The Biochemistry of the Nitrifying Organisms

4. THE RESPIRATION AND INTERMEDIARY METABOLISM OF *NITROSOMONAS*

BY T. HOFMAN AND H. LEES
Department of Biological Chemistry, University of Aberdeen

(*Received* 13 *November* 1952)

Nitrosomonas is an autotrophic micro-organism which depends for its free energy supply on the free energy released by the reaction

$$NH_4^+ + 1{\cdot}5\ O_2 = 2H^+ + NO_2^- + H_2O + 66\ 500 \text{ cal.}$$

Meyerhof (1917) found that the oxygen uptake of suspensions of the organism provided with ammonia agreed well with nitrite production in the suspensions. Bömecke (1939) established that the organisms had an oxygen uptake in the absence of ammonia; however, this endogenous respiration is very small and, although of considerable biochemical significance, is quantitatively negligible in ordinary respirometric studies in the presence of appreciable quantities of ammonia. The respiration of *Nitrosomonas* suspensions thus offers a very simple means of studying the metabolism of the organism since there is only one normal substrate and one end product; nevertheless, the studies of Meyerhof (1917) and Bömecke (1939, 1951) represent the bulk of the work that has been published in this field.

MATERIALS AND METHODS

The method used for the estimation of nitrite nitrogen (NO_2-N) has already been described (Lees, 1952*a*). NH_4-N was determined by the microdiffusion method, Conway (1950). Hydroxylamine was estimated by the method of Csaky (1949) in which the hydroxylamine is oxidized to nitrite by iodine and the nitrite so formed measured colorimetrically by the normal procedure.

Oxygen uptake was measured by the normal Warburg technique, using single side-arm vessels, 15–20 ml. total capacity, with 0·2 ml. 10% (w/v) KOH in the centre well. All experiments were carried out at 30°.

The organisms were grown as previously described (Lees, 1952*a*; Hofman & Lees, 1952) and the volume of the cultures was 400 ml. When the NO_2-N concentration of a culture had risen to approximately 100 μg./ml. the culture was centrifuged to separate the precipitate of carbonates and phosphates of Mg and Ca (the 'inorganic precipitate') initially present in the medium and to which the organisms adhere (Winogradsky, 1890; Meiklejohn, 1950; Lees, 1952*a*). The inorganic precipitate, with its adherent organisms, was then washed three times at the centrifuge with 200 ml. lots of distilled water; it was finally suspended in a suitable volume of distilled water and distributed by pipette into the Warburg vessels. The pH of the final suspension was that of the original medium, i.e. pH 8·5, the residual NO_2-N was about 0·2 μg./ml. and the residual NH_4-N about 1·0 μg./ml. (the NH_4-N was presumably mainly in the form of insoluble complex magnesium and calcium phosphates). It will be noted that the suspensions were washed with water and not with saline or any other iso-osmotic solution. Water washing was used because it was found that washing with NaCl, KCl, Na_2SO_4 or K_2SO_4 solutions, even at millimolar concentrations, caused loss of activity in the final suspension and that the loss of activity became progressively greater as the salt concentration was increased. This loss of activity was finally traced to dispersal of zooglea of the organisms in the salt solutions; no appreciable dispersal took place when water washing was employed.

For the preparation of washed suspensions at pH's different from pH 8·5, the cultures were first centrifuged to separate the inorganic precipitate, which was then suspended in 200 ml. distilled water and its pH (glass electrode) adjusted to the required final value by cautious dropwise addition of 0·1 N-HCl or 0·1 N-NaOH followed by vigorous shaking. This suspension was again centrifuged, the precipitate taken up in fresh distilled water, and the pH of the suspension readjusted to the required final value as before. After three such adjustments of pH the precipitate was taken up in buffer solution (0·01 M-KH_2PO_4 + 0·005 M-$Na_2B_4O_7$) which had already been adjusted to the required pH by means of 0·1 N-HCl or 0·1 N-NaOH. The suspension was then ready for use. This prolonged and cautious method of obtaining the required pH value in the final suspension ensured first that the slowly reached equilibrium between the suspension fluid and the carbonate of the inorganic precipitate was attained, and secondly that the organisms were never subjected to high and possibly injurious local concentrations of either acid or alkali during the course of pH adjustment.

The washed suspension from a single culture was usually distributed among eight to twelve Warburg vessels. Since the cultures were used when they had formed about 100 μg. NO_2-N/ml., it may be calculated from the results of Hofman & Lees (1952) that each vessel thus contained approximately 0·5–1·0 mg. dry weight of organism.

RESULTS

Table 1 shows that, within experimental error, washed suspensions of *Nitrosomonas* oxidize ammonia quantitatively to nitrite. Agreement between oxygen consumption and nitrite production was checked in all experiments; and such agreement is therefore to be inferred even where it is not specifically mentioned.

Table 1. *Ammonia oxidation, oxygen consumption, and nitrite formation by* Nitrosomonas

(Washed suspensions (3 ml.) of *Nitrosomonas* made up in (approximately) 10^{-3}M-$(NH_4)_2SO_4$ and placed in the main compartments of the Warburg vessels. Temp. 30°; after temperature equilibration one vessel was removed and sampled for NH_4-N and NO_2-N. The taps of the remaining vessels were closed and the experiment continued, one vessel being removed every 20 min. and sampled for NH_4-N and NO_2-N as before.)

Time after vessel removed (min.)	O_2 uptake (μmoles)	NH_4 consumed (μmoles)	NO_2 formed (μmoles)	
			Calculated*	Observed
20	1·76	1·05	1·17	1·22
40	3·25	2·00	2·17	2·19
60	4·45	3·22	2·97	3·00
80	5·95	4·10	3·97	3·85
100	6·75	4·42	4·50	4·50
120	7·80	4·72	5·20	5·15

* Calculated from O_2 uptake.

The initial rate of oxygen consumption by the washed suspensions was reasonably linear over a wide range of ammonium sulphate concentrations. Curves corresponding to the extreme concentrations of ammonium sulphate tested are shown in Fig. 1. When almost all the ammonia initially present had been oxidized, the rate of oxygen consumption fell sharply and dropped practically to zero when ammonia oxidation was complete.

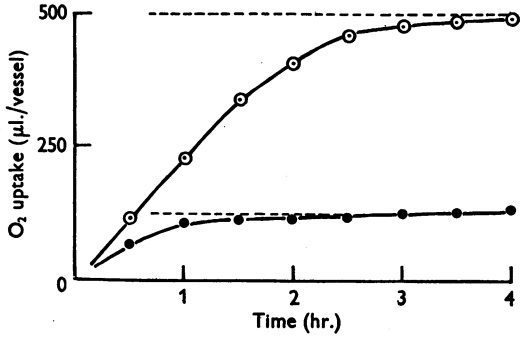

Fig. 1. Oxygen uptake by *Nitrosomonas* in the presence of different concentrations of ammonia. Each Warburg vessel contained 3 ml. washed suspension of *Nitrosomonas*. Ammonium sulphate (0·1 ml.) was added from the side arms after temperature equilibration to give initial $(NH_4)_2SO_4$ concentrations of $2·5 \times 10^{-3}$M (curve —⊙—) or $6·25 \times 10^{-4}$M (curve —●—) in the suspensions.

The influence of both the ammonia concentration and the pH of the suspension on the rates of oxygen uptake are shown in Figs. 2 and 3. The results of Meyerhof (1917) are included for comparison.

In previous incubation experiments with *Nitrosomonas* (Lees, 1952*a*) nitrite formation was markedly inhibited by allylthiourea. In the present work a similar inhibition of oxygen consumption by the allylthiourea was noted (Fig. 4). It was found that the inhibition of oxygen uptake caused by allylthiourea was completely abolished by washing the suspensions with water. The inhibition was not competitive with respect to ammonia over a wide range of ammonia concentrations (Table 2).

It has already been shown (Lees, 1952*b*) that hydroxylamine can be oxidized by *Nitrosomonas*, although the oxidation of hydroxylamine proceeds more slowly than that of ammonia if the hydroxylamine and ammonia are compared at nitrogen concentrations greater than about 2 μg./ml. It has now been found that although, as expected, hydroxylamine at 14 μg. nitrogen/ml. was oxidized more slowly than ammonia at the same nitrogen concentration, the oxidation of hydroxylamine remained virtually unaffected by concentrations of allylthiourea that completely inhibited oxidation of ammonia (Table 3). On the other hand, hydroxylamine oxidation was inhibited by hydrazine more strongly than was ammonia oxidation, and in the presence of hydrazine ammonia oxidation resulted in a small but definite accumulation of hydroxylamine (Table 4). The method used for hydroxylamine estimation involved a measurement of the difference in nitrite concentration before and after oxidation with iodine. It was therefore necessary, in order to demonstrate that without hydrazine no hydroxylamine accumulated during ammonia oxidation, to limit the amount of nitrite formed in these experiments and so minimize the possibility of masking small quantities of hydroxylamine by large quantities of nitrite. The experiments were therefore run for only a short period; as a consequence the oxygen uptakes were too small to be measured accurately and are omitted from Table 4.

DISCUSSION

The results of Table 1 show, as Meyerhof (1917) found, a quantitative agreement between oxygen uptake and nitrite formation in *Nitrosomonas* suspensions. The table also shows that nitrite formation closely paralleled ammonia disappearance; this fact proves that there could have been little, if any, accumulation of compounds intermediate between ammonia and nitrite during oxidation of the ammonia.

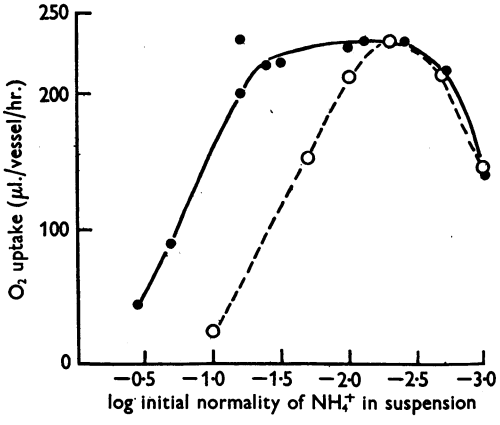

Fig. 2. The relation between ammonia concentration of the medium and rate of O_2 uptake by *Nitrosomonas*. Each Warburg vessel contained 3 ml. washed suspension of *Nitrosomonas*. Ammonium sulphate solution (0·1 ml.) was added from the side arms after temperature equilibration to give in the suspensions the initial ammonia concentrations indicated. Duration of experiment, 120 min. Dotted curve represents the results of Meyerhof (1917) redrawn to the same maximum rate of O_2 uptake as was observed in the present experiments.

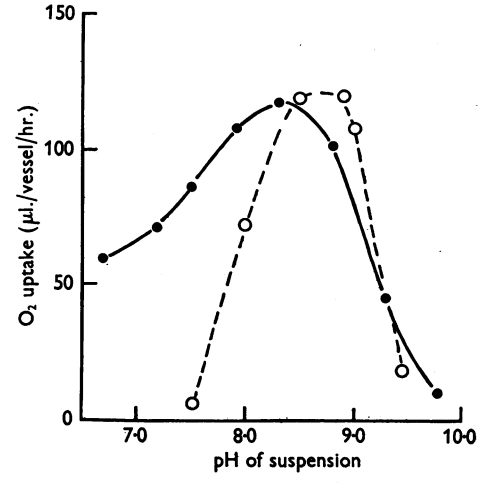

Fig. 3. The relation between pH of the suspension medium and rate of O_2 uptake by *Nitrosomonas*. Each Warburg vessel contained 3 ml. washed suspension of *Nitrosomonas* made up in phosphate-borate buffer of the required pH (for details of preparation see Materials and Methods section). Ammonium sulphate solution (0·1 ml.) was added from the side arm after temperature equilibration to give an initial concentration in the suspension of 10^{-2}M-$(NH_4)_2SO_4$. Duration of experiment, 120 min. Dotted curve represents the results of Meyerhof (1917) redrawn to the same maximum rate of O_2 uptake as was observed in the present experiments.

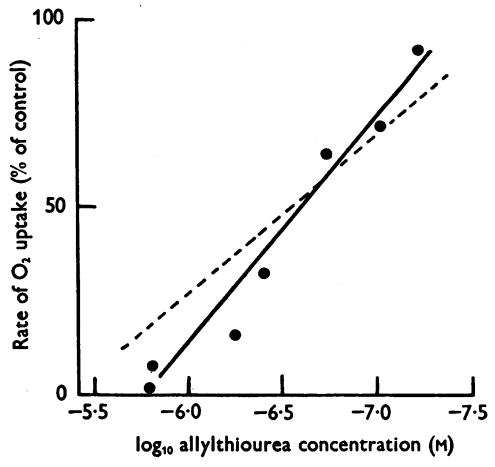

Fig. 4. The inhibition by allylthiourea of ammonia oxidation by *Nitrosomonas*. Each Warburg vessel contained 3 ml. washed suspension of *Nitrosomonas* made up in either water (control vessel) or in allylthiourea solutions of various strengths. Ammonium sulphate solution (0·1 ml.) was added from the side arms after temperature equilibration to give an initial concentration in the suspension of 5×10^{-3}M-$(NH_4)_2SO_4$. Observed rates of O_2 uptake (μl./vessel/hr.) were converted to percentages of the rate observed in the control vessel. Duration of experiment, 120 min. Dotted curve is the corresponding curve redrawn from Lees (1952*a*).

Table 2. *The absence of competition between allylthiourea and ammonia*

(Washed suspensions (3 ml.) of *Nitrosomonas* made up in water or in 2×10^{-7}M-allylthiourea and placed in the main compartments of the Warburg vessels: 0·1 ml. $(NH_4)_2SO_4$ solution added from side arms after temperature equilibration to give in the suspensions the initial $(NH_4)_2SO_4$ concentrations shown in the table. Temp. 30°; duration of experiment, 120 min.)

Nitrosomonas suspended in	Final $(NH_4)_2SO_4$ concentration (M)	O_2 uptake (μl./vessel/hr.)	Inhibition due to allylthiourea (%)
Water	$1·8 \times 10^{-1}$	31	—
Allylthiourea	$1·8 \times 10^{-1}$	14	55
Water	$3·0 \times 10^{-2}$	172	—
Allylthiourea	$3·0 \times 10^{-2}$	90	48
Water	$5·0 \times 10^{-3}$	167	—
Allylthiourea	$5·0 \times 10^{-3}$	86	48
Water	$8·0 \times 10^{-4}$	111	—
Allylthiourea	$8·0 \times 10^{-4}$	51	54

The present results on the influence of pH on the respiration rate (Fig. 1) agree well with Meyerhof's results above pH 8·0. At pH's lower than 8·0, however, Meyerhof noted a far greater diminution of respiration than we observed. This discrepancy between our results and his was probably due to the fact that Meyerhof reduced the pH of his sus-

Table 3. *The effect of allylthiourea on the oxidation of ammonia and of hydroxylamine*

(Washed suspensions (3 ml.) of *Nitrosomonas* made up in water or in 10^{-5}M-allylthiourea and placed in the main compartments of the Warburg vessels; 0·1 ml. $(NH_4)_2SO_4$ solution or $NH_2OH.HCl$ solution (neutralized with NaOH immediately before use) added from side arms after temperature equilibration to give in the suspensions initial concentration of 5×10^{-4}M-$(NH_4)_2SO_4$ or 10^{-3}M-NH_2OH, temp. 30°. There was no appreciable O_2 uptake in the absence of ammonia or hydroxylamine nor in their presence when boiled suspensions of *Nitrosomonas* were used. Duration of experiment, 120 min.)

Composition of suspension fluid	O_2 uptake (μmoles)	NO_2 formed during experiment: Calculated* (μmoles)	NO_2 formed during experiment: Observed (μmoles)
Ammonium sulphate	4·60	3·08	3·13
Hydroxylamine hydrochloride	1·83	1·83	1·84
Ammonium sulphate + allylthiourea	0·09	0·06	0·06
Hydroxylamine hydrochloride + allylthiourea	1·47	1·47	1·63

* Calculated from O_2 uptake

pensions from an initial value of 8·5 to lower values merely by adding hydrochloric acid directly to the suspension in the Warburg flask. To obtain a final pH of 7·6 he was compelled to add 0·45 ml. of 0·5N-hydrochloric acid to 1·5 ml. of *Nitrosomonas* suspension. The addition of such a relatively high concentration of acid must have caused a profound fall in pH of the suspension before the slow equilibration of the hydrochloric acid with the alkaline earth carbonates, present in the suspension, raised the pH again to its final value of 7·6. It is therefore probable that most of the organisms were killed by the temporarily extremely acid medium before the experiment began. The present results, but not Meyerhof's, conform with the well recognized fact that in soils nitrification proceeds readily at pH's as low as 5·5. Above pH 8·5, Meyerhof's results and our own were in excellent agreement; at pH's above 8·5 the suspension medium exerts no buffering effect and final pH values were obtained as soon as the necessary trace of alkali was added. Agreement between our results and his was therefore to be expected.

Table 4. *The effect of hydrazine on the oxidation of ammonia and of hydroxylamine by* Nitrosomonas

(Washed suspensions (3 ml.) of *Nitrosomonas* made up in water or 3×10^{-3}M-hydrazine sulphate and placed in main compartments of Warburg vessels; 0·1 ml. $(NH_4)_2SO_4$ solution, $NH_2OH.HCl$ solution, or water, added from side arms after temperature equilibration to give concentrations of (approximately) 4×10^{-4}M-$(NH_4)_2SO_4$ or 8×10^{-4}M-$NH_2OH.HCl$ in the suspensions. The hydrazine sulphate and hydroxylamine hydrochloride solutions were neutralized with NaOH immediately before use. Duration of experiment, 30 min. Initial NO_2-N$\simeq$0·3 μg./vessel. The formation of NO_2-N and $NH_2.OH$-N in the control vessels was from traces of ammonia in the washed suspensions (see Materials and Methods section).)

Nitrosomonas suspended in	μg. N per vessel found at the end of experiment as: NO_2-N	NH_2OH-N
Water	1·3	0
Ammonium sulphate	13·5	0
Hydroxylamine	9	21
Hydrazine	0·3	0·9
Hydrazine + ammonium sulphate	0·3	3·3
Hydrazine + hydroxylamine	0·3	29

A comparison between the present results and those of Meyerhof on the effect of ammonia concentration on oxygen consumption (Fig. 2) shows that Meyerhof found high ammonia concentrations somewhat more inhibitory than we did. The discrepancy is, however, not great and may well be due to a slight difference in ammonia tolerance between our strain of organisms and his.

The results given in Tables 3 and 4 are in agreement with the theory (Kluyver & Donker, 1926) that the oxidation of ammonia by *Nitrosomonas* proceeds via hydroxylamine. The steps envisaged by these workers were:

$$\underset{A}{NH_3} \rightarrow \underbrace{NH_2OH}_{B} \text{—} (H_2N_2O_2) \rightarrow HNO_2$$

We have now obtained experimental support for this theory as far as hydroxylamine is concerned. We have found that step *A* is inhibited by allylthiourea (Table 3) and that step *B* is inhibited by hydrazine (Table 4). Step *A* is also inhibited to some extent by hydrazine, perhaps indirectly by the accumulation of hydroxylamine resulting from the blockage of step *B*. The accumulation of hydroxylamine in the presence of hydrazine is shown by the results of Table 4. These results also show that in the absence of hydrazine no hydroxylamine accumulates during ammonia oxidation, thus confirming the results of Table 1.

The inhibition of step *A* by allylthiourea at very low concentrations (Fig. 4) suggests that, as previously postulated (Lees, 1946, 1948, 1952*a*), a metal enzyme is involved in ammonia oxidation and

that allylthiourea inhibition is due to chelation of the inhibitor with the essential metal. It now seems certain that the inhibition is not due to competition with ammonia, a theory adumbrated by Quastel & Scholefield (1951) since, as found previously by a different technique (Lees, 1952*a*) no competition between ammonia and allylthiourea could be detected (Table 2). Since the inhibition of nitrite formation by thiourea and allylthiourea is far more intense than the inhibition by any other of a wide range of chelating agents (Lees, 1952*a*) and since the thioureas are known to have a peculiarly high affinity for copper, there is good presumptive evidence for the theory already advanced (Lees, 1946) that the enzyme concerned in the primary ammonia oxidation by *Nitrosomonas* is a copper enzyme.

SUMMARY

1. The metabolism of cultures of *Nitrosomonas* has been investigated by the Warburg technique.

2. In contrast to results previously obtained by Meyerhof, the ammonia-oxidizing activities of *Nitrosomonas* in culture was found to be affected by change in pH in the same way as it is affected in soil.

3. Evidence is presented to show that the oxidation of ammonia to nitrite by *Nitrosomonas* proceeds via hydroxylamine. Hydrazine has been found to inhibit selectively the oxidation of hydroxylamine. In the presence of hydrazine, hydroxylamine accumulated, and was detected, during ammonia oxidation.

4. The oxidation of ammonia to hydroxylamine was completely inhibited by allylthiourea at concentrations which did not affect oxidation of the hydroxylamine.

We wish to thank Prof. W. O. Kermack, F.R.S., for his advice and encouragement throughout this work. One of us (T.H.) also wishes to acknowledge the receipt of a grant from the Agricultural Research Council.

REFERENCES

Bömecke, H. (1939). *Arch. Mikrobiol.* **10**, 385.
Bömecke, H. (1951). *Arch. Mikrobiol.* **15**, 414.
Conway, E. J. (1950). *Microdiffusion Analysis and Volumetric Error*, 3rd ed. London: Crosby Lockwood and Son Ltd.
Csaky, T. Z. (1949). *Acta chem. scand.* **2**, 450.
Hofman, T. & Lees, H. (1952). *Biochem. J.* **52**, 140.
Kluyver, A. J. & Donker, H. J. (1926). *Chem. Zelle*, **13**, 134.
Lees, H. (1946). *Nature, Lond.*, **158**, 97.
Lees, H. (1948). *Biochem. J.* **42**, 534.
Lees, H. (1952*a*). *Biochem. J.* **52**, 134.
Lees, H. (1952*b*). *Nature, Lond.*, **169**, 156.
Meiklejohn, J. (1950). *J. gen. Microbiol.* **4**, 185.
Meyerhof, O. (1917). *Pflüg. Arch. ges. Physiol.* **166**, 240.
Quastel, J. H. & Scholefield, P. G. (1951). *Bact. Rev.* **15**, 1.
Winogradsky, S. (1890). *Ann. Inst. Pasteur*, **4**, 257, 760.

Return of Cholinesterase Activity in the Rat after Inhibition by Organophosphorus Compounds

1. DIETHYL *p*-NITROPHENYL PHOSPHATE (E600, PARAOXON)

By A. N. DAVISON
Medical Research Council Unit for Research in Toxicology, Serum Research Institute, Carshalton, Surrey

(*Received* 16 *December* 1952)

The inhibition of cholinesterase *in vitro* by organophosphorus compounds has generally been considered to be an irreversible process. However, many workers have shown that cholinesterase activity does return both *in vivo* and in isolated tissue preparations (Koelle & Gilman, 1946; Mazur & Bodansky, 1946; Grob, Lilienthal, Harvey & Jones, 1947; Dubois, Doull, Salerno & Coon, 1949; Freedman, Willis & Himwich, 1949; Hobbiger, 1951; Burgen & Hobbiger, 1951; Frawley, Hagan & Fitzhugh, 1952). Two alternative mechanisms of this return of activity have been considered. Koelle & Gilman (1946), Mazur & Bodansky (1946) and Grob *et al.* (1947) have suggested that this return is due to the resynthesis of new enzyme, and that the original inhibition is irreversible. However, the difference between the rates of return of activity *in vivo* after inhibition by various organophosphorus compounds indicate that this view cannot be of general application (Dubois *et al.* 1949; Callaway, Davies & Risley, 1952). An alternative mechanism is based on the view that during inhibition the enzyme is phosphorylated. On the basis of experiments with electric-eel cholinesterase inhibited by