Studies on the Oxidation of Ammonia by *Nitrosomonas*

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1. Free-energy calculations for pH 7 showed that the oxidation of ammonia to hydroxylamine is endergonic and that the oxidations of hydroxylamine to nitrite and hydrazine to nitrogen are exergonic. It is suggested that the oxidation of ammonia requires the expenditure of energy. 2. The anaerobic dehydrogenation of hydrazine to nitrogen by extracts of the autotrophic nitrifying micro-organism, *Nitrosomonas*, in the presence of methylene blue as electron acceptor, was less rapid than the anaerobic dehydrogenation of hydroxylamine to nitric oxide. The inhibition by hydrazine of the dehydrogenation of hydroxylamine was attributed to substrate competition. 3. Whole cells in air did not produce nitrite from hydrazine. They produced nitrite from low concentrations of hydroxylamine more rapidly than from equimolar concentrations of ammonia; this result is consistent if hydroxylamine is an intermediate of the oxidation of ammonia. 4. The production of nitrite from hydroxylamine by whole cells was slightly inhibited by hydrazine, but the production of nitrite from ammonia was greatly inhibited and small amounts of hydroxylamine were formed. These results suggested that the dehydrogenation of hydroxylamine supplied energy required for the oxidation of ammonia and that hydroxylamine appeared because the energy production was replaced by that of the dehydrogenation of hydrazine. 5. The oxidation of hydroxylamine by whole cells was not inhibited by thiourea, but micromolar concentrations of the metal-binding agent markedly inhibited the oxidation of ammonia to hydroxylamine, suggesting that the oxidation of ammonia involved copper. A possible mechanism for the activation of ammonia is suggested.

The autotrophic micro-organism *Nitrosomonas* derives its energy for growth from the oxidation of ammonia to nitrite. Hofman & Lees (1953) found that hydroxylamine was an intermediate in the stepwise oxidation of ammonia. The evidence was that hydrazine inhibited the formation of nitrite from hydroxylamine by whole cells. Hydroxylamine was formed from ammonia in the presence of hydrazine.

Similar results were recorded in a preliminary communication (Anderson, 1964b). Free-energy calculations showed that the oxidations of hydrazine to nitrogen and of hydroxylamine to nitrite were exergonic at pH 7 ($\Delta G_0'$ values: -144 and -69.1 kcal./mole respectively) but the oxidation of ammonia to hydroxylamine was endergonic ($\Delta G_0'$ value: $+3.7$ kcal./mole); it was suggested that the oxidation of ammonia required the expenditure of energy. The anaerobic dehydrogenation of hydrazine to nitrogen by extracts in the presence of methylene blue as electron acceptor competed with

the anaerobic dehydrogenation of hydroxylamine to nitric oxide (Anderson, 1964a). It was suggested (Anderson, 1964b) that the dehydrogenation of hydroxylamine supplies the energy required for the oxidation of ammonia and that hydroxylamine appeared because the energy production was replaced by that of the dehydrogenation of hydrazine.

The present paper reports further investigations on the formation of hydroxylamine from ammonia by whole cells. Extracts did not oxidize ammonia but the dehydrogenation of hydrazine by extracts paralleled that of hydroxylamine.

MATERIALS AND METHODS

Reagents. Reagents were the same grade as those employed by Anderson (1964a). Mammalian cytochrome *c* was type II (obtained from Sigma Chemical Co., St Louis, Mo., U.S.A.).

The pH was adjusted with HCl to prepare 0.2M-tris (pH 7.5–8.5) and 0.2M-2-amino-2-methylpropane-1,3-diol (pH 8.5–9.7) buffers; NaOH was used to prepare 0.2M-phosphate (pH 5.5–8.0) buffers from KH_2PO_4 solution.

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Methods

The methods employed were those of Anderson (1964a) with the following modifications.

Estimation of hydrazine. Hydrazine was estimated manometrically by quantitative oxidation to nitrogen with alkaline permanganate. Samples of enzymic reaction mixtures containing hydrazine were diluted to 2.3 ml. with water in a Warburg flask. After temperature equilibration at 30° for 5 min. with shaking at 80 strokes/min., 0.3 ml. of a solution of 125 mM-KMnO₄ in N-NaOH was added from the side arm. The formation of nitrogen from 1–7.5 μ moles of hydrazine sulphate was complete in 5 min. After subtracting the small gas change produced when hydrazine was replaced by water, the formation of gas, calculated as nitrogen, accounted for 103–107% of the hydrazine N added. Hydroxylamine was mostly oxidized to gas, but none was formed from cell material or 5 μ moles of nitrite, nitrate or ammonia. Since the concentration of hydroxylamine in samples was small (0.3 mM) the gas formed by its oxidation with permanganate was not taken into account. Experiments to investigate the metabolism of hydrazine were buffered with phosphate, since alkaline permanganate attacked tris or 2-amino-2-methylpropane-1,3-diol, resulting in diminished oxidation of hydrazine to nitrogen. The course of the oxidation of hydrazine shown in Fig. 2 includes the period of 5 min. in which the samples were temperature-equilibrated in Warburg flasks. In other investigations it was found convenient to perform the enzymic experiments in Warburg flasks shaken at 80 strokes/min. The reactions were stopped by adding 0.3 ml. of alkaline permanganate from the side arm.

Estimation of hydroxylamine. Hydroxylamine was estimated according to the method of Anderson (1964a). In certain experiments the colorimetric method of Frear & Burrell (1955) was used, except that the addition of trichloroacetic acid was omitted.

Estimation of dry weight. Whole cells were washed twice with water by centrifuging at 10000g for 10 min., and dried *in vacuo* over P₂O₅.

Preparation of extracts. Sucrose-phosphate, which was previously used as a suspending medium for cells during ultrasonic disintegration and for the preparation of extracts (Anderson, 1964a), was replaced by 20 mM-phosphate buffer, pH 8.0. The supernatant and particulate fractions were prepared by centrifuging the extracts at 144000g for 2 hr.

Incubation techniques. Experiments to investigate the formation of hydroxylamine were carried out at 30° in 100 ml. conical flasks containing buffer and washed-cell suspensions. Reactions in batches of incubation mixtures were initiated in serial order by adding solutions of hydrazine sulphate (adjusted to pH 8 with NaOH), followed after 2 min. by hydroxylamine hydrochloride (pH 8) or by ammonium sulphate.

Spectrophotometric experiments to investigate the reduction of mammalian cytochrome *c* were carried out with the supernatant fraction. The amounts used were small and contained negligible cytochrome-oxidase activity. The incubations were carried out in glass cuvettes with a 1 cm. light-path containing in 3 ml.: supernatant fraction, 0.5 μ -mole of mammalian cytochrome *c* and 200 μ moles of buffer. Reactions were started by adding solutions of hydrazine sulphate or hydroxylamine hydrochloride adjusted to pH 8. These substrates were replaced by water in the spectro-

photometric blank. The rates of reduction at 551 m μ were calculated from the initial linear portions of progress curves. Experiments to investigate the rate of anaerobic reduction of methylene blue were carried out *in vacuo* as described by Umbreit, Burris & Stauffer (1957) in 18 ml. Thunberg tubes, which fitted the Evans Electro-selenium Ltd. colorimeter. A calibration curve for 5–300 μ moles of methylene blue in 5 ml. of water was prepared by using a green filter (OGRI; maximum transmission 530 m μ) with water as the blank. The reaction mixtures contained in 5 ml.: extract, 200 μ -moles of buffer and 300 μ moles of methylene blue. After evacuation for 5 min. with a rotary vacuum pump the tubes were temperature-equilibrated at 20°. Reactions were started by adding solutions of hydrazine or hydroxylamine at pH 8 (2 μ moles) from the side arm. The dye and substrate were replaced by water in the control. The rates of reduction of the dye were calculated from the initial linear portions of the progress curves.

RESULTS

Experiments with whole cells

Relative rates of oxidation of ammonia, hydroxylamine and hydrazine. The production of nitrite by whole cells at pH 8 was optimum with 5 mM-ammonia when the concentration of ammonia was increased from 0.5 to 10 mM. This production decreased continuously when corresponding amounts of hydroxylamine were used as substrate (Fig. 1). The nitrite produced from 1 mM-hydroxylamine was 295 μ moles/ml./30 min., which was 74% greater than that produced from 1 mM-ammonia (170 μ moles/ml./30 min.). This finding is consistent with hydroxylamine's being an intermediate of the oxidation of ammonia. Equal rates

Fig. 1. Relation between nitrite formation by whole cells and the initial concentrations of hydroxylamine and ammonia. Incubation was for 30 min. in 100 ml. conical flasks containing in 2.5 ml.: whole cells (approx. 0.12 mg. dry wt./ml.) and 200 μ moles of phosphate buffer, pH 8.0. Reactions were started by adding hydroxylamine hydrochloride or ammonium sulphate. Nitrite production: ▲, from ammonia; ●, from hydroxylamine.

of nitrite production were obtained with about 2mM-hydroxylamine and -ammonia. The slow rate of metabolism of high concentrations of hydroxylamine may be attributable to the inhibition of metalloenzymes, e.g. the terminal oxidation system (cytochrome oxidase) of the dehydrogenation of hydroxylamine (Anderson, 1964a). The optimum concentration of ammonia measured by oxygen uptake in the Warburg was 6mM at pH 8.5. The uptake was $5.3 \mu\text{moles of oxygen/30 min./mg. dry wt.}$, which corresponds to Q_{O_2} 237. The oxidation of ammonia produced corresponding amounts of nitrite. When up to 2mM-hydroxylamine was used as substrate, the nitrite production accounted for more than 90% of the hydroxylamine disappearing.

The experiments recorded in Fig. 2 were performed at pH 8 in conical flasks shaken at 80 strokes/min. The oxidations of 2mM-ammonia and 2mM-hyd-

roxylamine were linear with time. The initial rate of oxidation of 2mM-hydrazine was less rapid but increased as the hydrazine disappeared; when the cells were replaced by boiled cells, negligible amounts of hydrazine disappeared. Hydrazine, like hydroxylamine, probably inhibited a terminal oxidation pathway. No nitrite or ammonia was produced, and no change in gas volume was found when hydrazine was metabolized aerobically in Warburg respirometers. Since the oxidation of hydrazine according to the reaction:

does not involve a net change of gas volume, the sole product of the reaction was nitrogen. Since the pK of the dissociation:

is 8, incubation mixtures at pH 8 contain equal amounts of the singly charged and uncharged forms.

Before investigation of the effect of hydrazine on the oxidation of ammonia, the effect of ammonia on the oxidation of hydrazine and hydroxylamine at pH 8 was examined. The rate of oxidation of 2mM-hydrazine was not affected by 8mM-ammonia. Table 1 shows that 5mM-ammonia did not affect the disappearance of 2mM-hydroxylamine. The nitrite production attributable to the oxidation of ammonia was 8% of that produced from ammonia alone. These results show that 2mM-hydroxylamine inhibited the oxidation of ammonia by 92%.

Effect of hydrazine on the oxidation of hydroxylamine and ammonia. Fig. 2 shows that 2mM-hydrazine partially inhibited the production of nitrite from 2mM-hydroxylamine (42% inhibition after 60 min.). The same concentration of hydrazine greatly inhibited the production of nitrite from 2mM-ammonia for the first 10 min. and small amounts of hydroxylamine ($110 \mu\text{moles/ml.}$) were produced. The dehydrogenation of hydrazine probably competed with that of hydroxylamine. If the dehydrogenation of hydroxylamine supplied the energy required for the oxidation of ammonia, then presumably hydroxylamine appeared because the energy production was replaced by that of the dehydrogenation of hydrazine. Identical formation of hydroxylamine was obtained when the specific estimation of Frear & Burrell (1955) was used to check the method of Anderson (1964a). The ammonia disappearing was equal to the production of hydroxylamine plus nitrite.

Effect of inhibitors on the oxidation of ammonia and hydroxylamine. The oxidation of 2mM-hydroxylamine was markedly inhibited by 0.2mM-cyanide, *o*-phenanthroline and diethyldithiocarbamate, but was less markedly inhibited by 0.2mM-2,2'-bipyridyl and EDTA (Table 2). Thiourea (0.2mM) was slightly inhibitory but in other experiments it stimulated

Fig. 2. Time-course of hydroxylamine formation by whole cells from ammonia in the presence of hydrazine. Incubation was in conical flasks containing in 7.5 ml.: whole cells (approx. 0.2 mg. dry wt./ml.) and 300 μmoles of phosphate buffer, pH 8.0. Reactions were started by adding hydrazine sulphate followed by hydroxylamine hydrochloride or ammonium sulphate. The flasks were shaken at 80 strokes/min. Nitrite formation ($\mu\text{moles/ml.}$): ■, from 2mM-hydroxylamine; △, from 2mM-hydroxylamine + 2mM-hydrazine; □, from 2mM-ammonia; ●, from 2mM-ammonia + 2mM-hydrazine. ○, Hydroxylamine formation ($\mu\text{moles/ml.}$) from 2mM-ammonia + 2mM-hydrazine. ▲, Hydrazine disappearance ($\mu\text{moles/ml.}$) (initial concn. 2mM).

Table 1. *Inhibition of the oxidation of ammonia by hydroxylamine*

Incubation was for 45 min. in conical flasks containing in 5 ml.: whole cells (approx. 0.16 mg. dry wt./ml.), 200 μ moles of phosphate buffer, pH 8.0, and additions as indicated. The amount of ammonia oxidized in the presence of hydroxylamine was calculated as the difference between the hydroxylamine disappearance and the nitrite production.

Preparation	Additions	Hydroxylamine disappearance (m μ moles/ml.)	Nitrite produced (m μ moles/ml.)	Ammonia oxidized (m μ moles/ml.)
Cells	Hydroxylamine (2 mM)	500	450	—
Cells	Ammonia (5 mM)	—	480	480
Cells	Hydroxylamine (2 mM) + ammonia (5 mM)	500	540	40
Boiled cells	Hydroxylamine (2 mM) + ammonia (5 mM)	35	0	—

Table 2. *Effect of metal-binding agents on the oxidation of hydroxylamine to nitrite by whole cells*

Incubation was for 60 min. in conical flasks containing in 5 ml.: whole cells (approx. 0.2 mg. dry wt./ml.), 200 μ moles of phosphate buffer, pH 8.0, and additions as indicated. The reactions were started by adding hydroxylamine hydrochloride (2 mM).

Preparation	Additions	Nitrite production (m μ moles/ml.)
Cells	None	860
Boiled cells	None	5
Cells	<i>o</i> -Phenanthroline (0.2 mM)	300
Cells	Cyanide (0.2 mM)	80
Cells	Diethyldithiocarbamate (0.2 mM)	335
Cells	2,2'-Bipyridyl (0.2 mM)	510
Cells	EDTA (0.2 mM)	800
Cells	Thiourea (0.2 mM)	840

the reaction by 7%. These metal-binding agents (0.2 mM) inhibited by 95% the oxidation of ammonia to nitrite in the absence of hydrazine, and to nitrite plus hydroxylamine in the presence of hydrazine. The system used was that described in Table 3. Table 4 shows that 0.3 μ M-thiourea inhibited the reactions by approx. 50%. These findings suggest that the oxidation of ammonia involves a metal.

The oxidation of ammonia to hydroxylamine plus nitrite in the presence of hydrazine, and to nitrite in the absence of hydrazine, was slightly inhibited by 0.05 mM-*p*-chloromercuribenzoate, 0.1 mM-iodoacetate and 1 mM-arsenite; the oxidation of hydroxylamine to nitrite was also slightly inhibited by these compounds (Table 3).

Carrier amounts of methylene blue catalysed the oxidation of hydroxylamine, but the nitrite formation (Table 3) was only 51% of the hydroxylamine disappearing, since nitrous oxide and nitrate are produced in these conditions (Anderson, 1964a). The dye markedly inhibited the oxidation of ammonia to hydroxylamine. Similar results were obtained when phenazine methosulphate, thionine or 2,6-dichlorophenol-indophenol replaced methylene blue. These dyes are in equilibrium with a reduced hydrogen carrier, possibly a flavine, of the

electron-transport chain of the dehydrogenation of hydroxylamine. The oxidation of the reduced dye produces peroxide. The decomposition of 0.9 mM-hydrogen peroxide, which was preincubated with whole cells, was complete in 5 min. The system oxidizing ammonia was found to be almost completely inactivated, but the oxidation of hydroxylamine was less markedly affected (Table 3). Carrier amounts of methylene blue did not increase the rate of oxidation of hydroxylamine by peroxide-treated cells. These results suggest that the inhibitions by dyes of the oxidation of ammonia are caused by the production of peroxide.

Effect of pH and of the concentrations of hydrazine and ammonia on the production of hydroxylamine. The optimum pH for the oxidation of ammonia (5 mM) was 8.6 (Fig. 3). Hydrazine (3 mM) inhibited the production of nitrite from ammonia throughout the range pH 6.6–9.5; the optimum pH values for the formations of hydroxylamine and nitrite were 8.6 and 7.2 respectively (Fig. 3). The formation of 285 m μ moles of hydroxylamine/ml. at pH 8.6 was the maximum found in the present work.

At pH 8, 1 mM-hydrazine inhibited the oxidation of 1 mM-ammonia to hydroxylamine plus nitrite by 58%, but the oxidation of 1 mM-hydroxylamine to

Table 3. *Inhibition of the oxidation of ammonia and hydroxylamine*

Incubation was for 30 min. in conical flasks containing in 2.5 ml.: whole cells (0.2-0.4 mg. dry wt./ml.), 200 μ moles of phosphate buffer, pH 8.0, and additions as indicated. Reactions were started by adding hydrazine sulphate followed by ammonium sulphate or by adding hydroxylamine hydrochloride. The oxidations of hydroxylamine and ammonia were studied with different suspensions of cells.

Preparation	Additions	5 mM-Ammonia as substrate			5 mM-Ammonia + 3 mM-hydrazine as substrate			2 mM-Hydroxylamine as substrate	
		Nitrite production (m μ moles/ml.)	Nitrite production (m μ moles/ml.)	Nitrite production (m μ moles/ml.)	Nitrite production (m μ moles/ml.)	Hydroxylamine production (m μ moles/ml.)	Ammonia oxidized (m μ moles/ml.)	Nitrite production (m μ moles/ml.)	
Cells	None	540	80	250	330	0	0	620	
Boiled cells	None	0	0	0	0	0	0	5	
Cells	<i>p</i> -Chloromercuribenzoate (0.05 mM)	350	130	145	275	145	275	480	
Cells	Iodoacetate (0.1 mM)	470	120	160	280	160	280	480	
Cells	None	635	240	200	480	200	480	550	
Cells	Arsenite (1 mM)	635	220	180	400	180	400	500	
Cells	Hydrogen peroxide (0.9 mM)	50	45	20	65	20	65	160	
Cells	Methylene blue (0.1 mM)	10	25	45	70	45	70	310

Fig. 3. Relation between pH and hydroxylamine formation by whole cells from ammonia and hydrazine. Incubation was for 30 min. in conical flasks containing in 5 ml.: whole cells (approx. 0.2 mg. dry wt./ml.) and 200 μ moles of phosphate, tris or 2-amino-2-methylpropane-1,3-diol buffer. Reactions were started by adding hydrazine sulphate (3 mM) followed by ammonium sulphate (2.5 mM). $\square$, Nitrite formation from ammonia; $\circ$, hydroxylamine formation from ammonia + hydrazine; $\bullet$, nitrite formation from ammonia + hydrazine.

nitrite was not affected (Fig. 4). In the presence of hydroxylamine as substrate, a high concentration of hydrazine (9 mM) caused the disappearance of a small amount of hydroxylamine (approx. 50 m μ -moles/ml.), which was not accounted for as nitrite. No hydroxylamine disappeared when the cells were replaced by boiled cells. Probably a factor, X, in the cells was reduced by the oxidation of hydrazine to nitrogen and oxidized by the reduction of hydroxyl-

Table 4. *Relation between the concentration of thiourea and the formation of hydroxylamine from ammonia by whole cells in the presence of hydrazine*

Incubation was for 30 min. in conical flasks containing in 5 ml.: whole cells (approx. 0.14 mg. dry wt./ml.) and 200 μ moles of phosphate buffer, pH 8.0. Reactions were started by adding hydrazine sulphate followed by ammonium sulphate.

Concn. of thiourea (μ M)	10 mM-Ammonia as substrate	10 mM-Ammonia + 3 mM-hydrazine as substrate		
	Nitrite production (μ moles/ml.)	Nitrite production (μ moles/ml.)	Hydroxylamine production (μ moles/ml.)	Ammonia oxidized (μ moles/ml.)
0	750	200	210	410
0.3	360	140	120	260
1.0	120	70	55	125
3	60	30	20	50
10	30	30	0	30

Fig. 4. Relation between the concentration of hydrazine and the rate of metabolism of hydroxylamine and ammonia by whole cells. Incubation was for 30 min. in conical flasks containing in 5 ml.: whole cells (approx. 0.2 mg. dry wt./ml.) and 200 μ moles of phosphate buffer, pH 8.0. Reactions were started by adding hydrazine sulphate followed by hydroxylamine hydrochloride (1 mM) or ammonium sulphate (0.5 mM). ▲, Nitrite formation from hydroxylamine; ●, nitrite formation from ammonia; ○, hydroxylamine formation from ammonia.

amine to ammonia, as indicated by eqns. (3) and (4):

At pH 8 the production of hydroxylamine from ammonia in the presence of 3 mM-hydrazine increased from 100 to 220 μ moles/ml. when the concentration of ammonia was increased from 0.5 to 5 mM, but no further increase was found when the concentration of ammonia was increased from 5 to 10 mM (Fig. 5).

Aleem & Lees (1963) reported that whole cells catalysed the reduction of mammalian cytochrome *c* by hydroxylamine. In the present work, hydrazine (2 μ moles) was about one-quarter as effective as 2 μ moles of hydroxylamine.

Experiments with extracts

Inability to metabolize ammonia. Extracts of whole cells, or whole cells that had been rapidly frozen at -20° and thawed at 30° , did not produce nitrite from ammonia. They produced traces of nitrite from hydroxylamine. The metabolism of hydroxylamine was catalysed by carrier amounts of methylene blue. The inability to oxidize ammonia may be the result of disruption of energy-coupling mechanisms.

Requirement for added electron carriers for the dehydrogenation of hydrazine. Spectrophotometric experiments showed that 2 μ moles of hydrazine reduced mammalian cytochrome *c* in the presence of the supernatant fraction 39% as rapidly as did 2 μ moles of hydroxylamine (Table 5). The reductions were inhibited by mepacrine and quinine. These inhibitions were overcome by FAD, which by itself stimulated the reductions. Anaerobically, 2 μ moles of hydrazine reduced methylene blue 57% as rapidly as did 2 μ moles of hydroxylamine (Table 6). These results suggest that the reduction of the bacterial cytochrome, with which the mammalian form is in equilibrium, involves a flavine. The dye is in equilibrium with a reduced hydrogen carrier of the respiratory chain, possibly the flavine. Hydrazine replaces hydroxylamine in these systems,

The slow oxidation of hydrazine to nitrogen in the Warburg was catalysed by carrier amounts of methylene blue (Table 7). Carrier amounts of cyto-

chrome *c* (0.1 mM) had little effect, since 40 mM-phosphate, which was used as buffer, inhibited the reaction. Anaerobically the formation of nitric oxide plus nitrous oxide (or nitrogen) from 7.5 μ -moles of hydroxylamine and 22 μ -moles of methylene blue was complete in 25 min. (Fig. 6). The initial rates of formation of nitrogen from 1.25, 2.5 and

Fig. 5. Relation between the concentration of ammonia and hydroxylamine formation by whole cells in the presence of hydrazine. Incubation was for 30 min. in conical flasks containing in 5 ml.: whole cells (approx. 0.2 mg. dry wt./ml.) and 200 μ -moles of phosphate buffer, pH 8.0. Reactions were started by adding hydrazine sulphate (3 mM) followed by ammonium sulphate. $\blacktriangle$, Nitrite formation in the absence of hydrazine; $\bullet$, hydroxylamine formation in the presence of hydrazine; $\circ$, nitrite formation in the presence of hydrazine.

Fig. 6. Inhibition by hydrazine of the anaerobic formation of gas from hydroxylamine by extracts in the presence of methylene blue. Incubation was in Warburg flasks containing in 2.5 ml.: extract (approx. 2 mg. of protein), 200 μ -moles of phosphate buffer, pH 8.0, and 22 μ -moles of methylene blue. Reactions were started by adding hydroxylamine hydrochloride and hydrazine sulphate from the side arm. The centre well contained 0.2 ml. of 2N-KOH. The atmosphere was nitrogen. Gas formation from hydrazine was calculated as nitrogen (μ -moles): A, from 1.25 μ -moles of hydrazine; B, from 2.5 μ -moles of hydrazine; C, from 3.75 μ -moles of hydrazine. Gas formation calculated as nitrous oxide was: D, from 7.5 μ -moles of hydroxylamine; E, from 7.5 μ -moles of hydroxylamine + 1.25 μ -moles of hydrazine; F, from 7.5 μ -moles of hydroxylamine + 2.5 μ -moles of hydrazine; G, from 7.5 μ -moles of hydroxylamine + 3.75 μ -moles of hydrazine.

Table 5. Effect of FAD, mepacrine and quinine on the reduction of cytochrome *c* by hydroxylamine and hydrazine

Incubation was in cuvettes containing in 3 ml.: supernatant fraction (approx. 15 μ g. of protein), 200 μ -moles of tris buffer, pH 8.5, 0.5 μ -mole of cytochrome *c*, and additions as indicated. Reactions were started by adding 2 μ -moles of hydroxylamine hydrochloride or hydrazine sulphate.

Preparation	Additions	Rate of reduction of cytochrome <i>c</i> (μ -moles/3 min.)	
		With hydroxylamine as substrate	With hydrazine as substrate
Supernatant fraction	None	75	29
Boiled supernatant fraction	None	3	3
Supernatant fraction	Quinine (0.5 mM)	39	11
Supernatant fraction	Mepacrine (0.3 mM)	51	20
Supernatant fraction	FAD (0.3 mM)	78	65
Supernatant fraction	Quinine (0.5 mM) + FAD (0.3 mM)	43	21
Supernatant fraction	Mepacrine (0.3 mM) + FAD (0.3 mM)	64	35

Table 6. *Rate of anaerobic reduction of methylene blue by hydrazine and hydroxylamine*

Incubation was in Thunberg cuvettes containing in 5 ml.: supernatant fraction (approx. 0.1 mg. of protein), 200 μ moles of 2-amino-2-methylpropane-1,3-diol buffer, pH 8.5, and 300 m μ moles of methylene blue. Reactions were started by adding hydrazine sulphate and hydroxylamine hydrochloride from the side arm.

Preparation	Additions (μ moles)		Rate of reduction of methylene blue (m μ moles/4 min.)
	Hydroxylamine	Hydrazine	
Supernatant fraction	2	0	210
Supernatant fraction	0	2	120
Supernatant fraction	2	2	115
Boiled supernatant fraction	2	2	5

Table 7. *Aerobic metabolism of hydrazine by extracts*

Incubation was in Warburg flasks containing in 2.5 ml.: extract (approx. 1 mg. of protein), 200 μ moles of phosphate buffer, pH 8.0, and additions as indicated. Reactions were started by adding 5 μ moles of hydrazine sulphate from the first side arm and were stopped by adding 0.3 ml. of alkaline permanganate from the second side arm. The atmosphere was air. The centre well contained 0.2 ml. of 2N-KOH or, for cyanide poisoning, a mixture of Ca(OH)₂ and Ca(CN)₂ (Robbie, 1948).

Preparation		Additions	Hydrazine disappearance (μ moles/flask)	
			At 30 min.	At 60 min.
Extract	None		0.7	1.3
Boiled extract	None		—	0.6
Extract	Methylene blue (0.2 mm)		3.5	5.0
Extract	Methylene blue (0.2 mm) + cyanide (1 mm)		0.9	1.6

3.75 μ moles of hydrazine were slow (about 0.9 μ -mole/15 min.). The reaction with 1.25 μ moles of hydrazine was complete in 35 min., but the reactions with 2.5 and 3.75 μ moles of hydrazine were not complete in 3 hr. When the extract was replaced by boiled extract, the nitrogen and nitrous oxide produced from 3.75 μ moles of hydrazine and from 7.5 μ moles of hydroxylamine were 0.3 and 0.4 μ -mole/3 hr. respectively. The total amount of nitrogen formed after adding alkaline permanganate to partially completed reactions was equivalent to the hydrazine added. Partial conversions of 5 μ -moles of hydrazine into nitrogen were obtained when 20 μ moles of thionine, phenazine methosulphate and 2,6-dichlorophenol-indophenol were used as electron acceptors. Occasionally, freshly prepared extracts completely metabolized 5 μ moles of hydrazine in the presence of methylene blue; the reactions were complete in 45 min.

After storage at 2° for a week, extracts and supernatant fractions lost about two-thirds of their ability to metabolize hydrazine and about one-third of their ability to metabolize hydroxylamine in the system given in Table 6. Structural changes of the enzyme possibly occurred during storage.

Effect of hydrazine on the metabolism of hydroxylamine. The oxidation of hydroxylamine in the presence of methylene blue as carrier produced only

small amounts of nitrite (Fig. 7), since nitrate and nitrous oxide are produced in these conditions (Anderson, 1964a). The disappearance of hydroxylamine and the production of nitrite were increasingly inhibited by 0.5–5 mm-hydrazine. Similar results were obtained when methylene blue was replaced by cytochrome *c* as carrier. The presence of 1.25, 2.5 and 3.75 μ moles of hydrazine produced increasing inhibition of the anaerobic gas formation from 7.5 μ moles of hydroxylamine in the presence of methylene blue (Fig. 6). In the presence of the supernatant fraction, hydrazine (2 μ moles) plus hydroxylamine (2 μ moles) reduced the dye at the same rate as did 2 μ moles of hydrazine alone (Table 6). These results suggest that the dehydrogenation of hydrazine competed with that of hydroxylamine.

Distribution of activity. Enzyme fractions obtained by centrifuging an extract that had been stored for 5 days at 2° were examined in the system given in Table 6. The rates of reduction of methylene blue by hydroxylamine and hydrazine respectively in the presence of the supernatant fraction were 210 and 65 m μ moles of dye/3 min./0.2 mg. of protein, and were greater than the rates of reduction found in the presence of the particulate fraction (50 and 15 m μ -moles of dye/3 min./0.2 mg. of protein). Hydrazine reduced the dye about one-third as rapidly as did hydroxylamine in the presence of either fraction.

Fig. 7. Relation between the concentration of hydrazine and the rate of oxidation of hydroxylamine by extracts in the presence of methylene blue. Incubation was for 45 min. in conical flasks containing in 5 ml.: extract (approx. 0.25 mg. of protein), 200 μ moles of tris buffer, pH 8, and methylene blue (0.05 mM). Reactions were started by adding hydrazine sulphate followed by hydroxylamine hydrochloride (1 mM). ●, Hydroxylamine disappearance; ○, nitrite formation.

Effect of inhibitors. Aleem & Lees (1963) found that the reduction of cytochrome *c* by hydroxylamine in the presence of extracts was stimulated by iron and copper and inhibited by metal-binding agents. In the following experiments the rate of anaerobic reduction of methylene blue in the presence of supernatant fractions or extracts at pH 8.5 was studied. The experiments with hydrazine contained up to three times as much protein as did those with hydroxylamine. Low concentrations of copper sulphate (1–3 μ M) stimulated the reactions by 5–10%. Slight inhibitions were obtained with 100 μ M concentrations of copper sulphate, ferrous sulphate, cobalt sulphate, nickel sulphate and manganese sulphate. The following compounds had negligible effects: 1 mM-thiourea, 1 mM-EDTA, 1 mM-diethyldithiocarbamate or 1 mM-8-hydroxyquinoline; 1 mM-arsenite, 0.5 mM-*p*-chloromercuribenzoate or 0.5 mM-iodoacetate; 10 mM-ammonia, 2 mM-nitrate, 2 mM-nitrite or 2 mM-*O*-methylhydroxylamine. The last compound was not metabolized. The reactions were inhibited by 0.2 mM-cyanide and 1 mM-2,2'-bipyridyl and slightly stimulated by 1 mM-GSH (Table 8). Cyanide inhibited the aerobic metabolism of hydrazine in the presence of methylene blue (Table 7). Preliminary attempts to remove metal ions from the supernatant fraction by dialysis with mechanical stirring at 2° for 14 hr. against a solution at pH 8.0 containing cyanide (20 mM), GSH (1 mM) and tris buffer (100 mM), followed by further dialysis against tris buffer, resulted in a negligible diminution of the activities when tested in the system given in Table 6.

Effect of pH and of substrate concentration. Extracts catalysed the anaerobic reduction of methylene blue by hydroxylamine or hydrazine from pH 5.7 to 9.5 (Fig. 8). At pH 8.5, which was the optimum for each reaction, the rate of metabolism of hydrazine was 60% of that of hydroxylamine.

Table 8. *Inhibition of the anaerobic dehydrogenation of hydroxylamine and hydrazine*

Incubation was in Thunberg cuvettes containing in 5 ml.: supernatant fraction, 200 μ moles of tris buffer, pH 8.5, 300 μ moles of methylene blue, and additions as indicated. Reactions were started by adding 2 μ moles of hydroxylamine hydrochloride or hydrazine sulphate. The experiments with hydroxylamine contained approx. 0.1 mg. of protein; those with hydrazine contained twice as much.

Preparation	Additions	Rate of reduction of methylene blue (m μ moles/5 min.)	
		With hydroxylamine as substrate	With hydrazine as substrate
Supernatant fraction	None	110	150
Boiled supernatant fraction	None	5	0
Supernatant fraction	2,2'-Bipyridyl (1 mM)	65	60
Supernatant fraction	GSH (1 mM)	145	175
Supernatant fraction	None	200	145
Supernatant fraction	Cyanide (0.2 mM)	110	65

Fig. 8. Relation between pH and the rates of anaerobic reduction of methylene blue by hydrazine and hydroxylamine. Incubation was for 2 min. in Thunberg cuvettes containing in 5 ml.: extract (approx. 0.4 mg. of protein), 200 μ moles of phosphate, tris or 2-amino-2-methylpropane-1,3-diol buffer and 300 μ moles of methylene blue. Reactions were started by adding 2 μ moles of hydroxylamine hydrochloride or hydrazine sulphate from the side arm. Rate of reduction of methylene blue: ●, by hydrazine; ○, by hydroxylamine.

Fig. 9. Relation between the rate of anaerobic reduction of methylene blue and the concentrations of hydrazine and hydroxylamine. Incubation was for 4 min. in Thunberg cuvettes containing in 5 ml.: extract (approx. 0.3 mg. of protein), 200 μ moles of tris buffer, pH 8.5, and 300 μ moles of methylene blue. Reactions were started by adding solutions of hydroxylamine hydrochloride or hydrazine sulphate adjusted to pH 8 from the side arm. Rate of reduction of methylene blue: ●, by hydroxylamine; ○, by hydrazine.

The rate of anaerobic reduction of methylene blue at pH 8.5 was maximal with 0.6 mM-hydroxylamine (Fig. 9). The K_m of this reaction was 1.1×10^{-4} M. No optimum was found when hydroxylamine was replaced by hydrazine; the rate of reaction with

0.04–2 mM-hydrazine was about 15% of the maximum found with hydroxylamine. This result might be attributable to the effects of storage of the enzyme.

DISCUSSION

The electron-transport chain of the dehydrogenation of hydroxylamine contains a cytochrome, which is replaceable by the mammalian form, and probably contains a flavine since the reduction of cytochrome *c* by hydroxylamine in the presence of extracts was inhibited by mepacrine and the inhibition was overcome by FAD. In these respects the metabolism of hydrazine paralleled that of hydroxylamine but, since no nitrite was formed in air, hydrazine is not an intermediate of the oxidation of ammonia. Methylene blue is in equilibrium with a reduced natural hydrogen carrier in the chain, possibly the flavine. Anaerobic experiments to study the reduction of the dye showed that the dehydrogenations of hydrazine and hydroxylamine were inhibited by 2,2'-bipyridyl and cyanide, and slightly stimulated by micromolar concentrations of copper salts. Since the preliminary dialysis experiments with cyanide did not affect the enzyme activities, further work is necessary to confirm that the system reducing the bacterial cytochrome contains a metal. Inhibition by hydrazine of the anaerobic dehydrogenation of hydroxylamine to nitric oxide may be attributable to substrate competition; possibly one of the amino groups of hydrazine replaces that of hydroxylamine. Since *O*-methylhydroxylamine was not metabolized it seems possible that the hydroxyl moiety of hydroxylamine is also bound by the enzyme. The finding that during storage extracts became less capable of metabolizing hydrazine may account for the inability to obtain an optimum concentration of hydrazine in Fig. 9 and for the slow rates of reaction found in previous work (Anderson, 1964a; Aleem & Lees, 1963).

The oxidation of ammonia to hydroxylamine plus nitrite by whole cells in the presence of hydrazine, and to nitrite in the absence of hydrazine, was almost completely inhibited by low concentrations of metal-binding agents. With the exception of the effects of cyanide, the oxidation of hydroxylamine was only partially inhibited. These results show that the oxidation of ammonia involves a metal. The 50% inhibition by 0.3 μ M-thiourea is in agreement with the 50% inhibition by 0.2 μ M-allylthiourea found by Hofman & Lees (1953). These authors suggested that the metal was copper.

Possibly hydroxylamine and hydrazine inhibited the oxidation of ammonia by maintaining copper in the reduced form, as indicated by eqns. (5) and (6):

Scheme 1. Hypothetical scheme for the relationship between the intermediates of the oxidation of ammonia to nitrite.

The oxidation of ammonia was sensitive to added peroxide and to that produced by the oxidation of reduced methylene blue. Gould (1959) found that substances which decompose peroxide, e.g. catalase, peroxidase, ferric salts and dithionite, increased the initial rate of growth of cultures of *Nitrosomonas* that were contaminated with only very small numbers of heterotrophic bacteria.

Lees (1960) suggested that the inability of extracts to metabolize ammonia was caused by disruption of energy-coupling mechanisms when the cell was disintegrated. Possibly the freezing of cells disrupts these mechanisms. Anderson (1964b) suggested that the activation of ammonia required a form of 'high-energy' oxygen, which was produced by the oxidation of a reduced hydrogen carrier, YH, of the electron-transport chain of the dehydrogenation of hydroxylamine. The decomposition of peroxide to oxygen and water is exergonic ($\Delta G_0'$ value at pH 7: -25.2 kcal.; Anderson, 1964b), but it seems unlikely that peroxide is the energy source since it inhibited ammonia oxidation. A possible mechanism for the activation of ammonia (eqn. 9) involves the reduction of cupric copper (associated with the oxidation of ammonia) by YH (eqn. 7); activated oxygen, (OH), is then formed from cuprous copper and oxygen (eqn. 8):

Cuprous copper could also be produced according to eqns. (5) and (6). The formation of nitroxyl, (NOH), in eqn. (5) is based on chemical reactions (Anderson, 1964c). If eqn. (5) were enzymic then presumably nitroxyl could be metabolized to nitrite.

The oxidation state of nitrogen in nitric oxide differs from that in nitrite by one unit of charge, which suggests that the oxidation of ammonia to nitrite involves a series of single-electron steps. Since the intermediates, hydroxylamine and nitric oxide, contain single nitrogen atoms it seems likely that the other intermediates also contain single nitrogen atoms, as shown in Scheme 1. Aleem &

Lees (1963) found that *Nitrosomonas* oxidized nitrohydroxylamine, H₂N₂O₃, to nitrite. It is assumed that nitrohydroxylamine replaces nitric oxide since the oxidation state of nitrogen in each compound is the same. Nitroxyl, however, is not replaceable by hyponitrite since the latter is not metabolized by *Nitrosomonas* (Lees, 1954; Anderson, 1964a). The natural intermediates in Scheme 1 are presumably in equilibrium with the enzyme-bound forms; (NH₂), (NHOH) and (NOH) are given in parentheses since they are probably unstable and possibly free-radical in character. Binding on the surface of the enzyme probably stabilizes (NHOH) and (NOH) against spontaneous and irreversible electronic changes that result in the respective formations of nitrogen and nitrous oxide. The intermediate, (NH₂), is probably completely stabilized by its enzyme. Decomposition (e.g. to hydrazine and thence to nitrogen) apparently does not occur because the products of the oxidation of ammonia were equal to the ammonia disappearing, even when the reaction was inhibited by hydrazine. Although both hydrazine and (NH₂) contain nitrogen in the same oxidation state, their metabolic roles are different: the metabolism of hydrazine involves a dehydrogenation that is not inhibited by thiourea. The metabolism of (NH₂) is associated with the oxidation of ammonia, a reaction that requires oxygen and is sensitive to thiourea.

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