PURIFICATION AND CHARACTERIZATION OF AN ENZYME INVOLVED IN THE METABOLISM OF NITRILOTRIACETIC ACID

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ABSTRACT

PURIFICATION AND CHARACTERIZATION OF AN
ENZYME INVOLVED IN THE METABOLISM
OF NITRILOTRIACETIC ACID

By

Mary Kathryn Firestone

An enzyme believed to be involved in the metabolism of nitrilotriacetate (NTA) by a Pseudomonas sp. was purified 70-fold and partially characterized.

The activity of the enzyme was monitored by spectrophotometric measurement of NTA-dependent NADH oxidizing activity. Purification procedures included DEAE anion exchange chromatography and Sephadex gel filtration. Phenylmethylsulfonylfluoride stabilized the NADH oxidizing activity and hence was included in all buffer solutions throughout the purification procedure.

The NTA-dependent NADH oxidizing activity was found in cells grown on NTA or iminodiacetate (IDA) but not in cells grown on glycine and glyoxylate. Addition of FMN, Mn^{++} and either NTA or IDA was necessary to obtain the NADH oxidizing activity. Km values of 32 μM for NTA and 500 μM for IDA were determined using purified protein.

SDS polyacrylamide gel electrophoresis of the protein purified through a DEAE column and a Sephadex column showed four bands of protein. Passage of the purified protein through another DEAE column

or Sephadex column did not significantly alter the size or position of the four bands observed after gel electrophoresis.

The purified enzyme apparently did not alter the NTA molecule. Quantities of NADH oxidized in the presence of NTA were so large as to exclude any reasonable stoichiometric relationship between the two. The NTA-dependent NADH oxidation proceeded under anaerobic conditions. When cytochrome c was added, two molecules of this acceptor were reduced for each NADH molecule oxidized.

The FMN requirement for NTA-dependent NADH oxidation indicated that the flavin component of the enzyme was lost during purification.

These observations support the conclusion that the enzyme isolated had been altered by partial proteolytic fragmentation resulting in modification of its structural integrity and enzymatic capability.

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Mary Kathryn Firestone

A THESIS

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To my mother

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I am very grateful to Dr. J. M. Tiedje for his support and encouragement throughout the course of this work.

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I thank my husband Richard for bravely facing hundreds of quarter-pounders with cheese.

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INTRODUCTION

Nitrilotriacetic acid (NTA), $N(CH_2COOH)_3$ is a synthetic organic chelant with a variety of uses in agriculture and industry. It is currently used as a detergent "builder", to partially replace phosphates, in Canada and Sweden. The fact that NTA is being added to the water and soil environments has stimulated much work on the fate and environmental effects of the compound, including investigation of biodegradability and pathways of degradation.

Bacteria have been isolated which can utilize NTA as sole carbon source under aerobic conditions (6, 7, 16). Focht and Joseph, 1971 and Tiedje et. al., 1973, have shown that NTA is degraded to CO_2 and NH_4^+ by isolated Pseudomonas species, but no metabolic intermediates have been established in the whole cell systems (4, 6, 16).

The pathway of NTA degradation has been described by two groups, Cripps and Noble (4) and Tiedje et. al. (15) who both worked with crude cell-free extracts of a Pseudomonas species induced for NTA metabolism by growth on the substrate. The pathway proposed is shown in Figure 1. NTA undergoes cleavage to form the secondary amine, iminodiacetic acid (IDA) and the keto-acid, glyoxylate. The addition of NADH and O_2 (and Mg^{++} according to Cripps and Noble) is required for the production

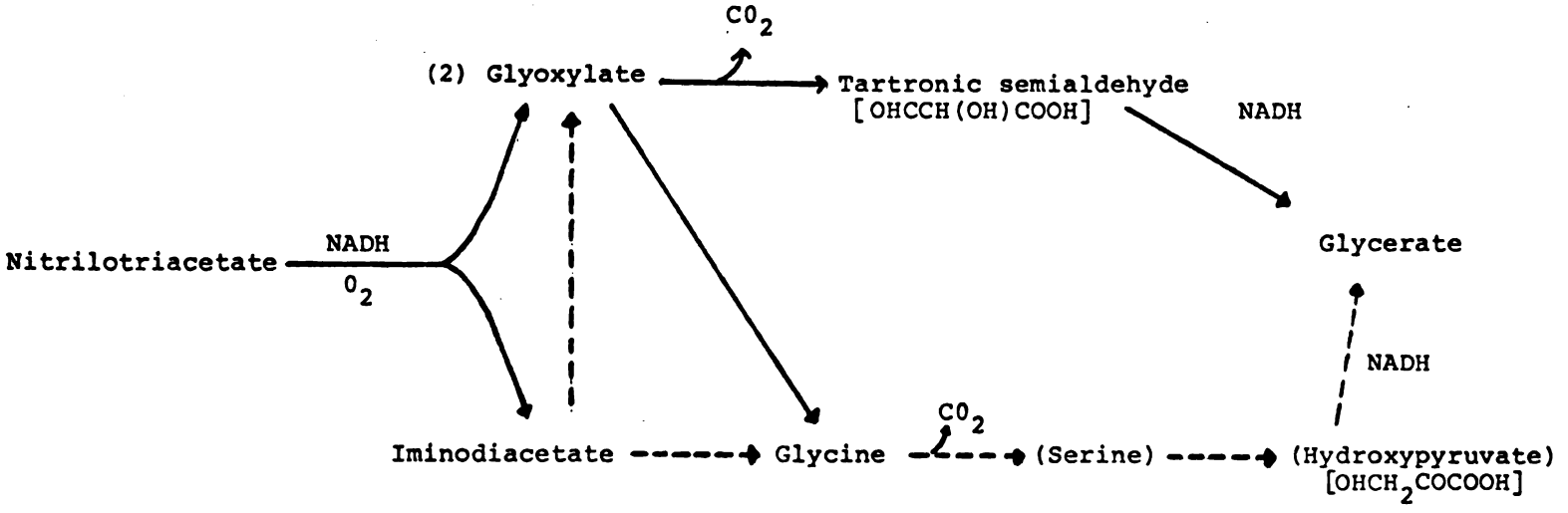


Figure 1. Pathway of NTA degradation. Intermediates in parenthesis have not been isolated.

of these two intermediates.

Previous work with crude cell-free extracts which provided information on the pathway is summarized below; the data accumulated by Tiedje and co-workers are reported in the appendix. In these crude extracts, IDA accumulates and is not further metabolized. However, that fact that IDA grown cells are induced for the NTA cleavage reaction suggests that in whole cell systems IDA undergoes similar cleavage to form glyoxylate and glycine. It appears that most of the glyoxylate produced is converted to tartronic semialdehyde by a glyoxylate carboligase with the production of 0.5 mole of CO_2 for each mole of glyoxylate. The tartronic semialdehyde in the presence of NADH is converted to glycerate. In this crude enzyme system a portion of the glyoxylate is transaminated to form glycine.

Cripps and Noble observed elevated activities of glycine decarboxylase, serine hydroxymethyltransferase, serine-oxaloacetate aminotransferase and hydroxypyruvate reductase in NTA grown cells which suggests the conversion of glycine to glycerate and CO_2 in whole cell catabolism of NTA. This sequence is included in Figure 1.

Cripps and Noble suggest that the enzyme responsible for the cleavage of NTA to IDA and glyoxylate is a Mg^{++}-requiring NADH-dependent mono-oxygenase. Other tertiary-amine mono-oxygenases of microbial origin have been reported (1, 3, 11) which are active on trimethylamine. The electron donor in these cases is NADPH and the conversion to the dimethylamine is a two-stage process. The first product is trimethylamine

N-oxide produced by a mono-oxygenase; this product is then non-oxidatively demethylated by trimethylamine N-oxide demethylase to form dimethylamine and formaldehyde (1, 3, 11). The partially purified enzyme oxidizes a wide range of tertiary and secondary amines but not NTA (1).

Colby and Zatman (2, 3) have also described a trimethylamine dehydrogenase which converts this tertiary amine to formaldehyde and dimethylamine. The stoichiometry found suggests the following sequence:

$$(CH_3)_3NH^+ + H_2O + PMS \rightleftarrows (CH_3)_2NH_2 + HCHO + PMSH_2$$

$$PMSH_2 + O_2 \rightleftarrows PMS + H_2O_2$$

(PMS = phenazine methosulfate)

In this system then, the oxygen atom found in the aldehyde probably comes from H_2O and the dye (PMS) is required as electron acceptor. Large (10) has reviewed the characteristics of the enzymes known to be involved in oxidative-cleavage of tertiary and secondary amines in microorganisms.

The present study was undertaken to isolate and characterize the enzyme responsible for the first step in the degradation of NTA and to identify the products of this reaction. The degradation pathway for NTA shown in Figure 1 was found in the Pseudomonas species previously described by Tiedje et. al. (16). This organism was used for all of the following studies including those found in the appendix.

The previously unpublished data in the appendix is included because of its direct bearing on the problem under investigation in this thesis. The data included in the appendix

resulted from work done by S. D. Aust or B. B. Mason and myself working under J. M. Tiedje.

MATERIALS AND METHODS

Culture and Medium. A 50-ml exponential phase culture of the Pseudomonas was used to inoculate a 14-liter Microferm fermentor (New Brunswick Scientific Co.) at 28 C. The 10 liters of medium contained the following mineral salts: 10.8 g K_2HPO_4; 19.2 g KH_2PO_4; 5.0 g NH_4NO_3; 2.0 g $MgSO_4 \cdot 7H_2O$; 0.25 g $CaCl_2 \cdot 2H_2O$; 0.025 g $FeCl_3 \cdot 6H_2O$; and 1.0 mg each $CuSO_4$, $MnCl_2$, H_3BO_4, Na_2Mo_4, $ZnSO_4$. The final pH was 6.9. The sole carbon source was 15 g of nitrilotriacetic acid, trisodium salt monohydrate, Gold Label from Aldrich Chemical Co.

One liter batches of cells were grown in the same medium but incubated on a rotary shaker at room temperature for the induction studies. In these experiments iminodiacetic acid disodium salt (Aldrich Chemical Co.) and glyoxylate-glycine (2:1), at a 0.1% concentration (wt/vol) were substituted for NTA as the sole carbon source.

Cells were harvested by centrifugation at 4 C in late exponential phase when the turbidity reached 0.3 to 0.4 absorbance units at 600 nm on a Spectronic 20 spectrophotometer (Bausch & Lomb, Inc.). The cells were washed once in cold 0.1 M tris(hydroxymethyl) aminomethane-hydrochloride (Tris) buffer pH 7.3. Before breaking, the cells were resuspended to 20 ml with 0.1 M Tris-HCl buffer containing 1mM dithiothreitol

(Sigma Chemical Co.) and 1mM phenylmethylsulfonylfluoride (PMSF, Sigma Chemical Co.) added to buffer in a small volume of 100% ethanol. This dithiothreitol, PMSF-containing buffer was used in all of the following purification procedures.

The cells were broken by twice passing through a cold Aminco French pressure cell (American Instrument Co.) at 20,000 PSI. Cellular debris was removed by centrifugation at 20,000 g for 20 min at 2 C. The yield was about 0.4 g protein in 17 ml of supernatant.

Enzyme Purification. Fifteen milliliters of the crude cell-free preparation were applied to a diethylaminoethyl-cellulose column (2x18 cm; Cellex-D anion exchange cellulose, BIO-RAD Laboratories). After passing 150 ml of buffer through the column, the desired protein was eluted with a 360-ml linear gradient of zero to 0.3 M KCl in buffer. NTA-dependent NADH oxidizing activity did not appear until the KCl gradient reached about 0.16 M (195 ml into the KCl buffer gradient). The protein elution pattern, determined by absorbance at 280 nm, is shown in Figure 6 in the appendix. After assaying, the pooled active fractions yielded approximately 40 ml. The pH of this pooled material was adjusted to 6.0 by dropwise additions of 0.2 M acetic acid while the protein mixture was being stirred in an ice bath. After overnight incubation at 2 C, protein precipitate was removed by centrifugation at 4000 g for 20 min at 2 C. After readjusting the pH to 7.3 with 0.1 M NaOH, an Amico Ultrafiltration cell (50 ml, Amicon Corp.) with a PM30

membrane operating at 30 PSI was used to reduce the volume to about 5 ml. This preparation was then applied to a Sephadex G-100 column (3x85 cm, Pharmacia Fine Chemicals Inc.) and eluted at a flow rate of 1.5 ml/hr. At this slow flow rate, four days were required for the protein to elute from the column, but the resolution was excellent. The protein elution pattern, determined by absorbance at 280 nm is shown in Figure 7 in the appendix. The fractions were assayed for NTA-dependent NADH oxidizing activity and pooled to give about 10 ml. When further purification was desired the post-sephadex protein was applied to another DEAE column (9x120 mm) and after 50 ml of buffer was passed through the column a 60-ml linear gradient of zero to 0.3 M KCl was used to elute the desired protein. The assayed and pooled fractions (about 7 ml) were then run through a 5 ml Amicon Ultrafiltration cell, PM30, 30 PSI, to give a volume of about 2 ml. This concentrated fraction was then reapplied to the Sephadex G-100 column (as before) and the protein collected from the tubes with activity. Samples from each step in the purification procedure were assayed for NTA-dependent NADH oxidizing activity and for protein by the tannic acid method (13).

Polyacrylamide-gel Electrophoresis. The methods used for sodium dodecyl sulfate (1%) polyacrylamide gel electrophoresis are similar to those used by Fairbanks (5) and are described elsewhere (18). The destained gels were scanned using an Isco Model UA-5 Absorbance Monitor with a Model 659 Gel scanner

(Instrumentation Specialties Company Inc.). When molecular weight standards were required the following protein standards were used: catalase (60,000, beef liver), bovine serum albumin (68,000), alcohol dehydrogenase (37,000, yeast) and trypsin (23,300, bovine pancreas), all from Sigma Chemical Co.

Enzyme Assay. The NADH oxidizing activity of the enzyme was determined by monitoring the rate of decrease in absorbance of the assay mixture at 340 nm on a Coleman 124 Perkin Elmer double beam spectrophotometer (Perkin Elmer Corp.). The standard assay mixture (1ml) contained 20 μM FMN (Sigma Chemical Co.), 0.1 mM NADH (disodium salt, grade III, Sigma Chemical Co.), 1.0 mM NTA, 2.0 mM $MnCl_2$, and 0.1 M Tris-HCl buffer. Enzyme activity is expressed in terms of units (micromoles of NADH oxidized to NAD^+ per minute) using a molar extinction coefficient for NADH of 6.22×10^6 cm^2/mole. Specific activities of the enzyme preparations are expressed as units of enzyme activity per milligram of protein.

When anaerobic conditions were required the assay mixture was added to a Thunberg cuvette which was then evacuated and gassed ten times using argon from which 0_2 had been removed by bubbling through two dithionite traps.

FMN reduction was assayed under anaerobic conditions by following the decrease in absorbance (450 nm) of the assay mixture (2ml) containing the same component proportions used for the NADH assay except that the concentration of FMN was increased to 50 μM.

Increasing absorbance at 550 nm was used to follow cytochrome c reduction using the same assay mixture as that used for NADH with the addition of 73 μm cytochrome c (horse heart, Type VI, Sigma Chemical Co.). An extinction coefficient of 2.1×10^4 cm^2/mmole (difference between reduced and oxidized cyt c) was used for activity calculations (12). Superoxide dismutase used was previously purified from bovine red blood cells.

Product Determination Procedures. NTA was determined by the zinc-Zincon procedure (14). Glyoxylate production was followed by the glyoxylate-hydrazone colorimetric procedure described by Trijbels and Vogels (17).

Product formation from NTA was also followed by thin layer chromatography and subsequent autoradiography. After protein removal from the assay mixtures by TCA precipitation, samples of 10-50 ul were spotted on Avicel thin layer plates (Analtech Inc.) and the plates developed in two dimensions with (i) HCl (1M):isopropanol:butanone (25:60:15) and (ii) T-butyl alcohol:butanone:acetone:ammonium hydroxide:methanol (40:20:20:19:1). The developed plates were placed on Kodak X-ray film for 10 days and the film developed by standard development procedures.

^{14}C-Labeled CO_2 production was followed by trapping $^{14}CO_2$ in NaOH as previously described (16). The ^{14}C-carboxyl labeled NTA (3.35 mCi/mmole) used here and in autoradiography was a gift from Proctor and Gamble Co.

RESULTS

The increasing specific activity of the NTA-dependent NADH oxidation during the purification procedure is shown in Table 1. A 71-fold purification resulted from the procedure but in the process over 95% of the enzyme activity was lost.

SDS polyacrylamide disc gel electrophoresis was run on samples during the later stages of purification. Gels run on samples purified through the first Sephadex procedure had four distinct bands of protein visible, one rather diffuse major band and three bands of less intensity. A second DEAE column was added to the purification procedure and the resulting gels had four distinct protein bands, at apparently the same positions as on the preceeding gels except that the three less intense bands appeared more concentrated relative to the major band. From comparison of the R_f values of these four bands to standard proteins run simultaneously, a molecular weight of 34,000 ($\pm$1,000) for the major band and 68,000, 56,000 and 14,500-15,200 (all $\pm$1,000) for the three bands of less intensity was derived (Figure 2). The molecular weight of the largest protein was difficult to determine accurately. When run on gels separate from the molecular weight standards, the protein had an R_f identical to that of catalase (68,000); but when the unknown was combined with the

Table 1. Specific activity of NTA-dependent NADH oxidation during protein purification.

Stage in purification	Specific Activity	Fold Purification	Total Activity Recovered (Enzyme Units)
Cell-free prep.	0.45	--	175.
After 1st DEAE	4.0	8.9	155.
After acid ppt.	4.6	10.2	151.
After 1st Sephadex	24.1	53.6	48.
After 2nd DEAE	26.5	59.0	19.1
After 2nd Sephadex	32.0	71.1	7.8

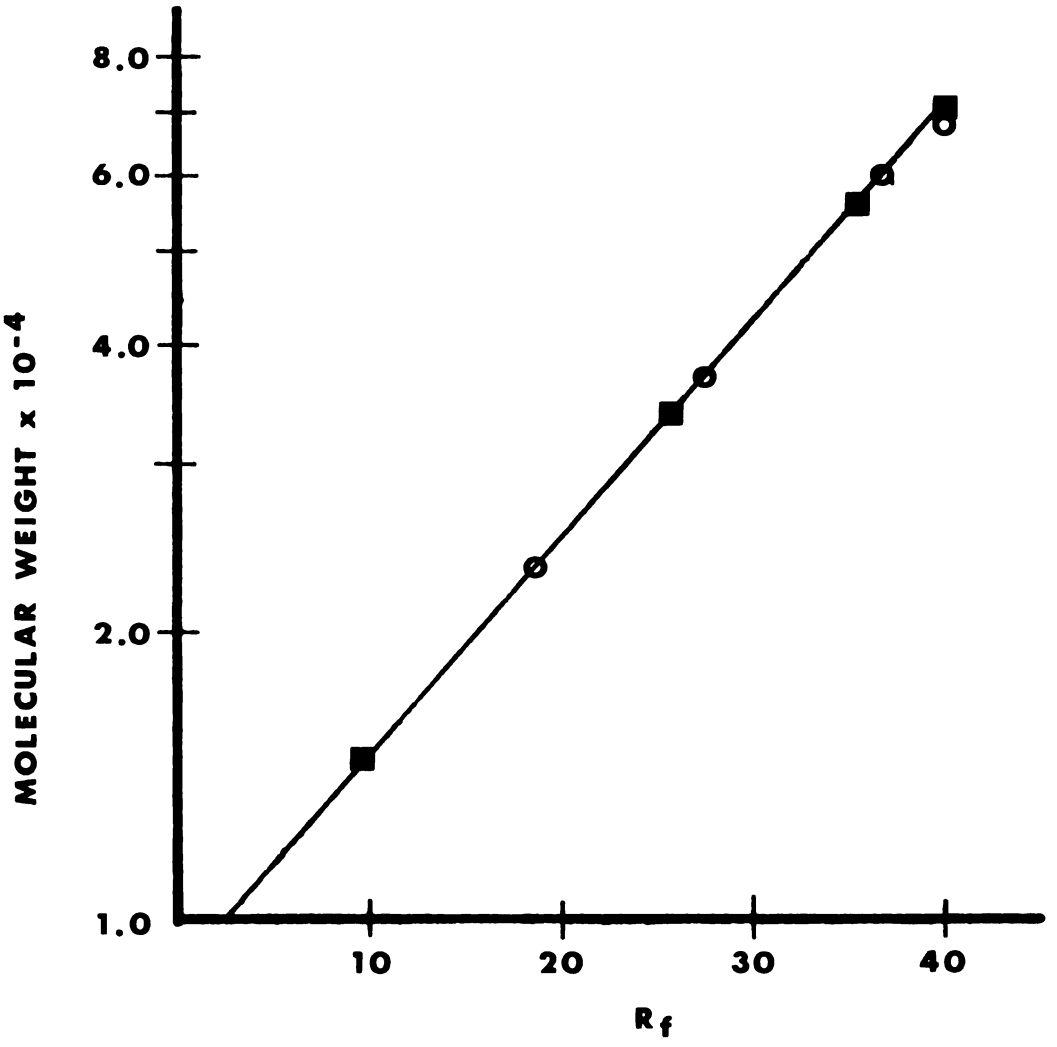


Figure 2. SDS polyacrylamide disc gels of molecular weight standards (O) (68,000; 60,000; 37,000; 23,300) and the four proteins found after purification through a second DEAE column (■).

protein standards for electrophoresis the band resulting from catalase and unknown together had peak absorbance at 66,000, indicating that the unknown protein was smaller than the 68,000 standard.

A second Sephadex column was added to the purification procedure and the resulting gels showed the same four proteins present; the only difference was that the band at 56,000 had decreased in intensity. Scans of the gels run after the 2nd DEAE and 2nd Sephadex purifications are shown in Figure 3.

During the preliminary attempts at purifying the protein, the specific activity decreased with progressing purification. To determine if the instability of the protein resulted from proteolytic degradation, partially purified preparations were incubated at 4 C with and without PMSF, a serine protease inhibitor. From the data in Table 2 it is evident that the NTA-dependent NADH oxidizing activity of the enzyme was stabilized by the presence of PMSF. Inclusion of dithiothreitol or incubating under anaerobic conditions appears to have no stabilizing effect on the activity.

The induction of the substrate-dependent NADH oxidation was investigated by growing the cells on NTA, IDA and glyoxylate-glycine (2:1 mixture). The resulting enzyme induction, as shown by assay of NADH oxidation in crude cell-free preparations, is revealed in Table 3. Growth on NTA or IDA is equally effective in inducing the substrate-dependent NADH oxidizing activity; while cells grown on glyoxylate-glycine contain essentially no enzyme activity.

Figure 3. Gel scans of: A) Protein purified through 2nd Sephadex B) Protein purified through 2nd DEAE C) Standard proteins: (1) BSA, 68,000; (2) catalase, 60,000; (3) alcohol dehydrogenase, 37,000; (4) trypsin, 23,300.

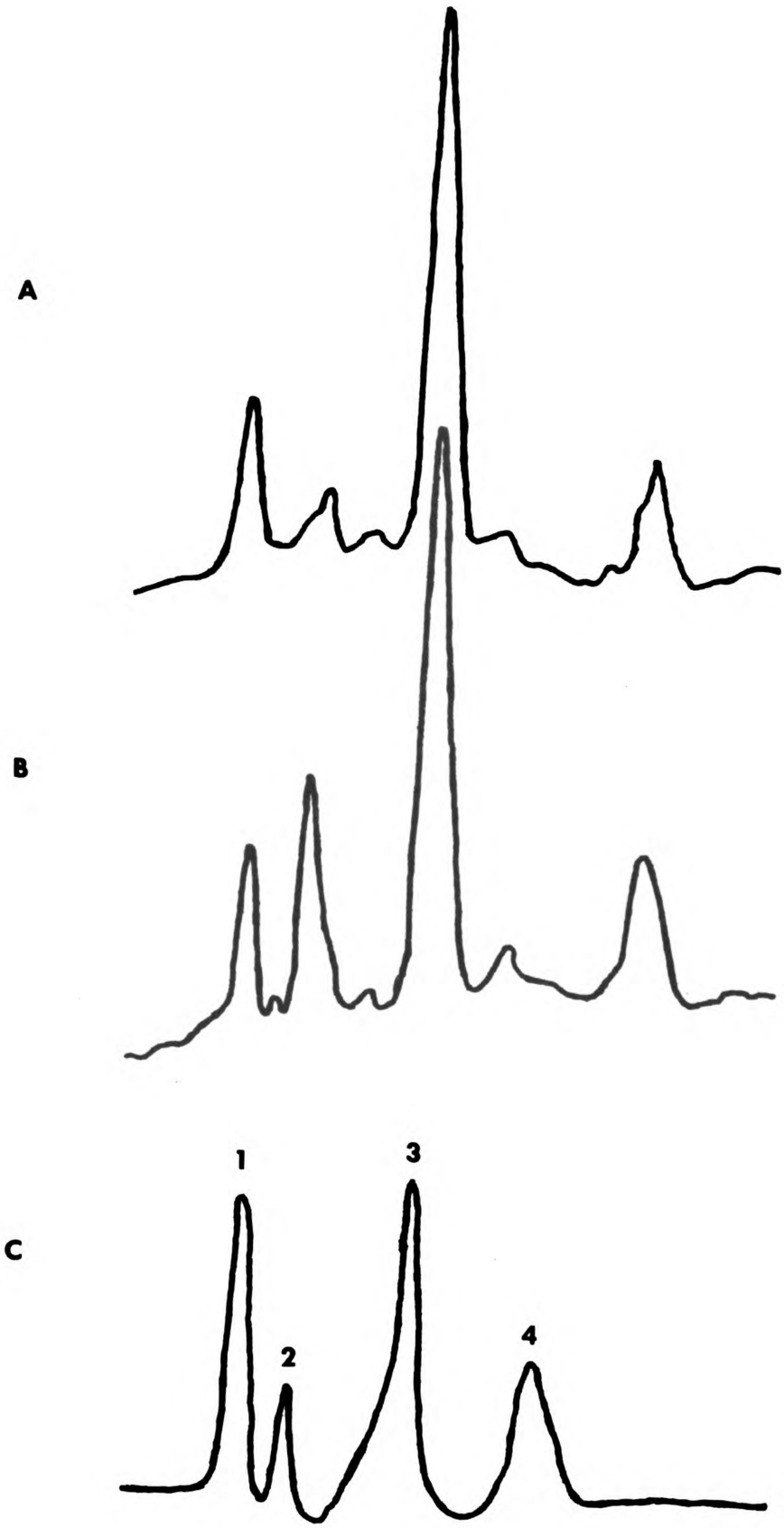

A
B
C
1
2
3
4

Table 2. Decrease in NTA-dependent NADH oxidizing activity under several incubation conditions.

	Incubation period (days)		
	0	14	29
Conditions of Incubation[a]	NADH Oxidation Activity (units x 10^2)		
Anaerobic[b]	1.85	1.45	1.09
Aerobic	1.93	1.45	1.13
Anaerobic + 1 mM dithiothreitol	1.93	1.37	1.13
Aerobic + 1 mM dithiothreitol	1.93	1.29	1.01
Anaerobic + 1 mM PMSF	1.85	1.93	1.74
Aerobic + 1 mM PMSF	1.85	1.93	1.82

[a]Protein had been purified through first DEAE step prior to incubations. All incubations at 4 C.

[b]Flushed with helium before capping.

Table 3. Substrate-dependent NADH oxidizing activity in cell-free preparations induced by growth on several carbon sources.

Carbon source[a] on which cells were grown	Protein concentration (mg/ml)	Substrate added for NADH oxidizing activity	NADH oxidation units x 10^2
NTA	8.3	NTA	5.1
NTA	8.3	IDA	4.2
NTA	8.3	None	0.5
IDA	7.9	NTA	5.0
IDA	7.9	IDA	4.3
IDA	7.9	None	0.3
Glyoxylate-Glycine	7.7	NTA	0.2
Glyoxylate-Glycine	7.7	IDA	0.3
Glyoxylate-Glycine	7.7	None	0.1

[a]All substrates at 0.1% concentration; glyoxylate-glycine was in a 2:1 ratio.

During the initial stages of this study the spectrophotometric assays for NADH oxidation were initiated by the addition of enzyme to the otherwise complete assay mixture. A lag of 1 to 2 min was observed before the NADH oxidation rate reached the maximum linear rate. Preincubation of the enzyme with either FMN, Mn^{++}, NTA, or NADH gave no reduction in the hysteresis, but preincubation of the enzyme with Mn^{++} and NTA together, totally eliminated the observed lag (Figure 4). The necessity for preincubation with Mn^{++} and NTA to obtain initially linear rates was observed for all preparations in the purification sequence.

Values for Km were determined for the required components of the assay mixture using protein which had experienced the complete purification procedure. NADH oxidation rates were determined for different concentrations of each component. Rates and concentrations were plotted in the standard double-reciprocal format and the line through the resulting data points was determined by a least squares fit using linear regression. The double-reciprocal plot for NTA is shown in Figure 5. The resulting kinetic values are shown in Table 4.

Km values similarly determined for NTA and IDA using crude cell-free preparations from NTA and IDA grown cells are given in Table 5.

Several attempts have been made to identify the product(s) that result from the activity of the purified enzyme on the NTA molecule. No products have been found. Incubations set up parallel to or followed directly by spectrophotometric assay of NADH oxidation have shown no glyoxylate or glycolate production

Figure 4. Effect of enzyme preincubation on hysteresis: A) No preincubation B) Enzyme preincubated with Mn^{++} C) Enzyme preincubated with NTA D) Enzyme preincubated with Mn^{++} and NTA.

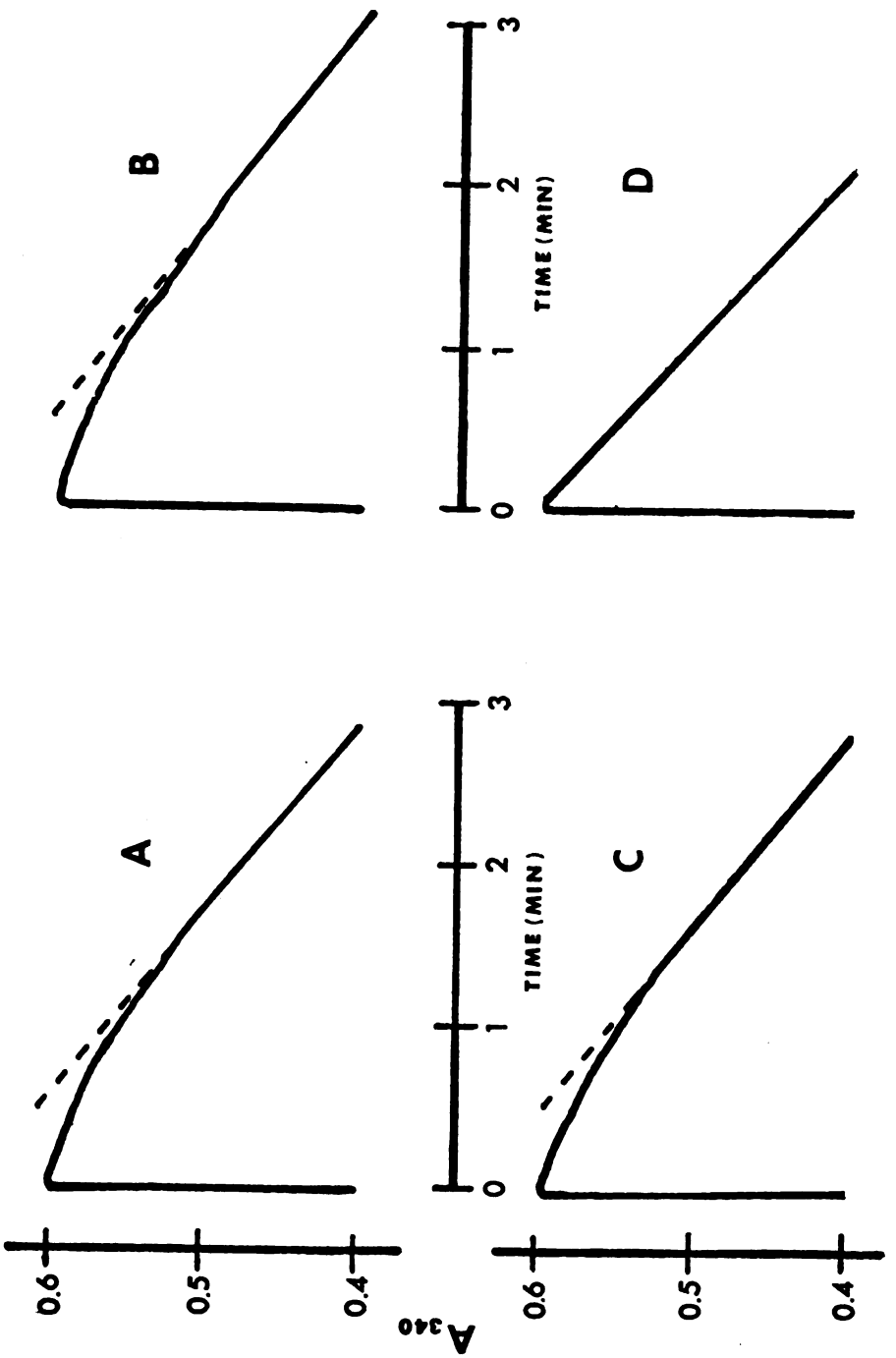

A
B
C
D
0.6
0.5
0.4
A_{340}
0
1
2
3
TIME (MIN)

Figure 5. Double reciprocal plot of NTA-dependent NADH oxidation using protein purified through 2nd Sephadex column.

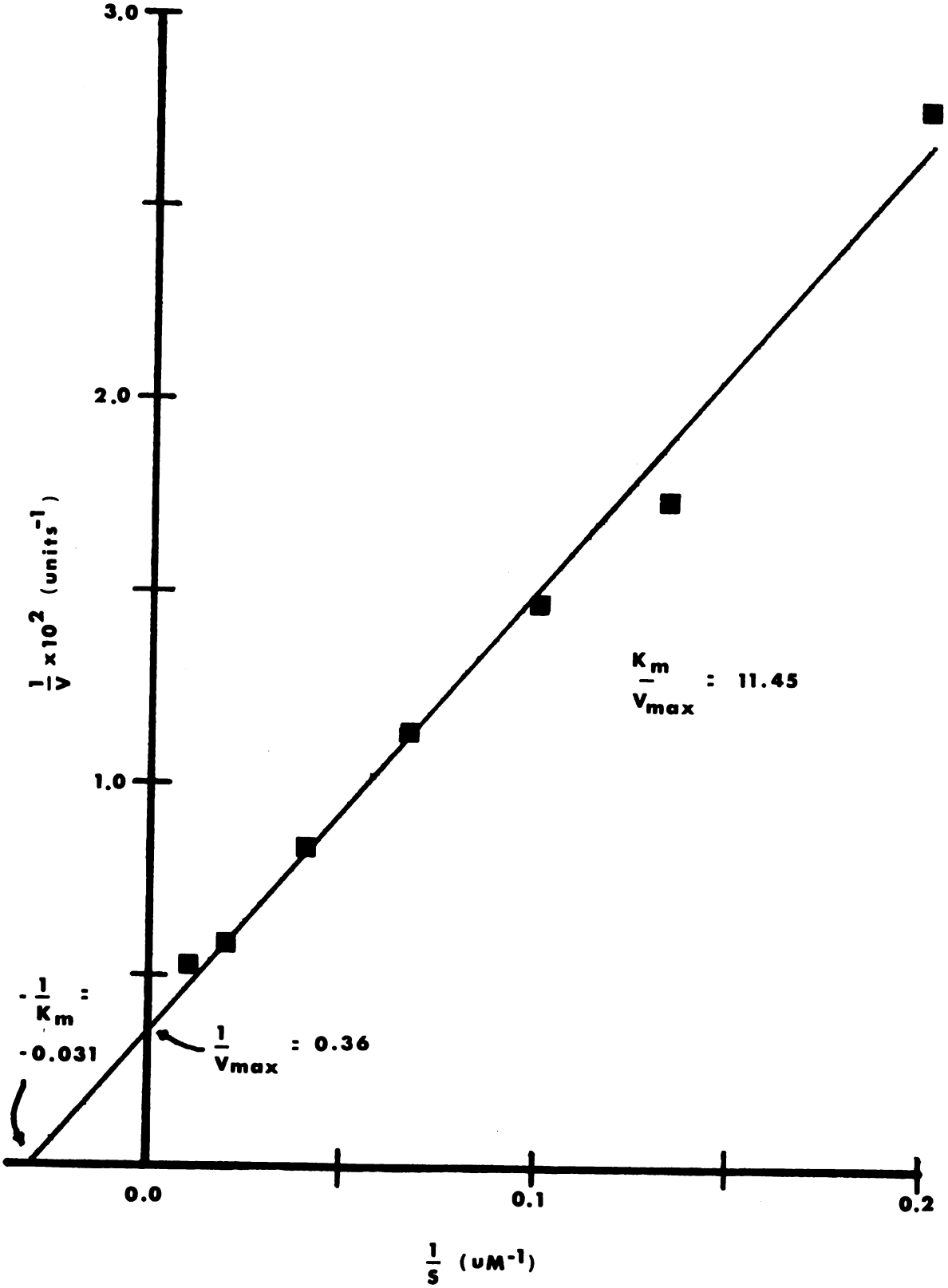

3.0
2.0
1.0
0.0
0.1
0.2
$\frac{1}{V} \times 10^2$ (units^{-1})
$\frac{1}{S}$ (uM^{-1})
$\frac{K_m}{V_{max}}$: 11.45
$-\frac{1}{K_m}$: -0.031
$\frac{1}{V_{max}}$: 0.36

Table 4. Km and Vmax values from purified protein[a].

Substrate	Km (μM)	Vmax (units x 10^2)
NTA	32.0	2.80
IDA	500.	2.36
FMN	0.92	1.84
FAD	3.8	1.62
Mn	29.3	1.92
Fe	no activity	-

[a]The quantity of protein in each assay was 6.6×10^{-2}μg.

Table 5. Km and Vmax values for NTA and IDA determined for crude cell-free preparations from cells grown on NTA or IDA.

Substrate for Growth	Substrate for Km	Km μM	Vmax units x 10^2	Protein μg/assay
NTA	NTA	29.9	5.63	83.
IDA	NTA	27.3	4.41	79.
NTA	IDA	688.	6.96	83.
IDA	IDA	589.	3.86	79.

as determined by colorimetric methods. Analysis of these assay mixtures by two-dimensional thin-layer chromatography and autoradiography resulted in total label accumulation in one spot with an R_f identical to that of NTA. In the TLC system used, IDA, glycine, serine, and glycerate would have been detected if produced in quantities greater than 5% of that predicted by the proposed NTA cleavage reaction. Products of NTA degradation from the crude cell-free preparation, identified by thin-layer chromatography-autoradiography are shown in Figure 11 in the appendix.

When 10 µl (0.2 mg protein) of the cell-free enzyme preparation was included in a ^{14}C-NTA containing assay mixture, the $^{14}CO_2$ produced was equivalent to 29% of the CO_2 expected, assuming the pathway shown in Figure 1. After the first step in purification (DEAE) the capacity to produce ^{14}C-CO_2 from labelled NTA was lost.

NADH oxidation under anaerobic conditions was followed using the standard assay mixture in a Thunberg cuvette. The anaerobic rate of NADH oxidation was 0.79×10^{-2} units while the corresponding aerobic rate was 1.25×10^{-2} units.

To determine the stoichiometric relationship between the quantity of NADH oxidized and the amount of NTA added, NADH oxidation was followed at 340 nm while sequentially adding NADH. In the presence of 0.5 µmoles of NTA, under aerobic conditions, using purified protein, the oxidation of 2.5 µmoles of NADH was recorded before the experiment was terminated. The molar quantity of NADH oxidized was greater than

five times the molar quantity of NTA present.

In an attempt to determine the fate of the electrons leaving NADH, the reduction of FMN to $FMNH_2$ was determined spectrophotometrically at 450 nm, under anaerobic conditions in a Thunberg cuvette. No FMN reduction was observed. During the course of each assay, 0.2 μmoles of NADH was oxidized, enough to theoretically reduce the 0.1 μmole of FMN twice.

Cytochrome c (7.3×10^{-2} μmole) was added to the standard NADH assay mixture and its reduction recorded at 550 nm. The rates of cytochrome c reduction under varying conditions are shown in Table 6. The corresponding rate of NADH oxidation in the standard assay mixture was 0.67×10^{-2} units. Cytochrome c was reduced in the absence of O_2 and in the presence of superoxide dismutase. In the absence of NTA the reduction of cytochrome c did occur but at a substantially reduced rate.

Table 6. Cytochrome c reduction by the purified protein in various assay mixtures.

Conditions	Units of activity x 10^2
Standard assay mixture	1.32
Minus O_2	1.24
Minus NADH	0.0
Minus NTA	0.27
Plus superoxide dismutase	1.32
Assay run for 1 min before cyt c addition	1.32

DISCUSSION

Purification. A 71-fold purification of the enzyme was achieved by the procedure used but in the process 95% of the original activity was lost. The two steps during which the greatest percentage of activity was lost were the two Sephadex procedures during which more than 60% of the remaining activity disappeared.

SDS polyacrylamide gels showed four protein bands of molecular weights: 15,000, 34,000, 56,000 and 68,000 after purification through the first Sephadex column, as well as after the second DEAE column and the second Sephadex column. It is highly unlikely that proteins with a molecular weight range of 15,000 to 68,000 could escape resolution by two Sephadex G-100 columns. The persistence of the four protein bands on SDS gels suggests two possibilities: that the protein is composed of subunits or that the protein is experiencing proteolytic cleavage. It is not probable that the four bands on the gels result from four subunits which are associated in the protein's native conformation. The four proteins do not appear to be present in equal concentrations. The concentration of the proteins relative to each other varies to some extent with varying purification procedures. It is likely that the protein is experiencing proteolysis at some time prior to SDS gels.

This proteolytic cleavage could be occurring early in the procedure with most of the protein fragments remaining associated during the purification as a result of disulfide bonds, H-bonding and hydrophobic attractions. This possibility will be discussed to greater extent in later sections.

Characterization. Growth of cells on IDA or NTA results in induction of the enzyme isolated; but when grown on glyoxylate-glycine, two of the breakdown products of NTA, the cells do not possess the NTA-dependent NADH oxidizing activity. Hence the enzyme of study is induced not constitutive.

The enzyme responsible for the NTA-dependent NADH oxidation does not appear to be a component of the cell membrane. It is shown in Table 7 of the appendix that there is no NTA-dependent NADH oxidizing activity associated with the cell debris removed from freshly broken cells by centrifugation. The enzyme responsible for NTA cleavage may be characterized as soluble in that the particulate proteins removed by centrifugation at 144,000 g possess no NTA degrading activity (Table 8, appendix).

There seems to be no pronounced activation or deactivation of the enzyme isolated by other components present in the crude system. The Km for NTA with purified protein is 32.0 compared to 29.9 with the crude cell-free preparation.

The enzyme is further characterized by data in Figures 8, 9 and 10 of the appendix. NTA-dependent NADH oxidizing activity has a pH optimum at about 7.4; the protein isolated has an isoelectric pH of 4.5. The NTA-dependent NADH oxidizing activity

is inhibited by ionic concentrations above 0.05M NaCl.

It is interesting that a flavin containing cofactor must be added to the assay for the NTA-dependent NADH oxidation to proceed. Flavin components of enzymes are rarely lost in purification procedures because of the high affinity of most flavin containing enzymes for the flavin moiety. It is possible that partial proteolytic fragmentation of the enzyme isolated, is responsible for the loss of the required flavin component. With the purified enzyme, the Km value for FMN (0.92 μM) is lower than the Km for FAD (3.8 μM). This does not indicate that FMN is the flavin bound to and utilized by the enzyme in the whole cell system. FMN, as the smaller molecule, may be reintroduced into the enzyme more readily than FAD.

The lag (hysteresis) observed in NTA-dependent NADH oxidation is eliminated by preincubation of the enzyme with Mn^{++} and NTA. It is possible that a conformational change in the enzyme occurs in the presence of NTA and Mn^{++}. The chelator characteristics of NTA make it likely that the Mn^{++} is involved in the binding of NTA to the enzyme (stability constant, log K_1, of Mn^{++} and NTA is 4.6). Because of the similarity of the Km values for Mn^{++} and NTA (29.9 and 32.0 μM) and their joint requirement for elimination of hysteresis, it can be reasoned that the Mn^{++} and NTA are bound in sequence or as one unit by the enzyme.

Products. To date no NTA products have been found to result from the activity of the purified enzyme. It is surprising to note that while the capacity to produce ^{14}C-labelled CO_2 from labelled NTA is lost in the first step of purification, work done in the early stages of this project (Table 9, appendix) showed that the enzyme preparation, purified through the 1st Sephadex, retains a capacity to produce 14-C-labelled CO_2 from glyoxylate. With certain cofactors present the quantity of ^{14}C-labelled CO_2 produced from labelled glyoxylate exceeds that which could be produced by a glyoxylate carboligase. Furthermore the production of CO_2 from glyoxylate requires the presence of FMN and Mn^{++}.

There are four possible explanations for the fact that no products have been detected from the action of the enzyme with NTA: (i) the enzyme isolated is not involved in NTA metabolism (ii) a product is being formed which cannot be detected by the methods used (iii) during the purification process a component is lost which is required for NTA alteration (iv) proteolytic fragmentation results in an incomplete enzyme incapable of altering NTA.

The induction of the enzyme by NTA and the NTA requirement for NADH oxidation, both indicate that the isolated enzyme is involved in NTA metabolism.

Cleavage of the NTA molecule to form glyoxylate and IDA is not occurring. Other possible intermediates in a two-step cleavage mechanism which have been considered are NTA N-oxide, α-keto NTA and α-hydroxyl NTA. NADH oxidation proceeds in

the absence of O_2 and it is difficult to explain the production of any one of these three compounds in the absence of O_2. The Pseudomonas grows rapidly when NTA N-oxide is the sole carbon source and the resulting cells are induced for NTA cleavage (Table 10, appendix). But this induction could result from NTA present as an impurity in the NTA N-oxide. NTA N-oxide incubated with the crude cell-free preparation and cofactors results in no glycerate production (NTA under same conditions always results in glycerate) but does produce a compound which readily reacts with chromotropic acid to form a highly colored derivative. The identity of this compound has not been established. The α-keto NTA has also been included in crude cell-free incubations. This compound was not metabolized, as indicated by no change in substrate concentration during incubation (Table 11, appendix). The α-hydroxyl NTA is unstable and should spontaneously decompose to NTA and glyoxylate (personal communication C. W. Warren) thus making it an unlikely candidate for a yet undetected product.

It is possible that a component is being lost during purification which is required for NTA alteration. If other avenues of investigation prove fruitless this will be investigated further.

When the other unusual characteristics of this enzyme are included in the consideration, it seems likely that proteolytic fragmentation results in an incomplete enzyme which is incapable of altering the NTA molecule.

The fate of the electrons leaving the NADH remains a mystery. The electron acceptor does not appear to be O_2, as NADH oxidation proceeds in its absence. Quantities of $FMNH_2$ do not build up in the system under anaerobic conditions. Cytochrome c will act as the terminal electron acceptor when added to the system; but the flow of electrons to cytochrome c does not involve superoxide formation. Further investigation of this problem would be interesting, but compared to the other questions remaining to be answered, the fate of the electrons in this possibly fragmented system is of secondary importance.

Summary. A protein has been isolated which when run on SDS gel electrophoresis results in four protein bands. The enzyme is incapable of modifying the substrate (NTA) which induces its synthesis and which it requires for NADH oxidation. The flavin component of the enzyme is lost in purification, an unusual result when dealing with such a tightly bound cofactor. The NTA-dependent NADH oxidizing activity is highly susceptible to inactivation by relatively low ionic strength solutions. The NTA-dependent NADH oxidizing activity proceeds to an unexplainable degree under illogical conditions, implying that the flow of electrons has been short circuited. All of these observations are consistent with an enzyme rendered incompetent by partial proteolytic fragmentation.

Enzymes have previously been isolated, which as a result of proteolytic modification, display incomplete enzymatic

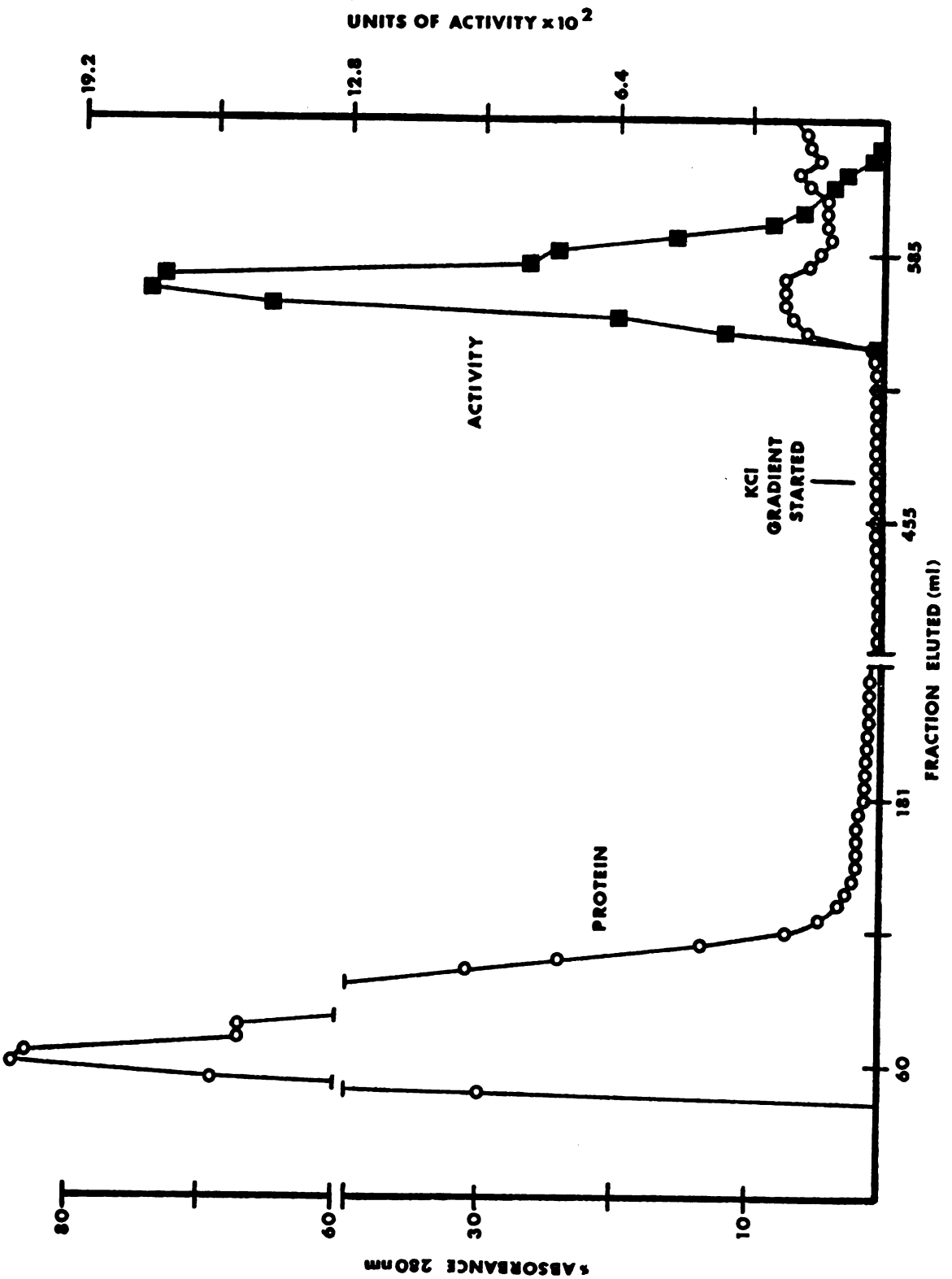
capability and changes in the parameters by which they are characterized (8, 9).

APPENDIX A

APPENDIX A

The data contained in this appendix are referenced in the materials and methods or discussion sections of this thesis. Appendix A contains data concerning the recovery of enzyme activity, conditions for optimum enzyme activity, characterization of the enzyme and products obtained from the enzyme's activity under varying conditions with numerous substrates. The data obtained on enzyme purification were obtained by S. D. Aust. The remainder of the data were from the work of J. M. Tiedje, M. K. Firestone, B. B. Mason of Michigan State University and C. B. Warren of Monsanto Chemical Company.

Figure 6. Protein elution pattern from 1st DEAE cellulose column.


UNITS OF ACTIVITY x 10 2
19.2
12.8
6.4
ACTIVITY
KCl GRADIENT STARTED
PROTEIN
% ABSORBANCE 280 nm
80
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10
60
181
455
585
FRACTION ELUTED (ml)

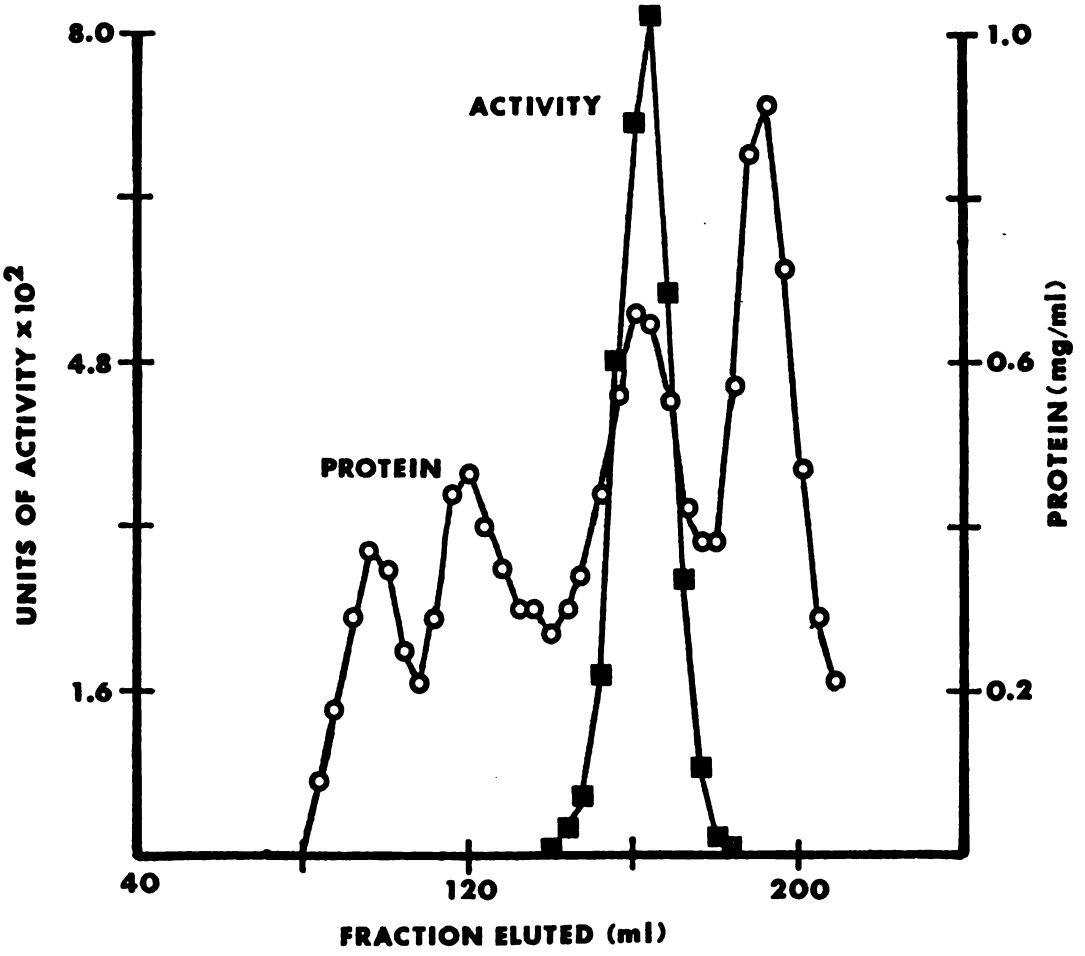


Figure 7. Protein elution pattern from 1st Sephadex gel filtration column.

Table 7. Location of NTA-dependent NADH oxidizing activity in freshly broken cells.

Fraction	Activity (units)
Crude preparation containing cell debris	5.04
Supernatant of crude after 48,000 g centrifugation	3.83
Resuspended pellet after first centrifugation	0.49
2nd supernatant from pellet wash centrifugation 48,000 g	0.49
Resuspended 2nd pellet after wash	0.0

Table 8. Fraction of crude cell-free preparation containing NTA degrading enzymes[a].

Fraction	µmoles O_2 uptake[e]	% ^{14}C label release as CO_2[f]
Crude[b]	2.0	5.0
Soluble[c]	2.2	6.5
Particulate[d]	0.0	0.6

[a]Determined by substrate-dependent O_2 uptake and ^{14}C-labelled CO_2 production from labelled NTA.

[b]Crude protein was prepared by removing cell debris by centrifugation at 20,000 g.

[c]Soluble protein was that remaining in the supernatant after centrifugation of the crude at 144,000 g.

[d]Particulate protein was the resuspended pellet from 144,000 g centrifugation.

[e]6 µmoles NTA and 6 µmoles NADH incubated with the protein fraction for 10 min.

[f]After 60 min.

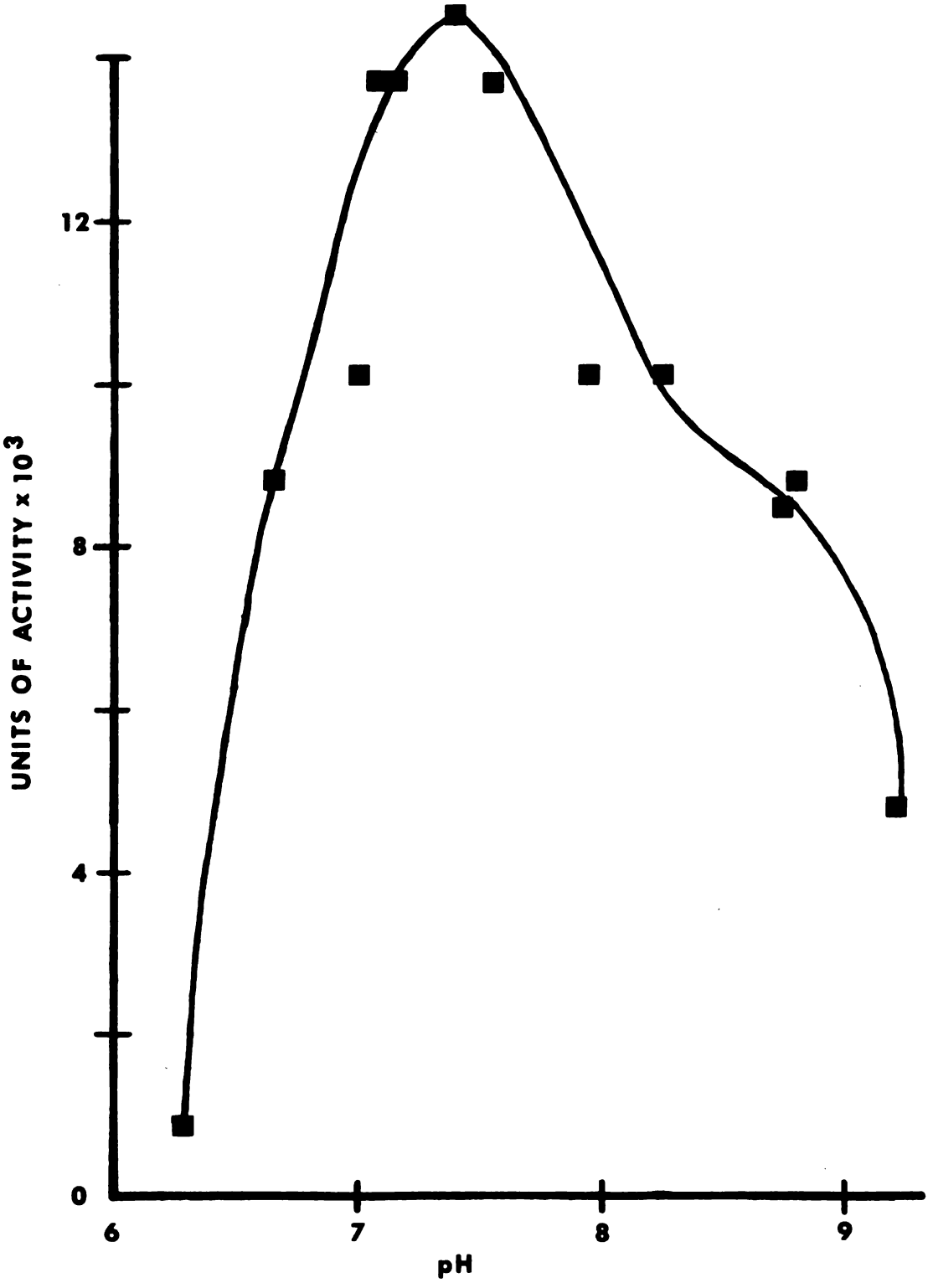
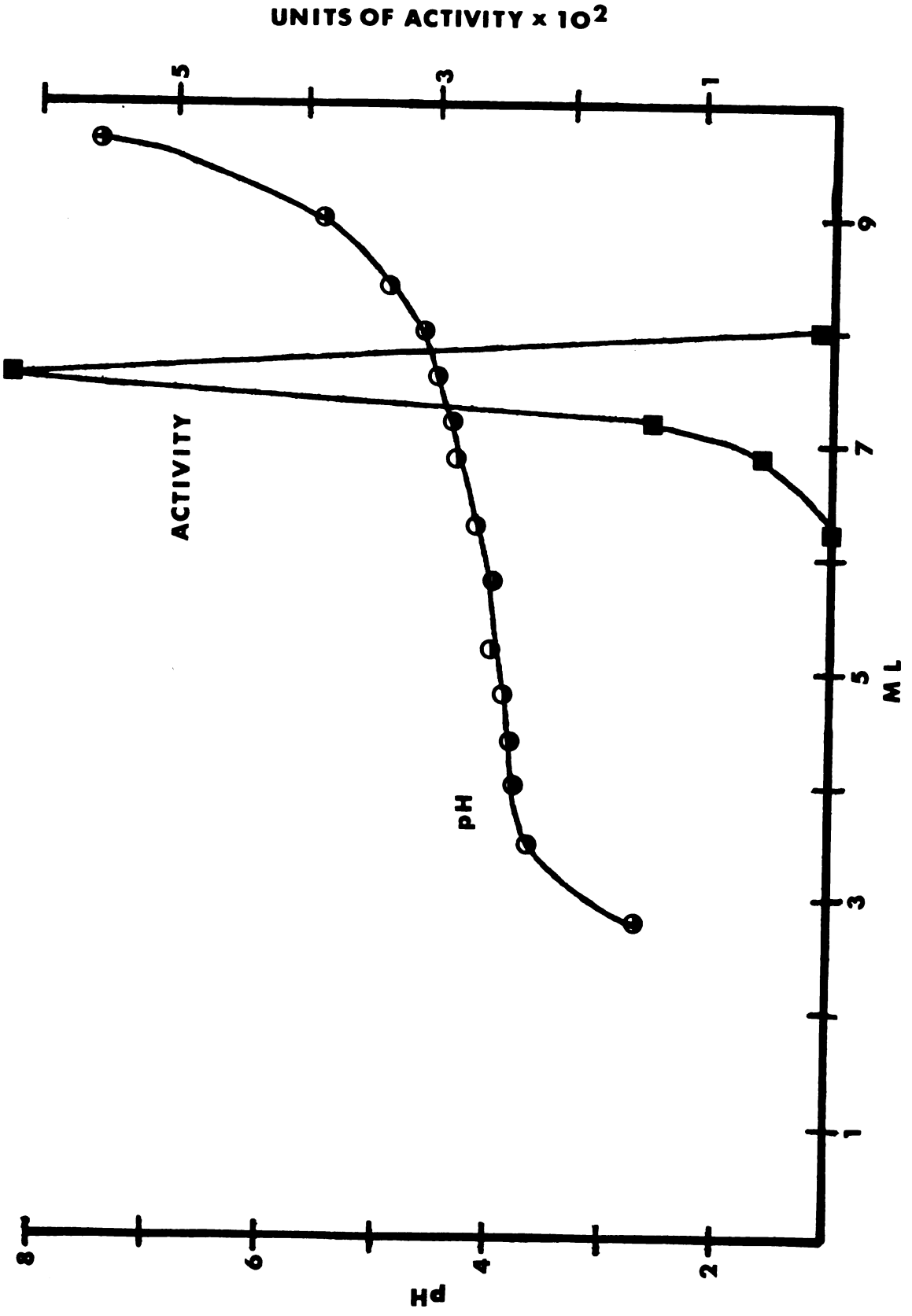


Figure 8. pH optimum for NTA-dependent NADH oxidizing activity.

Figure 9. Microelectrofocusing of protein in a 10 ml sucrose, stabilizing density gradient. Procedure given in Ph.D. thesis, Albert Chou, Dept. Biochemistry, M.S.U., 1973.


UNITS OF ACTIVITY x 10²
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3
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9
7
5
3
1
ML
ACTIVITY
pH
8
6
4
2
Hd

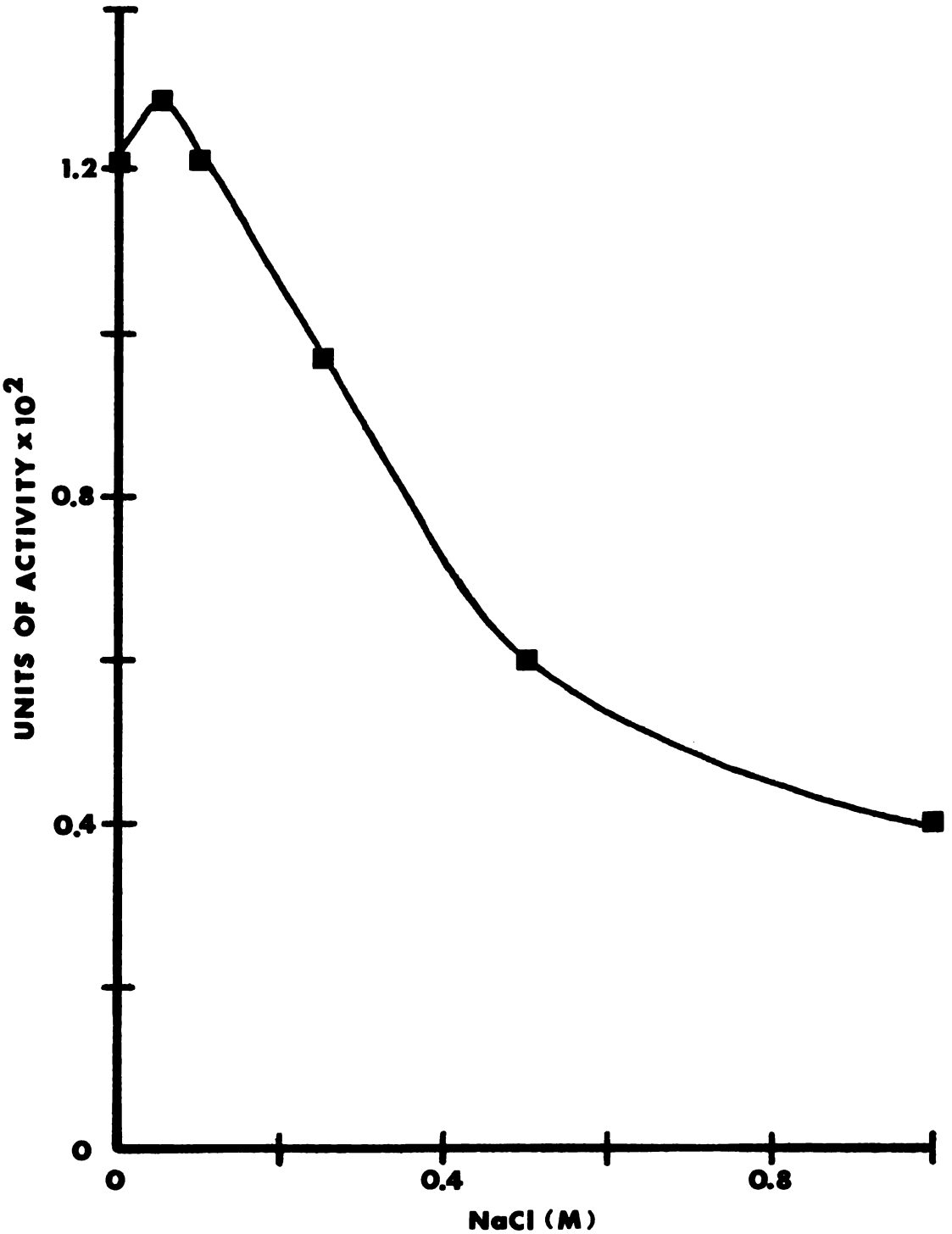

Figure 10. Effect of ionic strength on NTA-dependent NADH oxidizing activity.

Table 9. Products of enzyme purified through 1st Sephadex gel filtration.

Substrate	Cofactors added[b]	Glyoxylate[c] µmoles	% ^{14}C-label as CO_2
[a]NTA	NADH, FMN, Mn^{++}	0.0	0.04
Glyoxylate	NADH, FMN, Mn^{++}	0.67	22
Glyoxylate	NADH, Mn^{++}	1.13	6.6
Glyoxylate	NADH, FMN, Mn^{++} (no enzyme)	1.18	1.9
[d]NTA	NADH, FMN, Mn^{++}	0.0	--
IDA	NADH, FMN, Mn^{++}	0.0	--
Glyoxylate	NADH, FMN, Mn^{++}	0.09	71.7
Glyoxylate	NADH	1.71	29.2

[a] 1.2 µmoles substrate present.

[b] Cofactor quantities: 1.2 µmole NADH; 0.01 µmole FMN; 0.1 µmole $MnCl_2$

[c] Glyoxylate present at the end of 1 hr.

[d] 3.0 µmoles substrate present. Cofactor quantities: 12 µmoles NADH; 0.03 µmole FMN; 0.5 µmole Mn^{++}.

Table 10. Metabolic products from crude cell-free preparation from cells grown on NTA N-oxide.

Substrate 6 μmoles	% ^{14}C-label as CO_2	% Glycerate produced[a]
NTA	11.8%	97%
Glyoxylate	22.3%	82%
O-NTA	--	0%

[a]Percent of glycerate possible assuming the pathway shown in Figure 1.

Table 11. Range of substrates degraded by crude cell-free preparation.

Substrate[b]	Cofactor	Analysis		
		Disappearance[a]	% $^{14}CO_2$[c]	O_2 uptake
NTA	NADH	+	32%	+
Acetate	0	-	0.5%	0
Glyoxylate	0	+	24%	0
Glycolate	NADH	-	-	0
IDA	NADH	0%	0.4%	0
α-Keto NTA	NADH	5%	-	-
Sarcosine	NADH	0%	-	-
N-Methyl-IDA	NADH	13%	-	-
NTP	NADH	0%	-	-

[a]Substrate disappearance determined by gas chromatographic analysis, courtesy of C. B. Warren.

[b]6 μmoles of substrate and cofactor when added.

[c]% of that possible, if proceeding according to the pathway proposed.

APPENDIX B

APPENDIX B

The data contained in this appendix are a major portion of the work done by J. M. Tiedje, M. K. Firestone and B. B. Mason which resulted in the proposal of the NTA degradation pathway shown in Figure 1. The conclusions from some of this work have been presented in an abstract (15).

Table 12. Oxygen and NADH requirements for NTA disappearance.

NADH[a]	O_2	Incubation (hours)	NTA disappearance[c] (μmoles)
+	+	0	0.0
+	+	3	2.0
-	+	0	0.0
-	+	3	0.0
+	-[b]	3	0.0

[a] 2 μmoles NTA and NADH added.

[b] Run in Thunberg tube.

[c] Determined by zinc-Zincon analysis.

Table 13. Cofactor requirement for NTA cleavage.

Time (hours)	Cofactor (12 µmoles)	NTA disappearance (µmoles)	% label released as CO_2	O_2 uptake
[a]0	NAPH	1.0	0.0	-
0	NAD^+	0.3	0.0	-
1	NADH	4.7	8.9	yes
1	NAD^+	1.2	0.8	no
[b]0	NADH	0.0	-	-
0	NADPH	0.0	-	-
5	NADH	5.8	-	yes
5	NADPH	5.1	-	yes

[a]Samples incubated with 11.6 mg of soluble protein.

[b]Samples incubated with 4.8 mg of crude protein.

Table 14. Metabolic products of crude cell-free preparation[a].

Substrate (6 µmoles)	Cofactors added (6 µmoles)	Glyoxylate (µmoles)	Glycerate (µmoles)
Glyoxylate	NADH	0.81	2.2
Glyoxylate	--	0.72	0.5
Glycerate	NAD^+	0.00	4.5
Glycine	0,*	0.00	0.0
Serine	0,**	0.00	0.0

[a]Determined by glyoxylate and glycerate colorimetric analysis after an incubation of 60 min. 10.9 mg protein/sample.

*NAD^+, FAD, α-ketoglutarate, pyruvate.

**NADH, THF, α-ketoglutarate, pyruvate.

Table 15. Metabolic products of crude cell-free preparation[a].

Substrate (6 µmoles)	Cofactors (6 µmoles)	Glycine (µmoles)	Glyoxylate (µmoles)	Glycerate (µmoles)	Serine (µmoles)	% of label[b] as $^{14}CO_2$
IDA	NADH	0.00	0.00	0.00	0.00	0.1
Glyoxylate	NADH	0.23	0.59	2.25	0.00	41.9
Glyoxylate	--	0.14	0.32	0.10	0.00	65.2
Serine	NADH, THF	0.27	0.00	0.00	1.80	0.5
Serine	0,*	0.00	0.00	0.00	3.10	2.5
Glycine	0,**	3.4	0.00	0.00	0.00	1.0

[a]Determined by ^{14}C label on thin layer chromatography after an incubation of 60 min. 10.9 mg protein/sample.

[b]% of total label.

*α-ketoglutarate and pyruvate.

**NAD^+, FAD, α-ketoglutarate and pyruvate.

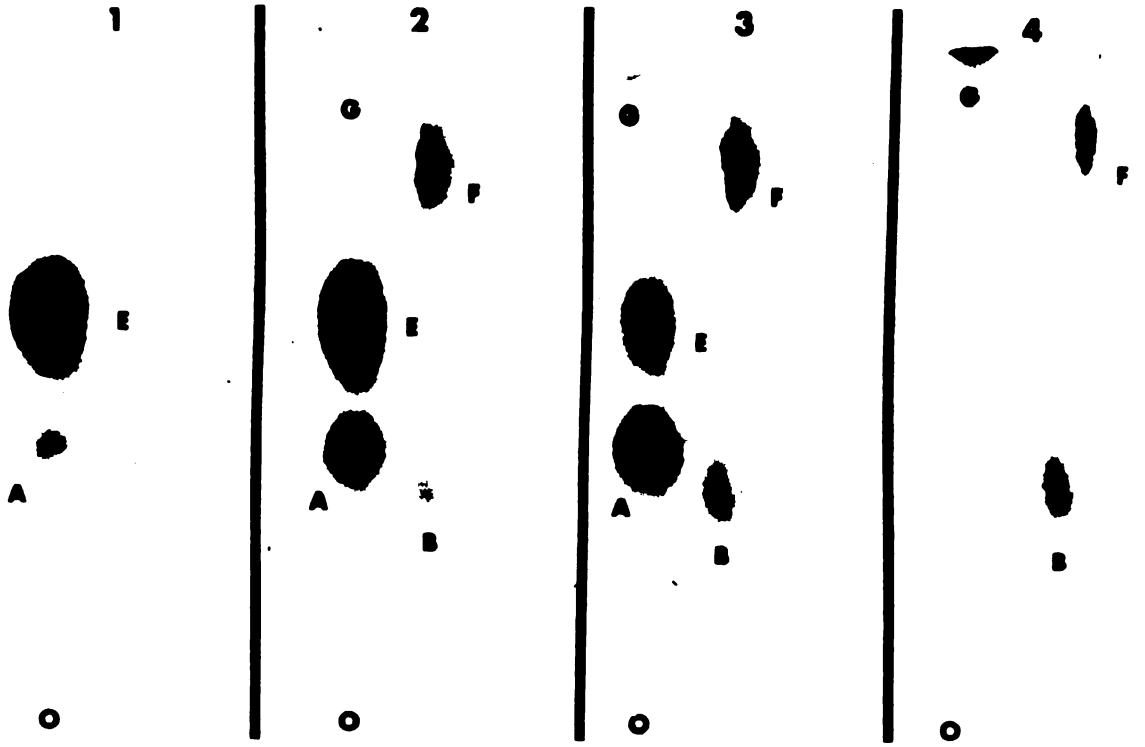

Figure 11. Thin layer chromatography and autoradiography to identify breakdown products from NTA incubated with crude cell-free preparation and cofactors. 1) 0 time *NTA 2) 10 min *NTA 3) 60 min *NTA 4) 60 min *glyoxylate. The identities of the spots are as follows: A - IDA; B - glycine; E - NTA; F - glycerate; G - glyoxylate; O - origin.

Table 16. Characterization of substrate metabolism of crude cell-free preparations from cells grown on NTA and IDA.

Cells grown on	IDA	NTA
% ^{14}C released as CO_2 from labelled NTA[a,b]	7.7	6.5
µmoles O_2 uptake on NTA[a,b]	3.1	2.8
µmoles O_2 uptake on IDA[a,b]	0.0	0.0
% ^{14}C released as CO_2 from glyoxylate[a]	61.	38.
µmoles O_2 uptake on glyoxylate[a]	0.0	0.0
Metabolic products[c] from NTA[a,d] (µmoles)	0.90 NTA 3.42 IDA 0.96 Glycerate 1.44 Glycine	1.56 NTA 2.97 IDA 1.22 Glycerate 0.09 Glycine

[a] 6 µmoles substrate run for 10 min.

[b] 6 µmoles NADH added.

[c] Products determined by label position on TLC.

[d] 12 µmoles NADH added for NTA grown, 6 µmoles NADH for IDA grown.

Table 17. Stoichiometry of NTA cleavage.

Time min	NTA degraded[a] (µmoles)	O_2 uptake (µmoles)	O_2/NTA	% CO_2[b]	% Glycerate[b]
4	2.41	0.56	0.23	26	26
8	2.98	1.55	0.52	36	35
12	4.20	2.77	0.66	62	44
16	5.42	4.22	0.73	81	62
20	5.42	4.58	0.85	81	64

[a] 6 µmoles NTA, 12 µmoles NADH and 7.3 mg soluble protein.

[b] % of possible, if proceeding according to the pathway proposed.

LIST OF REFERENCES

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1. Boulton, C. A., J. C. Crabbe and P. L. Large. 1974. Microbial oxidation of amines: Partial purification of a trimethylamine mono-oxygenase from Pseudomonas aminovorans and its role in growth on trimethylamine. Biochem. J. 140:253-263.

2. Colby, J. and L. J. Zatman. 1971. The purification and properties of a bacterial trimethylamine dehydrogenase. Biochem. J. 121:9P-10P.

3. Colby, J. and L. J. Zatman. 1973. Trimethylamine metabolism in obligate and facultative methyltrophs. Biochem. J. 132:101-112.

4. Cripps, R. E. and A. S. Noble. 1973. The metabolism of nitrilotriacetate by a pseudomonad. Biochem. J. 132: 1059-1068.

5. Fairbanks, G., T. L. Steck and D. F. H. Wallach. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. Biochem. 10:2606-2617.

6. Focht, D. D. and H. A. Jospeh. 1971. Bacterial degradation of nitrilotriacetic acid (NTA). Can. J. Microbiol. 17:1553-1556.

7. Forsberg, C. and G. Lindquist. 1967. Experimental studies on bacterial degradation of nitrilotriacetate, NTA. Vatten. 23:265-277.

8. Juni, E. and G. A. Heym. 1968. Properties of yeast pyruvate decarboxylase and their modification by proteolytic enzymes. I. Stability of decarboxylases from wild-type and mutant strains. Arch. Biochim. Biophys. 127: 79-88.

9. Juni, E. and G. A. Heym. 1968. Properties of yeast pyruvate decarboxylase and their modification by proteolytic enzymes. II. Selective alteration by yeast proteases. Arch. Biochim. Biophys. 127:89-100.

10. Large, P. L. 1971. The oxidative cleavage of alkyl-nitrogen bonds in micro-organisms. Xenobiotica. 1:457-467.

11. Large, P. L., C. A. Boulton and M. J. C. Crabbe. 1972. The reduced nicotinamide-adenine dinucleotide phosphate- and oxygen-dependent N-oxidation of trimethylamine by Pseudomonas aminovorans. Biochem. J. 128:137P-138P.

12. Massey. V. 1959. The microestimation of succinate and the extinction coefficient of cytochrome c. Biochim. Biophys. Acta. 34:255-256.

13. Mejbaum-Katzenellenbogen, W. and Dobryszycka. 1959. New method for the quantitative determination of serum proteins separated by paper electrophoresis. Clinica Chemica Acta. 1:515-522.

14. Thompson, J. E. and J. R. Duthie. 1968. The biodegradability and treatability of NTA. J. Water Pollut. Contr. Fed. 40:306-319.

15. Tiedje, J. M., M. K. Firestone, B. B. Mason and C. B. Warren. 1973. Abstr. Annu. Meet. Am. Soc. Microbiol. P183. p. 171.

16. Tiedje, J. M., B. B. Mason, C. B. Warren and E. J. Malec. 1973. Metabolism of nitrilotriacetate by cells of Pseudomonas species. Appl. Microbiol. 25:811-818.

17. Trijbels, F. and G. D. Vogels. 1966. Degradation of allantoin by Pseudomonas acidovorans. Biochim. Biophys. Acta. 113:292-301.

18. Welton, A. F. and S. D. Aust. 1974. The effects of 3-methylcholanthrene and phenobarbital induction on the structure of the rat liver endoplasmic reticulum. Biochim. Biophys. Acta. 373:197-210.