Properties of some Reductase Enzymes in the Nitrifying Bacteria and their Relationship to the Oxidase Systems

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The reductase enzymes in *Nitrosomonas* and *Nitrobacter* were studied under anaerobic conditions when the oxidase enzymes were inactive. The most effective electron-donor systems for nitrate reductase in *Nitrobacter* were reduced benzyl viologen alone, phenazine methosulphate with either NADH or NADPH, and FMN or FAD with NADH. Nitrite and hydroxylamine reductases were found in both nitrifying bacteria, and optimum activity for each enzyme was obtained with NADH or NADPH with either FMN or FAD. The product of both these enzymes was identified as ammonia. In extracts of *Nitrosomonas* the ammonia was further utilized by an NADPH-specific glutamate dehydrogenase. ^{15}N-labelled nitrite, hydroxylamine and ammonia were rapidly incorporated into cell protein by *Nitrosomonas*, and *Nitrobacter* in addition incorporated [^{15}N]nitrate. Relatively gentle methods of cell disruption were compared with ultrasonic treatment, to enable a more exact study to be undertaken of the intracellular distribution of the oxidase and reductase enzymes. The functional relationship of these opposing enzyme systems in the nitrifying bacteria is considered.

The nitrifying bacteria *Nitrosomonas* and *Nitrobacter* oxidize ammonia to nitrate. This eight-electron oxidation process occurs in two stages. *Nitrosomonas* converts ammonia (oxidation state for nitrogen -3) into nitrite (oxidation state $+3$); hydroxylamine (-1) is a well-established intermediate in this reaction, but there is still some doubt about the nature of the compound at the $+1$ oxidation state in the system (Falcone, Shug & Nicholas, 1963). Nitrite is further oxidized to nitrate ($+5$) by *Nitrobacter*.

Cell-free nitrification was first demonstrated in *Nitrobacter* by Aleem & Alexander (1958), and with *Nitrosomonas* extracts Nicholas & Jones (1960) obtained a conversion of hydroxylamine into nitrite in the presence of a suitable electron acceptor such as cytochrome *c* or PMS.* It was subsequently shown that these oxidase reactions were linked to phosphorylation processes (Aleem & Nason, 1960; Ramaiah & Nicholas, 1964). In these chemo-autotrophic bacteria a reversal of the electron-transfer system produces NADH (Aleem, Lees & Nicholas, 1963; Aleem, 1965).

Studies on the inorganic nitrogen metabolism of the nitrifiers have been mainly concerned with the oxidation reactions and very little work has been done on the reductase pathways and related

reactions whereby nitrogen is assimilated into amino acids and into cell nitrogen. In this paper the reductase enzymes identified in *Nitrosomonas* and *Nitrobacter* are described and their relation to the oxidase enzymes is considered. Cells of both nitrifiers were exposed to ^{15}N-labelled nitrate, nitrite, hydroxylamine and ammonia, and the incorporation of each substrate into protein was determined. In studies on the fine structure of the nitrifying bacteria, Murray & Watson (1965) showed that there is a considerable structural difference between *Nitrosomonas* and *Nitrobacter*. The latter has a more marked cell wall and plasma membrane and a more extensive intracellular membrane system. In most previous studies on these organisms ultrasonic treatment has been used to disrupt the cells. As a preliminary to this work a number of more gentle fractionation procedures were tested for their effectiveness in disrupting the cells of *Nitrosomonas* and *Nitrobacter*. In the Discussion section an attempt is made to correlate the enzyme activities *in vitro* with their intracellular distribution and functional significance.

MATERIALS AND METHODS

Materials. NADH, NADPH, NADP+, FMN, FAD and lysozyme were obtained from Calbiochem (Los Angeles, Calif., U.S.A.); ADP, ATP, PMS, cytochrome *c*, firefly

* Abbreviations: PMS, phenazine methosulphate; BV, benzyl viologen.

lanterns, hexokinase, glucose 6-phosphate dehydrogenase and yeast concentrate were from Sigma Chemical Co. (St Louis, Mo., U.S.A.); BV, riboflavine, o-phenanthroline and 8-hydroxyquinoline were from British Drug Houses Ltd. (Poole, Dorset); deoxyribonuclease was from Koch–Light Laboratories Ltd. (Colnbrook, Bucks.); menadione (vitamin K) was from Nutritional Biochemicals Corp. (Cleveland, Ohio, U.S.A.); $^{15}NH_4NO_3$, $K^{15}NO_3$ and $Na^{15}NO_2$ were from the Office National Industriel de l'Azote (Paris, France); and hydroxyl[^{15}N]amine hydrochloride was from Merck, Sharp and Dohme (Montreal, P.Q., Canada).

Culture of organisms. *Nitrosomonas europaea* and *Nitrobacter agilis* were grown in batch cultures, as described by Nicholas & Rao (1964) for *Nitrosomonas*. For *Nitrobacter* the ammonia in the culture medium was replaced by nitrite. The cells were harvested with a Sorvall RC-2 refrigerated centrifuge at 35000g with a continuous-flow head. A low rate of flow (12l./hr.) was used to obtain maximum yields. Cell samples were stored at 0° in ice. The *Nitrosomonas* cell pellet was reddish pink and that of *Nitrobacter* was brownish pink. The cells were washed three times in cold 0·05M-phosphate buffer, pH7·8, before use.

Methods for disrupting the cells. Full experimental details are given in the Results section, but a brief description is given here of the equipment used. An MSE ultrasonic disintegrator (20kcyc./sec.) was used, and the sample (cell pellet with 0·05M-phosphate buffer, pH7·8, 1:3, w/v) was placed in a double-walled glass container through which ice-cold water was continuously circulated. An Aminco French pressure cell (40ml. capacity) was used at 0° with an Aminco motor-driven press, giving a pressure inside the cell of 25000lb./in.^{2}. When the Bühler homogenizer was used the sample container was enclosed in an ice jacket and treatment was given over 30sec. periods. The sample for the pressure cell and the homogenizer consisted of cell pellet with 0·05M-phosphate buffer, pH7·8 (1:7, w/v). For centrifugation studies on cell extracts a Sorvall SS-1 (30000g centrifuge) and a Spinco model L (50 rotor; 225000g) ultracentrifuge were used.

Enzyme assays. All reductase assays were done in Thunberg tubes (20ml.). The enzyme fraction, cofactors and phosphate buffer, pH7·8, to give a final volume of 2·5ml. were placed in the main tube. The substrate (nitrate, nitrite or hydroxylamine) and the NADH or NADPH were placed in the side arm. The hydroxylamine solution was prepared just before use and the pH adjusted to 6·5 with 2N-NaOH. The electron donor and the enzyme were added to the tubes just before evacuation with a Speedivac high-vacuum pump. After a 5min. preincubation the contents of the tubes were mixed, and all assays were run for 30min. at 30° in a water bath with shaking at 50oscillations/min. When the *Azotobacter* transhydrogenase system (Naik & Nicholas, 1966b) was used, BV and the *Azotobacter* particles were placed in the main tube and NADH was added to the side arm as described previously. Full details of the assays for the reductase enzymes are given with each experiment. Chemical determinations are described in the following section.

(*a*) *Nitrite oxidase.* The assay, in an open tube, contained: nitrite, 0·4mM; enzyme, 0·75mg. of protein; phosphate buffer, pH7·8, 40mM; in a total volume of 2·5ml. The assay was for 30min. at 30°. The activity was determined as nitrite utilization.

(*b*) *Hydroxylamine oxidase.* The assay, in an open tube,

contained: hydroxylamine, pH6·5, 0·5mM; PMS, 7·5μM; cell extract, 0·01mg. of protein; phosphate buffer, pH7·8, 40mM; in a total volume of 2·5ml. Nitrite and hydroxylamine were determined at 0, 15 and 30min. The assay was at 30°.

(*c*) *Glutamate dehydrogenase.* The assay was by the amination reaction (W. Wallace & D. J. D. Nicholas, unpublished work) in a 3ml. cuvette containing: NADPH, 0·2mM; (NH$_4$)$_2$SO$_4$, 16mM; α-oxoglutarate, pH7·0, 3mM; enzyme, 0·2mg. of protein; phosphate buffer, pH7·8, 100mM. The assay was by the initial (1min.) decrease in E_{340} at 30°.

Chemical determinations. Internal standards (0·4mM) for nitrite, hydroxylamine and ammonia were used in the assays.

(*a*) Nitrite was determined by the Griess Ilosvay colorimetric method. To a portion (0·5ml.) of the assay mixture were added 0·03ml. of M-zinc acetate and 0·47ml. of ethanol to remove any residual NADH or NADPH that would interfere with the subsequent diazotization of nitrite (Medina & Nicholas, 1957a). After standing for 10min. the samples were centrifuged at 5000g for 5min., 0·1ml. of the clear supernatant fraction was diluted to 1ml. with water, and 1ml. of 1% (w/v) sulphanilamide in 1·5N-HCl (A.R.) and 1ml. of 0·02% N-(1-naphthyl)ethylenediamine dihydrochloride were added. The colour, which was allowed to develop for 20min., was determined in a Hilger colorimeter with filter 55.

(*b*) Direct methods for the determination of hydroxylamine, the 8-hydroxyquinoline method (Frear & Burrell, 1955) and the o-phenanthroline method (Medina & Nicholas, 1957b), were not found to be sufficiently quantitative for the present study. The Csáky method, where hydroxylamine is oxidized with iodine and measured as nitrite, was therefore used. A portion (0·2ml.) of the assay mixture was taken and the modified Csáky procedure described by Hewitt & Nicholas (1964) followed.

(*c*) Ammonia was assayed by the Russell alkaline phenoxide method after distillation by the Conway (1962) procedure: 1ml. of the assay mixture was added to the outer well of a prepared Conway unit; 1·5ml. of 0·01N-HCl was present in the centre well and 1ml. of saturated K$_2$CO$_3$ soln. was restricted to one side of the outer well. The unit was sealed and the contents of the outer well were mixed. After 3hr. diffusion at room temperature a portion (1ml.) was taken from the centre well and chilled in an ice bath. The following reagents were then added, with mixing: 0·01ml. of 3mM-MnCl$_2$, 1ml. of alkaline phenoxide [27% (w/v) phenol in 2·7N-NaOH]; 0·2ml. of sodium hypochlorite (1M in 0·1N-NaOH). The tubes were immersed in a boiling-water bath for 5min. and, after cooling, 5ml. of water was added and the colour was measured at 625mμ.

(*d*) Protein precipitated with 5% (w/v) trichloroacetic acid was determined by the Folin method of Lowry, Rosebrough, Farr & Randall (1951).

Assay procedure for adenylate kinase and phosphorylation reactions in Nitrobacter. In the main Thunberg tube were: phosphate buffer, pH7·8, 4mM; MgCl$_2$, 0·4mM; cell extract; tris buffer, pH7·8, 40mM; ADP was in the side arm to give a final concentration of 0·4mM in total tube contents of 3·0ml. The assay was for 15min. at 30°, and the reaction was stopped with 0·1ml. of 70% (w/v) HClO$_4$. ATP was determined by the firefly luciferase method (McElroy, 1963). ATP was also assayed by the glucose–

hexokinase–glucose 6-phosphate dehydrogenase–NADP+ method; in this case glucose ($4\,\mu M$) and hexokinase (13 units) were also included in the Thunberg tube. The glucose 6-phosphate formed was assayed with glucose 6-phosphate dehydrogenase (13 units) by the method of Naik & Nicholas (1966a).

^{15}N experiments. The incubation mixture contained: washed cell suspension, 30mg. of protein; ^{15}N-labelled substrate, 1mg. of N, in solution at pH7·5; $KHCO_3$, 1·66mM; in a total volume of 3ml. of 0·05M-phosphate buffer, pH7·5. Incubation was for 2hr. in a 100ml. conical flask kept in a shaker in a water bath at 30°. The experiment was stopped by transferring the reaction mixture to a chilled polyethylene tube and adding 20ml.of cold 0·05M-phosphate buffer, pH7·5. The mixture was centrifuged at 10000g and the pellet was washed with two 20ml. lots of 0·05M-phosphate buffer, pH7·5. The pellet was then suspended in 10ml. of 0·05M-phosphate buffer, pH7·5, and 10ml. of 20% (w/v) trichloroacetic acid was mixed in vigorously. After being left overnight the precipitate was washed with a further quantity of 10% (w/v) trichloroacetic acid. The protein precipitate was then prepared for ^{15}N-enrichment analyses by the procedure described by Brownell & Nicholas (1967).

RESULTS

Methods of disrupting the bacteria. Cells of *Nitrosomonas* were readily broken in the French pressure cell, as shown in Table 1. When this extract was centrifuged and the pellet of unbroken cells and cell debris was treated ultrasonically for 10min. there was a further extraction of cell protein. This amount, together with that in the first supernatant fraction, accounted for most of the protein present in the intact cells. An equally satisfactory result was achieved by treating the cells ultrasonically for 10min. A good extraction of *Nitrosomonas* cells was also obtained by the lysozyme technique of Rees & Nason (1965). The cells, after treatment for 2hr. at 30° in 0·25M-sucrose–1mM-EDTA containing lysozyme (1mg./ml.), were disrupted by suspension in 5mM-phosphate buffer, pH7·8. Extracts of *Nitrosomonas* prepared either by this method or in the French pressure cell were extremely viscous, indicating that the cell nucleoplasm had not been disrupted. These extracts were readily clarified by adding deoxyribonuclease ($1\,\mu g./ml.$). When the *Nitrosomonas* cells were treated ultrasonically the extracts were non-viscous since the nucleoplasm was dispersed.

The thick-walled *Nitrobacter* cells, even after treatment in the French pressure cell followed by ultrasonic treatment for 40min. (Table 1), were only partially broken. The lysozyme-disruption technique was also ineffective. Lysozyme concentrations of 0·5–5·0mg./ml. and EDTA concentrations of 3·0–10·0mg./ml. were tested in 0·05M-tris buffer, pH7·5. It was found, however, that *Nitrobacter* cells, treated first with lysozyme, as described for *Nitrosomonas* above, and then suspended in 0·05M-tris buffer, pH7·5, were partially disrupted in the French pressure cell (Table 2).

The extraction of nitrite oxidase, nitrate reductase and glutamate dehydrogenase from *Nitrobacter* varied greatly with the method of cell disruption. The mortar-and-pestle grinding technique gave a low yield of all these enzymes, especially the nitrite oxidase. The cell extract prepared with the homogenizer and glass beads was dark brown in colour and had a low nitrate reductase activity. Ultrasonic treatment of the cells gave an extract that had the highest activity of each of the three enzymes tested. The lysozyme–French-pressure-cell technique and ultrasonic treatment gave the same yield of nitrite oxidase (Table 2) but the latter gave a greater than twofold increase in nitrate reductase.

Other cell-disruption methods tested and found to be ineffective were acetone treatment (Nicholas & Mabey, 1960) and an osmotic-shock procedure (Pangborn, Marr & Robrish, 1962). In the experiments to be described, unless otherwise stated, cells of *Nitrosomonas* and *Nitrobacter* were disrupted by ultrasonic treatment for 10 and 20min. respectively.

Intracellular distribution of the oxidase and reductase enzymes. When the *Nitrosomonas* cells

Table 1. *Comparison between the French-pressure-cell method and the ultrasonic method for the extraction of protein from cells of* Nitrosomonas *and* Nitrobacter

	Total protein (mg.)	
Treatment	*Nitrosomonas*	*Nitrobacter*
Intact cells (2·0g. wet wt.)	256	206
(1) Passed through a French pressure cell three times. Supernatant fraction (10000g for 15min.) taken	167	23
(2) Pellet from (1) treated ultrasonically for 10min. Supernatant fraction (10000g for 15min.) taken	63	38
(3) Pellet from (2) treated ultrasonically for 30min. Supernatant fraction (10000g for 15min.) taken	—	42

Table 2. *Effect of various methods of cell disruption on the extraction of nitrite oxidase, nitrate reductase and glutamate dehydrogenase from* Nitrobacter

Washed-cell samples of *Nitrobacter* were used (2·0g. wet wt., 180mg. of protein) and the extraction medium was tris buffer, pH7·5, (0·05M) containing EDTA (1mM) and GSH (1mM). The amounts of protein and enzyme activities were determined in the supernatant fraction left after centrifugation of the extracts at 10000g (15min.). Nitrite oxidase and glutamate dehydrogenase were assayed as described in the text. Nitrate reductase was assayed as described in Table 5, with NADH (0·64mM) in the side arm and FMN (4μM) in the main tube.

			Total enzyme activity (μmoles/30min.)		
	Method of cell disruption	Total protein (mg.)	Nitrite oxidase	Nitrate reductase	Glutamate dehydrogenase
(a)	Lysozyme treatment in a sucrose medium (0·25M) followed by treatment of the resultant cell pellet (2000g for 10min.), in the tris buffer, with the French pressure cell	69	20·4	20·7	65·1
(b)	Grinding with a mortar and pestle in the presence of liquid N_2	58	4·4	26·0	26·0
(c)	Homogenizing with glass beads (size 16) for 5min.	57	12·8	8·6	57·9
(d)	Ultrasonic treatment for 20min.	57	21·1	50·3	74·5

Table 3. *Distribution of glutamate dehydrogenase and hydroxylamine oxidase and reductase in* Nitrosomonas

Hydroxylamine oxidase and glutamate dehydrogenase were assayed as described in the text. Hydroxylamine reductase was assayed as described in Table 9 with NADPH (0·64mM) in the side arm and FMN (4μM) in the main tube. (a) Cells (306mg. of protein) were treated in 0·3M-sucrose–1mM-EDTA containing lysozyme (1mg./ml.) for 2hr. and centrifuged at 2000g for 20min., and the pellet was suspended for 1hr. in 5mM-phosphate buffer, pH7·8, containing deoxyribonuclease (1μg./ml.). The cell extract was centrifuged at 10000g for 10min. and then at 144000g for 120min. (b) Cells (350mg. of protein) were suspended in 3M-glycerol (10min.) and then rapidly ejected into and mixed by Vortex action in 0·05M-phosphate buffer, pH7·8, containing deoxyribonuclease (1μg./ml.). After 1hr. the extract was centrifuged at 5000g and the pellet obtained was then treated ultrasonically for 10min. The centrifugation step was then repeated.

		Total activity (μmoles of substrate used/30min.)		
Fraction	Total protein (mg.)	Hydroxylamine oxidase	Hydroxylamine reductase	Glutamate dehydrogenase
(a)				
Pellet (10000g for 10min.)	6·5	65	3·1	72
Pellet (144000g for 120min.)	4·4	26	1·0	22
Soluble (144000g for 120min.)	97·0	15326	19·6	1183
(b)				
Supernatant (5000g for 60min.) after treatment below				
(1) Osmotic shock treatment	180	12620	22·6	8422
(2) Ultrasonic treatment (10min.) of pellet from (1)	200	4350	3·7	507

were disrupted by the lysozyme–osmotic lysis technique (Table 3a), hydroxylamine reductase, hydroxylamine oxidase and glutamate dehydrogenase were found mainly in the supernatant fraction after centrifugation at 144000g for 2hr. This supernatant fraction, which was reddish pink in colour, was very turbid. In earlier work it was shown that hydroxylamine oxidase (Falcone *et al.* 1963) and glutamate dehydrogenase (W. Wallace & D. J. D. Nicholas, unpublished work) were sedimented by centrifugation for 16hr. at 100000g; in those studies the cells were disrupted ultrasonically and the extracts were not treated with

deoxyribonuclease. The osmotic-shock method of cell rupture (Table 3b) also released a high proportion of the enzymes from *Nitrosomonas*. If the cell pellet obtained by centrifuging the extract at 5000g for 1hr. was then treated ultrasonically, a further amount of hydroxylamine oxidase was released (35% of that obtained with osmotic treatment); the corresponding values for hydroxylamine reductase and glutamate dehydrogenase were 16% and 6% respectively. It was also shown (Table 3b) that treatment of the cell pellet ultrasonically after the osmotic-shock treatment provided for a complete extraction of the protein

Table 4. *Distribution of nitrite oxidase, nitrate reductase and glutamate dehydrogenase in* Nitrobacter

Cells (2·0g. wet wt.) were disrupted by the lysozyme–French-pressure-cell method (Table 2). Enzyme assays were as described in Table 2.

Cell fraction	Total protein (mg.)	Total enzyme activity/30 min.		
		Nitrite oxidase (μmoles of NO_2^- oxidized)	Nitrate reductase (μmoles of NO_2^- formed)	Glutamate dehydrogenase (μmoles of NADPH oxidized)
Intact cells	248	1167·8	27·2	0
Supernatant fraction (2000g for 20 min.)	140	72·0	21·8	40·6
Supernatant fraction (10000g for 15 min.)	114	66·0	13·0	42·2
Pellet (10000g)	14	13·0	2·8	0
Supernatant fraction (144000g for 60 min.)	86	18·6	13·7	44·7
Pellet (144000g)	42	44·0	4·6	0

from the *Nitrosomonas* cell. A much smaller amount of protein was extracted by osmotic lysis of the lysozyme-treated cells (Table 3a).

A similar study was undertaken on *Nitrobacter* cells disrupted by the lysozyme–French-pressure-cell technique (Table 4). A better extraction of protein was obtained (2000g supernatant) than in Table 2; in the latter the 10000g supernatant was assayed. After centrifugation of the *Nitrobacter* cell extract at 144000g for 60 min., most of the nitrite oxidase (Table 4) was localized in the pellet fraction (see also Aleem & Alexander, 1958). When the cells were disrupted by the lysozyme–French-pressure-cell technique (Table 4), nitrate reductase was distributed mainly in the soluble fraction, but when the cells were broken by ultrasonic treatment this enzyme was also found associated with the particulate material.

In the lysozyme–French-pressure-cell extract of *Nitrobacter*, glutamate dehydrogenase was found exclusively in the soluble fractions (Table 4). The highest specific activity recorded for this enzyme in *Nitrosomonas*, 47 μmoles/mg. of protein (Table 3b), was more than 80 times the highest value obtained for *Nitrobacter* (Table 4: 144000g supernatant, 0·52 μmole/mg. of protein). In *Nitrosomonas*, even after ultrasonic treatment, the enzyme was sedimented only by prolonged high-speed centrifugation. Glutamate dehydrogenase, of the enzymes studied in the cells of the nitrifying bacteria, was thus the least associated with intracellular membranes. A high activity of nitrate reductase was detected with the intact cells (Table 4), but the activity was largely independent of the addition of the electron-donor system.

Properties of the oxidase and reductase enzymes

Nitrite oxidase. A rapid oxidation of nitrite occurred in a washed suspension of *Nitrobacter* cells:

156 mμmoles of nitrite oxidized/min./mg. of cell protein. In cell-free extracts, however, the highest specific activity recovered was 39 mμmoles/min./mg. of protein. No significant increase in activity was found with the addition to the assay of yeast extract (3 mg.), pig heart extract (3 mg.), ferric chloride (2 mM), cytochrome c (40 μM), PMS (6 μM), FMN or FAD (5 μM), riboflavine (10 μM) or vitamin K (0·2 mM). When NADH and FMN were added to the nitrite oxidase system, at the concentration used for nitrate reductase, a 24% inhibition of the oxidase was recorded.

Nitrate reductase. Under anaerobic conditions, when nitrite oxidase was inhibited, a reduction of nitrate to nitrite was demonstrated in cell extracts of *Nitrobacter*. The reductase activity was stimulated by adding NADH and FMN to the extracts (Table 5). Only a quarter of the activity was obtained when NADPH was used instead of NADH, but FAD was found to be only slightly less effective than FMN. When reduced BV was used as the electron donor there was a greatly increased nitrate reductase activity. The BV was reduced with the *Azotobacter* transhydrogenase enzyme, and with this system the control was an assay with boiled *Nitrobacter* enzyme. Another very effective electron-donor system for the nitrate reductase was PMS with either NADH or NADPH. The addition of PMS even without NADH or NADPH also stimulated the nitrate reductase, but this may have been due to amounts of endogenous nucleotides in the extract. Nitrate reductase was not detected in *Nitrosomonas*.

Phosphorylation activity associated with nitrate reductase in Nitrobacter. In preliminary studies an active adenylate kinase system was found in the *Nitrobacter* cell extract (Table 6). The enzyme was found mainly in the supernatant fraction, its activity was directly related to the amount of its substrate, ADP, included in the assay, and a

Table 5. *Nitrate reductase in* Nitrobacter

The assay was in a Thunberg tube for 30 min. at 30°. Side arm: nitrate (0·8 mM) and NADH or NADPH as indicated. Main tube: *Nitrobacter* enzyme (10 000g supernatant), 3·35 mg. of protein; phosphate buffer, pH 7·8, 40 mM; FMN, FAD or PMS. (When the *Azotobacter* transhydrogenase system was used to reduce BV, it was added to the main tube.) All concentration values are for the final 2·5 ml. reaction mixture.

Assay system		Nitrite produced (mμmoles/mg. of protein)
Electron donor	Cofactor	
None	None	20
NADH (0·64 mM)	None	49
NADH (0·64 mM)	FMN (4 μM)	219
NADH (0·64 mM)	FAD (4 μM)	161
NADPH (0·64 mM)	None	13
NADPH (0·64 mM)	FMN (4 μM)	53
NADPH (0·64 mM)	FAD (4 μM)	57
BV (0·13 mM)–*Azotobacter* transhydrogenase NADH (0·64 mM)		1064
Azotobacter transhydrogenase NADH (0·64 mM) (with boiled *Nitrobacter* enzyme)		56
None	PMS (50 μM)	89
NADH (0·64 mM)	PMS (50 μM)	680
NADPH (0·64 mM)	PMS (50 μM)	789

Table 6. *Adenylate kinase in* Nitrobacter

Cells were disrupted by ultrasonic treatment, and the cell-free extract was centrifuged at 144 000g for 1 hr. The assay method was as described in the text.

Enzyme sample	ADP included in assay (μmoles)	ATP at end of assay (mμmoles)
144 000g supernatant (0·21 mg. of protein)	0	0·5
	0·5	31·7
	1·0	66·9
	2·0	120·9
Boiled enzyme	1·0	19·4
144 000g pellet (0·40 mg. of protein)	0	0·3
	0·5	2·8
	1·0	3·7
	2·0	8·2
Boiled enzyme	1·0	3·4

relatively high activity was also found in the boiled-enzyme samples. This heat-stability is an important characteristic of the adenylate kinase system (Dixon & Webb, 1964). Thus to decrease these adenylate kinase effects a washed-pellet fraction was used (Table 7) in the investigation for phosphorylation activity associated with nitrate reductase in *Nitrobacter*. As a consequence of using the 144 000g pellet fraction a lower specific activity was found for the nitrate reductase than in Table 5, where the 10 000g supernatant was studied.

As Kiesow (1964) described a nitrate reductase in *Nitrobacter winogradskyi* linked with a phosphoryla-tion system, his preparation procedure was followed. An extraction medium containing 0·3 M-sucrose, 1 mM-EDTA and 1 mM-GSH was used, and the cells were disrupted with the homogenizer–glass bead technique. With a shorter treatment period (3 min.) the cell extract was not brown as in the experiment reported in Table 2. The results in Table 7(a) show that the amount of ATP at the end of the 15 min. assay was independent of the nitrate reductase activity in the *Nitrobacter* 144 000g washed-pellet fraction. Omission of the substrate or the coenzyme, or the inclusion of arsenite in the system, inhibited nitrate reductase but no significant decrease occurred in the final amount of ATP.

These results, and the relatively great formation of ATP in the boiled enzyme assay, suggest that all the phosphorylation activity indicated was due to the adenylate kinase system. An adenosine triphosphatase was present both in the washed pellet and in the supernatant fraction. The hexokinase–ATP trap (Table 7b) would provide a more reliable measure of the ATP formed in the assay but adenylate kinase, if present, would still interfere. With this procedure (Table 7b), greater amounts of ATP were found at the end of the assay, but significantly more ATP accumulated in the assay that did not contain nitrate. Similar results were obtained when reduced BV was used instead of NADH as the electron donor.

Hydroxylamine oxidase. The rapid oxidation of hydroxylamine to nitrite by intact cells of *Nitro-somonas* (Table 8) was not stimulated by PMS. This electron carrier greatly accelerated the oxidation of

Table 7. *Phosphorylation activity associated with nitrate reductase in* Nitrobacter

Nitrate reductase was assayed as described in Table 5 with NADH (0·64mM) in the side arm and FMN (4μM) in the main tube, and additional components for the phosphorylation study as described in the text. A washed-pellet enzyme preparation (144000g for 2hr.) was used.

Expt.	Nitrate reductase (mμmoles of NO_2^- formed/mg. of protein)	ATP at end of assay (mμmoles)
(a) ATP determined by the firefly-luciferase method		
Complete reaction mixture	96·0	11·8
Complete reaction mixture + arsenite (4mM)	0	15·1
Reaction mixture without nitrate	15·0	15·0
Reaction mixture without nitrate and NADH	0	10·9
Complete reaction mixture with boiled pellet enzyme	0	5·1
(b) ATP assayed by the glucose–hexokinase–glucose 6-phosphate dehydrogenase–NADP⁺ method		
Complete reaction mixture	99·0	52·0
Reaction mixture without nitrate	0	74·0

Table 8. *Hydroxylamine oxidase in* Nitrosomonas

Washed cells with 0·05M-phosphate, pH7·8 (1:7, w/v), and cell extracts (10000g supernatant) were prepared by ultrasonic treatment. Hydroxylamine oxidase was assayed as described in the text, and enzyme activities are expressed per ml. of cell suspension or cell extract.

Expt.	Dilution tested	Enzyme activity	
		Hydroxylamine oxidized (μmoles/ml.)	Nitrite formed (μmoles/ml.)
Nitrosomonas			
Whole cells	100	88·6	93·3
Whole cells + PMS	100	83·5	76·4
Cell extract		6·3	0
Whole cells + PMS	500	434·5	226·5
Nitrobacter			
Cell extract	10	0·6	1·0
Cell extract + PMS	10	1·2	1·4

hydroxylamine by cell extracts, but only a 50% conversion of hydroxylamine into nitrite was found. PMS concentrations in the range 7·5–150μM all gave optimum activity for hydroxylamine oxidase. In the absence of PMS very little hydroxylamine was oxidized by the cell extract and there was no concomitant production of nitrite. A cell extract of *Nitrobacter* was also tested with and without PMS but no oxidation of hydroxylamine occurred.

Hydroxylamine reductase. Under anaerobic conditions an active hydroxylamine reductase was demonstrated in *Nitrosomonas* (Table 9). The highest activity was obtained with NADPH and FMN and a somewhat lower activity when NADH was substituted for NADPH and FAD for FMN. Mn^{2+} ions gave a further stimulation of the hydroxylamine reductase. About 60% of

the hydroxylamine added was recovered as ammonia. Under the assay conditions used, chemical breakdown of hydroxylamine was less than 5%. Hydroxylamine was not oxidized to nitrite but some intermediate compounds might have been formed (Falcone et al. 1963). The effect of varying the concentration of FMN is shown in Table 10. As shown in Table 11, there was a considerable utilization of ammonia by *Nitrosomonas* extracts under anaerobic conditions. This effect, however, was not found in *Nitrobacter* extracts and a better stoicheiometry was established for the nitrite and hydroxylamine reductase enzymes in this organism. The specific activity of the hydroxylamine reductase in *Nitrosomonas* was four times that in *Nitrobacter*. The *Azotobacter* transhydrogenase system, used to generate the

Table 9. *Hydroxylamine reductase in* Nitrosomonas

The assay was in a Thunberg tube as described in Table 5 except that hydroxylamine (0·4 mM) was used as substrate and *Nitrosomonas* cell-free extract (5·15 mg. of protein) and $MnCl_2$ (0·6 mM) were present. The concentrations used were: NADH or NADPH, 0·64 μM; FAD or FMN, 4·4 μM.

	Total enzyme activity	
	Hydroxylamine used	Ammonia formed
Electron-donor system	(mμmoles)	(mμmoles)
None	129	70
NADPH	343	197
NADPH+FAD	439	288
NADPH+FAD+Mn^{2+}	463	300
NADPH+FMN	850	488
NADPH+FMN+Mn^{2+}	956	647
NADH	218	91
NADH+FAD	239	194
NADH+FAD+Mn^{2+}	338	188
NADH+FMN	703	404
NADH+FMN+Mn^{2+}	867	583

Fig. 1. Lineweaver–Burk plots for the effect of substrate concentration on nitrite reductase ($\square$) and hydroxylamine reductase ($\bigcirc$) in *Nitrosomonas*. Assays and enzyme activity are as described in Table 10. v, Velocity (μmoles/substrate).

reduced BV, could not be used in the hydroxylamine reductase assay owing to high rates of hydroxylamine breakdown in the assay with boiled extracts of *Nitrosomonas* or *Nitrobacter*. Further investigation showed that the loss of hydroxylamine was due to a reductase enzyme in the *Azotobacter* particle (Bulen, 1956) and not to a chemical reaction between reduced BV and hydroxylamine.

Nitrite reductase. This enzyme was identified in both the nitrifying bacteria and had electron-donor and cofactor requirements similar to those of the hydroxylamine reductase in *Nitrosomonas* (Table 9). Mn^{2+} ions also stimulated the nitrite reductase enzyme. Reduced BV was a suitable donor for the nitrite reductase, giving four times the activity of the NADPH–FMN system. When the *Nitrosomonas* cell was disrupted with the French pressure cell and the nucleoplasm was not treated with deoxyribonuclease the nitrite reductase was found exclusively within the nucleoplasm. When the cells were broken by the alternative ultrasonic treatment, the nitrite reductase was not sedimented by centrifugation at 226 000g for 2 hr.

It is shown in Fig. 1 that nitrite and hydroxylamine reductases in *Nitrosomonas* had similar substrate K_m values: nitrite, 0·48 mM; hydroxylamine, 0·72 mM. The specific activity of the hydroxylamine reductase was always twice that of nitrite reductase. The activity of both enzymes in *Nitrosomonas* was shown to be markedly stimulated by FMN (Table 10). Sulphite (1 mM) did not inhibit nitrite reductase or hydroxylamine reductase in the nitrifying bacteria.

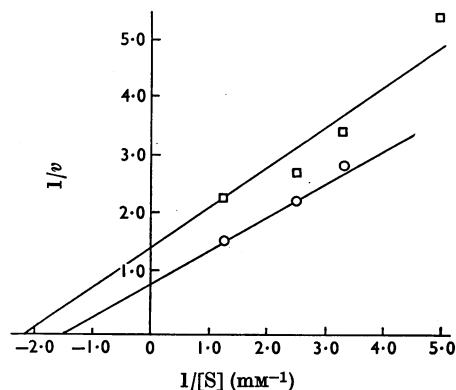*Further utilization of ammonia.* With a cell-free

Table 10. *Effect of the concentration of FMN on the assay of nitrite and hydroxylamine reductase in* Nitrosomonas

Hydroxylamine reductase was assayed as described in Table 9 with NADPH (0·64 mM) in the side arm and FMN in the main tube. Separately, but with the same FMN concentrations, a similar assay was used for nitrite reductase except that nitrite (0·4 mM) was used as the substrate. The enzyme preparation was the 10 000g supernatant (6·15 mg. of protein).

FMN in assay (mμmoles)	Nitrite reductase: nitrite disappearance (mμmoles)	Hydroxylamine reductase: hydroxylamine disappearance (mμmoles)
0	172	318
1·3	143	278
6·4	230	428
12·7	368	452
127·0	558	889

extract of *Nitrosomonas* tested under the assay conditions (NADPH and FMN) used for the reductase enzymes (Table 11), a quarter of the ammonia added (1 μmole) was used in 30 min. This utilization of ammonia was markedly stimulated by α-oxoglutaric acid, but a negligible loss of ammonia was detected when NADH was substituted for NADPH. Control checks with α-oxoglutaric acid alone eliminated any direct effect due to the keto acid, and a check with boiled *Nitrosmononas* extract indicated only a small loss of ammonia that could be accounted for by chemical reactions. With the *Nitrobacter* extract, tested with

Table 11. *Disappearance of ammonia under the assay conditions used for the characterization of nitrate, nitrite and hydroxylamine reductases*

The assay was in a Thunberg tube as described in Table 5; NH_4Cl (0·4mM) was added from the side arm, α-oxoglutarate (0·4mM) was in the main tube, and cell extract (5·0mg. of protein) was used.

Assay mixture	Total ammonia loss (mμmoles)
Nitrosomonas	
Boiled cell-free extract (BE)	0
Cell-free extract (E)	68
E + NADPH + FMN	248
E + α-oxoglutarate	92
E + NADPH + FMN + α-oxoglutarate	742
E + NADH + FMN + α-oxoglutarate	80
BE + NADPH + FMN + α-oxoglutarate	36
Nitrobacter	
E + NADPH + FMN + α-oxoglutarate	69
E + NADH + FMN + α-oxoglutarate	112

Table 12. *Incorporation of* [15]N-*labelled inorganic compounds into the cell protein of* Nitrosomonas *and* Nitrobacter

The methods used were those described in the text. The cell suspensions of *Nitrobacter* and *Nitrosomonas* contained 3·0mg. of protein N. All ^{15}N-labelled substrates used contained approx. 30% atom excess of [15]N.

Substrate	Nitrogen supplied (mg.)	Incorporation of ^{15}N into protein (μg./mg. of ^{15}N supplied)	
		Nitrosomonas	*Nitrobacter*
[15]NH₄NO₃	1·4	2·69	4·84
[15]NH₂.OH	1·0	4·63	4·52
Na[15]NO₂	1·6	0·41	0·91
K[15]NO₃	1·1	0	0·75

α-oxoglutaric acid and NADPH or NADH, there was much lower utilization of ammonia.

Utilization of [15]N-*labelled inorganic substrates by* Nitrosomonas *and* Nitrobacter. Based on the atom % excess of the substrate supplied and the value recorded in the extracted cell protein the results are expressed as uptake of [15]N (μg./mg. of ^{15}N supplied). Both *Nitrosomonas* and *Nitrobacter* (Table 12) showed incorporation of nitrite, hydroxylamine and ammonia into protein after 2hr. Much higher values were obtained for uptake into hydroxylamine and ammonia. With *Nitrobacter*, nitrate was also incorporated into protein at a rate similar to nitrite. No nitrate uptake into protein was recorded with *Nitrosomonas*.

DISCUSSION

In the present study it is shown that when the oxidase enzymes in the nitrifying bacteria are inactivated under anaerobic conditions, systems for reducing the inorganic nitrogen metabolites can be demonstrated. It is possible to detect nitrite and hydroxylamine reductase in *Nitrosomonas* and *Nitrobacter*, and a nitrate reductase is also present in the latter. The reductase enzymes, which are readily extractable in soluble form and require added NADH or NADPH with FMN or FAD or alternatively reduced BV, are similar in their electron-donor requirements to the assimilatory enzymes known to occur in a range of heterotrophs (Nicholas, 1963). Further evidence for the reductase enzymes in the nitrifiers being assimilatory systems is the rapid incorporation of ^{15}N-labelled nitrate, nitrite and hydroxylamine into the cell protein. In contrast with the nitrate reductase in crude extracts of higher plants (Wallace & Pate, 1965, 1967) the activity of the reductase enzymes in the nitrifying bacteria with NADH or NADPH as a donor is markedly stimulated by FMN or FAD. In these organisms it appears that the flavine reduced by the NADH or NADPH acts as the electron carrier. The high reductase activities with reduced BV and the absence of a requirement for additional cofactors with this donor indicates a more direct link with the terminal acceptor. It is also suggested that PMS, which is reduced by NADH or NADPH under anaerobic conditions, serves as a direct reductant for the nitrate reductase.

In previous work on the heterotrophs full stoicheiometry was not established for the nitrite and hydroxylamine reductases (Spencer, Takahashi & Nason, 1957; Roussos & Nason, 1960; Walker & Nicholas, 1961). The ammonia loss, under the assay conditions used for the reductase enzymes in *Nitrosomonas*, was stimulated by adding α-oxoglutaric acid but was much decreased when NADH rather than NADPH was the electron donor. This suggested that the ammonia was further utilized by the active NADP-specific glutamate dehydrogenase in *Nitrosomonas* (W. Wallace & D. J. D. Nicholas, unpublished work). With extracts of *Nitrobacter*, where there is a relatively low activity of glutamate dehydrogenase, a correspondingly low amount of ammonia was utilized. When this further utilization of ammonia is taken into account, for the *Nitrosomonas* extract reasonable stoicheiometry could be established for both nitrite and hydroxylamine reductase in the nitrifying bacteria.

In this study a comparison has been made between hydroxylamine oxidase in *Nitrosomonas* and nitrite oxidase in *Nitrobacter*. The nitrite oxidase was not stimulated by a range of added

cofactors and the oxidation of nitrite to nitrate was stoicheiometric. By contrast, in extracts of *Nitrosomonas* the oxidation of hydroxylamine to nitrite could be demonstrated only when an electron carrier such as PMS was added and only 50% of the hydroxylamine oxidized was recovered as nitrite. It was shown by Falcone *et al.* (1963) that the four-electron hydroxylamine oxidase reaction probably occurs in two steps.

Hydroxylamine has been well established as an intermediate in the oxidation of ammonia to nitrite by intact cells of *Nitrosomonas* (Lees, 1955). In previous work on the reduction of nitrite to ammonia in other organisms, there was doubt as to the identity and role of intermediate compounds, and the relation between the nitrite and hydroxylamine reductase enzymes is unclear. Lazzarini & Atkinson (1961) and Kemp & Atkinson (1966) established that, for *Escherichia coli*, hydroxylamine was not a free intermediate in the reduction of nitrite to ammonia. The substrate K_m value for hydroxylamine reductase was over 100 times that for nitrite reductase. In *Nitrosomonas*, however, the substrate K_m value for nitrite was similar to that of hydroxylamine, suggesting the prior reduction of nitrite to hydroxylamine, which, in turn, is reduced to ammonia. The hydroxylamine formed as an intermediate in the oxidation of ammonia may also be used by its reductase enzyme.

Kiesow (1964) suggests that the nitrate reductase in *Nitrobacter* is an anaerobic 'back reaction' associated with a phosphorylation system. This finding was not verified in the present study (Table 7). Indeed, NADH oxidase activity, which is mandatory for Kiesow's (1964) hypothesis, was not found in the *Nitrobacter* extract. This result is in agreement with recent observations in other chemoautotrophs (Smith, London & Stanier, 1967). A very active adenylate kinase was found in extracts of *Nitrobacter* (Table 6) and this could account for the apparent esterification of inorganic phosphate reported by Kiesow. It is not possible to assay nitrate reductase in *Nitrobacter* extracts under aerobic conditions, where the nitrite oxidase is also active. It is therefore difficult to interpret the data of Straat & Nason (1965), who used an aerobic assay procedure to characterize nitrate reductase in *Nitrobacter* with reduced cytochrome *c* as an electron donor.

The specific activity of glutamate dehydrogenase in extracts of *Nitrosomonas* is over 80 times that in *Nitrobacter*. In *Nitrobacter*, however, the activity of glutamate dehydrogenase is comparable with the rate of reduction of nitrite to ammonia. This apparent excess of glutamate dehydrogenase in *Nitrosomonas* agrees with the calculations of Hooper, Hansen & Bell (1967), who estimated that the activity was also 80 times that required to

provide for the organic nitrogen requirements of the organism.

It is interesting to correlate these enzyme studies with the electron-microscopic investigations on the nitrifying bacteria (Murray & Watson, 1965). *Nitrosomonas* was shown to have a thin cell wall, a few circumferential membranes and an extensive area of nucleoplasm, whereas the *Nitrobacter* cell had a dense inner layer as a component of its cell wall and plasma membrane, and a conspicuous intracellular membrane system. These observations may explain the relative ease with which the *Nitrosomonas* cell can be disrupted, e.g. by osmotic shock treatment, whereas the *Nitrobacter* cell, even after ultrasonic treatment for 40 min., was only partially broken. The cell extracts of *Nitrosomonas* were extremely viscous owing to the preponderance of nucleoplasm in the cell.

The nitrite oxidase in *Nitrobacter* was associated with particles, whereas the nitrate, nitrite and hydroxylamine reductases were relatively soluble enzymes. On this basis it is likely that the oxidase enzyme is located on the intracellular membranes whereas the reductases are in the cytoplasm. When the *Nitrobacter* cell was disrupted by ultrasonic treatment the nitrate reductase was also found in the particulate fraction. Rees & Nason (1965), in their studies on a soluble terminal oxidase in *Nitrosomonas*, made a similar observation, and suggested that ultrasonic treatment causes a vesicularization of the cell membranes and a consequent trapping of the soluble components of the cell. Based on the total extraction of nitrite oxidase, nitrate reductase and glutamate dehydrogenase from *Nitrobacter*, ultrasonic treatment was the most effective cell-disruption technique tested. Where the cellular distribution of an enzyme was sought, a more gentle fractionation procedure was required, such as the lysozyme–French-pressure-cell technique described in this paper. The direct lysozyme–osmotic-lysis technique was developed especially for the thicker-walled Gram-negative bacteria (Repaske, 1956). That it was completely unsatisfactory for disrupting *Nitrobacter* further emphasizes the unique nature of the cell wall and plasma membrane in this organism (Murray & Watson, 1965).

The distribution of the oxidase and reductase enzymes in the *Nitrosomonas* cell is not as well defined as in *Nitrobacter*. This organism has only a few peripheral membranes, but the oxidase enzyme again appears to be more closely associated with membranes than are the reductases. Murray & Watson (1965) established that the intracellular membranes are part of the plasma membrane and thus the oxidase reactions would be localized near the cell surface. In the *Nitrobacter* cell the oxidation of the toxic nitrite ion would then be restricted to

the outer membrane region of the cell and the nitrate ion produced, with the energy produced from the oxidation reaction, would be released into the cytoplasm. The nitrate reductase in the cytoplasm would thus have a role as the first enzyme in the assimilation of nitrate by the *Nitrobacter* cell.

In *Nitrosomonas* the system oxidizing ammonia to hydroxylamine is believed to be localized in the region of the cell wall (Lees, 1955). Thus although an active glutamate dehydrogenase is present in the cell that could utilize the ammonia directly, hydroxylamine and nitrite may be the substrates available for the assimilation reactions in the cytoplasm. Consequently the ammonia produced by the nitrite and hydroxylamine reductase enzymes may be linked to at least part of the amino acid synthesis (glutamate dehydrogenase) and protein synthesis. Since nitrite and hydroxylamine are known toxic compounds to cell metabolism an alternative explanation for the nitrite and hydroxylamine reductase in *Nitrosomonas* may be a detoxication system to control the concentration of these intermediates in the oxidation reactions.

REFERENCES

Aleem, M. I. H. (1965). *Biochim. biophys. Acta*, **107**, 14.

Aleem, M. I. H. & Alexander, M. (1958). *J. Bact.* **76**, 510.

Aleem, M. I. H., Lees, H. & Nicholas, D. J. D. (1963). *Nature, Lond.*, **200**, 759.

Aleem, M. I. H. & Nason, A. (1960). *Proc. nat. Acad. Sci., Wash.*, **46**, 763.

Brownell, P. F. & Nicholas, D. J. D. (1967). *Plant Physiol.* **42**, 915.

Bulen, W. A. (1956). *Plant Physiol.* **31**, xxix.

Conway, E. J. (1962). *Microdiffusion Analysis and Volumetric Error*, 5th ed,, pp. 90–97. London: Crosby, Lockwood and Son Ltd.

Dixon, M. & Webb, E. C. (1964). *Enzymes*, 2nd ed., pp. 146–147. London: Longmans, Green and Co. Ltd.

Falcone, A. B., Shug, A. L. & Nicholas, D. J. D. (1963). *Biochim. biophys. Acta*, **77**, 199.

Frear, D. S. & Burrell, R. C. P. (1955). *Analyt. Chem.* **27**, 1664.

Hewitt, E. J. & Nicholas, D. J. D (1964). In *Modern Methods of Plant Analysis*, vol. 7, p. 67. Ed. by Paech, K. & Tracey, M. V. Berlin: Springer-Verlag.

Hooper, A. B., Hansen, J. & Bell, R. (1967). *J. biol. Chem.* **242**, 288.

Kemp, J. D. & Atkinson, D. E. (1966). *J. Bact.* **92**, 628.

Kiesow, L. (1964). *Proc. nat. Acad. Sci., Wash.*, **52**, 980.

Lazzarini, R. A. & Atkinson, D. E. (1961). *J. biol. Chem.* **236**, 3330.

Lees, H. (1955). *Biochemistry of Autotrophic Bacteria*, pp. 44–58. London: Butterworths Scientific Publications.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.

McElroy, W. D. (1963). In *Methods in Enzymology*, vol. 6, p. 445. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Medina, A. & Nicholas, D. J. D. (1957a). *Biochim. biophys. Acta*, **23**, 440.

Medina, A. & Nicholas, D. J. D. (1957b). *Nature, Lond.*, **179**, 533.

Murray, R. G. E. & Watson, S. W. (1965). *J. Bact.* **98**, 1594.

Naik, M. S. & Nicholas, D. J. D. (1966a). *Biochim. biophys. Acta*, **113**, 490.

Naik, M. S. & Nicholas, D. J. D. (1966b). *Biochim. biophys. Acta*, **118**, 195.

Nicholas, D. J. D. (1963). *Biol. Rev.* **38**, 530.

Nicholas, D. J. D. & Jones, O. T. G. (1960). *Nature, Lond.*, **185**, 512.

Nicholas, D. J. D. & Mabey, G. L. (1960). *J. gen. Microbiol.* **22**, 184.

Nicholas, D. J. D. & Rao, P. S. (1964). *Biochim. biophys. Acta*, **82**, 394.

Pangborn, J., Marr, A. G. & Robrish, S. A. (1962). *J. Bact.* **84**, 669.

Ramaiah, A. & Nicholas, D. J. D. (1964). *Biochim. biophys. Acta*, **86**, 459.

Rees, M. & Nason, A. (1965). *Biochem. biophys. Res. Commun.* **21**, 248.

Repaske, R. (1956). *Biochim. biophys. Acta*, **22**, 189.

Roussos, G. & Nason, A. (1960). *J. biol. Chem.* **235**, 2997.

Smith, A. J., London, J. & Stanier, R. Y. (1967). *J. Bact.* **94**, 972.

Spencer, D., Takahashi, H. & Nason, A. (1957). *J. Bact.* **73**, 553.

Straat, P. A. & Nason, A. (1965). *J. biol. Chem.* **240**, 1412.

Walker, G. C. & Nicholas, D. J. D. (1961). *Biochim. biophys. Acta*, **49**, 361.

Wallace, W. & Pate, J. S. (1965). *Ann. Bot., Lond., N.S.*, **29**, 655.

Wallace, W. & Pate, J. S. (1967). *Ann. Bot., Lond., N.S.*, **31**, 213.