CCLIX. THE REDUCTION OF NITRATE TO AMMONIA BY *CLOSTRIDIUM WELCHII*

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MANY bacteria are able to reduce nitrate to nitrite and this reaction has long been used in the identification and classification of these organisms. Quastel *et al.* [1925] showed that the reduction took place as a result of a coupled reaction with some H donator (e.g. lactate) and it was demonstrated that certain facultative anaerobes could grow anaerobically in the presence of nitrate on media which, in the absence of nitrate, could only support aerobic growth. The nitrate replaces oxygen as the final H acceptor [Quastel & Stephenson, 1925]. Green *et al.* [1934], working with toluene-treated *Bact. coli* and formate and lactate as H donators, concluded that the H transfer to nitrate takes place via a carrier; the nature of the natural carrier is so far unknown. Organisms possessing the hydrogenase enzyme [Stephenson & Stickland, 1931] as well as the nitrate-activating enzyme can utilize molecular H_2 as reducing agent.

Stickland [1931] made a detailed study of the reduction of nitrates by suspensions of *Bact. coli*. With lactate or molecular hydrogen as donator nitrate was reduced quantitatively to nitrite. With toluene-treated cells the H_2 uptake was also theoretical. There was no indication of any further reduction of nitrite with either plain or toluene-treated cells.

Reduction of nitrate to NH_3 has been obtained by Stocklasa [1908] with *Azotobacter chroococcum* and *radiobacter* and by Stocklasa & Vítek [1905] with four other organisms. In growth experiments, with *azotobacter* and *radiobacter* on an inorganic medium plus mannitol and nitrate, there was a disappearance of nitrate and formation of nitrite and NH_3 both aerobically and anaerobically. The effect was most marked in the case of *radiobacter* where, after 20 days, the inorganic N fraction consisted solely of NH_3; there was also considerable denitrification. With washed suspensions of *Bact. coli* and nitrate Aubel *et al.* [1937] found a small production of NH_3 in addition to some nitrite, when glucose was used as H donator.

In the course of a general investigation of the enzymic make up of washed cells of *Cl. welchii*, hydrogenase was detected and nitrate was found to be reduced in the presence of H_2. The H_2 uptake was greatly in excess of that required for the formation of nitrite. The work described in the present paper shows that nitrate, nitrite and hydroxylamine are reduced quantitatively to NH_3 by H_2 in the presence of washed suspensions of *Cl. welchii*. Similar results have been obtained with one strain of *Bact. coli*. Whilst this work was in progress a further paper by Aubel [1938] appeared, in which the quantitative reduction of nitrite to ammonia by suspensions of *Bact. coli* with glucose as H donator was demonstrated.

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EXPERIMENTAL

Methods of estimation

(1) *Hydrogen.* H_2 uptakes were measured in Warburg manometers. CO_2 absorbers were placed in the inner tubes as the bacterial suspensions produced a little CO_2 when incubated. The main cup usually contained buffer and bacterial suspension and the substrate was tipped in from a side bulb after equilibration. The manometers were filled with H_2 or N_2 purified by passage over heated copper.

(2) *Ammonia.* NH_3 was determined on 1 or 2 ml. samples from the manometer vessels by the Conway & Byrne [1933] technique followed by nesslerization. Since NH_2OH ($\equiv 5\mu$g. N) produces a yellow opalescence and finally a dark precipitate in Nessler's reagent it was necessary to ascertain whether any distillation of NH_2OH occurred in the Conway apparatus. After distilling 56 μg. NH_2OH-N for 3 hr. at 37° the distillate gave no trace of reaction with Nessler's reagent, whilst 56 μg. NH_3-N were distilled quantitatively in 1 hr. at 37°.

(3) *Nitrite.* Nitrite was estimated colorimetrically by the Griess-Ilosvay reagent as described by Stickland [1931]. Standard solutions of nitrite were checked by this method or volumetrically with $KMnO_4$.

(4) *Hydroxylamine.* Tests for NH_2OH were made and standard solutions checked by oxidation with I_2 to NO_2 and estimation of the latter colorimetrically [Endres & Kaufman, 1937].

The dry wt. of bacterial suspensions was estimated by means of a photoelectric turbidimeter [Clifton *et al.* 1935].

Unless otherwise stated all experiments were carried out at 37° and in phosphate buffer *p*H 7·1 of final concentration 0·05–0·1 *M*. To facilitate comparison quantitative data are all expressed in μl., making the assumption, in the case of solids and liquids, that 1 g. mol. $\equiv$ 22·4 l. Thus 1 μg. NH_3-N, NO_3-N, NO_2-N or NH_2OH-N $\equiv 1{\cdot}6\mu$l. KNO_3 (B.D.H. "Analar") and $NaNO_2$ (Kahlbaum *pro analyse*) were used in this work. Hydroxylamine solutions were freshly prepared daily from the pure hydrochloride (Fraenkel and Landau) and neutralized to *p*H 7·1.

Growth of organisms

The strain of *Cl. welchii* used in this work was that of the National Collection of Type Cultures No. 273 *Bacillus welchii S.R. 9* and isolated by Robertson from a fatal case of gas gangrene in 1914. Cultures of this organism have a tendency to "rope" and to form rough colonies; the preparation of suspensions is then difficult. The following procedure (Robertson, personal communication) was adopted for the maintenance of stock cultures. The organism was plated anaerobically on tryptic caseinogen broth-agar, a smooth colony picked off, and, after a few rapid subcultivations through Robertson's meat medium, sown into a number of tubes of alkaline egg medium. The latter were incubated 48 hr. anaerobically, sealed and stored in the dark at room temp. A working stock culture for sowing bulk cultures was maintained by daily subcultivation from one tube of Robertson's meat to another and incubation for 10–12 hr. anaerobically. After 7–12 such subcultivations the culture usually "roped" and a new subculture from the stock on alkaline egg was taken. Every 4–6 weeks the organism was replated and a new stock of alkaline egg cultures put up.

For the preparation of suspensions 900 ml. tryptic caseinogen digest broth *p*H 7·5, containing a few pieces of meat from Robertson's meat medium, were autoclaved, cooled and sown at once with 1 ml. fluid from a 10 hr. culture on Robertson's meat medium. The culture was incubated at 37° in a McIntosh and

Fildes anaerobic jar for 10–12 hr. The following dry wt. determinations show that the growth obtained under these conditions is fairly uniform:

No. determinations	24
Mg. dry wt. organism/ml. culture:	
Range	0·15–0·27
Mean	0·21
Standard deviation	0·035

Longer incubation does not improve growth and the suspensions are less active. The culture was centrifuged, and the cells washed twice on the centrifuge with *M*/50 phosphate, *p*H 7·1; for washing the concentration of the organism should not exceed 1 mg. dry wt./ml., otherwise sedimentation is not sharp. The cells were finally suspended in water or buffer to give a concentration of *ca.* 10 mg. dry wt./ml. Addition of glucose to the culture medium gives even more rapid growth and greater bulk of organisms, but production of acid is very rapid and difficult to control and suspensions were less active from the point of view of the work in the present paper.

Two strains of *Bact. coli* have also been used: (*a*) *Escherichia coli* N.T.C. No. 86 (Strain I) and (*b*) a strain originating from the Bacteriological Department of Sheffield University (Strain II). Both were grown aerobically in flasks of tryptic caseinogen broth for 16 hr. at 37° and suspensions prepared in the usual way.

Experiments with *Cl. welchii*

The reduction of nitrate

The course of the H_2 uptake by suspensions of *Cl. welchii* in the presence of nitrate is shown in Fig. 1. There is a small blank H_2 output (rarely exceeding

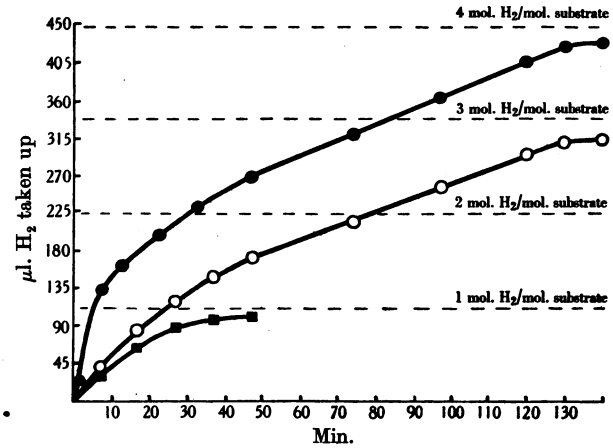

Fig. 1. Course of H_2 uptake. • nitrate, o nitrite, ■ hydroxylamine. 1 ml. bacterial suspension (6·2 mg./ml.), 1 ml. *M*/5 phosphate buffer *p*H 7·1, 0·1 ml. *M*/20 substrate, 0·4 ml. water.

15 µl./hr.) with the organisms alone; a correction for this has been made. It will be seen that there is an initial rapid uptake of H_2 equivalent to about 1 mol. per mol. NO_3 followed by a slower uptake which continues until approx. 4 mol.

H_2 have been absorbed and then ceases abruptly. The reduction of nitrate to nitrite requires only 1 mol. H_2:

$$HNO_3 + H_2 = HNO_2 + H_2O, \qquad \text{.....(1)}$$

whilst an uptake of 4 mol. H_2 would correspond to a complete reduction to NH_3:

$$HNO_3 + 4H_2 = NH_3 + 3H_2O. \qquad \text{.....(2)}$$

The presence of a volatile alkali giving the reaction with Nessler's reagent characteristic of NH_3 is readily demonstrated at the end of such an experiment. A number of experiments in which both H_2 uptake and NH_3 formation were estimated quantitatively have been carried out and the results of three such experiments are summarized in Table I. Controls were put up as follows: (*a*) without NO_3, (*b*) without H_2 (gas phase N_2), (*c*) without organisms. The quantitative data for the complete system NO_3-H_2-organism are in close agreement with the requirements of (2). The "H_2 uptake" in the N_2 gas phase experiments is due to the fact that there is a small H_2 evolution by the organisms alone in N_2 which is partially or completely suppressed in the presence of nitrate. It will be seen that there is also a small NH_3 production from nitrate in N_2; this production is always more than can be accounted for by the blank H_2 uptake in N_2 assuming that 4 mol. H_2 are required for formation of 1 mol. NH_3 (see last column $\frac{\mu l.\ H_2}{4}$ and cf. $\mu l.$ NH_3 found). It would seem, therefore, that some unknown H donators, apart from H_2, are present in the cell which can also reduce NO_3 to NH_3. It will be shown later that this effect is more marked in the case of NO_2 and NH_2OH.

Table I

Theoretical values are those required by equation (2). The organism blank has been corrected for where necessary. 1 ml. bacterial suspension (*ca.* 10 mg./ml.), 1 ml. *M*/5 phosphate buffer *p*H 7·1, 0·1 ml. *M*/20 or 0·2 ml. *M*/50 KNO_3, water to 2·5 ml.

System	Exp. no.	Gas phase	H_2 uptake ($\mu l.$) Found	Calc.	% calc	NH_3 production ($\mu l.$) Found	Calc.	% calc.	$\mu l.\ H_2$ found / 4
$NO_3 + H_2 + Cl.$ *welchii*	1	H_2	330	358	92	91	89·5	102	83
	2	H_2	443	448	99	115	112	102	111
	3	H_2	323	358	90	84	89·5	94	81
$NO_3 + Cl.$ *welchii*	1	N_2	9	358	2·5	6	89·5	6·8	2
	2	N_2	4	448	1·0	6·5	112	5·9	1
	3	N_2	26	358	7·3	9	89·5	10·0	7
$NO_3 + H_2$	1	H_2	0	358	0	0	89·5	0	—

Table II summarizes the results of a number of experiments on the quantitative relationship between H_2 uptake, NH_3 production and original NO_3 and shows close agreement with equation (2).

Table II

All values corrected for organism blank

	mol. H_2 per mol. NO_3	mol. NH_3 per mol. NO_3	mol. H_2 per mol. NH_3
No. of determinations	18	11	11
Range	3·58–3·96	0·89–1·03	3·52–4·29
Mean	3·75	0·98	3·81
Standard deviation	0·111	0·057	0·224
Theory (equation 2)	4·0	1·0	4·0

The reduction of nitrite

The initial rapid uptake of 1 mol. H_2 that occurs in the reduction of NO_3 (see Fig. 1) suggested that reduction of NO_3 to NO_2 might be the first stage in the production of NH_3 from NO_3. In this case NO_2 should also be reduced when incubated with suspensions of *Cl. welchii* in presence of H_2. This was found to be the case; a steady H_2 uptake reaching almost 3 mol. H_2 per mol. NO_2 being obtained (see Fig. 1). This would correspond to the final reduction of NO_2 to NH_3:

$$HNO_2+3H_2=NH_3+2H_2O. \quad \text{.....(3)}$$

NH_3 was again found to be present and Table III gives the results of three quantitative experiments. The data for the complete system are in good agreement with equation (3). As with NO_3 the production of NH_3 in N_2 is greater than can be accounted for by the H_2 uptake in N_2. The effect is more marked than with NO_3 owing to the larger NH_3 production in N_2.

Table III

Theoretical values are those required by equation (3). The organism blank has been allowed for where necessary. 1 ml. bacterial suspension (*ca.* 10 mg./ml.), 1 ml. *M*/5 phosphate buffer *p*H 7·1, 0·1 ml. *M*/20 or 0·2 ml. *M*/50 $NaNO_2$, water to 2·5 ml.

System	Exp. no.	Gas phase	H_2 uptake (μl.) Found	Calc.	% calc.	NH_3 production (μl.) Found	Calc.	% calc.	μl. H_2 found / 3
$NO_2+H_2+Cl.$ *welchii*	1	H_2	299	336	89	110	112	98	100
	2	H_2	255	269	95	85	89·5	95	85
	3	H_2	246	269	92	92	89·5	103	82
$NO_2+Cl.$ *welchii*	1	N_2	3	336	0·9	12	112	10·4	1
	2	N_2	6	269	2·2	7	89·5	8·0	2
	3	N_2	41	269	15·2	33	89·5	37·0	14
NO_2+H_2	1	H_2	0	336	0	0	112	0	—

A number of experiments in which the quantitative relations between the various reactants of the complete system were determined are summarized in Table IV. The close agreement with equation (3) is apparent.

Table IV

All values corrected for organism blank

	mol. H_2 per mol. NO_2	mol. NH_3 per mol. NO_2	mol. H_2 per mol. NH_3
No. of determinations	11	6	6
Range	2·53–2·86	0·95–1·03	2·49–3·0
Mean	2·70	0·99	2·69
Standard deviation	0·112	0·036	0·193
Theory (equation 3)	3·0	1·0	3·0

The reduction of hydroxylamine

The possibility that NH_2OH might be an intermediate in the reduction of NO_3 and NO_2 to NH_3 was at once apparent.

$$HNO_3+3H_2=NH_2OH+2H_2O \quad \text{.....(4)}$$
$$HNO_2+2H_2=NH_2OH+H_2O \quad \text{.....(5)}$$
$$NH_2OH+H_2=NH_3+H_2O. \quad \text{.....(6)}$$

It was found that NH_2OH was rapidly reduced with an uptake of approx. 1 mol. H_2 per mol. NH_2OH (see Fig. 1), and NH_3 was found present at the end of the

experiment. The results of three quantitative experiments are given in Table V and are in good agreement with (6). The production of NH_3 in N_2 is larger than with either NO_3 or NO_2 and again this NH_3 formation cannot be accounted for by the H_2 uptake in N_2 (see columns 3 and 6). The greater magnitude of the effect in this case may be due to the same amount of endogenous H donators being able to reduce 3 or 4 times as much NH_2OH as NO_2 or NO_3 respectively; alternatively, these donators may be able to reduce NH_2OH more rapidly than the other substrates. The almost quantitative H_2 uptake shows that the reduction by H_2 takes place preferentially to the reduction by cell donators.

Table V

Theoretical values are those required by equation (6). The organism blank has been allowed for where necessary. 1 ml. bacterial suspension (*ca.* 10 mg./ml.), 1 ml. *M*/5 phosphate buffer *p*H 7·1, 0·1 ml. *M*/20 or 0·2 ml. *M*/50 NH_2OH, water to 2·5 ml.

System	Exp. no.	Gas phase	H_2 uptake (μl.) Found	Calc.	% calc.	NH_3 production (μl.) Found	Calc.	% calc.
$NH_2OH + H_2$ + *Cl. welchii*	1	H_2	77	89·5	86	85	89·5	95·5
	2	H_2	75	89·5	84	91	89·5	101
	3	H_2	96	112	86	106	112	96
NH_2OH + *Cl. welchii*	1	N_2	2	89·5	2·2	18·5	89·5	20·7
	2	N_2	6	89·5	6·7	37	89·5	40·8
	3	N_2	4	112	3·6	25	112	22·7
$NH_2OH + H_2$	1	H_2	0	89·5	0	0	89·5	0

The quantitative relationships between the initial and final products of the reaction in a number of determinations are summarized in Table VI and agree fairly well with equation (6).

Table VI

All values corrected for organism blank

	mol. H_2 per mol. NH_2OH	mol. NH_3 per mol. NH_2OH	mol. H_2 per mol. NH_2OH
No. of determinations	13	6	6
Range	0·81–0·95	0·85–1·01	0·83–1·10
Mean	0·88	0·94	0·92
Standard deviation	0·046	0·054	0·094
Theory (equation 6)	1·0	1·0	1·0

Evidence that nitrite is an intermediate in the reduction of nitrate

The initial rapid uptake of about 1 mol. H_2 during the reduction of NO_3 suggests that there is a preliminary formation of NO_2 which is then further reduced. It is significant that, after the initial burst, the succeeding slower rate of H_2 uptake is equal, within experimental error, to the rate of H_2 uptake with NO_2 (Fig. 1); this result has always been obtained in experiments in which the same batch of organisms has been used with NO_3 and NO_2.

The comparative rates of NH_3 production from NO_3 and NO_2 provide further evidence that NO_2 is an intermediate. The rate of H_2 uptake with NO_2 is slower than the initial rate with NO_3; taking the figures for the first 7 min. of the experiment of Fig. 1 and dividing by 3 or 4 to correct for the difference in total H_2 uptake the resulting figures give a measure of the maximum possible rate of NH_3 formation:

	μl. H_2	μl. $H_2/4$	μl. $H_2/3$
NO_3	133	33	—
NO_2	40	—	13

If NO_2 is an intermediate the slower reduction of NO_2 should control the overall rate of NH_3 production from NO_3 and the rates of NH_3 production from the two substances should be equal. Fig. 2 shows that this is the case. For these determinations a series of manometers were set up containing NO_3, NO_2 or NH_2OH and bacterial suspension in a gas phase of H_2. The course of the reaction was followed by the H_2 uptake and at intervals manometers were rapidly disconnected, the cups plunged into ice-salt mixture to stop the reaction and NH_3 estimated on 2 ml. samples. Controls without substrate were carried out.

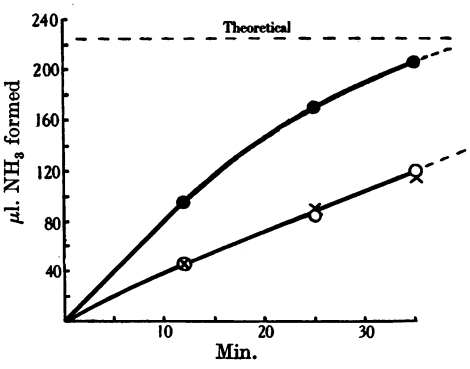

Fig. 2.

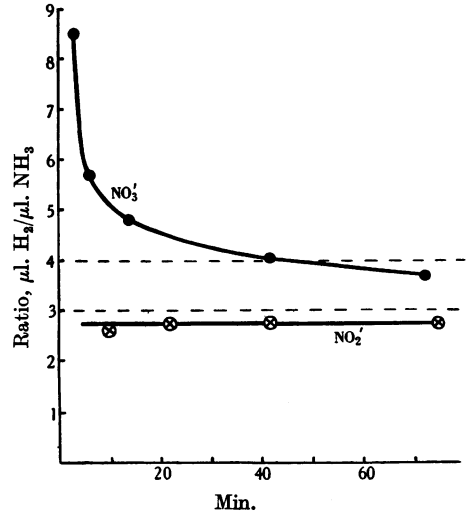

Fig. 3.

Fig. 2. Course of NH_3 production. × nitrate, o nitrite, • hydroxylamine. 1 ml. bacterial suspension (7·1 mg./ml.), 1 ml. *M*/5 phosphate buffer *p*H 7·1, 0·2 ml. *M*/20 substrate (224 μl.), 0·3 ml. water.

Fig. 3. Comparison of H_2 uptake with NH_3 production. 1 ml. bacterial suspension (10 mg./ml.), 1 ml. *M*/5 phosphate buffer *p*H 7·1, 0·2 ml. *M*/20 NO_3 or NO_2, 0·3 ml. water.

In Fig. 3 the ratio of H_2 uptake to NH_3 formation at various stages during the reduction of NO_3 is plotted. In order to obtain a more precise value for the H_2 uptake for the early samples it was necessary to stop the reaction by tipping in 0·2 ml. 10 % H_2SO_4 from a second side bulb as rapidly as possible after reading the manometer; even so the first two points may be up to 5 % lower than the true values. It will be seen that in the early stages the H_2 uptake is in excess of the 4 mol. required to reduce NO_3 to NH_3, but soon falls to approximately this value. The curve suggests that there may be an accumulation of a rapidly formed primary product of reduction (presumably NO_2) in the early stages. Experiments were carried out to test this possibility. A series of manometers containing NO_3 and *Cl. welchii* suspension in a H_2 gas phase were set up together with controls without NO_3. The NO_3 was tipped in from a side bulb after equilibration and the uptake of H_2 followed. At frequent intervals manometers were rapidly taken down and the cups plunged into ice-salt mixture. The contents were transferred to conical centrifuge tubes which were again cooled and then centrifuged at 0° till a sharp separation of the organisms was attained. 1 ml. of the clear supernatant was pipetted off (the organisms stir up easily) and used for nitrite

determination by the Griess-Ilosvay method. It was found impossible to use trichloroacetic acid as protein precipitant as there was a 20–30 % loss of NO_2 on acid treatment of quantities of the order found in these experiments. This loss was increased if the acid (though not the neutral) solution was filtered through kieselguhr.

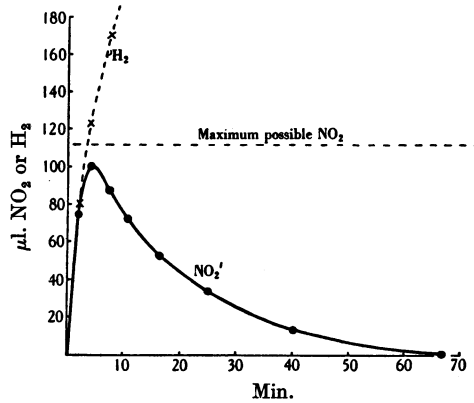

Fig. 4. Appearance and disappearance of nitrite. 1 ml. bacterial suspension (8·3 mg./ml.), 1 ml. *M*/5 phosphate buffer *p*H 7·1, 0·1 ml. *M*/20 KNO_3, 0·4 ml. water.

The curves given in Fig. 4 show the rapid production of NO_2 during the early stages and its removal as the reaction proceeds. The maximum NO_2 production reaches 89 % of that theoretically possible from the NO_3 added. During the period of rising NO_2 concentration the H_2 uptake is almost all accounted for by the NO_2 formed. Table VII shows that during the period of diminishing NO_2

Table VII

All values less organism blank

Time min.	μl. H_2 absorbed (*a*)	μl. NO_2 present	μl. H_2 to convert NO_2 into NH_3 (*b*)	(*a*) + (*b*)
2	80	75	—	—
4	123	100	300	423
7·5	170	87·5	262	432
11	211	72·5	218	429
16·5	263	53	159	422
25	321	34	102	423
40	383	13·5	40	423
70	421	0	0	421

concentration the H_2 already absorbed plus the H_2 required to reduce the NO_2 present to NH_3 agrees with the total H_2 uptake of the reaction. Thus, both qualitatively and quantitatively, these experiments show that NO_2 is formed as an intermediate product during the reduction of NO_3 by H_2.

Evidence in favour of hydroxylamine as an intermediate in the reduction of nitrite

It has already been shown that *Cl. welchii* reduces hydroxylamine to NH_3 by H_2 and the question arose as to whether it may be formed as an intermediate during the reduction of NO_2 and NO_3 (see equations (5) and (6)). If this is so it is necessary that the rate of NH_3 formation from NH_2OH should be at least as great as that from NO_3 and NO_2 with the same batch of organisms. Reference to

Fig. 2 shows that this is so, the rate in this particular experiment being twice that with NO_3 and NO_2. In nine similar experiments with different batches of organisms the ratio rate NH_3 from NH_2OH/rate of NH_3 from NO_2 varied from 1·2 to 4·6 (six values lay between 2·2 and 3·3) but was never less than 1. From this point of view therefore NH_2OH satisfies the conditions for an intermediate.

If NH_2OH is an intermediate it will be reduced to NH_3 as rapidly as it is formed and the rate of reduction of NO_2 to NH_2OH will set a limit to the overall rate of NH_3 production from NO_2; there would therefore be little possibility of a direct detection of NH_2OH during the reaction. This view is supported by data on the ratio H_2 uptake/NH_3 produced at various stages during the reduction of NO_2. Fig. 3 shows that this ratio remains constant at just below 3 so that throughout the reaction the H_2 uptake is completely accounted for by the NH_3 formed.

Attempts were therefore made by the addition of ketonic substances to trap any NH_2OH which might be formed. So far no fixative has been found which forms a sufficiently stable oxime and at the same time is not itself attacked by the organism. Diacetyl completely inhibited the reduction of NH_2OH to NH_3 and also brought about a partial inhibition of NH_3 formation from NO_3 and a corresponding partial inhibition of the H_2 uptake. The results are rendered somewhat unsatisfactory by the fact that diacetyl itself is decomposed by the organism with uptake of H_2; large corrections have therefore to be applied to the H_2 uptake figures and the possible effect on the NO_3 reaction of reduction products of diacetyl is unknown. Oxaloacetate, pyruvate and α-ketoglutarate formed insufficiently stable oximes.

Summarizing the position as regards the possibility that NH_2OH is an intermediate, it must be emphasized that whilst all the data so far obtained are in accordance with such a view, there is no conclusive evidence of its truth.

Effects of concentration of substrate, pH and age of suspension on rate of reduction of NO_3, NO_2 and NH_2OH

Concentration of substrate. Table VIII gives the Q_{H_2} values obtained for the 5 min. period immediately after tipping in the substrate. The value for NO_3 gives an approximate idea of the rate of the preliminary reduction of NO_3 to NO_2 as the period measured is within the initial burst of the H_2 uptake (Fig. 1).

Table VIII

All values corrected for organism blank. 1 ml. bacterial suspension (6·2 mg./ml.) 1 ml. *M*/5 buffer *p*H 7·1, 0·1, 0·2 or 0·5 ml. *M*/20 substrate, water to 2·5 ml.

	Initial Q_{H_2} values		
Conc. substrate	NO_3	NO_2	NH_2OH
0·01 *M*	−349	−95	−99
0·004 *M*	−339	−77	−66
0·002 *M*	−211	−49	−41

It will be realized from these figures that most of the quantitative work already described has been carried out with suboptimal concentrations (0·001–0·002 *M*) of substrate. This was done in order to be able to measure total H_2 uptakes without adding more H_2 and re-equilibrating during the reaction.

The figures of Table VIII also give some idea of the absolute activity of the organism in terms of dry weight. Q_{H_2} values of this order have always been obtained with fresh suspensions of organisms and are comparable in magnitude with Q_{O_2} values obtained with aerobic bacteria.

Age of suspension. The effect of storage in N_2 at 0° on the initial Q_{H_2} obtained with NO_3, NO_2 and NH_2OH is shown in Table IX. It will be seen that the activity is relatively stable for 10 hr., falls off seriously in 24 hr. and has almost disappeared in 46 hr. The activity falls off slightly more rapidly with NO_3 than with NO_2 and NH_2OH.

Table IX

All values less organism blank. 1 ml. bacterial suspension (8·6 mg./ml.; 1·7 mg./ml. with NO_3). 1 ml. *M*/5 buffer 7·1, 0·4 *M*/20 NO_3 or NH_2OH or 0·2 ml. *M*/20 NO_2, water to 2·5 ml.

	Nitrate		Nitrite		Hydroxylamine	
Age (hours)	Q_{H_2}	% Q_{H_2} at 2 hr.	Q_{H_2}	% Q_{H_2} at 2 hr.	Q_{H_2}	% Q_{H_2} at 2 hr.
2	−328	100	−70	100	−60	100
10	−293	90	−67	95	−57	95
24	−112	34	−30	43	−27	45
46	−4	1	−2	3	−2	3

Effect of p*H*. The *p*H curve with NH_2OH (Fig. 5) was rather unusual in that the rate of reduction increased steadily with rising *p*H throughout the range of phosphate buffer. Controls without bacterial suspension showed that there was no purely chemical reduction of NH_2OH at any *p*H used.

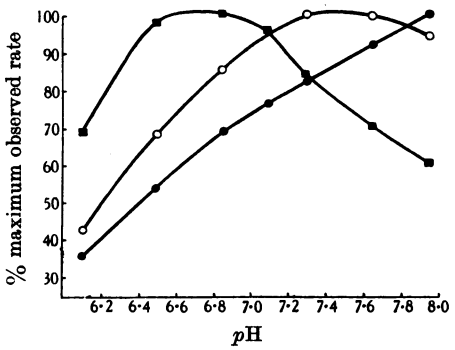

Fig. 5. Effect of *p*H. o nitrate, ■ nitrite, • hydroxylamine.

The relation between *p*H and rate of H_2 uptake with NO_2 is also shown in Fig. 5. The rates plotted are those for the first 5 min. period since at *p*H 6·1 and 6·5 the H_2 uptake fell off sharply and soon stopped completely (Fig. 6). It appears that the organism is rapidly inactivated by nitrite at *p*H 6·5 and below. At *p*H 7·95, although the initial rate of H_2 uptake is slower than at *p*H 6·5, there is no inactivation and the reduction proceeds to completion.

Experiments on the effect of *p*H on the initial rate of H_2 uptake with NO_3 yielded a curious result. It was found that below *p*H 7·3 there is a marked lag period of about 5 min. before the rate becomes linear (Fig. 7). This lag period is less marked or absent at *p*H 7·3 and above. This phenomenon has been observed with two other batches of organisms and no explanation can be offered for it. In the *p*H curve for NO_3 in Fig. 5 the rates are those for the second 5 min. period after tipping by which time the rate had become linear at all *p*H values. As with nitrite the reaction stops short of completion at *p*H 6·1 and 6·5, although complete inactivation took longer to occur. Thus the H_2 uptake became reduced to 1/10 of the initial value after 25 min. at *p*H 6·1 and after 35 min. at *p*H 6·5. As it

has already been shown that NO_2 is formed faster than it is removed this effect may be ascribed to the accumulation of NO_2. All the above experiments with NO_3 were carried out with 1/5 the usual concentration of organism (*ca.* 2 mg./ml.)

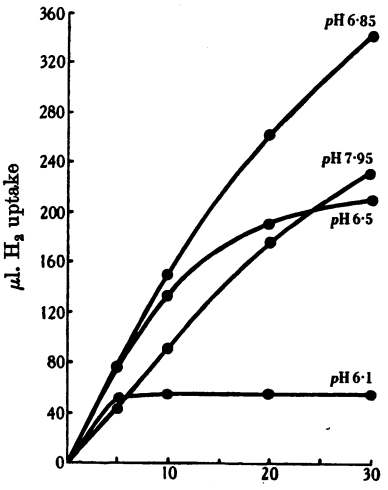

Fig. 6.

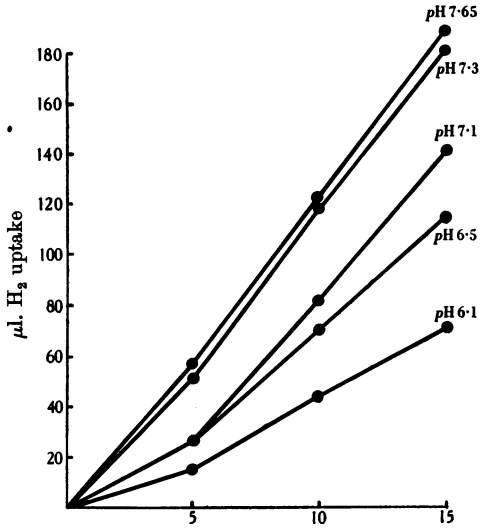

Fig. 7.

Fig. 6. Inactivation by nitrite at acid reaction. 1 ml. bacterial suspension (11·8 mg./ml.), 1 ml. *M*/5 phosphate buffer, 0·15 ml. *M*/20 $NaNO_2$, water to 2·5 ml.

Fig. 7. Lag period in H_2 uptake with nitrate. 1 ml. bacterial suspension (2 mg./ml.), 1 ml. *M*/5 phosphate buffer, 0·4 ml. *M*/20 KNO_3, water to 2·5 ml.

and a higher concentration of NO_3 (*M*/100) in order to confine the determinations to the initial rapid period during which the principal reaction is the reduction of NO_3 to NO_2.

Experiments with *Bact. coli*

Stickland [1931] has found that NO_3 is reduced quantitatively to NO_2 by H_2, in the presence of washed suspensions of *Bact. coli* (Esch.). His figures do not show any further reduction of NO_2. The chief experiments described above for *Cl. welchii* were repeated with two strains of *Bact. coli*; both reduced NO_3, NO_2 and NH_2OH to NH_3 in the presence of H_2 (Table XI), but quantitatively the rates of H_2 uptake with the various substrates varied greatly according to which strain was used (Table X). With strain II (see p. 2002) the rate of H_2 uptake, and

Table X

All values less organism blank. 1 ml. buffer *p*H 7·3, 0·1 *M*/20 substrate, 1 ml. bacterial suspension (exp. 1, 2·5 mg./ml.; exp. 2, 6·4 mg./ml.; exp. 3, 5·6 mg./ml.; exp. 4, 5·7 mg./ml.), water to 2·5 ml.

		Initial Q_{H_2}		
Strain	Exp. no.	NO_3	NO_2	NH_2OH
Strain II	1	−236	−248	−181
	2	−242	−253	−115
Strain I	1	−229	−45	−83
	2	−113	−6	−39

therefore NH_3 formation, is greater with NO_2 than with NO_3. Thus, unlike the case of *Cl. welchii*, NO_2 would be removed as fast as it is formed and the limiting factor in the rate of reduction of NO_3 to NH_3 becomes the rate of the preliminary reduction to NO_2. The rate of H_2 absorption by NH_2OH is also rapid and it follows from the data of Table X and equations (2), (3) and (6) that the rate of reduction of NH_2OH to NH_3 is even greater than that of NO_2 (as with *Cl. welchii*).

Quite different results are obtained with Strain I. Here the data resemble those for *Cl. welchii* more closely (see Tables VIII and X) except that the rate of H_2 uptake with NO_2 is slower.

Table XI

Theoretical values are those required by equations (2), (3) or (6). All values corrected for the organism blank. 1 ml. bacterial suspension (*ca.* 5 mg./ml.), 1 ml. *M*/5 phosphate buffer *p*H 7·3, 0·1 or 0·2 ml. *M*/20 substrate, water to 2·5 ml.

			H_2 uptake (μl.)			NH_3 production (μl.)			μl. H_2 found
Bact. coli	Substrate	Gas phase	Found	Calc.	% calc.	Found	Calc.	% calc.	1, 3 or 4
Strain II	NO_3	H_2	435	448	97	111	112	99	109
		N_2	1	448	0·2	0	112	0	0
	NO_2	H_2	328	336	97	114	112	102	109
		N_2	2	336	0·6	5	112	4·5	1
	NH_2OH	H_2	97	112	87	112	112	100	97
		N_2	1	112	0·9	24	112	21·3	1
Strain I	NO_3*	H_2	308	896	34·4	38	224	17	—
		N_2	14	896	1·6	14	224	6·3	—
	NO_2*	H_2	102	672	15·2	37	224	17	—
		N_2	18	672	2·7	6	224	2·6	—
	NH_2OH	H_2	97	112	87	113	112	101	97
		N_2	0	112	0	33	112	30	0

* Not taken to completion.

The quantitative data presented in Table XI show that with Strain II the H_2 uptake and NH_3 formation with NO_3, NO_2 and NH_2OH are in conformity with equations (2), (3) and (6). This is also true of Strain I with NH_2OH. Owing to the very slow reduction of NO_2 by the latter strain the experiments could not be taken to completion but the production of NH_3 and uptake of H_2 are demonstrated. Table XI also shows that with both strains NH_3 production in N_2 is similar to that obtained with *Cl. welchii* and the same possible explanations are applicable. It has not been possible to determine whether the failure of Stickland's [1931] strain to carry the reduction further than NO_2 is due to difference of strain or to different conditions of growth—both of which factors in influencing enzyme production were at that time imperfectly appreciated.

Discussion

The oxidations of NH_3 to NO_2 and NO_2 to NO_3 by the autotrophic soil organisms *nitrosomonas* and *nitrobacter* have long been known and considered as important stages in the circulation of N in nature. The reverse action—the reduction of NO_3 and NO_2 to NH_3—had until now only been demonstrated conclusively with *azotobacter* and *radiobacter* [Stocklasa, 1908] and for four other organisms [Stocklasa and Vítek, 1905]. The former are also soil organisms. The work reported in the present paper shows that such common organisms as *Cl. welchii* (one of the most widely distributed soil anaerobes) and some strains of *Bact.*

coli can also bring about this reduction energetically. It has been demonstrated that the reduction can be effected with the aid of molecular H_2; since *Cl. welchii* decomposes not only many carbohydrates but also some of the amino-acid constituents of tryptic caseinogen broth with evolution of H_2, a supply of this reducing agent would always be available. Furthermore, evidence was obtained that other unidentified H donators present in the cell can also bring about the reduction. Very recently Aubel [1938] has shown that washed suspensions of *Bact. coli* can reduce NO_2 (and therefore NO_3) to NH_3 using glucose as H donator. It is perhaps significant that glucose is fermented by *Bact. coli* with production of H_2. Lactate and succinate were unable to bring about the reduction of NO_2. Aubel also obtained some evidence that hyponitrite and hydroxylamine may be intermediates in the reduction of NO_2 by glucose with *Bact. coli*. The evidence is not very convincing as no data are given; it is simply stated "Il a été en outre possible de déceler des traces notables d'acide hyponitreux et d'hydroxylamine dans l'expérience *b*, en appliquant l'excellente technique de Lemoigne, Monguillon et Desvaux." It is not stated if HNO and NH_2OH can be reduced to NH_3 by this strain of *Bact. coli* in the presence of glucose.

It would seem that the reduction of NO_3 to NH_3 by bacteria is more general than supposed, and that the reduction must be seriously considered in assessing the importance of the oxidation of NH_3 to NO_3 by other micro-organisms in the general circulation of N in nature.

Summary

1. Washed suspensions of *Cl. welchii* are able to catalyse the reductions of nitrate, nitrite and hydroxylamine to NH_3 by molecular H_2.

2. During the reduction of nitrate an appearance and final disappearance of nitrite can be demonstrated. This and other evidence makes it clear that nitrite is an intermediate in the reduction of nitrate.

3. There is some evidence (though not conclusive) that hydroxylamine may be an intermediate in the further reduction of nitrite.

4. Two strains of *Bact. coli* tested also brought about the reductions of nitrate, nitrite and hydroxylamine to NH_3 in the presence of molecular H_2.

I wish to express my deep gratitude to Miss M. Robertson for directions as to the maintenance and growth of *Cl. welchii* cultures. I wish also to thank Dr P. Fildes for suggestions and Sir F. G. Hopkins and Dr M. Stephenson for their interest and advice.

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