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Nitroalkane Oxidation by Streptomycetes

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Crude cell-free extracts of nine strains of *Streptomyces* tested for nitroalkane-oxidizing activity showed production of nitrous acid from 2-nitropropane, 1-nitropropane, nitroethane, nitromethane, and 3-nitropropionic acid. These substrates were utilized in most strains but to a decreasing extent in the order given, and different strains varied in their relative efficiency of oxidation. *p*-Nitrobenzoic acid, *p*-aminobenzoic acid, enteromycin, and ω-nitro-L-arginine were not attacked. D-Amino acid oxidase, glucose oxidase, glutathione *S*-transferase, and xanthine oxidase, enzymes potentially responsible for the observed oxidations in crude cell-free extracts, were present at concentrations too low to play any significant role. A nitroalkane-oxidizing enzyme from streptozotocin-producing *Streptomyces achromogenes* subsp. *streptozoticus* was partially purified and characterized. It catalyzes the oxidative denitrification of 2-nitropropane as follows: $2CH_3CH(NO_2)CH_3 + O_2 \rightarrow 2CH_3COCH_3 + 2HNO_2$. At the optimum pH of 7.5 of the enzyme, 2-nitropropane was as good a substrate as its sodium salt; *t*-nitrobutane was not a substrate. Whereas Tiron, oxine, and nitroxyl radical acted as potent inhibitors of this enzyme, superoxide dismutase was essentially without effect. Sodium peroxide abolished a lag phase in the progress curve of the enzyme and afforded stimulation, whereas sodium superoxide did not affect the reaction. Reducing agents, such as glutathione, reduced nicotinamide adenine dinucleotide, and nicotinamide adenine dinucleotide phosphate, reduced form, as well as thiol compounds, were strongly inhibitory, but cyanide had no effect. The *S. achromogenes* enzyme at the present stage of purification is similar in many respects to the enzyme 2-nitropropane dioxygenase from *Hansenula mrakii*. The possible involvement of the nitroalkane-oxidizing enzyme in the biosynthesis of antibiotics that contain a nitrogen-nitrogen bond is discussed.

A number of strains of *Streptomyces* elaborate antibiotics containing a nitrogen-nitrogen bond (15, 16), and several of these are being studied in this laboratory to gain information on the pathways involved. N-N bond-containing compounds have been found also in other microorganisms (16), in mushrooms, and in plants (15), but despite the great interest that some of these compounds command as anticancer agents or as carcinogens, little is known about the biochemical steps involved in their formation (15).

It is conceivable that nitrous acid may participate in biological N-N bond formation. This assumption is based on the fact that nitrous acid is a well-recognized reagent in organic chemistry used to effect N-N bond formation, and it is supported by experiments on *N*-nitrosamine formation from a variety of amines and sodium nitrite by intact bacteria (32) and by bacterial cell-free extracts (5, 22). On the basis of these microbiological studies, it appears that nitrosation might proceed quite readily either as an enzyme-catalyzed process or possibly as an organic matter-catalyzed process (5, 22), provided that nitrous acid can be generated within the respective cells. Two main pathways that can lead to the formation of nitrous acid have been reported in microorganisms: reduction of inorganic nitrates (26) and degradation of organic nitro compounds (6, 9, 12, 17, 23, 33). The first pathway is probably not a general route in *Streptomyces* strains for nitrous acid formation since many of them cannot grow on nitrate as the sole nitrogen source. Therefore, the second pathway appears to be an attractive alternative.

Accordingly, we have studied the possible presence of nitroalkane-oxidizing enzymes in a number of strains of *Streptomyces* that elaborate antibiotics containing an N-N bond, and for comparison, we have studied their possible presence in a number of *Streptomyces* strains that do not produce any known N-N bond-containing compounds.

Bright and co-workers have shown that both D-amino acid oxidase (30) and glucose oxidase (29) are capable of oxidizing nitroalkanes to yield

nitrous acid, and Habig et al. (7) demonstrated that glutathione *S*-transferase catalyzes the formation of nitrite from aromatic nitro compounds and nitroalkanes. Thus, nitroalkane oxidation appears to be possible by several enzymes, and it was therefore necessary to consider the possible participation of these and of other enzymes.

MATERIALS AND METHODS

Chemicals, enzymes, and reagents. Nitroethane, 1-nitropropane, 2-nitropropane, 3,4-dichloronitrobenzene, 3-methyl-2-benzothiazolinone hydrazone-hydrochloride, *o*-dianisidine, pyrocatechol-3,5-disulfonate (disodium salt; Tiron), 3-hydroxyquinoline (oxine), 4-hydroxy-2,2,6,6-tetramethyl piperidino oxy radical (HTMP-oxy radical), and 1,10-phenanthroline were products of Aldrich Chemical Co. Nitromethane and sodium peroxide were obtained from Matheson, Coleman, & Bell. Horseradish peroxidase, D-amino acid oxidase (hog kidney), glucose oxidase (*Aspergillus niger*), xanthine oxidase (buttermilk), superoxide dismutase (bovine blood), catalase (beef liver), flavin mononucleotide, flavin adenine dinucleotide, NAD, NADH, NADP, NADPH, glutathione, reduced glutathione, ω-nitro-L-arginine, D-phenylalanine, xanthine, cytochrome *c*, bovine serum albumin fraction V, *N*-(1-naphthyl)-ethylenediamine dihydrochloride, sulfanilic acid, Sepharose 6B gel, and DEAE-cellulose were obtained from Sigma Chemical Co. Sodium superoxide was obtained from Ventron. 3-Nitropropionic acid was prepared from 3-bromopropionic acid by the method of Bush et al. (3). The sodium salt of 2-nitropropane was prepared by adding 2-nitropropane (1 mol) to sodium hydroxide solution (1 mol). The salt was reported to reprotonate very slowly (29) and was freshly prepared before use. Chromosorb 101 was obtained from Varian. The other general chemicals were analytical grade reagents and were obtained from Mallinckrodt.

Microorganisms. The microorganisms used were *Streptomyces achromogenes* subsp. *streptozoticus* NRRL-3125, *Streptomyces ambofaciens* FD-15663 and BA 8509 (from J. B. Routien), *Streptomyces gelaticus* NRRL-2425, *Streptomyces hinnulinus* NRRL-3592, *Streptomyces caespitosus* NRRL-2564, *Streptomyces verticillatus* ATCC-13495, *Streptomyces bottropensis* (from H. Umezawa), *Streptomyces griseus* ATCC-13741, *Streptomyces cannus* ATCC-12646, and *Hansenula mrakii* IFO-0895 (from K. Soda).

Growth of organisms and preparation of crude cell-free extract. All species of *Streptomyces* (except *S. achromogenes*) were grown in medium containing the following (in grams per liter): Trypticase peptone (Baltimore Biological Laboratory), 10; Phytone (Baltimore Biological Laboratory), 5; glucose, 5; $(NH_4)_2HPO_4$, 2; K_2HPO_4, 1.5; $MgSO_4 \cdot 7H_2O$, 0.25; NaCl, 2.0; and glycerol, 10. Also added was 1 ml of a trace element solution (14) per liter, which contained 0.015% $AlK(SO_4)_2$, 0.003% KI, 0.003% KBr, 0.04% $MnCl_2 \cdot 4H_2O$, 0.006% $ZnSO_4$, 0.006% $CaSO_4 \cdot H_2O$, 0.007% $CoCl_2$, 0.003% $(NH_4)_6Mo_7O_{24}$, 0.001% $K_2Cr_2O_7$, and 0.003% $CuSO_4 \cdot 5H_2O$. *S. achromogenes* cells were grown in the above medium, but with the Trypticase concentration reduced to 5 g/liter and with the addition of 4 g of $(NH_4)_2SO_4$ per liter. The pH of this medium was adjusted to 5.8. Ammonium sulfate helped to stabilize the pH between 4 and 5 during growth of the cells, a requirement which had to be met to obtain acceptable streptozotocin titers. *H. mrakii* cells were grown in a medium containing (in grams per liter): yeast extract, 3; malt extract, 3; peptone (Difco Laboratories), 5; and glucose, 10. All strains were grown in 100 ml of the respective medium in 500-ml Erlenmeyer flasks at 27°C on a reciprocating shaker (200 rpm) after inoculation with 2 ml each from an appropriate 2-day-old master culture. *Streptomyces* cells and *H. mrakii* cells were usually harvested by suction filtration after 96 to 120 and 18 h of growth, respectively. The average yield of cells was 2 g (wet weight) per 100 ml of medium harvested. Cells which had been washed once with 100 ml of 0.15 M potassium phosphate buffer, pH 7.5 (50 mM KCl, 1 mM magnesium acetate or magnesium chloride [buffer A]) and suspended in the same buffer in a ratio of 1 g of cells per 5 ml of buffer, were passed through a precooled (0 to 5°C) French pressure cell at 15,000 to 20,000 lb/in^2. The resulting solution was centrifuged at 18,000 × *g* for 20 min, and the supernatant was used as the source of enzyme and is referred to as crude extract.

Enzyme assay: nitroalkane oxidation. The enzyme was assayed by determining the amount of nitrite formed by the method of Ida and Morita (8). The standard reaction mixture contained 0.8 ml of 0.2 M sodium phosphate buffer (pH 9.0), 100 μmol of substrate (25 μmol of 3-nitropropionic acid), and 50 to 100 μl of enzyme in a final volume of 1 ml. The blank contained the enzyme boiled for 5 min. The reaction was initiated by adding substrate, and the reaction mixture was incubated for 1 h at 37°C. The reaction was stopped by adding 2 ml of color-forming reagent solution. The stock solution was obtained by mixing 25 ml of 0.5% sulfanilic acid in 30% acetic acid, 25 ml of 0.1% *N*-(1-naphthyl)-ethylenediamine dihydrochloride, and 10 ml of 6 N HCl. Precipitated protein was removed by centrifugation at 8,000 × *g* for 10 min in a Sorvall SS-3 centrifuge, and the color was read at 540 nm. One unit of activity was defined as the amount of enzyme that released 1 nmol of nitrite per min. Specific activity is expressed as units per milligram of protein. Acetone formation was determined spectrophotometrically with 3-methyl benzothiazolone hydrazone-hydrochloride by the method of Paz et al. (27) and by gas chromatography with a series 1400 Varian gas chromatograph equipped with a coiled glass column (2-mm ID by 2 m) packed with Chromosorb 101 (80 to 100 mesh) (11). Helium was used as the carrier gas at a flow rate of 30 ml/min. Injection port and detector temperatures were 140 and 150°C, respectively. The column was programmed from 60 to 140°C at a rate of 6°C/min. 2-Nitropropane consumption was measured on the same instrument under identical conditions. Oxygen consumption was measured with a Yellow Springs Instruments biological oxygen monitor. The reaction buffer was saturated with oxygen before use. The possible formation of hydrogen peroxide was determined by the method of Malmstadt and Djiioannou (20) by using *O*-dianisidine.

Other enzymes. D-Amino acid oxidase was assayed essentially by the method of Wellner and Lichtenberg

(34) by using D-phenylalanine as the substrate. Activity is expressed as units of absorbance at 300 nm per mg of protein per 30 min. Glucose oxidase was determined by titrating the gluconic acid formed from glucose in an NaCl-acetic acid (0.01 M, pH 5.1) buffer system (28). One unit of enzyme catalyzed the oxidation of 1 μmol of glucose to gluconic acid per min at 35°C. Xanthine oxidase was assayed spectrophotometrically at 292 nm by the formation of uric acid from xanthine. One milliunit of enzyme produced an absorbance change at 292 nm of 0.008 per min at 25°C in 0.2 M sodium phosphate buffer at pH 7.5. Xanthine dehydrogenase was assayed likewise by using NAD as the electron acceptor (25). Glutathione *S*-transferase was assayed by the method of Habig et al. (7) by using 3,4-dichloronitrobenzene as the substrate. Superoxide dismutase was assayed essentially by the method of McCord and Fridovich (19). One unit of enzyme inhibited the rate of reduction of cytochrome *c* in the presence of xanthine and xanthine oxidase by 50% as measured spectrophotometrically at 550 nm. Catalase was assayed by the method of Beers and Sizer (2), in which disappearance of peroxide is followed spectrophotometrically at 240 nm. One unit of enzyme decomposed 1 μmol of H_2O_2 per min at 25°C and pH 7.0.

Partial purification of the nitroalkane-oxidizing enzyme from *S. achromogenes*. All operations were carried out at 0 to 5°C.

Ammonium sulfate precipitation. The *S. achromogenes* crude supernatant was brought to 20% saturation by slow addition of solid ammonium sulfate with stirring. The precipitate was separated by centrifugation at 18,000 × *g* for 15 min in a Sorvall SS-3 centrifuge and discarded. The supernatant was brought to 65% saturation with ammonium sulfate with stirring. The protein was collected by centrifugation at 18,000 × *g* for 15 min, the supernatant was decanted and discarded, and the pellet was dissolved in buffer A.

Sepharose 6B fractionation. The dissolved pellet from the previous step was dialyzed overnight against two changes of 500 ml of buffer A and applied to a Sepharose 6B column (85 by 2.5 cm) pre-equilibrated with the same buffer. Protein was eluted with buffer A at a rate of 20 ml/h, and fractions (6.5 ml) were collected with a Gilson microfractionator. Fractions were assayed for nitroalkane-oxidizing enzyme activity. The most active fractions were pooled and concentrated by ammonium sulfate precipitation to 70% saturation. The protein pellet was dissolved in a small volume of buffer A and dialyzed overnight against 500 ml of 0.1 M sodium phosphate buffer, pH 8.5 (buffer B).

DEAE-cellulose chromatography. The enzyme solution from the previous step was loaded onto a DEAE-cellulose column (50 by 2.5 cm) equilibrated with buffer B. The column was washed with 140 ml of the same buffer and eluted with a 0 to 1 M sodium chloride gradient in buffer B. Fractions were collected with a Gilson microfractionator and assayed for nitroalkane-oxidizing enzyme activity. The most active fractions were pooled and concentrated by ammonium sulfate precipitation to 70% saturation. The protein pellet was dissolved in a small volume of buffer A and dialyzed against two 500-ml changes of the same buffer. All subsequent studies of the properties of the enzyme were performed with this preparation.

Protein determination. Protein was determined by the method of Lowry et al. (18) by using bovine albumin fraction V as a standard. Absorbance at 280 nm of column eluate fractions was recorded as a measure of protein concentration.

K_m for O_2. Oxygen concentrations required for the determination of the K_m of oxygen were adjusted by bubbling nitrogen through the oxygen-saturated enzyme-buffer solution in a Yellow Springs Instruments biological oxygen monitor cell for different periods of time, followed by recording of the oxygen concentration. Excess 2-nitropropane was then added, and the rate of nitrite formation was determined at each oxygen concentration.

RESULTS

Nitroalkane oxidation by *Streptomyces* strains. All crude cell-free extracts of the strains of *Streptomyces* investigated, irrespective of the ability or inability of the strain to produce N-N bond-containing compounds, as well as a crude cell-free extract of *H. mrakii* which was included for comparison, were capable of forming nitrous acid from a variety of aliphatic nitro compounds (Table 1). The crude cell-free extracts have shown nitroalkane oxidation with varying degrees of specific activity against primary and secondary nitroalkanes. 2-Nitropropane was the best substrate in the vast majority of the extracts, 3-nitropropionic acid was the least efficient substrate, and nitroethane appeared to be a slightly better substrate than 1-nitropropane in the strains that do not produce N-N bond-containing compounds. None of the following was a substrate: *p*-nitrobenzoic acid, *p*-aminobenzoic acid, enteromycin (15) (an antibiotic which is also elaborated by *S. achromogenes* subsp. *streptozoticus* and which contains an *O*-methyl-aci-nitro group), and ω-nitro-L-arginine and *t*-nitrobutane (tested only in *S. achromogenes*). Addition of 0.5% 2-nitropropane to the growth media of *S. achromogenes*, *S. ambofaciens*, *S. gelaticus*, *S. hinnulinus*, *S. caespitosus*, *S. bottropensis*, and *S. griseus* delayed the growth of all of these species by 3 to 4 days. However, nitroalkane-oxidizing activity of crude cell-free extracts made from these cultures showed only a slight increase in specific activity (in the range of up to 10%), and therefore it appears that the enzyme is not significantly inducible under these conditions (data not shown).

Since Porter et al. (29, 30) have demonstrated that D-amino acid oxidase from hog kidneys and glucose oxidase from *A. niger* can form nitrous acid from primary nitroalkanes, we investigated their possible role in nitroalkane oxidation in *Streptomyces*. D-Amino acid oxidase activity was detectable in crude cell-free extracts of all of the

TABLE 1. *Nitroalkane oxidation by crude cell-free extracts of Streptomyces species and H. mrakii*

Source of enzyme	Antibiotic[a]	Activity (U/mg of protein) with the following substrates:				
		2-Nitro-propane	1-Nitro-propane	Nitro-ethane	Nitro-methane	3-Nitro-propionic acid
S. achromogenes subsp. *streptozoticus*	Streptozotocin[b]	2.08	1.85	0.83	0.37	0.03
S. ambofaciens	Duazomycin[b]	0.2	0.11	0.11	0.07	0.01
S. gelaticus	Elaiomycin[b]	1.54	0.67	0.60	0.34	0.07
S. hinnulinus	LL-BH872α[b]	2.63	0.70	0.59	0.16	0.02
S. caespitosus	Mitomycin	2.27	1.07	1.38	0.50	0.06
S. verticillatus	Mitomycin	0.28	0.23	0.38	0.14	0.01
S. bottropensis	Bottromycin	2.11	0.56	0.67	0.44	0.03
S. griseus subsp. *farinosus*	Streptolin	0.65	0.14	0.19	0.14	0.02
S. canus	Telomycin	0.95	0.22	0.22	0.05	<0.01
H. mrakii		0.81	0.21	0.38	0.11	0.09

[a] For structure of antibiotics, see reference 16.
[b] Contains a nitrogen-nitrogen bond.

Streptomyces strains investigated. This activity ranged from 0.2×10^{-2} to 2.0×10^{-2} U/mg of protein. Assuming that D-amino acid oxidase from *Streptomyces* behaves similarly to hog kidney D-amino acid oxidase (1 U of which catalyzes the formation of 4 nmol of nitrite per min from 2-nitropropane), then the 0.02 U of D-amino acid oxidase present in the crude *S. achromogenes* extract can only account for maximally 4% of the nitroalkane-oxidizing activity reported in Table 1. Likewise, the D-amino acid oxidase activity seen in the crude cell-free extracts of the other strains can only account for maximally 15% of the observed nitroalkane oxidation. Glucose oxidase activity was measured at pH 5.1 in crude cell-free extracts of *S. achromogenes*, and an amount of 0.1 U was detected per 1.5 ml. This same volume produced 0.63 μmol of nitrite per min, whereas 0.1 U of commercial *A. niger* glucose oxidase, when assayed as a nitroalkane-oxidizing enzyme at pH 7.5, produced only 0.06 nmol of nitrite per min. Therefore, assuming that the glucose oxidases from *Streptomyces* and *Aspergillus* are not significantly distinct in their properties, it can be concluded that glucose oxidase cannot be responsible for the observed nitroalkane oxidation in *S. achromogenes* and presumably in the other *Streptomyces* strains. Likewise, we could not detect any appreciable level of glutathione *S*-transferase activity (7) measured with 3,4-dichloronitrobenzene as the substrate in crude cell-free extracts of *S. achromogenes*; therefore, this enzyme can also be excluded from consideration as a participant in nitroalkane oxidation in this strain and presumably also in the other strains. In addition to these enzymes, we investigated whether xanthine oxidase, which is generally known to have a rather broad substrate specificity, can generate nitrite from nitroalkanes. We found that xanthine oxidase from buttermilk has appreciable activity toward 2-nitropropane (1 U of enzyme can generate 10 nmol of nitrite per min; Dhawale and Hornemann, unpublished data), and therefore we examined whether xanthine oxidase or xanthine dehydrogenase, which in mammalian systems and presumably in microorganisms is closely related to xanthine oxidase, could be demonstrated in crude cell-free extracts of *S. achromogenes*. We could not detect these enzymes, and therefore they are not contributors in nitrite formation from nitroalkanes in *S. achromogenes* and presumably in the other strains as well. It is of interest to note that xanthine dehydrogenase was reported to be repressed in *Streptomyces* sp. (25) grown on media containing glucose and ammonium salts. All of the strains of *Streptomyces* under investigation were also grown on media rich in glucose and ammonium salts; therefore, there is good reason to believe that xanthine dehydrogenase and xanthine oxidase will also be repressed in these strains.

Characterization of the nitroalkane-oxidizing activity of *S. achromogenes*. The nitroalkane-oxidizing enzyme from *S. achromogenes* subsp. *streptozoticus* was partially purified (Table 2) by ammonium sulfate precipitation and Sepharose 6B gel filtration, as well as by DEAE-cellulose ion-exchange chromatography (Fig. 1); this preparation was used in characterizing the nitroalkane-oxidizing activity with 2-nitropropane as the substrate. The stoichiometry of the oxidation of 2-nitropropane was deduced from the data presented in Table 3, which fit the following equation: $2CH_3CH(NO_2)CH_3 + O_2 \rightarrow 2CH_3COCH_3 + 2HNO_2$ (equation 1). Hydrogen peroxide formation was not observed in the reaction. The de-

TABLE 2. *Partial purification of the nitroalkane-oxidizing enzyme from S. achromogenes*

Step	Vol (ml)	Total protein (mg)	Enzyme activity[a]		% Recovery
			U/mg	Total U	
Crude extract	63	2,394	8.4	20,110	100
			(0.9)	(2,155)	(100)
Ammonium sulfate precipitation	24	1,709	8.03	13,723	68
			(1.2)	(2,050)	(95)
Sepharose 6B filtration (concentrated and dialyzed solution)	23	362	34.0	12,308	61
			(1.8)	(651)	(30)
DEAE-cellulose ion-exchange chromatography	5	19	317	6,023	30
			(2.4)	(46)	(2)

[a] Measured with 2-nitropropane as substrate. Figures in parentheses represent activity measured with nitromethane as substrate.

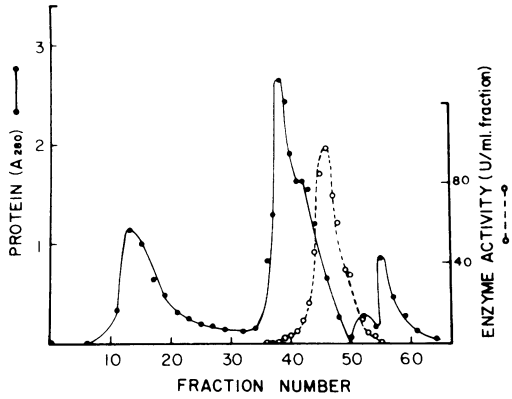

FIG. 1. *DEAE-cellulose chromatography of nitroalkane-oxidizing enzyme from S. achromogenes. Symbols:* ●, *absorbance at 280 nm for protein;* ○, *enzyme activity expressed in units per milliliter of fraction when 2-nitropropane was used as substrate.*

TABLE 3. *Stoichiometry of oxidation of 2-nitropropane by the partially purified nitroalkane-oxidizing enzyme from S. achromogenes*[a]

2-Nitropropane consumed (μmol)	Oxygen consumed (μmol)	Nitrite formed (μmol)	Acetone formed (μmol)	Hydrogen peroxide formed (μmol)
1.64	0.846	1.7	1.7	0

[a] A 5-ml amount of standard reaction mixture containing 60 μg of enzyme protein and 100 μmol of 2-nitropropane in the glass chamber of a Yellow Springs Instruments biological oxygen monitor were incubated at 37°C for 75 min. Oxygen consumption was calculated, and a part of the reaction mixture was used to determine the amount of nitrite, acetone, and H_2O_2 formed and the amount of 2-nitropropane consumed.

termination of the possible formation of this substance was not straightforward because the partially purified *S. achromogenes* enzyme contained catalase activity (16 U/mg of protein). However, we observed that cyanide, a potent inhibitor of catalase, did not inhibit either nitroalkane oxidation (Table 4) or the peroxidase needed in the assay for hydrogen peroxide formation. Despite a detection limit of 1 nmol, the formation of hydrogen peroxide could not be demonstrated in assay mixtures containing 0.025 mM sodium cyanide. According to equation 1 and in analogy to the results reported by Soda and co-workers on the nitroalkane-oxidizing enzyme from *H. mrakii* (13), the *S. achromogenes* enzyme is a nitroalkane dioxygenase. Soda and co-workers reported that the homogeneously purified *H. mrakii* enzyme was inactive toward nitromethane (13). The *S. achromogenes* enzyme appears to be similar in its reactivity to-

TABLE 4. *Effect of inhibitors and of superoxide dismutase on the nitroalkane-oxidizing enzyme of S. achromogenes*

Addition	% Activity[a]
None	100
Azide (1 mM)	7
Cyanide (1 mM)	100
Flavin adenine dinucleotide (20 μg)	96
NAD (20 μg)	100
Flavin mononucleotide (20 μg)	98
Glutathione (5 mM)	100
NADH (1 mM)	3
NADH (5 mM)	0
NADPH (5 mM)	6
Reduced glutathione (5 mM)	4
2-Mercaptoethanol (1 mM)	23
D- or L-Cysteine (5 mM)	20
Tiron (1 mM)	9
Tiron (5 mM)	1
Oxine (1 mM)	17
Oxine (5 mM)	4
HTMP-oxy radical (0.1 mM)	7
1,10-Phenanthroline (1 mM)	105
EDTA (1 mM)	89
$HgCl_2$ (1 mM)	6
Iodoacetate (1 mM)	95
Superoxide dismutase (3 μM)	107
Sodium peroxide (1 mM)	128
Sodium superoxide (1 mM)	100

[a] Activity without any addition was considered to be 100%.

ward nitromethane since during purification (Table 2) the reactivity toward this substrate decreased progressively.

Effect of pH and temperature. Nitroalkane-oxidizing activity was measured in sodium phosphate buffer solution in the pH range of from 4.3 to 9.5 (Fig. 2). An optimum was observed at pH 7.5; consequently buffers at this pH were used in all subsequent experiments. The temperature optimum of the enzyme was found to be at 37°C by incubating reaction mixtures at a range of temperatures in a water bath (±0.5°C).

Enzyme concentration curve and progress curve. A plot of enzyme activity against enzyme protein concentration showed a slightly convex curve, and a plot of enzyme activity against time (Fig. 3) shows an irregular progress curve. The progress curve is characterized by a short lag period before a quasi-linear stage is reached, which is followed by a time period in which enzyme activity decreases slightly. The decline in enzyme activity could be partially prevented, and the linearity of the enzyme concentration curve could be improved, by shaking the reaction mixture; however, bubbling oxygen gas or air through the reaction vessel led to an even more pronounced decline of activity. It is not known whether the slight decline in enzyme activity was due to denaturation of the enzyme or to other factors. It appears that product inhibition, as tested by the addition of 1 μmol of either nitrite or acetone, or a change in pH (the pH changed only from 7.50 to 7.42 during 75 min when 1.7 μmol of nitrite was formed) was not responsible for the observed decrease.

Km. A K_m of 1.78×10^{-2} M for 2-nitropropane and a K_m of 2.67×10^{-4} M for oxygen were obtained when 2-nitropropane was used as the substrate for the partially purified nitroalkane-oxidizing activity of *S. achromogenes*. These K_m values are very similar to those reported for the *H. mrakii* nitroalkane dioxygenase.

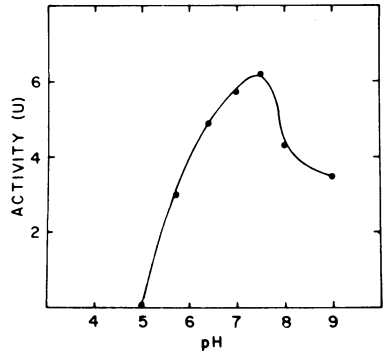

FIG. 2. *Effect of pH on the activity of the nitroalkane-oxidizing enzyme from S. achromogenes. All buffers contained 0.2 M sodium phosphate; adjustment to pH 9.0 was done with 0.1 N NaOH.*

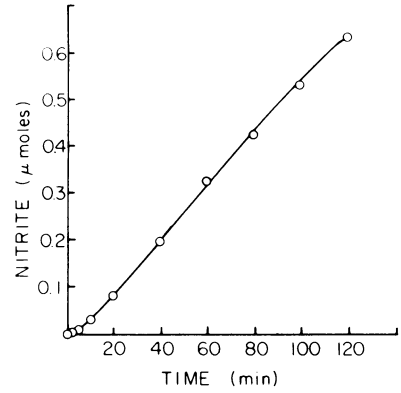

FIG. 3. *Relationship of nitrite formed and duration of incubation of nitroalkane-oxidizing enzyme from S. achromogenes.*

Effect of azide, cyanide, redox cofactors, thiol compounds, free radical scavengers, chelating agents, sulfhydryl reagents, superoxide dismutase, and sodium peroxide and superoxide on the nitroalkane-oxidizing enzyme of *S. achromogenes*. As shown in Table 4, the partially purified nitroalkane-oxidizing enzyme was inhibited by azide, but it was not affected by cyanide or by flavine adenine dinucleotide, NAD, or flavin mononucleotide and oxidized glutathione; however, almost complete loss of activity was seen with NADH, NADPH, and reduced glutathione. Like reduced glutathione, 2-mercaptoethanol and D- or L-cysteine were inhibitory. Tiron, oxine, and HTMP-oxy radical acted as potent inhibitors. 1,10-Phenanthroline was marginally stimulatory rather than inhibitory, and EDTA caused only slight inhibition. $HgCl_2$ was a potent inhibitor, whereas iodoacetate hardly showed any inhibition at all. When HTMP-oxy was added at a concentration higher than 0.1 mM, nonenzymatic oxidation of 2-nitropropane was observed. Superoxide dismutase, which was recently reported by Kido et al. (10) to inhibit 2-nitropropane dioxygenase of *H. mrakii*, was not inhibitory at concentrations up to 6 μM. Addition of sodium peroxide at a concentration of 1 mM to the standard reaction mixture and to the no enzyme blank resulted in the disappearance of the lag period in the progress curve (data not shown) and a 28% acceleration of the enzymatic reaction and significant stimulation of the chemical oxidation of the substrate, respectively. In contrast, the presence of 1 mM sodium superoxide, which was reported by Kido et al. (10) to stimulate the *H. mrakii* enzyme, had no effect.

In view of the report by Porter and Bright (29) that glucose oxidase shows a pronounced rate acceleration of oxidation (up to 6×10^3-fold) at pH 5.1 (the pH optimum for this enzyme) when the anion of nitroethane is offered as the substrate, we investigated the reactivity of the nitroalkane-oxidizing enzyme from *S. achromogenes* toward free 2-nitropropane and its potassium salt. We did not observe any significant rate enhancement at pH 7.5 with the salt as the substrate.

DISCUSSION

The results of this investigation show that the ability to generate nitrous acid from aliphatic nitroalkanes is widespread among streptomycetes. No major difference was found in crude cell-free extracts in the ability to oxidize these substrates when strains that produce and those that do not produce antibiotics containing an N-N bond were compared (Table 1). Whether the slight difference in the preferential utilization of either 2-nitropropane or nitroethane by the strains producing N-N bond-containing compounds and the nonproducers is significant cannot be evaluated at the present time.

Nitroalkane-oxidizing activity has been reported in many microorganisms (6, 9, 12, 17), but so far only 2-nitropropane dioxygenase from *H. mrakii* (10, 11, 13), a β-nitropropionate-oxidizing enzyme from *Penicillium atrovenetum* (6) and *Aspergillus flavus* (23), and a nitroalkane oxidase from *Fusarium oxysporium* (9) which yields nitrite, acetone, and hydrogen peroxide from 2-nitropropane and oxygen have been studied in more or less detail. We have considered the possibility that these three enzymes or a number of nonspecific enzymes, such as D-amino acid oxidase (30), glucose oxidase (29), glutathione *S*-transferase (7), and possibly xanthine dehydrogenase, could be responsible for the observed nitroalkane oxidations in the crude cell-free extracts of the *Streptomyces* strains investigated. The results of this paper suggest that all of the nonspecific activities, as well as the β-nitropropionate-oxidizing enzyme, can essentially be eliminated from consideration. Therefore, it is likely that a specific nitroalkane-oxidizing enzyme is responsible for the observed oxidations. This enzyme in *S. achromogenes* subsp. *streptozoticus* appears to be a dioxygenase and not an oxidase. This distinction cannot presently be made for the enzymes from the other *Streptomyces* species because they were not studied in sufficient detail. The *S. achromogenes* enzyme is very similar in many respects, but not in all respects, to the *H. mrakii* 2-nitropropane dioxygenase. Thus, both enzymes exhibit similar substrate specificity, stoichiometry, high substrate K_m values, and susceptibility to a number of inhibitors. However, these enzymes differ in their response to superoxide dismutase and sodium superoxide, but no reason for this difference is presently known. It may be assumed that the *S. achromogenes* nitroalkane-oxidizing enzyme, like the *H. mrakii* enzyme, is a flavin- and iron-containing dioxygenase; however, we have not studied the nature of any cofactors of this enzyme, nor have we studied the incorporation of ^{18}O into acetone, which would substantiate the latter assumption.

There is currently lively debate concerning the nature of the mechanism of flavin enzyme-catalyzed oxidations (29). T. C. Bruice and coworkers, who first reported on the use of the HTMP-oxy radical to investigate flavin-catalyzed oxidation reactions in their study of the oxidation of glucose analogs by glucose oxidase (4) and in their study of the oxidation of 9-hydroxy and 9-methoxy fluorene carbanions by flavin (24), favor a radical mechanism for all flavin-catalyzed oxidations. Others seem to favor mechanistic schemes which involve ionic rather than radical species, especially for flavin-catalyzed oxidations of physiological substrates (29). From the results of the inhibition studies, we can infer that both the *S. achromogenes* enzyme and the *H. mrakii* enzyme may act via a mechanism involving radical species. This inference is based on the findings that Tiron and oxine inhibit both enzymes, whereas the iron chelator 1,10-phenanthroline is without effect, and that the HTMP-oxy radical, whose effect on the *H. mrakii* enzyme has apparently not been studied, inhibits the *S. achromogenes* enzyme. Further presumptive evidence in favor of a reaction mechanism involving radical species can be seen in the fact that the progress curve shows a slight lag phase. This lag phase may be due to a requirement for the generation of a certain concentration of a radical species before the reaction shown in equation 1 can proceed. It is of interest to note that the chemical oxidation of 2-nitropropane shows a progress curve which also features a lag phase (31). The chemical reaction, like the enzymatic reaction described above, does not yield hydrogen peroxide, and, besides being considerably slower, it differs from the enzymatic reaction by a pronounced dependence on the presence of base. The chemical reaction in basic solution is not stimulated by added sodium peroxide. The lag phase was attributed to the requirement for the buildup of radical species to a sufficient level for the reaction to proceed by a radical chain mechanism. Our observation that sodium peroxide abolishes the lag

period of the enzymatic reaction is presently unexplained.

Assuming that the *S. achromogenes* enzyme that we have studied, despite its high K_m for 2-nitropropane, is a genuine nitroalkane-oxidizing enzyme and given the widespread occurrence of nitroalkane-oxidizing activity in strains of *Streptomyces* as well as the relative ease of formation of *N*-nitroso compounds (5, 22), it appears that the potential of a strain to produce a compound containing an N-N bond might be governed by the availability of a suitable aliphatic nitro compound within the cells of that strain. Streptomycetes elaborate a variety of compounds containing nitro groups (16), but the only one known to contain a primary nitro group is 3-nitropropionic acid (bovinocidin [1]). This acid is found to be a poor substrate for the nitroalkane-oxidizing enzymes of the streptomycetes investigated. Since aromatic nitro compounds, as tested with *p*-nitrobenzoate, are not attacked by the enzymes and since other potential donors of nitrous acid such as enteromycin and ω-nitro-L-arginine are not reactive, it appears that no good, naturally occurring substrate for nitrous acid formation with the nitroalkane-oxidizing enzymes is presently known in streptomycetes. The fact that with very few exceptions (1, 3, 16) aliphatic nitro compounds have not been reported in streptomycetes or in other microorganisms, although, for instance, 1-nitro-2-phenylethane appears to occur relatively frequently in plants (21), may be due to lack of systematic searches for these compounds, which by and large are relatively innocuous. We feel that in view of their potential role in the formation of antibiotics containing a nitrogen-nitrogen bond a search for the natural occurrence of aliphatic nitro compounds in streptomycetes and in other microorganisms may prove to be worthwhile.

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