of the colour.

Pyruvic acid determinations: In some kinetic experiments, pyruvic acid was estimated, as well as cell nitrogen, cell number, and both total and free hydrazine. In these experiments the volume of the sample was 20 ml and it was pipetted directly into dilute acetic acid (5 ml of 0.075 *M*). The suspension was centrifuged until clear and the supernatant decanted and used as follows: 20 ml were pipetted into 2,4-dinitrophenyl hydrazine reagent for the pyruvic acid estimation; 2 ml, after suitable dilution, were used for "free" and "total" hydrazine determination.

The cells remaining in the centrifuge tube were suspended in sterile medium

(5 ml) re-centrifuged and transferred into a micro Kjeldahl flask for cell nitrogen determination.

Reactions of supernatant liquids from hydrazine disappearance experiments

The cultures used in these experiments were prepared as for the kinetic experiments. A sterile solution of hydrazine sulphate (1 to 2 ml of $5 \cdot 10^{-2}$ *M*) was added to the culture 15–24 h after inoculation and the culture was shaken at 30° for a further 6–8 h. At the end of this time all the hydrazine had disappeared. The culture was then centrifuged and the clear supernatant decanted.

Reaction with picryl chloride: Supernatant liquid was mixed with half its volume of a 0.2 % alcoholic solution of picryl chloride, a small amount of solid sodium bicarbonate was added and the solution was allowed to stand overnight at room temperature. The almost opaque brown reaction mixture was then reduced to about one third of its volume by distilling under vacuum at 30 to 40°, made about 1 *M* with respect to hydrochloric acid, and extracted with chloroform. The yellow chloroform layer was in turn extracted with aqueous sodium bicarbonate (2 %, w/v) giving a dark brown solution which was acidified and extracted into a small volume of chloroform. This final extract, which contained all the reaction products, was investigated by paper chromatography using 0.5 *M* ammonium hydroxide and butanol–ammonia as solvents. The other layers, remaining after extraction, were found to contain only picryl chloride and its hydrolysis product, picric acid.

Reaction with 2,4-dinitrophenyl hydrazine: The supernatant liquid was mixed with one fourth of its volume of 0.01 *M* 2,4-dinitrophenyl hydrazine in 5 *M* hydrochloric acid and the solution allowed to stand at room temperature overnight. The reaction mixture was then extracted two or three times with ether and then with ethyl acetate until completely colourless. The combined ether layers were in turn extracted with 10 % aqueous sodium carbonate giving a reddish brown solution. The extracted ether layer, which was still yellow, and the ethyl acetate layer were examined by paper chromatography, after suitable concentration, and found to contain nothing but 2,4-dinitrophenyl hydrazine.

The reaction products were recovered from the aqueous carbonate layer by acidifying and extracting into ethyl acetate. Chromatograms of this final concentrate consisted almost entirely of the two isomeric hydrazones of pyruvic acid. Blank systems using water, 1.5 % aqueous mannitol, sterile medium and culture liquids from cultures to which no hydrazine was added, extracted in the above way, were found to contain a number of coloured spots. The intensity of these spots relative to that of the pyruvic acid hydrazones was very low, and they were hardly noticeable in chromatograms of culture liquids in which hydrazine had disappeared.

Reaction with picryl hydrazine: Culture liquids (25 ml) were reacted with a 0.14 % alcoholic solution of picryl hydrazine (15 ml) under acid conditions (2.5 ml of conc. HCl) at room temperature. After removing most of the ethanol by distilling *in vacuo* at room temperature, the reaction products, and some of the excess reagent, were extracted by shaking with chloroform. Extraction of this chloroform layer into aqueous alkali did not lead to a further separation as picryl hydrazine is also extracted.

The reaction products were identified as the picryl derivatives of pyruvic acid by comparison with similar extracts from reaction mixtures containing potassium

pyruvate and picryl hydrazine, and with synthetic picryl hydrazone of pyruvic acid. This latter was prepared by dissolving powdered picryl hydrazine (50 mg) in one to two drops of anhydrous pyruvic acid and diluting with water (2 ml). The yellow crystalline precipitate was crystallised repeatedly from water. Picryl hydrazine was prepared from picryl chloride and hydrazine hydrate[18].

RESULTS

Growth in hydrazine containing media

Small inocula of *A. vinelandii* fail to multiply in media containing initial hydrazine concentrations greater than $7\text{-}9 \cdot 10^{-6}$ *M*. This inhibitory effect is reversible and, as the hydrazine concentration is reduced by aerial oxidation to non-toxic levels, growth appears in tubes which initially contained concentrations of up to 10^{-4} *M* in hydrazine. The presence of a supply of fixed nitrogen (5 mg of ammonium acetate nitrogen per 100 ml) does not counteract this inhibition.

In cultures which are aerated by vigorous shaking the rate of hydrazine disappearance due to aerial oxidation is not affected by the presence of small inocula (10^8 cells/100 ml). Appearance of growth is delayed until the concentration of hydrazine is reduced to a non-toxic level; thus, growth in media initially 10^{-4} and $1.5 \cdot 10^{-5}$ *M* was delayed by 30 and 12 h respectively. In the latter case growth was accompanied by a sharp drop in hydrazine concentration as can be seen from Table II.

TABLE II

DISAPPEARANCE OF HYDRAZINE FROM INOCULATED AND BLANK MEDIUM AT 30°

Solution A: Inoculated with 10^8 cells at time 0. Volume 100 ml. C_0, 0.0000153 *M* hydrazine.
Solution B: Blank. C_0, 0.0000132 *M* hydrazine.

Time (h)	*Solution A* C_t/C_0	*Solution B* C_t/C_0
0	1.00	1.00
3	0.87	0.84
6	0.85	0.82
9	0.79	0.77
12	0.77	0.72
26 "Initial"	0.05 After 17 h 0.62	0.37
33 "Initial"	0.06 After 17 h 0.64	0.25

Disappearance of hydrazine in dense cultures

By the azine method: By adding small amounts of hydrazine to cultures already in an advanced stage of growth (5 to 10 mg of cell nitrogen/100 ml) and by following the concentration of hydrazine in the supernatant by the azine method, rapid disappearance was observed. The half-life of the reaction depends on initial concentration (*cf.* curves A and B, Fig. 1) and on bacterial population (*cf.* curves B and C, Fig. 1).

The O.D. of every point in curves A, B and C was determined 15 min after adding the azine reagent. Normally this time is sufficient for the colour intensity to become constant. In this case, however, the O.D. of all points, except those for time 0, increased with time, reaching, in all cases, the same equilibrium value within 24–48 h. This quantitative reappearance of the colour could also be achieved by hydrolysing

the sample with acid prior to adding the *p*-dimethylaminobenzaldehyde reagent. In this way, curve D, which is a composite from points from curves A, B and C, was obtained.

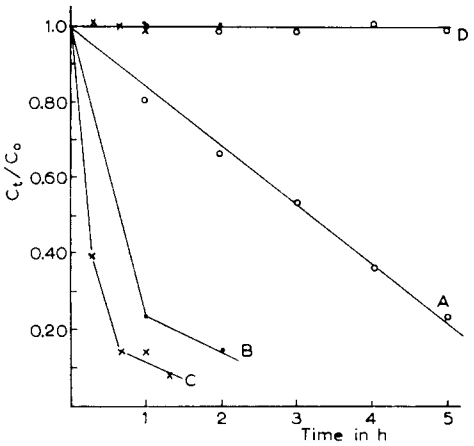

Fig. 1. Disappearance of hydrazine from cultures (by the azine method). A. $C_0 = 5.23 \cdot 10^{-4} M$; No. of cells/ml, $7.2 \cdot 10^8$; B. $C_0 = 1.04 \cdot 10^{-4} M$; No. of cells/ml, $8.0 \cdot 10^8$; C. $C_0 = 1.06 \cdot 10^{-4} M$; No. of cells/ml, $1.2 \cdot 10^9$; D. Points from A, B and C after acid hydrolysis.

The above results suggest that the observed disappearance of hydrazine is due to the formation of a compound which stays in the supernatant liquids, and from which hydrazine can be obtained quantitatively by acid hydrolysis. It is also worth noting that the presence of the bacteria protects hydrazine from aerial oxidation. Thus, the concentration of total hydrazine (*i.e.* free plus acid labile) is not appreciably different from the initial concentration for periods of time during which, in blank experiments using sterile medium, a 20 to 30 % reduction was observed.

By the picryl chloride method: A typical disappearance curve in which hydrazine was estimated by the picryl chloride method is shown in Fig. 2a. The O.D. of each point was determined 5 min after adding the picryl chloride reagent, this time being sufficient for the full production of the violet hexanitrohydrazobenzene. The accompanying Fig. 2b shows the subsequent variation in each point with time. However, in this case, the increase in O.D. is not due to the slow liberation of hydrazine by hydrolysis but to the production of new coloured compounds through a slow reaction between picryl chloride and other substances accumulating in the supernatant liquids. This was proved by allowing samples from various parts of the curve to react with picryl chloride for 12 h, extracting the products in suitable solvents and examining them by paper chromatography. Points from the limiting part of the disappearance curve showed a brown and an orange spot but no violet hexanitrohydrazobenzene; the initial sample contained only hexanitrohydrazobenzene while the intermediate points contained progressively more of the "brown" and "orange" components.

Corrected curves: Clearly, because of the contribution to the total colour made by the brown and orange components, disappearance curves obtained by the picryl chloride method do not give a true picture of hydrazine disappearance. Such curves can be corrected by subtracting the limiting value from the rest of the curve by proportional redistribution. The justification for such a correction is that the increase

in the O.D. of each point over an arbitrary period of time after the fifth minute (*i.e.* Δd_{5-15}), which is entirely due to the slow formation of the brown and orange components, varies inversely with the O.D. at the 5th min as shown below,

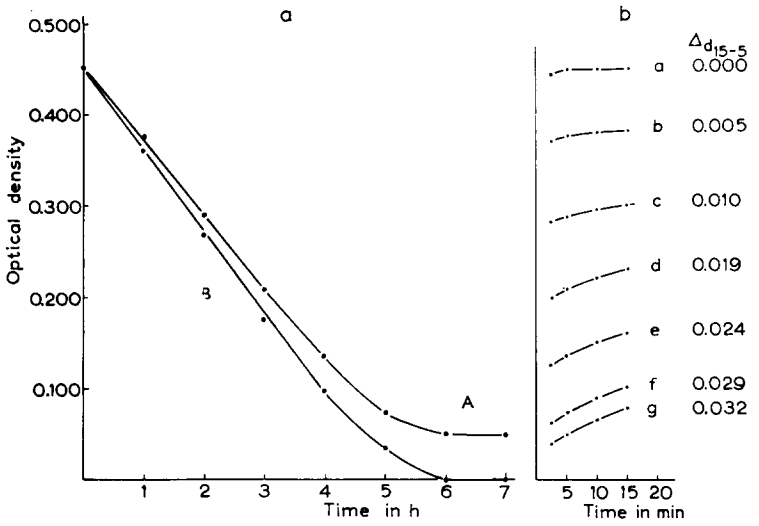

Fig. 2a. Disappearance of hydrazine by the picryl chloride method. Initial hydrazine concentration: $1.05 \cdot 10^{-3}$ *M*. Curve A. O.D. at the 5th min. Curve B. Corrected curve.
Fig. 2b. Variation in O.D. with time. Reagent added at time o.

O.D. at 5th min	Δd_{5-15}
0.450	0.00
0.380	0.005
0.290	0.010
0.210	0.019
0.130	0.024
0.080	0.029
0.050	0.032
0.050	0.032

It is reasonable to assume that the contribution due to these components at the 5th min (*i.e.* Δd_{0-5}) also follows the same relationship.

Curves corrected in this way are linear almost over the whole concentration range with a slope slightly greater than that of the uncorrected curves (*cf.* curve B, Fig. 2a).

Rate of hydrazine disappearance in relation to hydrazine concentration and bacterial population: The zero order reaction with respect to hydrazine concentration which is implied by the shape of the corrected curves was confirmed by measuring the initial rate of disappearance from cultures of identical strength at different hydrazine concentrations. As can be seen from Fig. 3, the time necessary to reduce the initial concentration by 5, 10 or 15 % is directly dependent on initial concentration. The linearity holds both for the corrected and uncorrected curves although the absolute rate is slightly different in the two cases. The cultures used in these experiments were of identical bacterial densities as they were obtained from the same master culture by subdivision.

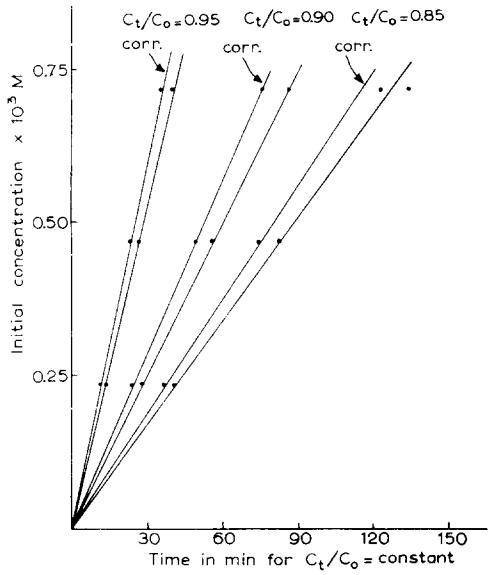

Fig. 3. Plots of C_t/C_0 = Constant, against initial hydrazine concentration.

The effect of bacterial density on the rate of hydrazine disappearance could not be demonstrated by such a direct experiment, as not only subdivision but volumetric dilution of the master culture with sterile medium would also be necessary. Such dilution led to lag periods and other irregularities which made the experiment impossible.

The relationship between rate of disappearance, initial hydrazine concentration and strength of culture is shown in Fig. 4. In curves A and B, $t_{0.50}$ and $t_{0.80}$ are plotted against C_0/N, where C_0 is the initial hydrazine concentration, and N is the nitrogen content, in mg, of the cells contained in 100 ml of culture.

The experimental points used for these curves were taken from Table III.

The relationship is linear, although the deviations shown by individual points are greater than those in Fig. 3. This is partly due to the additional experimental errors involved in estimating the nitrogen content of the cells, and partly because

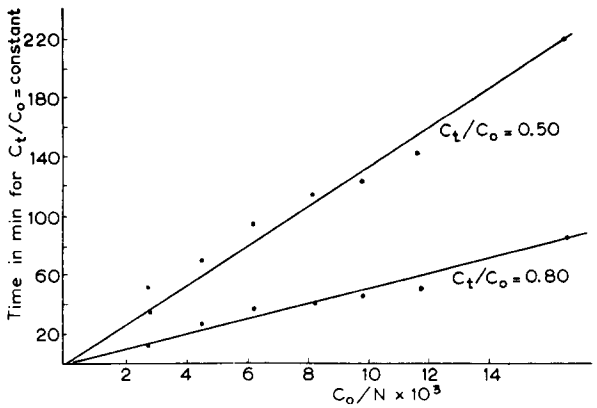

Fig. 4. Rate of hydrazine disappearance in relation to initial concentration and culture strength.

the results were obtained from experiments on individual cultures under, by necessity, slightly different experimental conditions. As the relationship between time t for C_t/C_0 constant, and C_0 is linear (*cf.* Fig. 3), it follows from curves A and B in Fig. 4 that the former quantity depends inversely on the strength of the culture, expressed as milligram of cell nitrogen per 100 ml.

The slopes of curves A and B are given by $N(1\text{-}K)/k$, where K is equal to 0.50 and 0.80 respectively, and k is the velocity constant of the zero order reaction. From the numerical value of the slope the rate constant of the reaction can be calculated as

$$k \text{ (Corrected)} = 4.0 \cdot 10^{-6} \text{ moles, litre}^{-1}\text{, minute}^{-1}$$

for a culture containing 10 mg of cell nitrogen per 100 ml; k varies directly with cell nitrogen content, at least over the range 5 to 12 mg of cell nitrogen per 100 ml.

From a similar plot, using values for $t_{0.50}$ and $t_{0.80}$ derived from the uncorrected curves, the value of $3.4 \cdot 10^{-6}$ was calculated for k (uncorrected).

Other effects of adding hydrazine to azotobacter cultures

Inhibition of growth: Addition of hydrazine to cultures of *A. vinelandii* arrests growth as measured by cell nitrogen content, total cell count or O.D. of the culture. Table IV shows the results from two typical experiments.

TABLE III

DISAPPEARANCE OF HYDRAZINE FROM *A. vinelandii* CULTURES USING THE PICRYL CHLORIDE METHOD

Culture	$C_0 \times 10^{-4}$ *M*	*N mg/100 ml*	$C_0/N \times 10^{-4}$	$C_t/C_0 = 0.50$ *Time (min)*		$C_t/C_0 = 0.80$ *Time (min)*	
				Exptl.	*Corr.*	*Exptl.*	*Corr.*
A	2.26	8.15	0.28	40	37	20	15
B	4.74	10.4	0.45	81	71	33	27
C	7.50	11.8	0.63	108	96	45	39
D	10.6	12.7	0.83	127	115	51	42
E	7.37	7.50	0.99	150	123	54	47
F	10.50	8.85	1.18	161	143	57	51
G	9.95	5.96	1.67	246	219	99	84
H	21.3	7.35	2.90	420		Abnormal	

TABLE IV

EFFECT OF HYDRAZINE ON GROWTH OF CULTURE AND ON PYRUVIC ACID CONTENT OF THE SUPERNATANT

Hydrazine added at time 0.

Time (h)	*Cell N mg/100 ml*	*O.D.* of culture*	*No. of cells/ml*	*Hydrazine concentration (corr.)*	*Pyruvic acid concentration*
Experiment 1					
0	7.40	0.430	$4.9 \cdot 10^8$	$7.37 \cdot 10^{-4}$ *M*	0.00
2	7.50	0.470	$5.6 \cdot 10^8$	$3.95 \cdot 10^{-4}$ *M*	$1.08 \cdot 10^{-4}$ *M*
4	7.40	0.465	—	$1.64 \cdot 10^{-4}$ *M*	$1.02 \cdot 10^{-4}$ *M*
6	7.55	0.450	$4.6 \cdot 10^8$	$0.26 \cdot 10^{-4}$ *M*	$0.93 \cdot 10^{-4}$ *M*
Experiment 2					
0	6.00	0.393	—	$9.95 \cdot 10^{-4}$ *M*	0.00
2	—	0.400	$3.6 \cdot 10^8$	$6.90 \cdot 10^{-4}$ *M*	$0.81 \cdot 10^{-4}$ *M*
4	6.20	0.397	$4.1 \cdot 10^8$	$4.65 \cdot 10^{-4}$ *M*	$0.73 \cdot 10^{-4}$ *M*
6	6.00	0.400	$3.3 \cdot 10^8$	$1.87 \cdot 10^{-4}$ *M*	$0.88 \cdot 10^{-4}$ *M*

* O.D. measured using 1-cm cells and a culture dilution of 4/5.

Growth, as measured by any of the above properties, is resumed after complete disappearance of hydrazine.

Production of coloured picryl derivatives: Supernatant liquids containing sequestered hydrazine react with picryl chloride to give dark brown solutions. Suitable extracts from such reaction mixtures were investigated chromatographically and found to contain the following spots:

Spot	R_F value			Colour	
	Solvent*			Acid	Alkali
	1	2	3		
a	0	0	0.96	Yellow	Orange
b	0.45	0.57	0.45	Yellow	Violet
c	0.55	0.66	0.88	Yellow	Brown
d	0.71	0.77	0.72	Yellow	Yellow

* Solvent 1: *n*-butanol–0.5 *M* ammonium hydroxide. Solvent 2: *n*-butanol–ethanol–0.5 *M* ammonium hydroxide (70:10:20). Solvent 3: 0.5 *M* ammonium hydroxide.

Of these spots b was identified as hexanitrohydrazobenzene by direct comparison with the reaction product of hydrazine and picryl chloride. This spot was only found in experiments in which hydrazine had not disappeared completely. Spot d was due to picric acid which is formed by the hydrolysis of picryl chloride.

Spots a and c are produced as a direct result of hydrazine disappearance and they are not found in blanks carried out with sterile medium, growth supernatant or supernatant to which hydrazine was added after the removal of bacteria by centrifugation.

If supernatant liquids containing sequestered hydrazine are hydrolysed with acid prior to reacting with picryl chloride, then no trace of spots a and c is found, the chromatograms consisting entirely of picric acid and hexanitrohydrazobenzene. This fact, which confirms the "reappearance" of hydrazine on acid hydrolysis observed by the azine method, also shows that the substances, which react with picryl chloride to give spots a and c are completely destroyed by acid.

Of the two coloured picryl derivatives, the orange decomposes slowly in the weakly alkaline chromatographic solvent. The brown, however, being more stable, can be eluted from the chromatograms and estimated colorimetrically; similarly, by eluting the violet hexanitrohydrazobenzene, an estimate can be made of the concentration of free hydrazine in the culture liquids. The values given in Table V below, obtained in this way, show that the concentration of the brown compound increases with decreasing concentration of free hydrazine.

TABLE V

Hydrazine added at time 0.

Time (h)	O.D. of eluted hexanitrohydrazobenzene spot × ¼	O.D. of eluted brown spot
0	0.410	0.000
2	0.220	0.080
4	0.145	0.230
6	0.025	0.420

In the same way, using cultures in which hydrazine had disappeared completely, it was found that the concentration of the brown component was independent of the density of the bacterial culture for a given initial hydrazine concentration, but that it varied, almost linearly, with initial hydrazine concentration.

Production of pyruvic acid: Supernatant liquids containing sequestered hydrazine were treated with 2,4-dinitrophenyl hydrazine under acid conditions and the products were extracted and investigated by paper chromatography using the standard procedures[11,12]. The original ethyl acetate layer, after extraction with sodium carbonate, contained only excess reagent while the sodium carbonate layer, on paper chromatography, was found to contain the two isomeric hydrazones of pyruvic acid. That the production of pyruvic acid is a direct result of hydrazine disappearance can be seen from Table VI below, which shows the O.D. of the carbonate layers, obtained under identical conditions, from the reaction mixtures of various blank systems with 2,4-dinitrophenyl hydrazine.

TABLE VI

Reaction: 2,4-dinitrophenyl hydrazine with		*O.D. (blue filter)*
1. Supernatant containing sequestered hydrazine		2.5·0.750
2. Supernatant containing no hydrazine	a	0.250
	b	0.248
3. Sterile medium	a	0.102
	b	0.125
4. 1.5% aqueous mannitol		0.070
5. Distilled water		0.068

The presence of pyruvic acid was confirmed by treating supernatant liquid containing sequestered hydrazine with an alcoholic solution of picryl hydrazine under acid conditions. Chloroform extracts of such reaction mixtures were investigated by paper chromatography and found to contain the following three spots,

Spot	*R_F value*		*Colour*			*Substance*
	*Solvent 3**	*Solvent 2**	*On paper (wet)*	*On paper (dry)*	*In NH_3 vapour*	
A	0.65	0.40	Orange	Yellow	Pink	Picryl hydrazine
B	0.76	0.60**	Brownish	Yellow	Brown	Due to pyruvic acid
C	0.85	0.20**	Brown	Yellow	Brown	Due to pyruvic acid

* Solvents as described for the chromatography of the picryl derivatives.
** These R_F values are approximate as substance is unstable in the chromatographic solvent and only short runs were possible.

Identical spots were obtained from chromatograms of synthetic pyruvic picryl hydrazone, or from the reaction mixture of pyruvic acid and picryl hydrazine treated under the same conditions as used when investigating the culture liquids.

Spot "B" decomposes slowly in solvent 2 to give some residue, which stays at the origin, and a long hydrolysis trail which contains spots A and C. The fact that

chromatograms of pyruvic picryl hydrazone, purified by repeated crystallizations from water, still contain picryl hydrazine, suggests that the latter is formed by the decomposition of the hydrazone.

It is significant that none of the reaction products with picryl hydrazine is identical with the "brown" or "orange" components observed in the picryl chloride reaction.

Production of pyruvic acid in relation to sequestered hydrazine and the picryl derivatives: Supernatant liquid containing sequestered hydrazine was treated with picryl chloride, and the brown and orange products were removed from the acidified reaction mixture by extracting with ethyl acetate. The remaining aqueous layer, on reacting with 2,4-dinitrophenyl hydrazine, was found to contain pyruvic acid.

Essentially the same experiment was repeated quantitatively. Three aliquots, A, B and C, of supernatant containing sequestered hydrazine were used. A was treated with picryl chloride and extracted with ethyl acetate until colourless. B was treated in exactly the same way but without using any picryl chloride, the purpose of this blank being to determine the amount of pyruvic acid lost into the ethyl acetate during the extraction of the coloured compounds. The extracted aqueous phases of A and B together with C (which was a normal aliquot, not treated in any way) were reacted with 2,4-dinitrophenyl hydrazine. By extracting the products, chromatographing, eluting the two isomeric hydrazones and determining their O.D. the following results were obtained:

Pyruvic hydrazone spots (solvent 2)	*Optical density of elute*		
	A	*B*	*C*
"Slow" (*trans*)	0.220	0.235	0.525
"Fast" (*cis*)	0.060	0.100	0.208

Clearly, solutions A and B contain substantially the same amount of pyruvic 2,4-dinitrophenyl hydrazone, but less than C, which was not submitted to an extraction process.

Using this method for determining the 2,4-dinitrophenyl hydrazone of pyruvic acid, the relationship between pyruvic acid and sequestered hydrazine concentration was investigated. As can be seen from Table IV, above, the production of pyruvic acid in cultures to which hydrazine is added, shows no relation to the hydrazine disappearance curve. The concentration of pyruvic acid is more or less constant after

TABLE VII

All concentrations measured 2 h after addition of hydrazine.

Culture	*Pyruvic acid* $\times 10^{-4}$ *M*	*Hydrazine concentration* $\times 10^{-4}$ *M*			*Cell nitrogen mg/100 ml*
		C_o	C_t	$C_o - C_t$	
1	1.08	7.37	4.00	3.37	7.4
2	0.81	9.95	6.93	3.02	6.0
3	0.81	7.50	2.85	4.65	11.9
4*	0.84	10.6	3.70	6.36	12.7

* Concentrations determined 2.5 h after hydrazine addition.

the second hour, and is at all times very small by comparison to the total, or sequestered hydrazine concentration.

From experiments carried out on different cultures no relation could be found between the amount of pyruvic acid formed and initial (C_0), free (C_t), or sequestered (C_0-C_t) hydrazine concentration; nor did the strength of the culture appear to have any significant effect on the pyruvic acid level, as can be seen from Table VII.

DISCUSSION

From the experimental evidence presented above it is clear that the observed hydrazine disappearance is in effect a detoxification process by which hydrazine is converted into an acid labile compound which accumulates outside the cells. The nature of this compound has not been elucidated, but the ease with which it liberates hydrazine on acid hydrolysis suggests that it contains hydrazine bound to an organic residue by a hydrazone ($R_1R_2C = NNH_2$) or hydrazide ($RCONHNH_2$) linkage. As hydrazine can react at both ends, ring formation is also possible; in such a case, a cyclic azine, hydrazide, or hydrazone-hydrazide may be formed.

Supernatant liquids containing bound hydrazine also contain some other compound or compounds which can react with picryl chloride to yield coloured picryl derivatives. The ability of these compounds to react with picryl chloride is destroyed under the acid conditions which liberate hydrazine from its bound form. Recently, HARRIS[19] has demonstrated that the converse is also true, *i.e.* formation of the picryl derivatives precludes liberation of hydrazine by acid hydrolysis. Moreover, the colour intensity of one of the picryl derivatives (brown) is directly proportional to the concentration of acid labile hydrazine. The above suggests that these compounds and "bound" hydrazine are identical, and that the coloured picryl compounds are in fact the picryl derivatives of sequestered hydrazine.

The accumulation of pyruvic acid in the surrounding medium on adding hydrazine to *A. vinelandii* cultures appears to be incidental to the phenomenon of hydrazine disappearance. The lack of stoichiometry between the concentration of pyruvic acid and acid labile hydrazine, and the simultaneous isolation of both pyruvic acid and the coloured picryl derivatives, show that pyruvic acid is not the organic acceptor responsible for binding hydrazine.

Failure to isolate any carbonyl compounds other than pyruvic acid, under conditions which liberate hydrazine, argues against the hydrazine being bound in a hydrazone or azine type of compound. Chromatographic investigation of synthetic N-acetyl picryl hydrazine[8], N-benzoyl picryl hydrazine[19] and the reaction product of α-glutamyl hydrazide with picryl chloride reveals two spots, one "orange" and one "brown" with R_F values similar to those of the picryl derivatives of sequestered hydrazine. The two coloured compounds are always present and appear to be either isomers, or else directly related to each other. The similarity between the chromatograms of the picryl derivatives of sequestered hydrazine and those of the three synthetic picryl hydrazides suggests that hydrazine disappears by combining with an acid residue to give a hydrazide.

The kinetic characteristics of the disappearance reaction, *i.e.* first order dependence on cell substance, and zero order with regard to hydrazine concentration, are consistent with an enzymic reaction in which hydrazine is used as a substrate.

These two conditions can also be satisfied by a scheme in which hydrazine reacts chemically with the products of an enzymic reaction of the cell,

$$\text{Enzyme} \longrightarrow X + NH_2\text{–}NH_2 \longrightarrow \text{sequestered hydrazine},$$

provided that the chemical reaction is fast and that the enzymic reaction leading to the formation of X is rate determining. These assumptions also imply that the system is in a "steady state" and that the concentration of "X" is much lower than that of free hydrazine.

It may be that the phenomenon of disappearance is directly responsible for the observed inhibition of growth in the presence of hydrazine. In such a case, inhibition could be due to competition of hydrazine for a metabolite vital to the growth of the organism. It is interesting to note that HARRIS[19] has found that ammonia can remove at least some of the inhibitory effects of hydrazine and at the same time reduce the rate at which hydrazine disappears from such systems. Competition of hydrazine with ammonia in enzymic amidation[20,21] reactions, and reactions involving imine formation is well known.

BACH[3] found that hydrazine disappeared from heavy suspensions of resting *Azotobacter* cells by combining with α-ketoglutaric acid to form 3,4-dihydropyrazinone-5-carboxylic acid (PCA) and other condensation products. However, the disappearance observed in our case must proceed by a different mechanism as no 2,4-dinitrophenyl hydrazone of α-ketoglutaric acid could be isolated from the acid hydrolysate of culture liquids containing sequestered hydrazine. Moreover, solutions of PCA, or solutions containing equimolar amounts of α-ketoglutaric acid and hydrazine, on treating with picryl chloride under the same conditions as used for the formation of the picryl derivatives of sequestered hydrazine gave no trace of the "brown" or "orange" compounds.

This difference in the mechanism of the disappearance reaction may be due to the fact that in BACH's experiments no outside supply of carbohydrate was available to the cells, whilst in our case mannitol was present, under conditions which inhibit the respiration of the culture only partially[2]. Clearly, in cultures in which oxidative glycolysis is taking place, organic acceptors (or the energy necessary for an enzymic reaction) may be available for the binding of hydrazine, which are not present in the resting systems used in BACH's experiments. That this may be the case is also suggested by the fact that the amount of hydrazine bound, over comparable periods of time, in relation to the quantity of the cell substance present, is much higher in our case (10^{-5} moles of hydrazine/mg of cell nitrogen as opposed to $3 \cdot 10^{-8}$ moles/mg of N in BACH's experiments).

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SOME PHYSICAL PROPERTIES OF DEOXYRIBONUCLEIC ACIDS DISSOLVED IN A HIGH-SALT MEDIUM: SALT HYPERCHROMICITY

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SUMMARY

1. The effects of very high salt concentrations upon the u.v. absorption, viscosity, and optical rotation of DNA were studied.

2. Concentrated NaBr (7.4 *M*) caused a pronounced hyperchromicity (measured at 260 mμ) which was entirely reversible when excess salt was removed. The increased absorption (salt hyperchromicity) was approximately linear with salt concentration.

3. Heating of a DNA solution in saturated NaBr produced a further increase in absorption which was irreversible and independent of DNA concentration.

4. Salt hyperchromicity, which was most pronounced in NaBr solutions, was less evident in other halide and alkali salts, and completely absent with LiCl solutions.

5. Gradually increasing NaBr concentration produced a loss in dextrorotation of DNA from an initial value of about + 117 degrees to a final value of + 67 degrees at 7.4 *M* salt concentration. The rate of change in specific optical rotation was most pronounced in 4 to 5 *M* NaBr concentrations.

6. Concentrated NaBr solutions of DNA showed a striking loss in viscosity as compared with DNA solutions in 0.1 *M* salt.

7. Neutral salt concentration in excess of about 13 molal caused quantitative precipitation of DNA. Among the salts which were tested, precipitation of DNA resulted only from the use of LiCl solutions.

8. Some structural implications of these phenomena were discussed, and an attempt was made to compare the various types of hyperchromicity ("hydrolytic", "water", and "salt") in nucleic acids.