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The Metabolism of Hydroxylamine to Nitrite by *Nitrosomonas*

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Hofman & Lees (1953) found that washed-cell suspensions of the autotrophic micro-organism *Nitrosomonas*, which derives the energy required for growth from the oxidation of ammonia to nitrite, was capable of metabolizing hydroxylamine to nitrite. Lees (1954) proposed that the metabolism of ammonia to nitrite proceeded via hydroxylamine and an unknown intermediate in a series of three two-electron steps in which the energy and reducing power released could be efficiently utilized by the cell. Since hyponitrite, $\text{H}_2\text{N}_2\text{O}_2$, was not metabolized to nitrite, he suggested that the hypothetical intermediate was nitroxyl, (NOH). Since the free energy released by the oxidation of

ammonia to hydroxylamine was small, the metabolism of hydroxylamine to nitrite provided the main free-energy requirements of the cell.

Nicholas & Jones (1960) found that cell-free extracts produced nitrite from hydroxylamine aerobically in the presence of mammalian cytochrome *c*. The enzymic reduction of the cytochrome by hydroxylamine was inhibited by mepacrine (Falcone, Shug & Nicholas, 1962). The reversal by FAD of the inhibition suggests that a flavin was involved.

Whole or crushed cells reduced methylene blue anaerobically in the presence of hydroxylamine; the disappearance of hydroxylamine was accom-

panied by the formation of a nitrogenous gas but no nitrite, suggesting that the metabolism of hydroxylamine to nitrite occurred in two stages (Anderson, 1959; see review by Lees, 1960). The first stage was a dehydrogenation to form an intermediate in the same oxidation state as nitroxyl which decomposed to nitrous oxide; the second stage was the conversion of nitroxyl into nitrite by an enzyme system requiring oxygen. In the present work the dehydrogenation of hydroxylamine anaerobically resulted in the formation of nitric oxide, which involves the removal of three electrons; the metabolism of nitric oxide to nitrite was a process requiring molecular oxygen. The enzymology of these reactions was investigated.

MATERIALS AND METHODS

Reagents

Water was deionized. Reagents were analytical-reagent grade. Tris was recrystallized three times from ethanol; 2-amino-2-methylpropane-1,3-diol was obtained from Eastman Kodak Co., Rochester, N.Y., U.S.A.

NAD, NADP, FMN, FAD (each 90–97% pure), type III mammalian cytochrome *c* and crystalline bovine serum albumin were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. NADH₂ and NADPH₂ were prepared by enzymic reduction of NAD and NADP respectively (Racker & Colowick, 1957).

The pH was adjusted with HCl to prepare 0.2M-tris (pH 6.0–9.0), 0.2M-2-amino-2-methylpropane-1,3-diol (pH 8.2–9.5) and 0.1M-sodium pyrophosphate–0.09M-borax (pH 5.5–9.8) buffers. NaOH was used to prepare 0.2M-phosphate buffer from KH₂PO₄ solution.

Preparation of inorganic nitrogen compounds

Sodium hyponitrite. The solid prepared according to the method of Addison, Gamlen & Thompson (1952) contained carbonate but no nitrite, nitrate, hydroxylamine or ammonia. The purity, calculated from the amount of nitrous oxide formed spontaneously at pH 8, according to the equation:

(Anderson, 1963), was 90%. Solutions in 0.1N-NaOH were stable; 0.1 ml. was added to biochemical experiments.

Nitric oxide. Nitric oxide was prepared under nitrogen according to the equation:

and collected over water in an aspirator. The concentration of nitric oxide was determined differentially. A known volume (approx. 20 ml.) in a gas burette was mixed with 10 ml. of pure oxygen. The nitrogen dioxide formed and excess of oxygen were absorbed by shaking vigorously with a saturated solution of pyrogallol in 20% (w/v) KOH from a reservoir. The contraction in volume represented the nitric oxide present.

Determinations

Determination of protein. The method of Lowry, Rosebrough, Farr & Randall (1951) was used, with crystalline bovine serum albumin as a standard.

Determination of inorganic nitrogen compounds. Ammonia was determined according to the method of Conway (1957).

Hydroxylamine was determined quantitatively by a modification of the method of Czaky (1949) by measuring the azo dye formed before and after oxidation of hydroxylamine to nitrite by iodine. Two samples each containing less than 0.2 μmole of hydroxylamine plus nitrite were diluted to 5.0 and 3.5 ml. respectively. Nitrite was determined in the 5 ml. sample as described by Lees (1952) except that the filter used for colorimetric determinations had a maximum transmission at 530 mμ. Hydroxylamine was determined in the 3.5 ml. sample by adding 1 ml. of sulph-anilic acid reagent (Lees, 1952) followed by 0.5 ml. of iodine reagent (0.5 g. of iodine/100 ml. of acetic acid). Precisely 4 min. after the addition of the iodine reagent, 1 ml. of 2% (w/v) sodium arsenite was added and the mixture was swirled vigorously until the colour of iodine had disappeared. After the addition of 1 ml. of 1-naphthylamine reagent (Lees, 1952) the extinction of the azo dye was determined colorimetrically. The concentration of hydroxylamine plus nitrite was found from a calibration curve for the nitrite determination.

Methylene blue did not interfere with the formation of azo dye, but since it interfered with the colorimetric determination it was necessary to use the following differential spectrophotometric method. Solutions containing 0.02–0.25 μmole of methylene blue were treated as described for the nitrite determination. The extinctions at 665 and 525 mμ, which are the peaks for methylene blue and azo dye respectively, were determined in glass cells of 1 cm. light-path in the Unicam SP. 500 spectrophotometer by reading against a reagent blank (no methylene blue added). By subtracting the extinction of methylene blue at 525 mμ (derived from the calibration curves at 525 and 665 mμ) from the observed reading at 525 mμ, the extinction due to the azo dye was obtained. The concentration of nitrite or hydroxylamine was determined from a nitrite calibration curve at 525 mμ.

Nitrate was determined by reduction to nitrite with zinc dust and ammonia (Anderson, 1963). Since traces of methylene blue interfered, the dye was completely adsorbed from the ammoniacal solution with 100 mg. of kaolin (from Hopkin and Williams Ltd.). Before the reduction, kaolin was removed by centrifuging. The recoveries of nitrate from solutions of methylene blue were quantitative. Nitrate could not be determined in the presence of hydroxylamine which itself formed small quantities of nitrite in ammonia.

Culture of *Nitrosomonas*

The strain employed and the method of batch culture (50 l.) were those of Nicholas & Jones (1960) except that the pH was maintained at 8.0–8.2 by continuous automatic addition of sterile 20% (w/v) K₂CO₃. Growth conditions were not completely aseptic because the carboy and the 48 l. of deionized tap water that it contained were not sterilized. The neck of the vessel was, however, plugged with sterilized cotton wool and the usual precautions of sterilizing inlet aeration tubes and reagents were taken. After about 5 days, the culture was harvested by siphoning it through a Servall model SS34 centrifuge at 34000g over a period of 3 hr. at room temperature by using a Szent-Györgi and Blum continuous-flow system (I. Servall Inc.,

Norwalk, Conn., U.S.A.). The pink suspension of cells was washed by centrifuging (at 10000g) with 0.02M-phosphate buffer, pH 8.0, until free from nitrite. Cells were finally suspended in this buffer and stored at 2° and used within 2 weeks. The yield of cells was 0.5–0.8 g. dry wt./50 l. After centrifugation, traces (approx. 1%) of a pale-yellow contaminant were observed as a lower layer.

Preparation of cells and extracts

Stored cell suspensions used during the 2-week period after harvest had an appreciable respiration (e.g. 0.6 μ mole of oxygen/mg. dry wt./hr.) owing to the oxidation of carbon compounds and ammonia, probably derived from the breakdown of cell constituents. Suspensions for biochemical experiments were prepared by shaking portions of the stored cell suspensions for 0.5–1.5 hr. in the Warburg apparatus at 30° until the uptake of oxygen was minimal (0.05 μ mole of oxygen/mg. dry wt./hr.). The suspension was then washed by centrifuging with 0.02M-phosphate buffer, pH 8, until free from nitrite, and the cells were finally suspended in this buffer.

Extracts were prepared from cells suspended in a solution of 0.25M-sucrose–0.02M-phosphate buffer, pH 8.0. The suspensions were disintegrated with a titanium ultrasonic probe at 4° for 5 min. (Nicholas & Jones, 1960). The red faintly-opalescent extract obtained by centrifuging the homogenate at 20000g for 20 min. at 0° contained 25–50% of the total cell N. It had no endogenous oxygen uptake.

After an extract had been centrifuged at 144000g for 2 hr. at 5°, a deep-red pellet of cell particles was deposited. The supernatant fraction, which was very pale pink but not completely free of particles, was removed and retained. A hazy red suspension above the pellet was discarded and the particles were then dispersed in the sucrose-phosphate solution at 2°.

Metabolic experiments

Rate experiments. Incubations were carried out at 30°. Reactions for studying the rates of disappearance of hydroxylamine and appearance of nitrite were carried out in 100 ml. conical flasks in which the reaction mixture (2.5 ml.) formed shallow well-aerated layers. Reaction mixtures contained 0.1–0.2 ml. of extract (diluted with phosphate buffer before addition) corresponding to a protein concn. of 0.2–1.0 mg./ml. of reaction mixture, or whole cells corresponding to approx. 0.1 mg. dry wt./ml. Boiled preparations were made by heating samples at 100° for 20 min., then cooling and diluting with water to the original volume. Before the addition of a solution of KCN to reaction mixtures it was adjusted to pH 8 with HCl.

Anaerobic spectrophotometric experiments were performed at room temperature in quartz cells of 1 cm. light-path fused to a Thunberg assembly with a total capacity of 7 ml. The atmosphere was nitrogen. The reaction mixtures contained 100 μ moles of tris buffer, pH 8, and approx. 0.05 ml. of extract. The reduction of methylene blue or cytochrome *c* was initiated by adding 0.2 ml. of substrate from the side arm, bringing the total volume to 3 ml. Reactions were followed spectrophotometrically at 665 or 551 μ respectively.

Nitrogen-balance experiments. Anaerobic and aerobic

nitrogen-balance experiments were performed in conventional Warburg respirometers with double-side-arm vessels of capacity 16 ml. The centre well contained filter paper plus 0.2 ml. of 2N-KOH. For cyanide poisoning, 0.2 ml. of a suspension of $\text{Ca}(\text{OH})_2$ in $\text{Ca}(\text{CN})_2$ was used (Robbie, 1948). To obtain anaerobic conditions flasks were gassed for 20 min. with nitrogen containing less than 0.05% of oxygen. For the experiments in Table 6 an atmosphere containing nitric oxide was subsequently introduced by diminishing the rate of flow of nitrogen to zero while increasing the flow of a nitric oxide plus nitrogen (5:95) mixture (0.5 l./flask in 5 min.). After temperature equilibration (at 30°) for 20 min., reactions were started by adding 0.2 ml. of substrate from the side arm, bringing the total volume to 2.5 ml. Flasks were shaken at 80 cycles/min.

The determination of nitric oxide formed by the anaerobic dehydrogenation of hydroxylamine in the presence of a hydrogen acceptor was based on the differential absorption by alkaline sulphite (Najjar & Allen, 1954). Identical enzymic reaction mixtures were prepared in flasks A and B. The centre well of flask A contained 0.2 ml. of 2N-KOH; that of flask B contained 0.2 ml. of a solution of 10% (w/v) Na_2SO_3 in 0.5N-NaOH. The alkaline sulphite solution was placed in the side arm when the $\text{Ca}(\text{CN})_2$ – $\text{Ca}(\text{OH})_2$ mixture was present in the well. After the addition of hydroxylamine the formation of nitric oxide plus nitrous oxide attained maximum values. In flask B, nitric oxide was rapidly absorbed and stationary manometric readings represented the residual nitrous oxide. In flask A, a slow absorption of nitric oxide (0.1–0.3 μ mole/hr.) occurred, probably as a result of reaction with traces of oxygen. The difference between the maximum formation of nitric oxide plus nitrous oxide in flask A and the stationary reading due to nitrous oxide in flask B represented the amount of nitric oxide formed. This was calculated according to the equation:

$$\text{NO } (\mu\text{moles}) = \frac{k_A}{22.4} \left[h_A - \left(\frac{h_B \times k_B^{N_2O}}{k_A^{N_2O}} \right) \right]$$

where k_A is the flask constant for nitric oxide of flask A, h_A is the maximum reading (mm. of manometric fluid) of nitric oxide plus nitrous oxide in flask A, h_B is the stationary reading (mm. of fluid) in flask B, $k_A^{N_2O}$ is the flask constant for nitrous oxide of flask A, and $k_B^{N_2O}$ is the flask constant for nitrous oxide of flask B. In the expression $h_B \times k_B^{N_2O}/k_A^{N_2O}$, which gives the nitrous oxide (mm. of fluid) in flask A, the value of the solubility of nitrous oxide at 30° used to calculate flask constants was 0.50 ml./ml. of water. The rate of gas production (nitric oxide plus nitrous oxide in flask A) was calculated from the initial 10 or 20 min. period, during which it was linear with time; the flask constant for nitrous oxide was used.

Cytochrome-oxidase activity. This was measured by following oxygen uptake in the presence of ascorbate and cytochrome *c*. The reaction mixture in Warburg flasks contained 100 μ moles of phosphate buffer, pH 7.5, 0.25 μ -mole of cytochrome *c* and extract corresponding to 2 mg. of protein. The reaction was initiated by adding 0.2 ml. of 114 mM-ascorbic acid adjusted to pH 7, bringing the total volume to 2.5 ml. The well contained 0.2 ml. of 2N-KOH. The oxygen uptake was corrected by subtracting the small uptake in a corresponding reaction mixture in which the extract was replaced by boiled extract.

RESULTS

Bacterial cytochrome

Spectroscopic examination of whole cells and extracts under anaerobic conditions showed the α - and β -bands of a cytochrome. These were intensified by adding solid dithionite or solutions of hydroxylamine or hydrazine, but not by hypoxanthine or ammonium sulphate. The intensity of the bands diminished on admitting air. These findings indicate that the metabolism of hydroxylamine involved the cytochrome component of a respiratory chain. The peaks of the cytochrome in extracts reduced by dithionite or hydroxylamine were: $\alpha_{\text{red.}}$, 551 $\mu\mu$; $\beta_{\text{red.}}$, 522 $\mu\mu$; $\gamma_{\text{red.}}$, 417 $\mu\mu$.

Experiments with whole cells

The metabolism of hydroxylamine by whole cells, measured manometrically by oxygen uptake, was independent of pH over the range 6.0–9.2. When the hydroxylamine (5 μ moles) had completely disappeared the recoveries of nitrite in residual reaction mixtures were 90–105 % of the theoretical value calculated from the oxygen uptake according to the equation:

The nitrite recoveries were 90–100 % of the hydroxylamine added. The recoveries with cells that had been stored for 3–4 weeks were only 60 % of the hydroxylamine added. Since the nitrite recovery was approximately equal to the oxygen uptake, and the formation of nitrous oxide according to the reaction:

does not involve a net gas change, it is probable that the hydroxylamine which could not be accounted for as nitrite was converted into nitrous oxide.

The oxidation of NH_4^+ ions by whole cells occurred from pH 6.5 to 9.5 with an optimum at

pH 8.5. In contrast with hydroxylamine metabolism, no oxidation occurred below pH 6.5. The disappearance of ammonia was accompanied by an equivalent appearance of nitrite and the theoretical oxygen uptake as found by Hofman & Lees (1953). Thus no nitrous oxide was formed by the oxidation of ammonia.

Hydroxylamine was not utilized anaerobically by whole cells, probably because oxygen was the obligatory terminal hydrogen acceptor (Table 1). In the presence of a substrate amount of methylene blue as hydrogen acceptor a mixture of nitrous oxide and nitric oxide was produced equivalent to the hydroxylamine added. The reaction occurred equally well in a gas phase of nitrogen or nitrous oxide. The recoveries of nitrite and hydroxylamine at the end of incubation were negligible (flask B in Table 1). The metabolism of hydroxylamine therefore involves a dehydrogenation represented by the equation:

in which X is a natural hydrogen carrier oxidized by the respiratory chain. The metabolism of nitric oxide to nitrite is a direct oxidation. If nitroxyl is the intermediate, as suggested by Lees (1954), the dehydrogenation of hydroxylamine involves two steps: (a) the formation of nitroxyl:

(b) the further dehydrogenation of nitroxyl:

The formation of nitrous oxide and possibly small amounts of nitrogen may be due to spontaneous decomposition of nitroxyl according to the equations:

The addition of methylene blue as carrier aerobically (Table 2) diminished the nitrite production. The oxygen uptake exceeded that calcu-

Table 1. *Products of the anaerobic dehydrogenation of hydroxylamine by cells and extracts in the presence of methylene blue as hydrogen acceptor*

Incubation was in Warburg flasks containing, in 2.5 ml., 100 μ moles of tris buffer, pH 8.0, and additions as indicated; 5 μ moles of hydroxylamine hydrochloride were added from the side arm. The centre well contained 0.2 ml. of $\text{Ca}(\text{OH})_2$ - $\text{Ca}(\text{CN})_2$, 2N-KOH or alkaline sulphite solution (see the Materials and Methods section). The atmosphere was nitrogen. The periods of reaction were: for extracts, 40 min.; for cells, 20 min.

Preparation	Methylene blue added (μ moles)	Concn. of cyanide (mm)	Recovery of inorganic N (μ moles/flask)				(NO + N ₂ O) N (% of NH ₂ OH N added)
			NH ₂ OH	Nitrite	NO	N ₂ O	
Cells	10	0	0.0	0.2	1.4	1.9	104
Cells	10	1	0.0	0.2	1.1	2.1	106
Cells	0	0	4.0	0.1	0.0	0.7	—
Boiled cells	10	0	4.5	0.1	0.0	0.3	—
Extract	10	0	0.0	0.3	1.8	1.5	96
Extract	10	1	0.0	0.2	1.6	2.1	116
Boiled extract	10	0	4.7	0.0	0.0	0.3	—

lated from the nitrite production in accordance with eqn. (1). Small amounts of nitrate were found which were approximately equal to that calculated by multiplying the excess of oxygen uptake by the factor $\frac{2}{3}$ derived from the equation:

Non-enzymic oxidation of nitric oxide might account for the nitrate formation and also for some of the nitrite produced. Nitrite might also be oxidized to nitrate with peroxide generated by the oxidation of reduced methylene blue.

Cyanide inhibited hydroxylamine disappearance aerobically (Table 2). In the presence of carrier amounts of methylene blue, cyanide partially inhibited hydroxylamine disappearance but (Table 2) the nitrite formation was diminished. In the presence of methylene blue, under anaerobic conditions where the terminal oxidation system was not operative, cyanide did not prevent the conversion of hydroxylamine into nitric oxide and nitrous oxide (Table 1) but the rate was inhibited by 30%.

The essential findings with whole cells were: (a) Anaerobically, in the presence of methylene blue, hydroxylamine was converted into nitric oxide plus nitrous oxide with negligible nitrite formation. (b) Aerobically hydroxylamine was oxidized quantitatively to nitrite. (c) This reaction was inhibited by cyanide. In the presence of methylene blue and cyanide, hydroxylamine was metabolized but only small amounts of nitrite were recovered.

Experiments with extracts

Requirement for added electron carriers for the dehydrogenation of hydroxylamine. Extracts slowly oxidized hydroxylamine at pH 8.0 (Table 3) but nitrite was found only with high concentrations of

extract (7 mg./ml.) and low amounts of hydroxylamine (0.2 mM). The inability of extracts to produce nitrite from ammonia is in agreement with previous results (Engel & Alexander, 1959; Anderson, 1959; Nicholas & Jones, 1960). The addition of carrier amounts of methylene blue or mammalian cytochrome *c* at pH 8 increased the rate of dehydrogenation of hydroxylamine aerobically and nitrite was produced (Table 3). Only traces of nitrate were found with cytochrome *c*; larger amounts were found with methylene blue as carrier (Table 2). Since 20 mM-phosphate or 20 mM-pyrophosphate inhibited the disappearance of hydroxylamine with cytochrome *c* as carrier, tris was used as buffer. Mepacrine inhibited the rate of dehydrogenation of hydroxylamine with cytochrome *c* as carrier (Table 3). FAD but not FMN reversed the inhibition produced by incubating extracts with mepacrine. Anaerobically nitric oxide and nitrous oxide were formed from hydroxylamine and substrate amounts of methylene blue (Table 1). Substrate amounts of cytochrome *c* (12.5 μ moles) anaerobically caused hydroxylamine to disappear with the production of nitrous oxide, as found by Falcone *et al.* (1962), but the reaction was probably non-enzymic since it proceeded at the same rate with boiled extract. These results suggest that the respiratory chain of the dehydrogenation involves a flavin and cytochrome.

At pH 8.0 thionine, 2,6-dichlorophenol-indophenol, pyocyanine and phenazine methosulphate (each 10 μ moles) and ferricyanide (50 μ moles) were effective as hydrogen acceptors for the formation of nitric oxide anaerobically. These acceptors were tested on the system shown in Table 1. Compared with methylene blue the rate of formation of nitric oxide plus nitrous oxide anaerobically with phenazine methosulphate was twice as rapid and the

Table 2. Nitrogen balance showing the products of the aerobic metabolism of hydroxylamine by cells and extracts

Incubation was for 1 hr. in Warburg flasks containing, in 2.5 ml., 100 μ moles of tris buffer, pH 8.0, and additions as indicated; 5 μ moles of hydroxylamine hydrochloride were added from the side arm. The centre well contained 0.2 ml. of 2N-KOH or $\text{Ca}(\text{OH})_2$ - $\text{Ca}(\text{CN})_2$ mixture. The atmosphere was air. The negative O_2 uptake denotes a gas output.

Preparation	Carrier	Concn. of cyanide (mM)	O_2 uptake (μ moles)	Recovery of inorganic N (μ moles/flask)			
				NH_2OH	Nitrite	Nitrate	
						Calc.	Found
Extract	None	0.0	-0.1	4.6	0.0	—	—
	Cytochrome <i>c</i> (0.1 mM)	0.0	1.1	0.2	1.4	—	0.2
	Methylene blue (0.05 mM)	0.0	3.5	0.0	2.7	0.5	0.5
	Methylene blue (0.05 mM)	1.0	5.8	0.0	2.6	2.1	2.3
Cells	None	0.0	4.7	0.1	4.6	0.1	0.0
	None	1.0	0.2	4.0	0.1	—	—
	Methylene blue (0.05 mM)	0.0	5.0	0.0	3.7	0.8	0.4
	Methylene blue (0.05 mM)	1.0	4.8	0.0	1.9	1.9	1.2

proportion of nitric oxide in the gas was about 50 % greater. NAD, NADP, FMN or FAD in substrate amounts anaerobically had negligible effect. In anaerobic spectrophotometric experiments, 2 μ moles of hydroxylamine did not reduce 2 μ moles of NAD or NADP in the presence of the extract at pH values from 6.0 to 8.5. Similar results were obtained when methylviologen or benzylviologen, which have low redox potentials, replaced the pyridine nucleotide. The extracts barely catalysed the reduction of cytochrome *c* or methylene blue by NADH₂ or NADPH₂ (Table 4).

Effect of cyanide and carbon monoxide. The cytochrome-oxidase activity of an extract (2.2 μ moles of oxygen/mg. of protein/30 min.) was inhibited 95 % by 2 mm-cyanide, 71 % by a carbon monoxide plus oxygen plus nitrogen (20:20:60) mixture in the dark, 86 % by 2 mm-hydroxylamine and 51 % by 0.5 mm-hydroxylamine. Cyanide inhibited the disappearance of hydroxylamine with cytochrome *c* as carrier aerobically (Table 3), but the carbon monoxide gas mixture in the dark had no effect. With methylene blue as carrier, cyanide partially inhibited hydroxylamine disappearance (Table 3);

nitrite was formed and the nitrate formation was markedly increased (Table 2).

Under anaerobic conditions with substrate amounts of methylene blue, nitric oxide and nitrous oxide were produced from hydroxylamine (Table 1) but the rate of gas formation was inhibited by about 30 %. Anaerobic spectrophotometric experiments (Table 4) showed that the reduction of methylene blue or cytochrome *c* by hydroxylamine was partially inhibited by cyanide. It seems that both cytochrome oxidase and the respiratory pathway that reduces cytochrome *c* are cyanide-sensitive.

Dehydrogenation of hydrazine. Hofman & Lees (1953) found that hydrazine was a selective inhibitor of hydroxylamine metabolism aerobically. Anaerobic spectrophotometric experiments (Table 4) showed that hydrazine reduced cytochrome *c* or methylene blue in the presence of the extract. The reactions, like those with hydroxylamine, were partially inhibited by cyanide. Anaerobically the extracts catalysed a slow gas formation from hydrazine and methylene blue, but no nitrite or nitric oxide was produced (Table 5). Aerobically

Table 3. *Rate of aerobic metabolism of hydroxylamine by extracts*

Incubation was for 20 min. in 2.5 ml. of a mixture containing 100 μ moles of tris buffer, pH 8.0, extract, and additions as indicated. The reaction was started by adding hydroxylamine hydrochloride to give a concn. of 1000 μ m-moles/ml.

Additions	NH ₂ OH disappearance (μ m-moles/ml.)	Nitrite appearance (μ m-moles/ml.)
None	25	0
Methylene blue (0.1 mm)	925	450
Methylene blue (0.1 mm) + cyanide (1 mm)	585	185
None	50	0
Cytochrome <i>c</i> (0.1 mm)	400	185
Cytochrome <i>c</i> (0.1 mm) + cyanide (1 mm)	80	10
Cytochrome <i>c</i> (0.1 mm)	670	280
Cytochrome <i>c</i> (0.1 mm) + mepacrine (0.5 mm)	360	0
Cytochrome <i>c</i> (0.1 mm) + mepacrine (0.5 mm) + FAD (0.5 mm)	600	80

Table 4. *Rate of spectrophotometric reduction of methylene blue or cytochrome c*

Incubation was in Thunberg cuvettes containing, in 3 ml., 100 μ moles of tris buffer, pH 8.0, 0.2 μ mole of methylene blue or 0.5 μ mole of cytochrome *c*, and additions as indicated. The atmosphere was nitrogen.

Preparation	Additions	Rate of reduction of hydrogen acceptor (μ m-moles/5 min.)	
		Cytochrome <i>c</i>	Methylene blue
Extract	Hydroxylamine (2 μ moles)	129	170
Extract	Hydroxylamine (2 μ moles) + cyanide (1 mm)	31	42
Boiled extract	Hydroxylamine (2 μ moles)	9	5
Extract	Hydrazine (2 μ moles)	94	105
Extract	Hydrazine (2 μ moles) + cyanide (1 mm)	58	33
Boiled extract	Hydrazine (2 μ moles)	7	5
Extract	NADH ₂ (2 μ moles)	15	130
Boiled extract	NADH ₂ (2 μ moles)	10	105
Extract	NADPH ₂ (2 μ moles)	11	188
Boiled extract	NADPH ₂ (2 μ moles)	8	170

Table 5. *Products of the anaerobic dehydrogenation of hydrazine*

Incubation was in Warburg flasks containing, in 2.5 ml., 100 μ moles of tris buffer, pH 8.0, 10 μ moles of methylene blue, and additions as indicated. Hydroxylamine and hydrazine were added separately from the side arms. The centre well contained 0.2 ml. of 2N-KOH or alkaline sulphite solution (see the Materials and Methods section). The atmosphere was nitrogen.

Preparation	Additions	Rate of gas formation
Extract	N_2H_4, H_2SO_4 (5 μ moles)	N_2 (0.13 μ mole/15 min.)
Boiled extract	N_2H_4, H_2SO_4 (5 μ moles)	N_2 (0.04 μ mole/15 min.)
Extract	NH_2OH, HCl (5 μ moles)	N_2O (1.4 μ moles/15 min.) + NO (2.1 μ moles/15 min.)
Extract	NH_2OH, HCl (5 μ moles) + N_2H_4, H_2SO_4 (1.25 μ moles)	NO + N_2O (total 0.6 μ mole/15 min.); after 50 min. the reaction was complete (0.8 μ mole of NO + 2.4 μ moles of N_2O formed)

there was no oxygen uptake or nitrite formation from 0.5 mM-hydrazine in the presence of the extract and 0.1 mM-methylene blue or cytochrome *c* as carrier. Probably the dehydrogenation of hydrazine produced nitrogen as indicated by the equation:

The presence of hydrazine inhibited the initial rate of anaerobic gas formation from hydroxylamine and methylene blue (Table 5). After 25 min. the inhibition rapidly diminished and the formation of nitric oxide plus nitrous oxide was complete in a further 25 min. Probably the dehydrogenation of hydrazine preceded that of hydroxylamine.

Formation of nitrous oxide from hyponitrite. The great rapidity with which the $HN_2O_2^-$ ion spontaneously decomposes to nitrous oxide (Anderson, 1963) suggested that it might be an intermediate in the formation of nitrous oxide in the gas produced from hydroxylamine. The decomposition of $HN_2O_2^-$ ions was a first-order reaction which at pH 8 was complete in 1 hr. Since at the same pH with increased amounts of extract (1 mg./ml.) or cells, the anaerobic conversion of hydroxylamine into nitric oxide plus nitrous oxide was complete in a shorter period (10 min.), it was necessary to find whether cell material catalysed the decomposition of hyponitrite. The rate of nitrous oxide formation from 6.4 μ moles of hyponitrite at pH 8 in air was not affected by the presence of cells or the extract with or without methylene blue or cytochrome *c*. It therefore seems unlikely that the $HN_2O_2^-$ ion is an intermediate in the conversion of nitroxyl into nitrous oxide. No nitrite or nitrate was produced; this is in agreement with the findings of Lees (1954).

Nitric oxide as substrate. Experiments investigating the further metabolism of nitric oxide were performed anaerobically in the Warburg apparatus with 5% nitric oxide as substrate and ferricyanide as hydrogen acceptor (Table 6). The addition of cytochrome *c* increased the rate of nitric oxide uptake and this reaction was inhibited by cyanide. Since ferricyanide interfered with the formation of

Table 6. *Metabolism of nitric oxide by extracts in the presence of ferricyanide as hydrogen acceptor anaerobically*

Incubation was in Warburg flasks containing, in 2.5 ml., 100 μ moles of tris buffer, pH 8, and additions as indicated; 50 μ moles of ferricyanide were added from the side arm. The atmosphere was nitric oxide plus nitrogen (5:95). The centre well contained 0.2 ml. of $Ca(OH)_2$ - $Ca(CN)_2$ mixture or 2N-KOH.

Preparation	Additions	NO uptake (μ moles/hr.)
Extract	—	1.2
Extract	Cytochrome <i>c</i> (0.1 mM)	3.1
Boiled extract	Cytochrome <i>c</i> (0.1 mM)	0.5
Extract	Cytochrome <i>c</i> (0.1 mM) + cyanide (2 mM)	1.1

azo dye, nitrite determinations were not made. There was negligible nitric oxide uptake when ferricyanide was replaced by ferrocyanide or by the two-electron acceptor methylene blue. Possibly a natural carrier of the direct oxidation system was in equilibrium with cytochrome *c* which was maintained in the oxidized state by ferricyanide.

Effect of pH. Anaerobically, with methylene blue, the rate of formation of nitric oxide plus nitrous oxide increased from pH 5.5 to 7.5 (Fig. 1). It was independent of pH in the range 7.5–9.2, then fell rapidly to pH 9.7. At this pH value the rate of gas formation (nitrous oxide) in the presence of boiled extract was about 80% of the enzymic rate. The gases formed (at pH 5.5–9.2) were equivalent to the hydroxylamine N added, and the amount of nitric oxide in the gas increased with increase of pH.

Distribution of activity. The distribution of the activities of some enzymes involved in the dehydrogenation of hydroxylamine was examined in the particulate and the supernatant fractions obtained by centrifuging freshly prepared extracts at 144000g (Table 7). The cytochrome-oxidase activity was mainly in the particulate fraction, whereas the anaerobic formation of nitric oxide plus nitrous oxide from hydroxylamine and methylene blue was catalysed predominantly by the supernatant fraction.

DISCUSSION

A hypothetical scheme for the stepwise metabolism of hydroxylamine to nitrite is given in Scheme 1. The overall process requires oxygen. Anaerobically the dehydrogenation of hydroxylamine by cells was inhibited and the reduction of the bacterial cytochrome was observed. Anaerobically the addition of substrate amounts of methylene blue to cells or extracts resulted in the reduction of the dye and a complete disappearance of hydroxyl-

amine; negligible amounts of nitrite were produced; nitric oxide and nitrous oxide were formed in amounts equivalent to the hydroxylamine added. Aerobically the addition of carrier amounts of methylene blue to cells, or of cytochrome *c* or methylene blue to extracts, increased the rate of dehydrogenation of hydroxylamine. Probably methylene blue and cytochrome *c* replaced a natural hydrogen carrier and the bacterial cytochrome in a respiratory chain. Possibly FAD is the natural carrier which is oxidized by cytochrome *c* and with which methylene blue is in equilibrium, since FAD overcame the inhibition by mepacrine. The formation of nitric oxide from hydroxylamine involves the production of three equivalents of reducing power. The natural carrier reduced by this reaction is probably capable of transmitting single equivalents through the respiratory chain.

The metabolism of hydroxylamine to nitrite probably provides most of the energy released by

Fig. 1. Relation between pH and (i) the rate of production of gas by the anaerobic dehydrogenation of hydroxylamine, (ii) the amount of nitric oxide in the gas. Incubation was in Warburg flasks containing, in 2.5 ml., 1 ml. of borax-pyrophosphate buffer, pH 5.5–9.7, 0.2 ml. of extract and 10 μmoles of methylene blue; 5 μmoles of hydroxylamine hydrochloride were added from the side arm. The atmosphere was nitrogen. The well contained 0.2 ml. of 2*N*-KOH or alkaline sulphite (see the Materials and Methods section). ○, Rate of formation of nitric oxide plus nitrous oxide (μmoles/20 min.); ●, formation of nitric oxide (μmoles) when the dehydrogenations were complete (70 min. at pH 5.5; 30 min. at pH 9.2).

Table 7. *Distribution of enzyme activities in particulate and supernatant fractions*

Cytochrome-oxidase activity was measured as described in the Materials and Methods section. The reactions for studying anaerobic gas formation were carried out in Warburg flasks containing, in 2.5 ml., 100 μmoles of tris buffer, pH 8.0, 10 μmoles of methylene blue, and particles or supernatant fraction (2.5 mg. of protein); 5 μmoles of hydroxylamine hydrochloride were added from the side arm. The centre well contained 0.2 ml. of 2*N*-KOH. The atmosphere was nitrogen.

Fraction	Cytochrome-oxidase activity (μmoles of O ₂ /mg. of protein/30 min.)	Anaerobic gas formation (μmoles of NO + N ₂ O/15 min.)
Particles	8.0	0.5
Supernatant	2.9	3.9

Scheme 1. Hypothetical scheme for the stepwise oxidation of hydroxylamine to nitrite.

ammonia oxidation (Lees, 1954), and is likely therefore to be coupled with systems that generate 'high-energy' phosphate bonds. Extracts in the presence of hydroxylamine did not, however, form reduced nicotinamide nucleotides, which are necessary for the reduction of carbon dioxide to the level of carbon in cell material.

The disappearance of hydroxylamine aerobically in the presence of cells or of extracts with cytochrome *c* was inhibited by cyanide. Since the cytochrome-oxidase activity in extracts was cyanide-sensitive it seems that the inhibitions were due to a block of the oxidation of the reduced cytochrome. In anaerobic conditions where the terminal oxidation system did not operate, cyanide partially inhibited the dehydrogenation, as shown by spectrophotometric experiments and the evolution of gas with methylene blue. Possibly the reduction of the cytochrome by hydroxylamine involves a metal. Cytochrome oxidase was also sensitive to hydroxylamine, in agreement with the findings of Takemori, Sekuzu & Okunuki (1960) with mammalian systems. Beinert, Griffiths, Wharton & Sands (1962) have suggested that hydroxylamine binds copper in the enzyme. Possibly the dehydrogenation in *Nitrosomonas* is inhibited because of poisoning of cytochrome oxidase by hydroxylamine.

Since the direct oxidation of nitric oxide to nitrite was associated with the formation of nitrate, attempts were made to study the reaction anaerobically with nitric oxide as substrate. The uptake of nitric oxide by extracts in the presence of ferricyanide as hydrogen acceptor was increased by adding cytochrome *c* which was probably oxidized by ferricyanide. The uptake in the presence of cytochrome *c* and ferricyanide was inhibited by cyanide, suggesting that the enzyme system contains a metal. These results may be compared with denitrification systems. For example, the reduction of nitrite to nitric oxide and nitrogen by extracts of *Pseudomonas stutzeri* involves a flavin as carrier and a reduced nicotinamide nucleotide as hydrogen donor (Chung & Najjar, 1956*a, b*).

Ammonia was not metabolized by extracts. Hydrazine, in which nitrogen is in an oxidation state intermediate between that in ammonia and in hydroxylamine, was dehydrogenated, possibly by the system that dehydrogenated hydroxylamine, and slowly formed nitrogen. Since no nitrite was formed aerobically, hydrazine is unlikely to be an intermediate in nitrification. Probably the enzyme concerned cannot break the single bond linking the two nitrogen atoms. Hydrazine inhibited the gas formation from hydroxylamine with methylene blue anaerobically, which is in agreement with the inhibition found by Hofman & Lees (1953) aerobically.

Aleem, Lees, Lyric & Weiss (1962) suggested that nitrohydroxylamine, $\text{H}_2\text{N}_2\text{O}_3$, is an inter-

mediate in hydroxylamine metabolism. They found that the oxidation of this compound to nitrite by whole cells was accompanied by a theoretical oxygen uptake. In the present work the dehydrogenation of hydroxylamine anaerobically in the presence of methylene blue as acceptor produced nitric oxide and nitrous oxide in amounts equivalent to the hydroxylamine added. It seems that nitroxyl and nitric oxide are the intermediates involved. Possibly the metabolism of nitrohydroxylamine involves its decomposition to nitric oxide.

SUMMARY

1. Washed-cell suspensions of the autotrophic micro-organism *Nitrosomonas*, which obtains its free energy for growth from the oxidation of ammonia to nitrite, rapidly oxidized low concentrations of ammonia and hydroxylamine, but not hyponitrite, to nitrite. Extracts were capable of producing small amounts of nitrite from hydroxylamine but did not retain the ability to produce nitrite from ammonia.

2. Anaerobically in the presence of the extract and methylene blue, hydroxylamine disappeared and negligible amounts of nitrite were formed. Nitric oxide and nitrous oxide were produced in amounts equivalent to the hydroxylamine added.

3. Aerobically in the presence of extracts, methylene blue or mammalian cytochrome *c* as carrier increased the rate of disappearance of hydroxylamine and increased the nitrite production. The disappearance of hydroxylamine in the presence of cytochrome *c* was inhibited by cyanide. In the presence of methylene blue, cyanide-poisoned extracts metabolized hydroxylamine and nitrite appeared.

4. These results suggested that the metabolism of hydroxylamine was a two-stage process. In stage 1 hydroxylamine was dehydrogenated to form nitroxyl which decomposed to nitrous oxide and which could be further dehydrogenated to nitric oxide. The oxidation of a reduced natural hydrogen carrier, with which methylene blue was in equilibrium, involved a cytochrome and cytochrome oxidase. Stage 2 was the conversion of nitric oxide into nitrite by an enzyme system requiring oxygen.

5. Stage 2 was further investigated anaerobically with extracts in the presence of nitric oxide as substrate, and ferricyanide as hydrogen acceptor. Cytochrome *c* as carrier increased the rate of nitric oxide uptake, suggesting that the metabolism of nitric oxide to nitrite aerobically involved a cytochrome.

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The Distribution of Reactive Disulphide Bonds in Wool

REDUCTIVE AND OXIDATIVE STUDIES

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Elsworth & Phillips (1938) and Middlebrook & Phillips (1942) showed that about 50 % of the disulphide bonds of some types of wool appear to be more readily reduced than the rest. There has since been considerable speculation on the significance of this finding. Although it was generally thought that these disulphide bonds are restricted to particular protein components, Gillespie & Springell (1961) showed that the more-readily-reducible disulphide bonds are distributed throughout the major components of wool. This has since been confirmed by Blackburn (1961) and is consistent with other findings by Crewther & Dowling (1961) and Springell (1963). In addition, Gillespie & Springell (1961) recorded and commented on the unexpected finding that the disulphide groups of the high-sulphur and low-sulphur proteins of wool react by the same fractional amount when treated with mercaptoacetate at pH 5.0 and alkylated with iodoacetate at pH 8.5. This is in spite of the fact that these proteins have widely differing compositions and structures (Corfield, Robson & Skinner,

1958; Gillespie & Simmonds, 1960; Sikorski & Woods, 1960; Fraser, MacRae & Rogers, 1962). It may be a coincidence that the two protein components have equally reactive disulphide bonds. On the other hand, it is possible that one protein is less extensively reduced at pH 5.0 than the other, and that subsequent reactions obscure this. The reactions that may be involved are those which promote disulphide interchange or the interchange of *S*-carboxymethyl groups between the two proteins. We have investigated the possible extent of these reactions, and have extended the studies to Lincoln wool as well as to the least-reactive disulphide bonds in Merino wool. In an attempt to eliminate the possibility of interchange reactions, the reactivity of disulphide bonds towards oxidation with performic acid was also examined.

MATERIALS

Wool. Medium Peppin B type 64s Merino wool top (batch MW129) and loose Lincoln wool (batch MW114) had thiol-plus-disulphide contents (Leach, 1960) of 470 and 409 μ moles/g. dry wt. of wool respectively.

Iodo[2- 14 C]acetic acid. This was prepared, as described by Gillespie & Springell (1961), from bromo[2- 14 C]acetic acid

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