Biochimica et Biophysica Acta, 462 (1977) 141–152

BBA 47366

HYDROXYLAMINE OXIDOREDUCTASE OF *NITROSOMONAS* OXIDATION OF DIETHYLDITHIOCARBAMATE CONCOMITANT WITH STIMULATION OF NITRITE SYNTHESIS.

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(Received February 25th, 1977)

SUMMARY

Although nitrate was not produced in the oxidation of ammonia or hydroxylamine as catalyzed by intact cells, hydroxylamine oxidoreductase from *Nitrosomonas europaea* catalyzed the aerobic oxidation of hydroxylamine to nitrite and nitrate. The reaction was most rapid in the presence of added electron acceptor. Nitrate was not produced from nitrite. The precursor of nitrate was an N-oxide of oxidation state between hydroxylamine and nitrite. In the presence of diethyldithiocarbamate, hydroxylamine was oxidized to nitrite but nitrate was not produced. For each mol of nitrite produced, approximately 1 mol of diethyldithiocarbamate was oxidized to bis(diethyldithiocarbamoyl)disulfide (Disulfiram). Oxidation of diethyldithiocarbamate required the concomitant oxidation of hydroxylamine. The enzyme did not catalyze the direct oxidation of diethyldithiocarbamate utilizing oxygen, H_2O_2, nitrite, nitrate or PMS as electron acceptor. The effect of structural analogs of diethyldithiocarbamate suggested that diethyldithiocarbamate was effective because it bound to a metal ion and had an oxidizable SH group. Diethyldithiocarbamate may act by either (a) reducing a reactive form of oxygen generated during the oxidation of hydroxylamine thus preventing the oxidation of an N-compound to nitrate, or (b) reducing an N-containing precursor of nitrate to an N-compound which was subsequently oxidized to nitrite.

Methods for the assay of diethyldithiocarbamate are described.

Abbreviations: DCIP, 2,6-dichlorophenolindophenol; PMS, phenazine methosulfate; Disulfiram, bis(diethyldithiocarbamoyl)disulfide; MTT-tetrazolium, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium chloride; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); Thiram, bis(dimethylthiocarbamoyl)disulfide; Tillam, *S*-propylbutylethylthiocarbamate; Eptam, *S*-ethyldipropylthiocarbamate; CDEC, 2-chloroallyldiethyldithiocarbamate.

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INTRODUCTION

The autotrophic bacterium *Nitrosomonas* derives energy from the oxidation of ammonia to nitrite. Hydroxylamine oxidoreductase is a cytochrome-containing enzyme which catalyzes the oxidation of hydroxylamine to nitrite in the presence of oxygen and a suitable electron acceptor such as phenazine methosulfate (PMS). Experimentally, 50 % or less of the hydroxylamine which is oxidized appears as nitrite [1–3] and a measured ratio of approximately 2 mol electron acceptor reduced per mol of hydroxylamine oxidized [2, 4] has indicated that a compound of the oxidation state of nitroxyl (HNO) is an intermediate produced by the dehydrogenation of hydroxylamine. Nitric oxide has also been identified as a product of hydroxylamine oxidation and possible precursor to nitrite [5, 6, 3] although some nitric oxide may also arise from the reduction of nitrite by nitrite-reductase activity closely associated with the hydroxylamine-oxidizing enzyme during purification [7, 8]. Anderson [5] first identified nitrate as an additional product of the oxidation of hydroxylamine in the presence of oxygen, methylene blue and extracts of *Nitrosomonas*. In the present work we show that both nitrite and nitrate are products of hydroxylamine oxidation as catalyzed by extracts of *Nitrosomonas* and that in the presence of diethyldithiocarbamate, nitrite is produced rather than nitrate with the concomitant oxidation of diethyldithiocarbamate to bis(diethyldithiocarbamoyl)disulfide (Disulfiram).

METHODS

Growth of cells and preparation of enzyme

Nitrosomonas europaea (Schmidt strain) was grown and the third ammonium sulfate fraction was prepared as previously described [9]. The third ammonium sulfate precipitate (3AS) was dialyzed, layered on a 3–20 % sucrose gradient contained in a 5/8 × 3 inch nitrocellulose tube, and centrifuged for 20 h at 201 000 × g in a Beckman Model SW 41 rotor. The fraction containing enzyme activity (SGI) was dialyzed and this step repeated to yield fraction (SGII), enriched 36-fold in specific activity.

The following enzyme activities were found in SGII (expressed as the change in the indicated substrate or product, μmol min^{-1} mg protein^{-1}): *p*-phenylenediamine oxidase (oxygen utilized), $4.2 \cdot 10^{-3}$; pyrogallol oxidase (oxygen utilized), 0.24; DCIP oxidase (DCIP oxidized), 0.55; mammalian cytochrome oxidase (cytochrome *c* oxidized), $8.2 \cdot 10^{-3}$; hydroxylamine-nitrite reductase (nitrite disappearance) $8 \cdot 10^{-4}$; catalase (H_2O_2 utilized), less than 0.3; peroxidase (peroxidation of *o*-dianisidine), 0.96. Recovery of hydroxylamine oxidoreductase was 33 %, nitrite reductase 0.2 %, pyrogallol oxidase 20 % and all other oxidase enzymes 3 % or less.

Chemical assays. Nitrite was estimated by diazotization [10]. Nitrate was assayed as nitrite, following reduction by one of three methods. For reduction on a copper-cadmium column [11], the pH of a 3 ml aliquot was adjusted to a value of 10 with EDTA sodium salt and the solution was passed through a 1 × 28 cm glass column filled with copper-amalgamated cadmium filings. The sample was eluted into a 25 ml volume with 20 μM EDTA sodium salt. The pH was adjusted to a value of approx. 7 before nitrite analysis. For reduction by zinc [12], the pH of a 3 ml aliquot was adjusted to a value of 10 with 4 M NH_4OH and 50 mg powdered metallic zinc was added with vigorous mixing. Aliquots were withdrawn and analyzed for nitrite content at six

separate 2 min intervals. The maximum nitrite value was used and adjusted for recovery by comparison to the value of nitrite obtained with 3-ml aliquots containing 10, 20 or 50 μM nitrate and treated in the same way. For reduction by nitrate reductase [13], 0.03 ml of 10 mM flavin adenine dinucleotide and 0.4 mg of *Neurospora* nitrate reductase (kindly supplied by Dr. R. Garrett, University of Virginia) were added to a 3 ml aliquot containing nitrate. Successive 0.02 ml aliquots of reduced nicotine adenine dinucleotide phosphate were added until the nitrite concentration ceased to increase. The copper-cadmium column assay was used for most of the experiments reported here. Reduction by zinc rapidly produced an N-oxide of a lower oxidation state than nitrite and was used only in those experiments with inhibitors, such as KCN, which inhibited nitrate reductase and poisoned the copper-cadmium column. Nitrate was also estimated by the phenoldisulfonic acid method [14]. Hydroxylamine was assayed by reaction with 8-hydroxyquinoline [15].

Thiocarbamate compounds (0.1 ml) were separated on precoated Silica G plates (Baker-Flex, J. T. Baker & Co., Phillipsburg, N. J.) by a solution of ether/benzene (1 : 1). After the solvent had migrated 15 cm the compounds were located as spots after spraying with 0.5 % ninhydrin in butanol, followed by heating to 100 °C for 5 min. The thiocarbamate compounds tested here were not reactive with ninhydrin in solution, however. In the presence of 2 % $CuSO_4$, diethyldithiocarbamate formed a brown chelation complex with maximum absorbance at 435 nm. The absorbance was proportional to diethyldithiocarbamate concentration over the range 10–100 μM diethyldithiocarbamate. Based on the amount of diethyldithiocarbamate present, the copper-diethyldithiocarbamate complex had an absorption coefficient of 3 $mM^{-1} \cdot cm^{-1}$. In addition, diethyldithiocarbamate reacted stoichiometrically with the sulfhydryl reagent 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) with the production of the yellow compound 5-thio-2-nitrobenzoic acid [16]. Reactivity of diethyldithiocarbamate with DTNB was approximately equivalent to that of equimolar glutathione with DTNB. Based on the amount of diethyldithiocarbamate present, the absorption coefficient at 410 nm was 11 $mM^{-1} \cdot cm^{-1}$. The absorbance was proportional to diethyldithiocarbamate concentration.

Enzyme assays. All enzyme reactions were incubated at 25 °C. For hydroxylamine oxidoreductase, the standard reaction mixture contained 100 μM hydroxylamine and 5 μM PMS in 0.05 M Tris(hydroxymethyl)aminomethane sulfate (Tris), pH 8.0, total volume 5–15 ml. The reaction was started by adding enzyme and samples were withdrawn at intervals and assayed for hydroxylamine, nitrite and nitrate. Oxygen utilization was measured polarographically with a Clark-type oxygen electrode (Yellow Springs Instrument). Measurement of hydroxylamine-DCIP reductase, catalase and peroxidase was as described earlier [9]. Hydroxylamine-cytochrome *c* reductase, *p*-phenylenediamine oxidase, pyrogallol oxidase, cytochrome oxidase and nitrite reductase were assayed as described previously [17].

RESULTS

Production of nitrate. As shown in Fig. 1, aerobic incubation of purified hydroxylamine oxido-reductase in the presence of 100 μM hydroxylamine resulted in the production of 0.36 mol nitrite and 0.27 mol nitrate and the utilization of 1.37 mol oxygen per mol of hydroxylamine oxidized. The addition of catalase at the time that

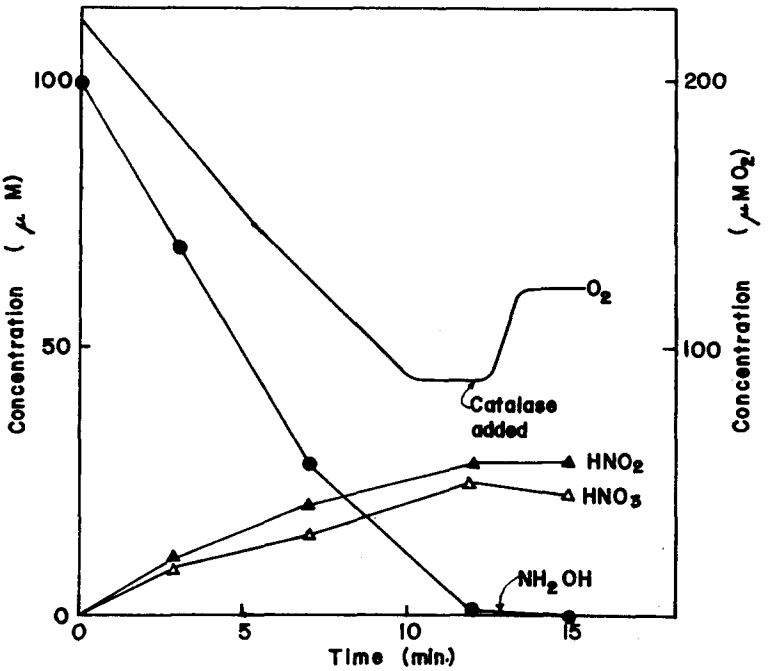

Fig. 1. Utilization of hydroxylamine and oxygen and production of nitrite and nitrate as catalyzed by *Nitrosomonas* hydroxylamine oxidoreductase. Enzyme (1.6 μg SGII) was added to 8 ml of the standard reaction mixture. Left ordinate, concentration of hydroxylamine, nitrite or nitrate. Right ordinate, concentration of oxygen.

hydroxylamine had been completely utilized resulted in the rapid production of 0.30 mol of oxygen per mol of hydroxylamine oxidized, demonstrating the production of hydrogen peroxide. Changes in concentration of hydroxylamine, nitrite and nitrate were linear with respect to time and the rates of hydroxylamine utilization and production of nitrite or nitrate were directly proportional to the amount of enzyme added.

Nitrate was identified as a product by the three procedures described in Methods. The possible production of pernitrous acid was not eliminated. Intact cells converted hydroxylamine stoichiometrically to nitrite and did not produce nitrate in the presence or absence of PMS (Table I).

Requirement for electron acceptor. As shown in Table I, nitrate was a product of the aerobic oxidation of hydroxylamine in the absence or presence of a variety of added electron acceptors including PMS, mammalian cytochrome *c*, DCIP or MTT-tetrazolium. Methylene blue also acts as electron acceptor during the oxidation of hydroxylamine to a mixture of nitrate and nitrite [5]. Although the rate of oxidation of hydroxylamine varied with electron acceptor (PMS > DCIP > cytochrome *c* = MTT-tetrazolium), the stoichiometry of nitrite produced : hydroxylamine utilized or nitrate produced : hydroxylamine utilized was approximately the same in each case. Characteristically 60–80 % of the hydroxylamine utilized was recovered as nitrite and nitrate in approximately equimolar quantities. The aerobic oxidation of hydroxylamine, as catalyzed by the enzyme in the absence of added electron acceptor, was extremely slow but also resulted in the production of both nitrite and nitrate (Table I).

TABLE I

EFFECT OF ADDED ELECTRON ACCEPTOR ON THE AEROBIC OXIDATION OF HYDROXYLAMINE TO NITRITE AND NITRATE

Reactions were carried out in 15 ml of 0.05 M Tris, pH 8.0, containing 100 μM hydroxylamine. Electron acceptors were added at the following concentrations: phenazine methosulfate 5 μM, mammalian cytochrome *c* (horse heart, from Sigma) 100 μM, dichlorophenolindophenol 200 μM, and MTT-tetrazolium 20 μg/ml. The reaction was started by adding enough enzyme (approximately 40 μg of fraction SGI) or intact cells to oxidize 0.5–3 μM hydroxylamine per min. Aliquots were withdrawn at intervals and analyzed for hydroxylamine, nitrite and nitrate concentrations. The ratios of product formed : hydroxylamine oxidized were calculated from values obtained at the end of the reaction when all hydroxylamine had been oxidized. It was not possible to assay nitrate in the presence of DCIP.

Catalyst	Added electron acceptor	Rate of hydroxylamine oxidation	Ratio of HNO_2 produced: NH_2OH oxidized	Ratio of HNO_3 produced: NH_2OH oxidized
		(μmol/min per mg protein		
Purified enzyme	Oxygen	$2.8 \cdot 10^{-4}$	0.21	0.09
Purified enzyme	Phenazine methosulfate	27.9	0.39	0.41
Purified enzyme	Dichlorophenolindophenol	7.23	0.32	–
Purified enzyme	Mammalian cytochrome *c*	4.24	0.29	0.26
Purified enzyme	MTT-tetrazolium	3.15	0.33	0.39
Intact cells	Oxygen	0.57	1.0	0
Intact cells	Phenazine methosulfate	4.56	1.0	0

Nitrogen-containing substrate for nitrate production. Nitrate was not produced by the oxidation of nitrite. The enzyme did not catalyze the aerobic oxidation of 50 μM nitrite to nitrate in the presence or absence of PMS nor did the presence of 50 μM nitrite in a reaction mixture containing 100 μM hydroxylamine and 5 μM PMS stimulate the rate of nitrate production or result in increased total production of nitrate at the time when hydroxylamine had been completely utilized. The incubation of 50 μM nitrite with 100 μM hydrogen peroxide resulted in the slow nonenzymatic disappearance of nitrite but nitrate was not produced in the presence or absence of enzyme. Hydroxylamine oxidoreductase is able to catalyze the oxidation of hydrazine to N_2 with the concomitant reduction of PMS. Hydrogen peroxide is generated during the nonenzymatic reaction of reduced PMS and oxygen and is thus present during the enzyme-catalyzed aerobic dehydrogenation of hydrazine (or hydroxylamine) in the presence of PMS (Fig. 1). The aerobic incubation of nitrite in the presence of the enzyme, PMS and hydrazine did not result in the production of nitrate, indicating that if reactive forms of oxygen were generated by autooxidation of PMS, they did not react with nitrite so as to produce nitrate.

Source of oxygen. Preliminary attempts to demonstrate the involvement of hydrogen peroxide in the production of nitrite or nitrate from hydroxylamine gave negative results. The presence of 100 μM H_2O_2 in the reaction mixture did not stimulate the production of nitrite or nitrate. The fact that the enzyme was able to catalyze the oxidation of hydroxylamine to nitrite and nitrate slowly in the absence of an added electron acceptor or rapidly in the presence of electron acceptors which are not rapidly autooxidized such as DCIP, cytochrome *c* or MTT-tetrazolium (Table I), indicated that autooxidation of the electron acceptor (with the possible generation of a reactive form of oxygen) was not a reaction necessary for nitrite or nitrate production.

Effect of diethyldithiocarbamate. As shown in Fig. 2 the presence of 10^{-4} M diethyldithiocarbamate in a reaction mixture carrying out the rapid oxidation of hydroxylamine resulted in complete inhibition of nitrate synthesis, stimulation of nitrite production and the concomitant utilization of diethyldithiocarbamate. The time-course of utilization of diethyldithiocarbamate paralleled that of changes in hydroxylamine, nitrite and nitrate. Rates of utilization of diethyldithiocarbamate and hydroxylamine, or production of nitrite or nitrate, were proportional to the enzyme concentration. The amount of diethyldithiocarbamate utilized was approximately equal to the amount of nitrite produced.

The product of diethyldithiocarbamate oxidation was identified as bis(diethyldithiocarbamoyl)disulfide (Disulfiram) (Table II). The product shared with Disulfiram the lack of cupric ion binding ability, the lack of reactive sulfhydryl groups, and an R_f of 0.88 on silica thin layer chromatography. The product or Disulfiram were converted stoichiometrically by reduction with sodium borohydride to a compound with the same mobility on silica thin layer chromatography as diethyldithiocarbamate ($R_f = 0$) and an amount of cupric ion binding activity and reactive sulfhydryl equal to 2 mol diethyldithiocarbamate. The compound produced by borohydride-reduction of either authentic Disulfiram or the product of enzymatic diethyldithiocarbamate oxidation was active in the stimulation of nitrite synthesis. The oxidation of diethyldithiocarbamate by equimolar quantities of *m*-chloroperbenzoic acid or hydrogen peroxide yielded a compound, presumed to be a sulfoxide or sulfone, which did not

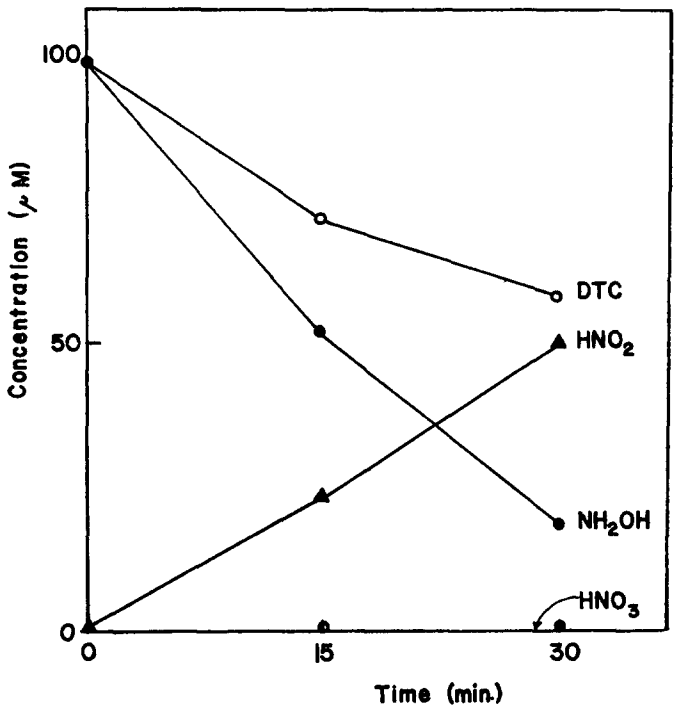

Fig. 2. Time-course of hydroxylamine oxidoreductase in the presence of diethyldithiocarbamate (DTC). The standard reaction mixture (15 ml) contained 100 μM diethyldithiocarbamate and 0.8 μg of SGII. Diethyldithiocarbamate was determined quantitatively as reactable sulfhydryl (Table II).

have cupric ion binding ability or reactive sulfhydryl groups, had an R_f value of 0.2 and could not be reduced to diethyldithiocarbamate by treatment with borohydride.

The stimulation of either the ratio of nitrite produced : hydroxylamine oxidized or rate of disappearance of diethyldithiocarbamate was at a maximum level or 50 % of maximum at concentrations of diethyldithiocarbamate of approximately 50 μM or 10 μM, respectively. The presence of diethyldithiocarbamate at concentrations greater than 10 mM resulted in equivalent inhibition of the rate of both hydroxylamine utilization and nitrite production.

Nature of electron acceptor for oxidation of diethyldithiocarbamate. The aerobic and enzymatic oxidation of diethyldithiocarbamate did not occur in the absence of NH_2OH and in the presence or absence of the following added electron acceptors: PMS, hydrogen peroxide (100 μM), nitrite and nitrate (50 μM). The presence of DCIP (100 μM) or 1 mM hydrogen peroxide caused the rapid nonenzymatic disappearance of diethyldithiocarbamate. Diethyldithiocarbamate was not oxidized in the presence of enzyme, hydrazine and PMS.

All attempts to demonstrate the presence of nitrate reductase activity were unsuccessful. The enzyme-catalyzed conversion of nitrate (50 μM) to nitrite did not occur in any of the following reaction mixtures: 50 μM hydroxylamine and 5 μM PMS; 100 μM diethyldithiocarbamate and 5 μM PMS; 50 μM hydroxylamine, 5 μM PMS and 100 μM diethyldithiocarbamate; or 50 μM hydrazine and 5 μM PMS.

TABLE II

IDENTIFICATION OF DISULFIRAM AS A PRODUCT OF THE HYDROXYLAMINE OXIDOREDUCTASE REACTION IN THE PRESENCE OF DIETHYLDITHIOCARBAMATE

Test compounds were dissolved in 0.05 M Tris, pH 8.0, at a concentration of 100 μM. For peroxidation, equimolar hydrogen peroxide or equimolar solid *m* chloroperbenzoic acid was added, followed by vigorous mixing on a Vortex mixer. For reduction, 50 mg solid $NaBH_4$ were added to the solution and the test tube containing the solution was rotated in a nearly horizontal position until excess $NaBH_4$ had completely decomposed. Chromatography of thiocarbamates and the assay for reaction with $CuSO_4$ or DTNB is described in Methods. To determine the effect on stoichiometry, hydroxylamine oxidoreductase was added to the standard reaction mixture in the presence of 100 μM of the test compound and a ratio of nitrite produced : hydroxylamine oxidized was determined ($R = 0.41$ in the absence of added compounds).

Compound tested	Cu binding	SH reaction (μM)	Chromatography on silica, R_f	Ratio of nitrite produced: hydroxylamine oxidized
DTC*	+	200	0	0.75
Product of DTC+peroxide	–	10	0.2	0.41
Product of DTC+peroxide subsequently reduced with $NaBH_4$	–	10	0.2	0.44
Disulfiram	–	9	0.88	0.41
Product of reduction of 100 μM Disulfiram with $NaBH_4$	+	160	0	0.74
Product of enzyme reaction of 200 μM DTC	–	36	0.88	0.41
Product of enzyme reaction reduced with $NaBH_4$	+	146	0	0.72

* DTC, diethyldithiocarbamate

TABLE III

EFFECT OF ANALOGUES OF DIETHYLDITHIOCARBAMATE ON THE REACTION CATALYZED BY HYDROXYLAMINE OXIDOREDUCTASE

The reactions were carried out by adding enzyme (3AS, 2–4 μg protein) to 5 ml of the standard reaction mixture, containing from 0.1 to 10 mM of test compound. Samples were withdrawn at 10 min intervals and assayed for hydroxylamine and nitrite as described in Methods. Symbols: ↑, increase or ↓, decrease of activity by 20 % or more; sl, increase or decrease of 20 % or less. Eptam and CDEC were dissolved in 0.5 % (final concentration) dimethyl sulfoxide. The data for diethyldithiocarbamate sulfoxide and Disulfiram is shown in Table II.

Common or trade name	Chemical formula	Effect of compound on: ΔNH_2OH	ΔHNO_2	Stoichiometry	Color with $CuSO_4$	SH reaction
Sodium diethyldithiocarbamate	$(CH_3CH_2)_2NC(S)SNa$	sl ↓	↑	↑	+	+
Eptam	$(CH_3CH_2CH_2)_2NC(O)SCH_2CH_3$	0	0	0	−	−
CDEC	$(CH_3CH_2)_2NC(S)SCHCClCH_3$	0	0	0	−	−
Sodium dithionite	NaO-S(O)-S(O)ONa	0	0	0	−	+
Allylthiourea	$CH_2 = CHCH_2NC(S)NH_2$	↓	↓	0	−	−
Thiosemicarbazide	$NH_2NHC(S)NH_2$	0	↓	↓	−	−
Ethyl xanthate	$CH_3CH_2OC(S)SH$	0	sl ↓	sl ↓	+	+
Glutathione	$NH_2(COOH)CHCH_2CH_2CONHCH(CH_2SH)CONHCH_2COOH$	0	0	0	−	+
Dithiothreitol	$SHCH_2CHOHCHOHCH_2SH$	0	↑	↑	+	+
EDTA Sodium salt	$(NaOOCCH_2)_2NCH_2CH_2N(CH_2COONa)_2$	0	0	0	0	0

Effect of analogs of diethyldithiocarbamate. A comparison of the effect of diethyldithiocarbamate to the effect of other metal binding agents or reducing agents suggested that diethyldithiocarbamate was effective at low concentrations because it bound metal ions and possessed an oxidizable sulfhydryl group. Other metal binding agents or analogs of diethyldithiocarbamate not possessing a free SH group (Eptam, CDEC, diethyldithiocarbamate-sulfoxide, allylthiourea, thiosemicarbazide, Disulfiram and EDTA) neither stimulated nitrite synthesis nor inhibited nitrate production (Tables II and III). This was also true of Thiram, Tillam and amino triazole (data not shown). The presence of reducing agents such as dithiothreitol, cysteine or sodium ascorbate, alone or in the presence of metal binding agents resulted in a slight increase in the ratio of nitrite synthesized: hydroxylamine oxidized but the required concentration of reducing agent was greater than the corresponding concentration of diethyldithiocarbamate by a factor of 10 or more, and the maximum stimulation of nitrite was less than 30 % of the corresponding stimulation observed in the presence of diethyldithiocarbamate. The presence of glutathione did not result in a stimulation of nitrite synthesis. Ethyl xanthate and dithiothreitol, the only compounds tested other than diethyldithiocarbamate which formed a colored chelation compound in the presence of cupric ion and had a reactive sulfhydryl group, stimulated nitrite production slightly.

Effect of inhibitors. KCN (1 mM) or hydrazine (100 μM) caused 50 % inhibition of the rate of utilization of hydroxylamine and diethyldithiocarbamate and the rate of production of nitrite and nitrate. Carbon monoxide (50 % CO : 50 % air) affected neither the rate of utilization of hydroxylamine or diethyldithiocarbamate nor the rate of production of nitrite or nitrate.

DISCUSSION

As shown by Anderson [5], the present results indicate that nitrate is a product of the aerobic oxidation of hydroxylamine as catalyzed by extracts of *Nitrosomonas.* The production of nitrate and hydrogen peroxide during the oxidation of hydroxylamine described here accounts for the amount of oxygen utilized in excess of the amount expected based only on the amount of nitrite produced, a phenomenon described in the first report of cell-free hydroxylamine oxidation [1].

Nitrate is not produced from ammonia [18] or hydroxylamine (present work) by intact cells. Thus, the capacity of isolated hydroxylamine oxidoreductase to produce nitrate is non-biological. Because nitrate is produced in the absence of added electron acceptor, production is not an artifact due to the addition of a compound such as PMS. The capacity to produce nitrate evidently results from a change in the enzyme itself or its association with other enzymes which occurred immediately upon cell breakage. In vivo, hydroxylamine oxidoreductase ultimately transfers electrons to a membrane-bound cytochrome a_1-containing terminal oxidase [17] and is therefore likely to be physically associated with the membrane. In the present work, the addition of the reductant diethyldithiocarbamate caused the enzyme to catalyze a reaction which, at least in overall stoichiometry, is the same as the reaction in vivo, suggesting the possible loss of access of the enzyme to a natural reductant during purification.

The production of both nitrate and nitrite was apparently initiated by the same hydroxylamine dehydrogenase; the rates of production of nitrite and nitrate were

affected equally when the rate of hydroxylamine oxidation varied as a result of heat treatment, aging of the enzyme, inactivation of hydroxylamine dehydrogenase activity by H_2O_2 [9], inhibition by high concentrations of KCN, or by comparison between enzyme fractions during purification. Nitrite was not oxidized to nitrate under any of the conditions of assay reported here so that the N-containing substrate for nitrate production is presumed to be an N-oxide of oxidation state between hydroxylamine and nitrite. The fact that nitrite and nitrate were produced in approximately equimolar quantities suggests three possible schemes: (a) the reaction of a dinitrogen compound such as N_2O_4, (b) the coordinate reaction of two separate N-oxides with a dioxygen compound, or (c) the production, during the generation of the intermediate common to production of nitrite and nitrate, of half the amount of oxidant required to produce 1 mol of nitrate.

To our knowledge, this is the first report of a reversible effect of diethyldithiocarbamate on an enzyme activity resulting in the stoichiometric oxidation of the dithiocarbamate to the disulfide, Disulfiram. Reversible effects of diethyldithiocarbamate on enzyme activity are often taken as an indication of the involvement of a metal ion such as Cu^{2+} in the action of the enzyme. Dithiocarbamates also act as sulfhydryl reagents. For example, zinc dimethyldithiocarbamate has been used as a sulfhydryl reagent in the inhibition of mitochondrial dehydrogenase activity [19]. Disulfiram is reported to form a stable disulfide with aldehyde dehydrogenase of sheep liver [20]. The sulfoxidation of thiocarbamate compounds such as Eptam to the sulfoxide has been demonstrated in plants [21], however, the product of oxidation of diethyldithiocarbamate in the presence of hydroxylamine oxidoreductase and hydroxylamine was shown here not to be sulfone or sulfoxide.

The present data do not reveal the mechanism of oxidation of diethyldithiocarbamate or production of nitrate. The oxidation of diethyldithiocarbamate required the accompanying oxidation of hydroxylamine. Diethyldithiocarbamate was not enzymatically oxidized with added oxygen, nitrate, nitrite, PMS, DCIP, mammalian cytochrome *c* or hydrogen peroxide as electron acceptors. A candidate for the compound acting as oxidant for diethyldithiocarbamate is a reactive form of oxygen (such as super-oxide, hydroxyl radical or peroxyl radical) which was produced by the reaction with oxygen of an electron originating from hydroxylamine. According to this hypothesis the reactive form of oxygen was able to react either (a) with diethyldithiocarbamate to produce Disulfiram and H_2O or H_2O_2, or (b) with an N-oxide precursor of nitrite to produce nitrate. Assuming that hydrazine and hydroxylamine are oxidized by a common dehydrogenase and considering the fact that diethyldithiocarbamate was not oxidized with hydrazine as substrate, the hypothesized reactive electron must have come from the oxidation of an N-oxide of oxidation state equal to or greater than HNOH.

The concentration (10 μM) of diethyldithiocarbamate which caused 50 % inhibition of ammonia oxidation (but not hydroxylamine oxidation) as catalyzed by intact cells of *Nitrosomonas* [22] is approximately the same as the concentration of diethyldithiocarbamate which resulted in 50 % of the maximum rate of oxidation of diethyldithiocarbamate or stimulation of nitrite production accompanying the oxidation of hydroxylamine by hydroxylamine oxidoreductase. It is thus a plausible hypothesis that a reactive form of oxygen necessary for ammonia oxidation is generated by hydroxylamine oxidoreductase in vivo during the oxidation of an N-oxide of oxidation state between that of NH_2OH and nitrite.

Rather than reacting with a species of oxygen generated during hydroxylamine oxidation, it is equally possible that diethyldithiocarbamate reacted directly with an N-oxide precursor of HNO_3 (such as NO, NO_2, N_2O_3, or N_2O_4) thus reducing the N-oxide to nitrite or a compound which was enzymatically oxidized to nitrite rather than to nitrate.

A survey of the relative ability of diethyldithiocarbamate and several structural analogs of diethyldithiocarbamate to cause stimulation of nitrite synthesis and inhibition of nitrate synthesis suggested that diethyldithiocarbamate bound to a metal ion in the active site of hydroxylamine oxidoreductase and reacted stoichiometrically with an oxidant generated during the reaction.

ACKNOWLEDGEMENTS

This research was supported by a research grant from the National Science Foundation (BMS73-00891 A01). We thank Sidney Simms for aid in growing bacteria.

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