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CHARACTERIZATION OF THE COMPONENT ENZYMES OF THE TNT DETECTION --ETC(U)
JUN 79 K DONOVAN , T GAWRONSKI , B SCOTT DAAK70-77-C-0129

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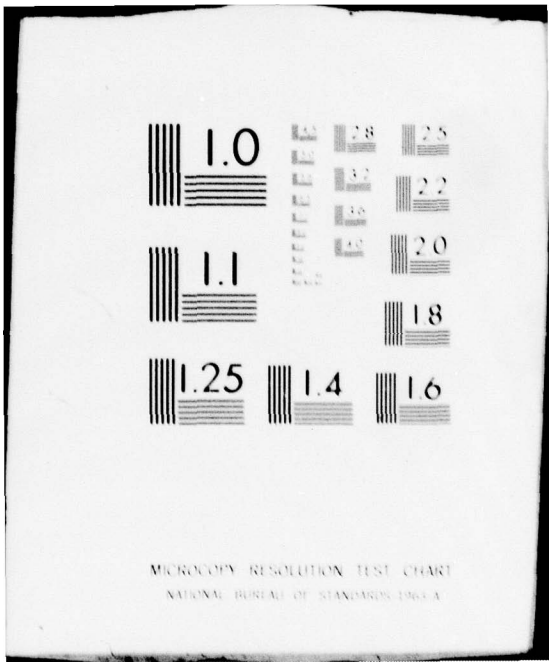
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CHARACTERIZATION OF THE COMPONENT ENZYMES OF THE TNT DETECTION SYSTEM- PHYSICAL AND CHEMICAL STUDIES

9 FINAL TECHNICAL REPORT,

BY

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U.S. Army Mobility Equipment
Research and Development Center

Beckman Instruments, Inc.
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1.0 INTRODUCTION

As noted in the contract proposal, more than 90 percent of the initial NADH in the TNT Reductase reagent is consumed in a reaction which is independent of TNT. This high rate of non-specific NADH oxidation, attributed to NADH Oxidase, results in less reproducible baseline NADH levels. Further, since the level of NADH Oxidase in the reagent varies directly with the level of TNT Reductase, the contaminant imposes a limit on the level of TNT Reductase in the reagent. The contaminant does have one advantage since it permits use of higher initial NADH concentrations which promote more rapid reaction rates for TNT reduction. However, these rapid reaction rates could also be attained by increasing the TNT Reductase concentration if the NADH Oxidase were eliminated.

Efforts have been made to reduce the level of NADH Oxidase in TNT Reductase preparations. The methods used have included further use of cellulose-based ion exchange chromatography as well as gel permeation chromatography. Those efforts were unsuccessful in reducing NADH Oxidase activity, as measured at saturating substrate concentration, to levels below the 0.1% usually obtained in the purification.

Although the purification methods employed have not exhausted the possibilities, it seemed clear that a more fundamental, rigorous approach to the problem should be taken. The identity of the non-specific NADH oxidizing activity has not been established, and the designation "NADH Oxidase" is one of convenience only. It seemed possible that NADH oxidation is an activity of TNT Reductase itself, possibly due to side-reaction of enzyme-NADH complexes. Before making further attempts at large-scale purification of TNT Reductase, it was necessary to explore this possibility.

There are two general methods of approaching this question. Powerful enzyme purification techniques may be used in attempts to separate the two activities. Alternatively, the identity of the enzyme activities may be explored by indirect methods. For example, the preparation may be subjected to partially denaturing conditions. Differential loss of the two activities is indirect evidence for non-identity of the two enzymes.

Thus far, much of the data indicates that the two enzyme activities may reside on a single macromolecular species. However, this coincidence of both activities on a single protein has not been shown directly.

2.0 INVESTIGATION

2.1 General

This section contains brief statements of all work accomplished during the contract period, together with the results obtained. A detailed discussion of the operational and technical problems encountered and the results achieved is presented in Section 3.0.

The work presented in this report deals primarily with the physical characterization and attempted separation of TNT Reductase and NADH Oxidase activities.

2.2 Attempted Physical Separation of TNTase and NADH Oxidase Activities

2.2.1 Isoelectric Focusing - Separation by Charge

TNTase preparations have been subjected to isoelectric focusing in sucrose density gradients. Separation by charge of TNT Reductase and NADH Oxidase has not been accomplished as both enzymes have apparent isoelectric points of 4.5 $\pm$ .05. Unfortunately, much of the TNTase activity is rapidly and irreversibly lost at this pH due to denaturation of the protein. Addition of TNT to the system did not protect the enzyme.

2.2.2 Gel Filtration - Separation by Size

Gel filtration, using Sephadex G-150 (Pharmacia Fine Chemicals, Piscataway, N.J.), was used to investigate the relative molecular weights of TNTase and NADH Oxidase. The peaks of both enzyme activities were in the same tubes, indicating that the hydrodynamic size of the molecular species with TNTase activity is very similar to that of the molecular species with NADH Oxidase activity.

2.2.3 DEAE-Cellulose Chromatography - Separation by Charge

In the course of enzymatic purification, some interesting data involving DEAE-cellulose column chromatography was accumulated. Peak TNT Reductase activity is eluted prior to elution of peak NADH Oxidase activity. NADH Oxidase is eluted at a slightly higher salt concentration.

Exposure of TNTase fractions with the lowest levels of NADH Oxidase contamination to 3 successive DE-52 Diethylaminoethyl Cellulose anion exchange columns produced a recurrent value of approximately 0.10% contamination.

2.2.4 Sepharose - Active Site Recognition

Work utilizing Pharmacia Blue Sepharose^{R} CL-6B affinity chromatography was undertaken in an attempt to separate NADH Oxidase from TNT Recutase. It appears either that the Blue Sepharose environment was unfavorable and that the enzymatic activities were in fact destroyed by exposure to the Blue Sepharose matrix or that the enzymes did bind to the matrix but were not eluted by the substrates used. Neither NaCl, NADH nor TNT appeared to displace the enzyme(s) such that the original level of pre-column enzymatic activity was recovered.

2.2.5 Polyacrylamide Gel Electrophoresis

A more sensitive NADH Oxidase assay system utilizing the Beckman Luminescent Photometer was developed for the purpose of detecting low levels of NADH Oxidase activity following subjection of the enzyme to polyacrylamide gel electrophoresis. The luminescent system utilizes 10^{-7} M NADH whereas the spectrophotometric system uses 10^{-4} M NADH. The luminescent system appears to be at least 35 times more sensitive in the detection of NADH Oxidase activity than the spectrophotometric system.

Approximately 6.6% of the original TNT Reductase and 1.7% of the original NADH Oxidase activity loaded on each respective polyacrylamide gel was detectable after exposure of the enzyme(s) to electrophoresis. The fastest migrating band in the polyacrylamide gel appears to contain the TNT Reductase units. The NADH Oxidase activity is located in the same region of the gel where the highest concentration of TNT Reductase activity is also located. This indicates that it is not possible to separate TNT Reductase and NADH Oxidase activities using the polyacrylamide gel electrophoresis technique of separation.

2.3 Attempted Identification of the Electron Acceptor in the NADH Oxidase Reaction

2.3.1 Elimination of Oxygen

There was no reduction in NADH Oxidase activity following the removal of dissolved molecular oxygen from solution, possibly indicating that the enzyme does not use molecular oxygen as hydrogen acceptor

2.3.2 Addition of Putative Inhibitors (CN^-, N_3^-)

NAD^+, sodium cyanide and sodium azide were examined as potential inhibitors of TNT Reductase and/or NADH Oxidase in an attempt to further characterize the physical properties of TNT Reductase and NADH Oxidase. It appears that none of these compounds substantially inhibit the activity of either TNT Reductase or NADH Oxidase. It appears that NADH Oxidase does not use molecular oxygen as hydrogen acceptor.

2.3.3 Use of Radioactive Tracer

Tritium labelled NADH(T) was prepared by an enzymatic procedure and added to a TNTase-NADH Oxidase preparation. After essentially complete reaction of NADH(T), only 50% of the label appeared in water. Approximately 25% of the label was retained in a nucleotide fraction with molecular size and spectrum characteristic of NAD^+, while the remainder of the label appeared in a lower molecular fraction which also had a characterisitic nucleotide spectrum. Interpretation of these data is somewhat difficult, but it appears that the NADH Oxidase reaction is not a simple electron transfer to molecular oxygen.

2.4 Effect of NADH and NADH Analogs on TNTase and NADH Oxidase Activities

2.4.1 Kinetics of TNTase Destruction in NADH

Kinetic methods were used to examine the relationship of TNT Reductase activity to NADH Oxidase activity. NADH Oxidase activity is stable in the presence of NADH, while TNT Reductase activity is rapidly lost. In addition, previous data concerning the concentration dependence of NADH-induced TNTase inhibition, were confirmed. The kinetics of TNTase destruction in 10^{-4}, 10^{-6}, and 10^{-7} M NADH show that the rate of NADH-induced inhibition of TNTase depends on the 0.07 power of the NADH concentration. This would appear to indicate that this process is an indirect one. It appears that TNT does protect TNT Reductase from NADH-induced inhibition, probably by binding at the active site.

2.4.2 Kinetics of TNTase Inactivation in 10^{-4} M NADH Analogs

The NADH analogs (α-NADH, Nicotinamide Hypoxanthine Dinucleotide, 3-Acetyl-pyridine-DPNH) were utilized in an attempt to further characterize the physical properties of TNT Reductase and NADH Oxidase. It was found that the NADH analogs used affect the activities of TNTase and NADH Oxidase in a similar manner, except that α-NADH is apparently not a TNTase substrate. The rate of TNTase inactivation in 10^{-4} M NADH analogs appears to be somewhat more rapid than in 10^{-4} M NADH.

It also appears that α-NADH is not a substrate for TNT Reductase. However, α-NADH does inactivate TNT Reductase activity. The rate of inactivation of TNT Reductase by 10^{-4} M α-NADH under pre-incubation conditions appears to be the same rate as that found for 10^{-4} M β-NADH. In this study, the $t_{\frac{1}{2}}$ for TNTase inactivation by both α- and β-NADH was approximately 70 seconds.

2.4.3 Kinetics of TNTase Inactivation in NADPH

The effects of substituting NADPH for NADH on the activities of TNTase and NADH Oxidase were determined. Both activities were increased in similar proportion.

2.4.4 Effect of Additives on Inactivation of TNTase

Addition of catalase, which destroys hydrogen peroxide, to NADH preincubations did not protect TNTase activity nor did N-methyl-L-tryptophan, which can react rapidly with highly reactive forms of oxygen which might be produced in this system.

2.4.5 Effect of Preincubation in 10^{-7} M Hydrogen Peroxide on TNTase Activity

Preincubation of enzyme preparations with hydrogen peroxide, a possible product of NADH oxidation, does not inhibit TNTase activity.

2.5 Effects of Group Specific Reactive Compounds on TNTase and NADH Oxidase

N-ethylmaleide (NEM), p-chloromercuribenzoate (PMB), phenylmethyl sulfonyl-fluoride (PMSF), acetic anhydride, iodoacetic acid, 3-bromo pyruvic acid and N-bromosuccinimide (NBS) were used as group specific reagents in an attempt to physically characterize the structure of the enzymatic active site(s).

NEM, PMB, PMSF, iodoacetic acid and 3-bromopyruvic acid did not appear to affect either TNT Reductase or NADH Oxidase activities. This possibly suggests that neither free sulfhydryl groups nor serine residues exist in the enzymatic active site(s).

Acetic anhydride inhibited both TNT Reductase and NADH Oxidase activity by approximately 50%. This suggests that lysine and/or tyrosine residues may contribute to the enzymatic activities.

N-bromosuccinimide inhibits both TNT Reductase and NADH Oxidase activity proportionately depending on the concentration of NBS used. This indicates that possibly a tryptophan, tyrosyl and/or histidyl residue contribute(s) to enzymatic activity in the active site(s).

2.6 Attempts to Differentially Denature TNTase and NADH Oxidase Using Physical Methods

2.6.1 Storage Effect

TNTase is quite stable when subjected to prolonged frozen storage, while a significant fraction of the NADH Oxidase activity is lost. After approximately one year of frozen storage, the TNTase lost 8.0% of its original activity whereas the NADH Oxidase lost 34.8% of its original activity.

2.6.2 Heat

Thermal denaturation experiments indicate that loss of TNTase activity, produced by storage at elevated temperatures, is paralleled closely by loss of NADH Oxidase activity. The correlation coefficient, obtained from a linear regression analysis, was 0.714.

2.7 Luciferase Glow Effect: Removal of DTE

Removal of dithioerythritol (DTE) by dialysis from the Luciferase reagent does not appear to eliminate the Luciferase glow effect.

3.0 DISCUSSION

3.1 Attempted Physical Separation of TNTase and NADH Oxidase Activities

3.1.1 Isoelectric Focusing - Separation by Charge

Isoelectric focusing was performed in sucrose density gradients using an LKB 8100 ampholine electrofocusing apparatus. The system was cooled using a recirculating refrigerated bath. System temperature was maintained at approximately 4°C. Power input was 4-6 watts for all runs. pH ranges tested were 3.5-10, 4-6 and 5-8. Determination of pH of respective column fractions collected was made using a Beckman Zeromatic pH meter equipped with a combination electrode. Where appropriate, fractions were concentrated using Amicon Minicon-B clinical sample concentrators and assayed for TNT Reductase and NADH Oxidase activity.

Separation by charge of TNT Reductase and NADH Oxidase was not accomplished using the isoelectric focusing technique as both enzymes have isoelectric points of 4.5 $\pm$ .05. Unfortunately, at this pH, much of the TNTase activity is rapidly and irreversibly lost due to denaturation of the protein. Addition of TNT to the system did not protect the enzyme.

3.1.2 Gel Filtration - Separation by Size

Gel filtration, using Sephadex G-150 (Pharmacia Fine Chemicals, Piscataway, N.J.), was used to investigate the relative molecular weights of TNTase and NADH Oxidase. A Sephadex G-150 column (2.5 x 29 cm) was prepared according to the maufacturer's directions. A 6.0 ml sample of TNTase was loaded on the column and eluted with 0.05 M potassium phosphate buffer, pH 7.0. The collected fractions were concentrated using Amicon Type CF25 Centriflo Membrane Cones (Amicon Corporation, Lexington, Mass.) and assayed spectrophotometrically for respective TNT Reductase and NADH Oxidase activities. The data are shown graphically in Figure 3-1. The peaks of both enzyme activities were in the same tubes, indicating that the hydrodynamic size of the molecular species with TNTase activity is very similar to that of the molecular species with NADH Oxidase activity.

3.1.3 DEAE-Cellulose Chromatography - Separation by Charge

A DE-52 Diethylaminoethyl Cellulose anion exchange column (1.0 x 16 cm) was prepared using 1 g DE-52 resin/15 mg total protein. A linear gradient of 0-0.4 M KCL in .05 M potassium phosphate buffer, pH 7.3, was established. The column was run in the cold (4°C). A total of 82 fractions were collected and assayed for TNT Reductase and NADH Oxidase activities. The data are shown graphically in Figure 3-2.

Peak TNT Reductase activity appears to elute from DE-52 at a slightly lower salt concentration than peak NADH Oxidase activity. Peak TNT Reductase activity eluted with approximately 0.12 M KCl whereas peak NADH Oxidase activity eluted with approximately 0.14 M KCl.

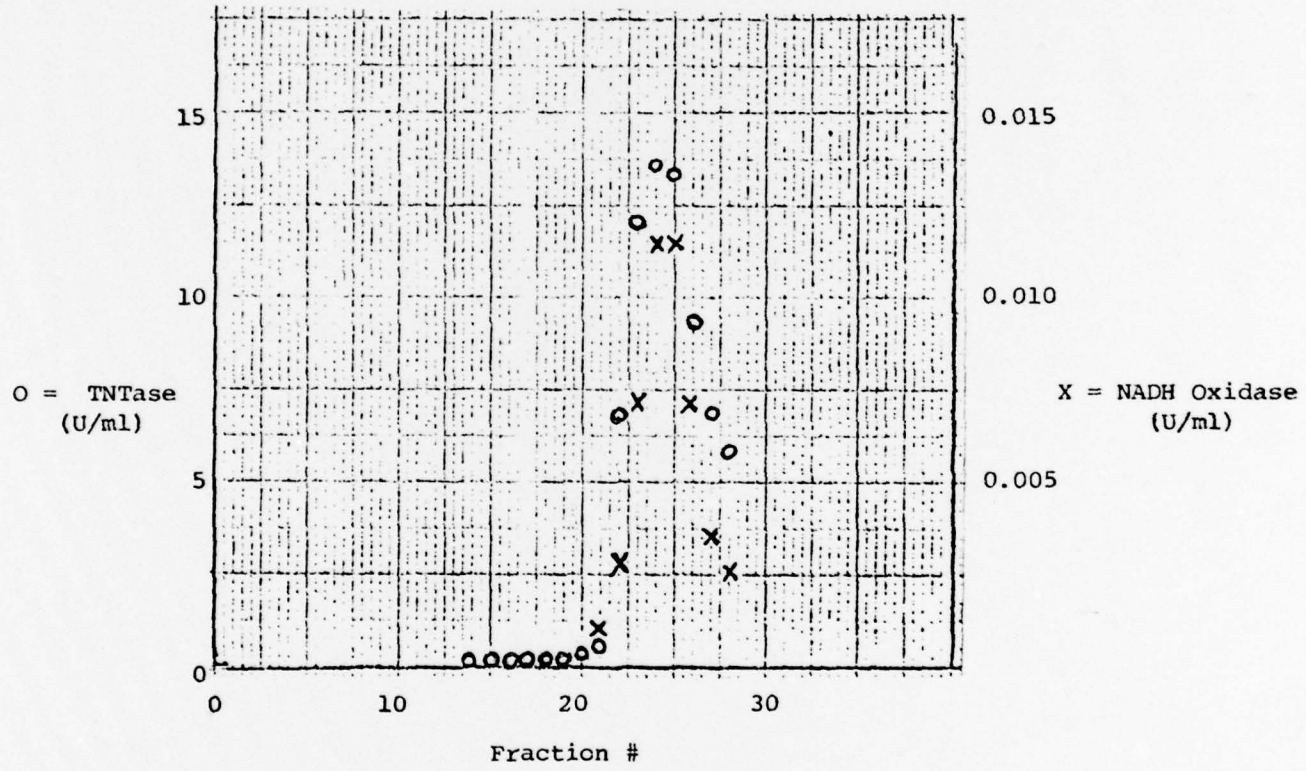

FIGURE 3-1: Elution of TNT Reductase and NADH Oxidase Activities from Sephadex G-150.

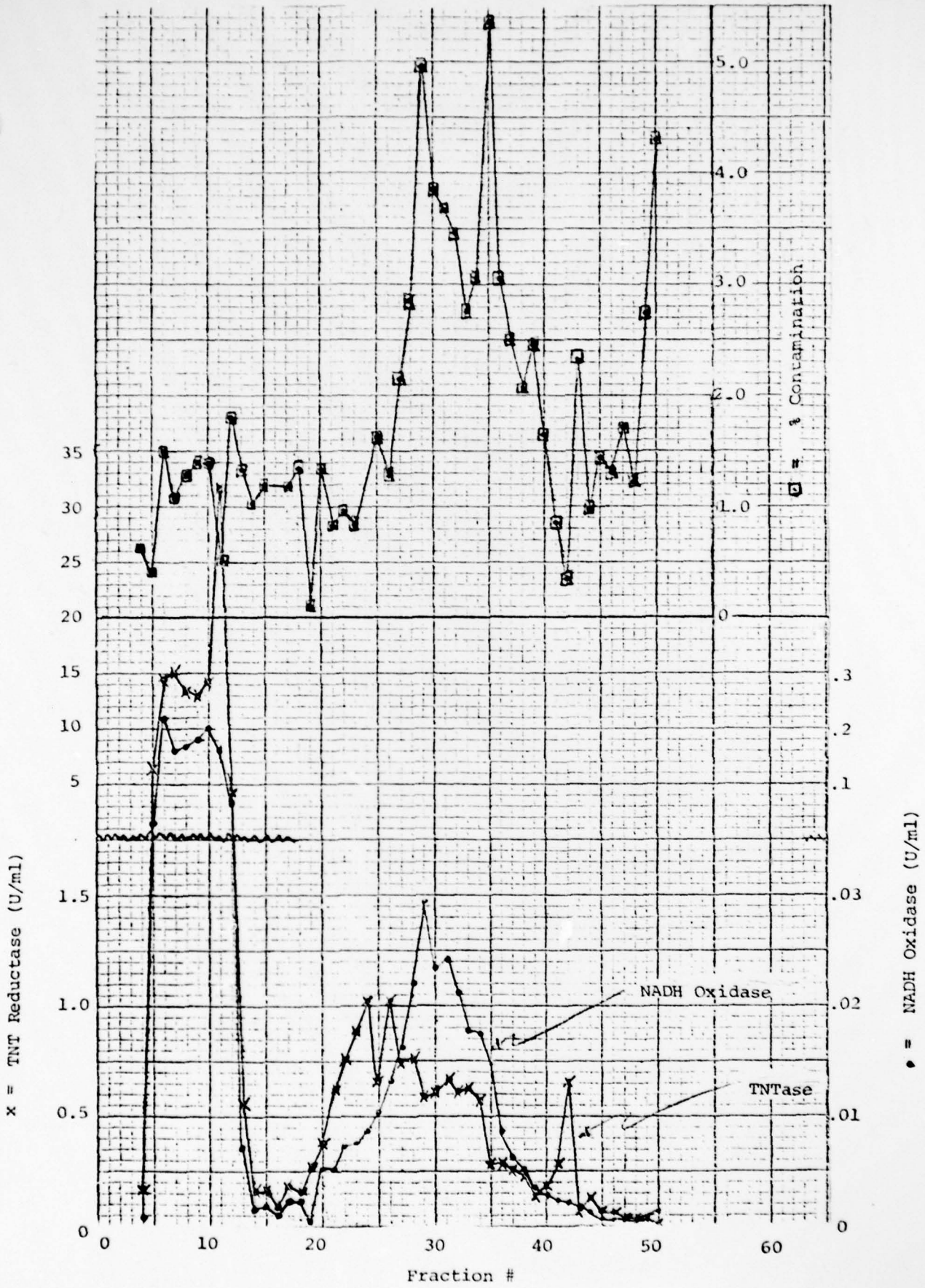

FIGURE 3-2: Elution of TNT Reductase and NADH Oxidase Activities from DE-52 Diethylaminoethyl Cellulose.

A post-DE-52 TNTase fraction from a main batch of purified enzyme containing 0.168% NADH Oxidase contamination was loaded on a 20.0 ml DE-52 column and eluted with a 100 ml linear gradient of 0-0.4 M potassium chloride in .05 M potassium phosphate, pH 7.3, buffer. Three consecutive fractions containing 0.10% contamination were then combined, loaded on a 5.0 ml DE-52 column, and eluted with a similar 50 ml linear gradient. All columns were run in the cold ($4^{o}C$). Approximately 1.0 g DE-52 resin/15 mg total protein was used. Exposure of TNTase fractions with the lowest levels of NADH Oxidase contamination to 3 successive DE-52 columns produced a recurrent value of approximately 0.10% NADH Oxidase contamination.

3.1.4 Sepharose - Active Site Recognition

Work utilizing Pharmacia Blue Sepharose^{R} CL-6B affinity chromatography was undertaken in an attempt to separate NADH Oxidase from TNT Reductase. Blue Sepharose was chosen because it is specific for binding the tertiary protein configuration known as the dinucleotide fold which forms the NAD-binding site in many proteins.

A 5 ml bed volume for every 20 mg of protein to be chromatographed was used. .05 M potassium phosphate buffer, pH 7.0, was used as the starting buffer. The chase buffers tried consisted of 1 mM NADH, 10 mM NADH, 10 µg/ml TNT and 100 µg/ml TNT in starting buffer. The first column was run at room temperature ($23^{o}C$), the second and third columns were run in the cold ($4^{o}C$). Fractions were collected and assayed for TNT Reductase and NADH Oxidase activity. None of these eluents displaced any substantial amount of enzymatic activity from any of the three columns run.

It, therefore, became necessary to search for an eluent which might displace enzymatic activity from the column matrix. 2.0 ml TNTase was added to 2.5 ml Blue Sepharose CL-6B suspended in .05 M potassium phosphate buffer, pH 7.0. The pellet was resuspended in 5.0 ml cold 0.5 M NaCl in buffer and centrifuged ($4^{o}C$). The pellet was then resuspended in 5.0 ml cold 1 mM NADH and 10 µg/ml TNT (for substrate protection) in buffer and centrifuged ($4^{o}C$). The supernatants were assayed for TNT Reductase and NADH Oxidase activity. The data are shown in Table 3-1. Neither NaCl, NADH nor TNT displaced the enzyme such that the original level of pre-column enzymatic activity was recovered.

It is possible that the TNT Reductase and NADH Oxidase were inactivated by exposure to the Blue Sepharose matrix and were either eluted in the void volume or bound (possibly irreversibly) to the column matrix. It is also possible that no loss of activity occurred on the column and that the enzymes did in fact bind (possibly irreversibly) to the column matrix but were not eluted by the substrates used as eluents. TNT protection was used in an attempt to protect the enzyme in the column environment as previous studies show that preincubation of TNTase in NADH-containing solution leads to enzymatic destruction whereas preincubation with TNT protects the enzyme against destruction (see Section 3.3.1.4).

TABLE 3-1: Effect of Various Eluents on TNTase Bound to Blue Sepharose^{R} CL-6B

		TNTase (IU/ml)	NADH Oxidase (IU/ml)	% Contamination
(1)	Positive Control	19.678	.0338	.172
(2)	Supernatant (post-Blue Sepharose spin)	2.391	.0142	.592
(3)	Supernatant (post-.5M NaCl spin)	1.050	.0035	.333
(4)	Supernatant (post-1 mM NADH/10 µg/ml TNT spin)	0.164	.0030	1.829

3.1.5 Polyacrylamide Gel Electrophoresis

3.1.5.1 Luminescent Photometer Assay System

A more sensitive NADH Oxidase assay utilizing the Beckman Luminescent Photometer was developed. This assay was required for the purpose of detecting low levels of NADH Oxidase activity following subjection of the enzyme to polyacrylamide gel electrophoresis. The luminescent photometer assay system differs from the normally used spectrophotometric assay system both in NADH concentration used and in relative sensitivity. In the photometer system the NADH Oxidase reaction is coupled with the luciferase light emission system. This reaction allows the detection of lower levels of enzymatic activity by monitoring relative changes in the system's light output. The light output is a function of the amount of NADH remaining after the interaction of a given enzyme sample. The spectrophotmetric system directly measures the rate of NADH consumption at a wavelength of 340 nm. The luminescent system utilizes 10^{-7} M NADH whereas the spectrophotometric system uses 10^{-4} M NADH. The luminescent system appears to be at least 35 times more sensitive in the detection of NADH Oxidase activity than the spectrophotometric system.

10 µl aliquots of various TNTase dilutions were allowed to preincubate for 5 minutes in 1.0 ml aliquots of 2.0×10^{-7} M NADH. 20 µl samples of each respective dilution were then injected into photometer cuvettes and assayed for light loss based on the amount of NADH consumed during the preincubation period. The cuvette contained 1.0 ml .05 M potassium phosphate, pH 7.0, 50 µl luciferase mix and 10 µl tetradecanal dissolved in ethanol. The data are shown graphically in Figure 3-3. The calibration curve constructed equates relative enzymatic activity with % light output in the luminescent photometer system.

Figure 3-4 is a flow diagram which illustrates the basic overall luminescent photometer assay system.

3.1.5.2 Use of Polyacrylamide Gel Electrophoresis

The polyacrylamide gels and respective electrophoresis solutions were prepared as described in Davis, B.J. (1964) "Ann. N.Y. Acad. Sci. 121", 404-427. .001% Bromphenol Blue in water was used as indicator solution during the electrophoresis run. 600 µg total protein was loaded on each respective gel in order to increase relative yield recoveries after exposure of the enzyme(s) to electrophoresis. The gels were run at 1 mA/tube until the sample band migrated into the upper gel surface; after which the gels were run at 4 mA/tube for 2.5 hours. The gels were then removed and subjected to one of two procedures; either preparation for assay or fixative staining.

Gels designated for fixative staining were stained with a 1% Coomassie Blue indicator solution (in 7% acetic acid) for approximately 20-25 minutes. The destaining process involved submersion of the gels in a series of 7% acetic acid solutions for several days at 37°C. The destaining process is necessary for the removal of excess dye from the gels.

Fixative staining results indicate that the fastest migrating band in the polyacrylamide gel appears to contain the TNT Reductase units. This band apparently is located approximately 1 cm ahead of the bulk of additional protein in the gel. Therefore, it appears to be a good technique for further purification of TNT Reductase although recovery of enzymatic activity is relatively low.

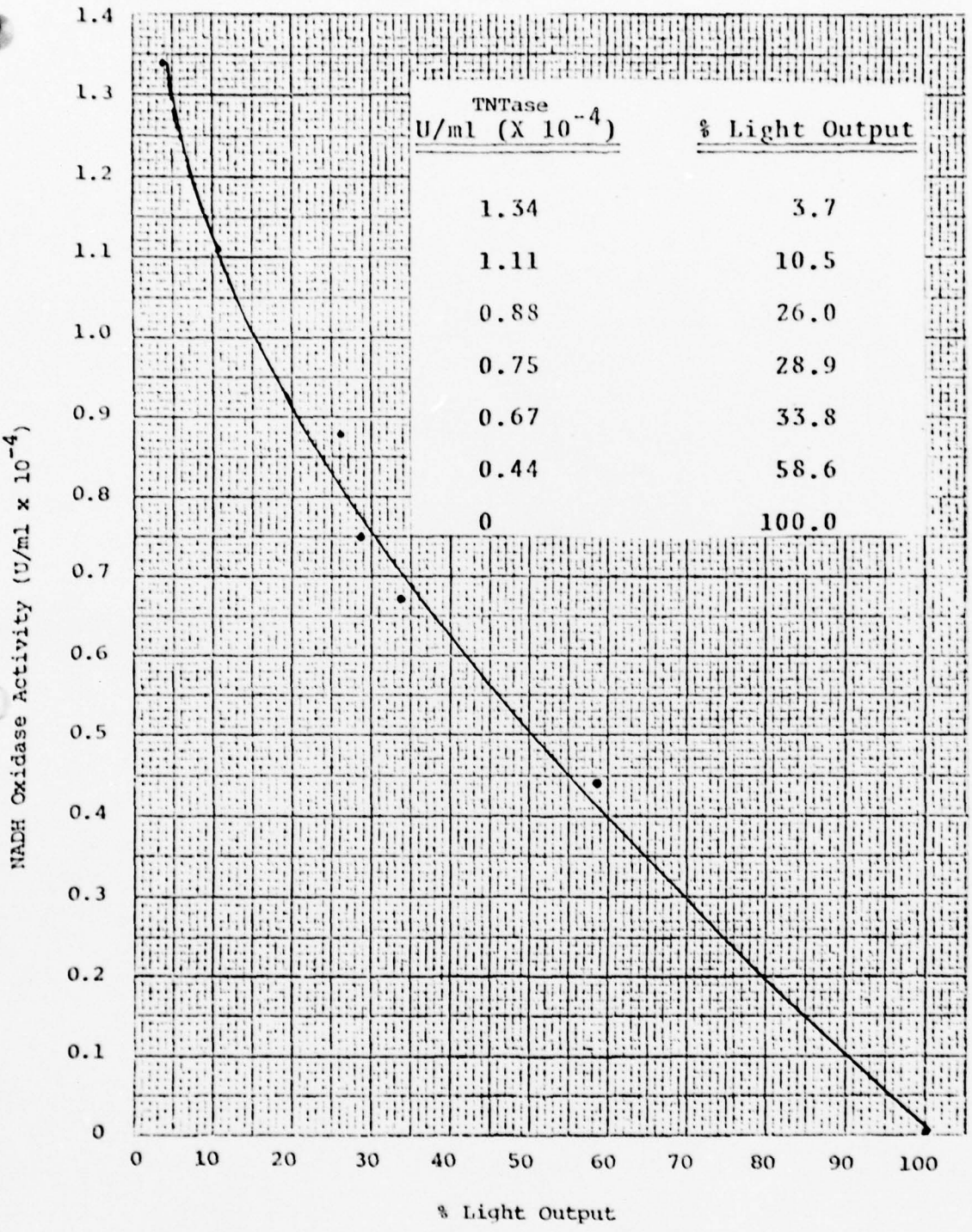

FIGURE 3-3: Calibration Curve which Equates Relative NADH Oxidase Activity with % of Light Output in the Luminescent Photometer Assay System.

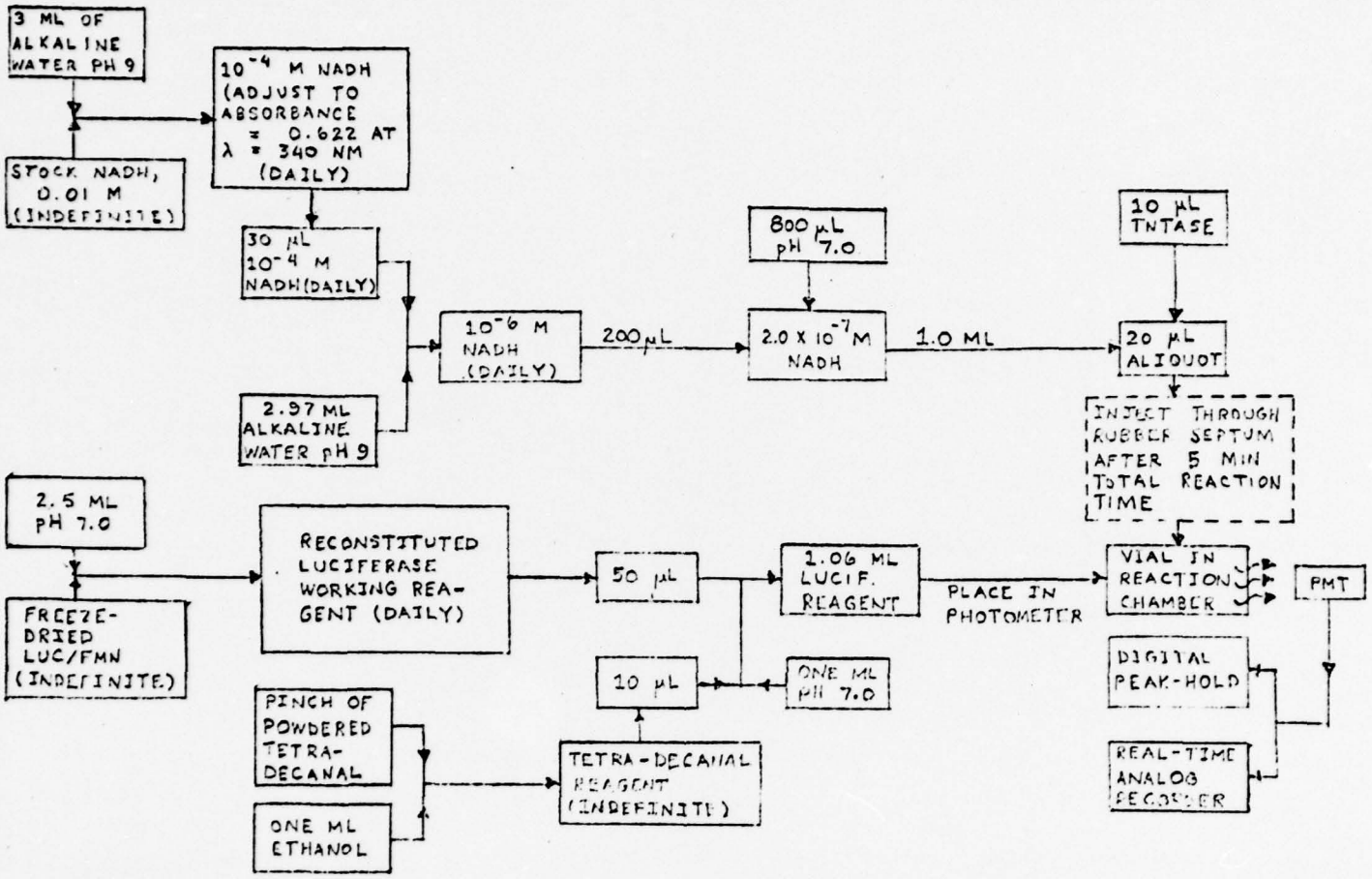

FIGURE 3-4: Luminescent Photometer Assay System

Gels prepared for assay were cut into 8 equal length segments. Each segment was resuspended in .05 M potassium phosphate buffer, pH 7.0, and allowed to sit in the cold (4°C) overnight. (Stability studies show that a gel segment containing TNT Reductase activity treated in this manner appears to retain the enzymatic activity for several days.) Each segment was then macerated in a test tube and centrifuged. The supernatant was then assayed spectrophotometrically at 340 nm for TNT Reductase activity. The supernatants from those gel segments showing activity were then assayed for NADH Oxidase activity using the luminescent photometer assay system since NADH Oxidase activity could not be detected using the conventional spectrophotometer assay system at 340 nm.

Approximately 6.6% of the original TNT Reductase and 1.7% of the original NADH Oxidase activity loaded on each respective polyacrylamide gel was detectable after exposure of the enzyme(s) to electrophoresis. There appears to be approximately a 17% loss of TNT Reductase and a 25% loss of NADH Oxidase activity due to interaction of the respective enzymatic activities with the marker dye (.001% Bromphenol Blue in water) used in the gels. The dye had no effect on the assay blank rates.

Injection of gel extraction from the banding region containing the highest concentration of TNT Reductase activity significantly decreases % of light output in the luminescent photometer assay system. It appears that this decrease is due to NADH Oxidase activity as none of the other variables tested in the assay system caused a similar reduction in light output. The polyacrylamide gel material, electrophoresis buffer (.05 M Tris, .4 M glycine in water, pH 8.3), and marker dye used in the gels (.001% Bromphenol Blue in water) were assayed to determine whether any of these components in the system were responsible for the decrease in % light output observed. The reproducibility of values obtained using the luminescent photometer assay system was checked on numerous occasions.

The fact that the NADH Oxidase activity is apparently located in the same region of the gel where the highest conentration of TNT Reductase activity is also located appears to indicate that it is not possible to separate TNT Reductase and NADH Oxidase activities using the polyacrylamide gel electrophoresis technique of separation. These data appear to support the hypothesis that both TNT Reductase and NADH Oxidase activities reside on a single macromolecule or macromolecular complex.

3.2 Attempted Identification of Electron Acceptor in the NADH Oxidase Reaction

3.2.1 Elimination of Oxygen

It is known that molecular oxygen serves as electron acceptor for some microbial NADH Oxidases. It seemed possible that elimination of oxygen from solution might eliminate the enzyme activity.

In an initial experiment, elimination of oxygen from 0.05 M potassium phosphate assay buffer, pH 7.0, was attempted by first boiling, then cooling while bubbling nitrogen through the solution. NADH and TNT Reductase were rapidly added (2% of total volume) and NADH Oxidase activity was measured. No reduction in NADH Oxidase activity was observed.

A further attempt to eliminate molecular oxygen from the NADH Oxidase assay system was made. It was proposed that removal of dissolved molecular oxygen was incomplete in the previous experiment. Therefore, a new oxygen scavenging procedure utilizing glucose and Glucose Oxidase was designed based upon the following reaction:

$$\text{Glucose} + O_2 \xrightarrow{\text{Glucose Oxidase}} \text{gluconic acid} + H_2O_2$$

Elimination of oxygen from solution was accomplished in .05 M potassium phosphate assay buffer, pH 7.0, in two steps. The buffer was first bubbled with nitrogen for 1 hour, after which .1 M glucose and Glucose Oxidase (Beckman Type III, 10 IU/ml) were introduced into solution. NADH and TNT Reductase were rapidly added (2% of total volume) and NADH Oxidase activity was measured. The results of this experiment (see Table 3-2) show no reduction in NADH Oxidase activity, possibly indicating that the enzyme does not use molecular oxygen as hydrogen acceptor. (Note that other studies (see Section 3.3.5) show that hydrogen peroxide does not interfere with enzymatic activity).

3.2.2 Addition of Putative Inhibitors (CN^-, N_3^-)

NAD^+, sodium cyanide and sodium azide were tested as potential inhibitors of TNT Reductase and/or NADH Oxidase activity in an attempt to further characterize the physical properties of TNT Reductase and NADH Oxidase.

The concentrations of NAD^+, sodium cyanide and/or sodium azide were 10^{-3}M. TNT Reductase and NADH Oxidase activities were assayed spectrophotometrically at 340 nm following addition of each respective potential inhibitor. The results appear in Table 3-3. It appears that none of these compounds substantially inhibit the activity of either TNT Redcutase or NADH Oxidase. NADH Oxidase does not appear to use molecular oxygen as hydrogen acceptor.

3.2.3 Use of Radioactive Tracer

In an attempt to identify the electron acceptor in the NADH Oxidase reaction, Tritium labelled NADH(T) was prepared by an enzymatic procedure and added to a TNTase - NADH Oxidase preparation. After essentially complete reaction of NADH(T), only 50% of the label appeared in water. Approximately 25% of the label was retained in a nucleotide fraction with molecular size and spectrum characteristic of NAD^+, while the remainder of the label appeared in a lower molecular weight fraction which also had a characteristic nucleotide spectrum. Interpretation of these data is somewhat difficult, but it appears that the NADH Oxidase fraction is not a simple electron transfer to molecular oxygen.

TABLE 3-2: Attempted Identification of Electron Acceptor in the NADH Oxidase Reaction: Elimination of Oxygen from Solution.

Solutions (10^{-4}M NADH)	STEP 1	STEP 2		NADH Oxidase
	Solution bubbled for 1 hr. with nitrogen	Addition of .1M glucose to solution	Addition of GO Type III (10 IU/ml) to solution	(IU/ml)
(1) Positive Control	No	No	No	.0061
	Yes	No	No	.0054
(3)	Yes	Yes	No	.0061
(4)	Yes	Yes	Yes	.0047

TABLE 3-3: Addition of Putative Inhibitors

	TNTase (IU/ml)	NADH Oxidase (IU/ml)	% Contaminant
(1) 10^{-3} M NAD^+			
(a) Positive Control, without NAD^+	9.839	.0068	.0691
(b) Experiment, with NAD^+	11.592	.0068	.0587
(2) 10^{-3} M Sodium Cyanide or Sodium Azide			
(a) Positive Control, without sodium cyanide, without sodium azide	7.642	.0057	.0746
(b) Experiment, with sodium cyanide	6.624	.0085	.1283
(c) Experiment, with sodium azide	7.452	.0057	.0765

3.2.3.1 Preparation of Labelled NADH(T)

Tritium label was enzymatically transferred from 1-^{3}H-D-Glucose to the PRO-S position of NAD^+ via the following reactions:

(I) Glucose + ATP $\xrightarrow{\text{hexokinase}}$ glucose-6-phosphate + ADP

(II) Glucose-6-phosphate + NAD^+ $\xrightarrow[\text{glucose-6 phosphate dehydrogenase}]{Mg^{+2}}$ 6-phosphogluconate + NADH + H^+

Glucose tritiated in the 1-position, was purchased from ICN. Additional quantities of NAD^+ and ATP were added to a Beckman Enzymatic Glucose Reagent Kit (HK-Endpoint) in order to optimize transfer of the ^{3}H-label from glucose to NAD^+ to make NAD(T)*. The initial concentrations of each component were as follows: 19.5 mM NAD^+, 20.1 mM ATP, 18.8 mM cold glucose, 10 µM 1-^{3}H-glucose, Hexokinase (2 IU/ml) and Glucose-6-Phosphate Dehydrogenase (5 IU/ml). All components were dissolved in 100 mM triethanolamine buffer, pH 7.85. These components were mixed and allowed to react for 30 minutes, 37°C. The pH of the system was then brought to approximately 9.5-10.0 with 1.0M NaOH so as to eliminate hexokinase and G-6-PDH activity (to insure they would not interfere with NADH Oxidase activity later) and to stabilize the NAD(T) formed during the reaction period.

3.2.3.2 Introduction of NAD(T)* into TNTase Preparation

A 0.5 ml aliquot of the NADH(T) solution described above was added to 2.5 ml TNTase containing 14.8 U/ml with 0.18% NADH Oxidase and 2.0 ml 50 mM potassium phosphate buffer, pH 7.0 and placed in a 30°C bath. Conversion of NADH (NADT*) to NAD^+ in the presence of NADH Oxidase was monitored spectrophotometrically at 0, 10, 30, and 50 minutes. TNT Reductase activity was also assayed at those time intervals so as to monitor the TNT Reductase inactivation in the presence of the NADH (NADT*). These data are given in Table 3-4. At the completion of the reaction (60 minutes) the sample was loaded on a Bio-Rad polyacrylamide gel (P-2) column.

3.2.3.3 Separation of Reaction Products

A Bio-Rad P-2 column was equilibrated with a 50 mM potassium phosphate, pH 7.0 solution. 5.0 ml of the reaction solution was loaded on the column and eluted overnight in the cold (4°C). 150 fractions were collected (each fraction was .72 ml) and then counted for radioactivity. Results are given in Figure 3-5.

3.2.3.4 Analysis of Fractions

A 10 µl aliquot of each fraction was added to 10.0 ml Beckman Ready-Solv™ HP (High Performance Pre-Mixed Liquid Scintillation Cocktail for Aqueous Samples) and counted in a Beckman LS-230 Liquid Scintillation System. Initially all fractions were counted for .1 min. Peak radioactivities were located and fractions containing significant radioactivity were then each counted for 10 minutes. Figure 3-5 shows the radioactivity profile. Three peaks of radioactivity were located. The first two peaks were scanned spectrophotometrically from 200-400 nm.

TABLE 3-4: Addition of TNTase to NAD(T)*

Time (min)	NADH (NADT)* (OD_{340})	NAD^+	TNT Reductase (u/ml)	
0	---		12.3	(calculated)
1	5.32		3.870	
10	3.56		1.705	
30	2.48		1.410	
60	2.30		2.230	
		P.C.	14.841	

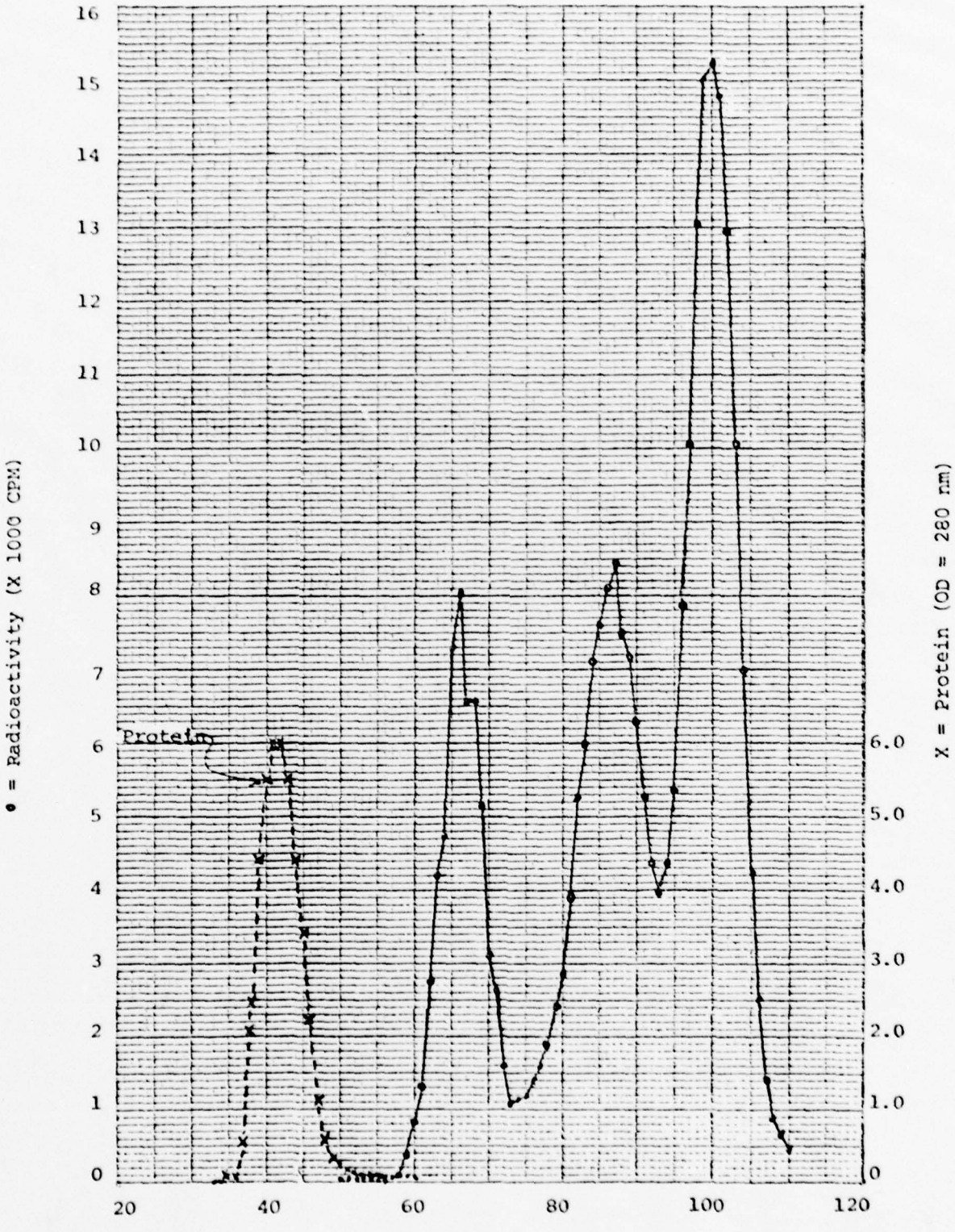

Figure 3-5: Elution of Isotopically Labelled NADH Oxidase Reaction Products From Bio-Gel P-2.

TABLE 3-5: NADH Oxidase assays (P-2 Column).

FRACTION #	NADH OXIDASE (u/ml)
40	.0042
41	.0051
42	.0044
43	.0037
P.C.	.0275

The absorption profile for peak #1 was similar to that obtained for an NAD^+ standard. Absorption at 340 nm, characteristic of NADH(T), was not observed. The scan for peak #2 was similar to that obtained for an ATP Standard. Hexokinase endpoint and 6-P-GDH assays indicate that labeled glucose accounts for only 12% of the radioactivity located in Peak #2. An aliquot of solution from peak #3 (fraction #101) was placed in a dessicator containing drierite in a 105°C oven for approximately 2 hours. All liquid was evaporated out of the fraction tube. The residue in the tube was reconstituted with 0.72 ml H_2O. A 10 µl aliquot of the solution was then recounted for respective radioactivity. 1.4% of the original counts remained indicating that this tube may have contained labeled H_2O. Note that 85% of the total counts loaded on the column were recovered.

Fractions containing protein were located spectrophotometrically by determining absorption at 280 nm. Protein was located in fractions #37-#50 (see Figure 3-5 for plot of this data). NADH Oxidase activity was also located, peak enzymatic activity being in fraction #41 (see Table 3-5). Note that no ^3H - counts were associated with NADH Oxidase

3.3 Effect of NADH and NADH Analogs on TNT Reductase and NADH Oxidase Activities

3.3.1 Kinetics of TNTase Inactivation by NADH

3.3.1.1 Post - NADH Treatment

TNTase was pre-incubated in a pre-warmed (30°C) assay cuvette containing 10^{-4}M NADH in .05M potassium phosphate assay buffer, pH 7.0. At appropriate intervals, the preparations were assayed for TNT Reductase or NADH Oxidase activity. The data are shown graphically in Figure 3-6.

Pre-incubation of TNT Reductase with 10^{-4}M NADH inactivates the enzyme; the degree of inactivation is a function of the pre-incubation time period. On the contrary, pre-incubation of NADH Oxidase with 10^{-4}M NADH does not inactivate the enzyme. These observations suggest that TNT Reductase and NADH Oxidase may indeed be two distinct active sites.

3.3.1.2 Kinetics of TNTase Inactivation in 10^{-4} or 10^{-6}M NADH

The experimental procedure is described in Section 3.3.1.1. The data for 10^{-4} and 10^{-6}M NADH are shown graphically in Figures 3-7 and 3-8 respectively.

The kinetic analysis of TNT Reductase inactivation at 10^{-4}M NADH indicated that the half time ($t_{\frac{1}{2}}$) was approximately 73 seconds. The $t_{\frac{1}{2}}$ for 10^{-6}M NADH was approximately 94 seconds.

3.3.1.3 Data Analysis of the Kinetics of TNTase Inactivation in NADH

Kinetic analysis of TNTase inactivation at various NADH concentrations (see Table 3-6) indicate that the rate of NADH - induced inhibition of TNTase depends on the 0.07 power of the NADH concentration. The data are shown graphically in Figure 3-9.

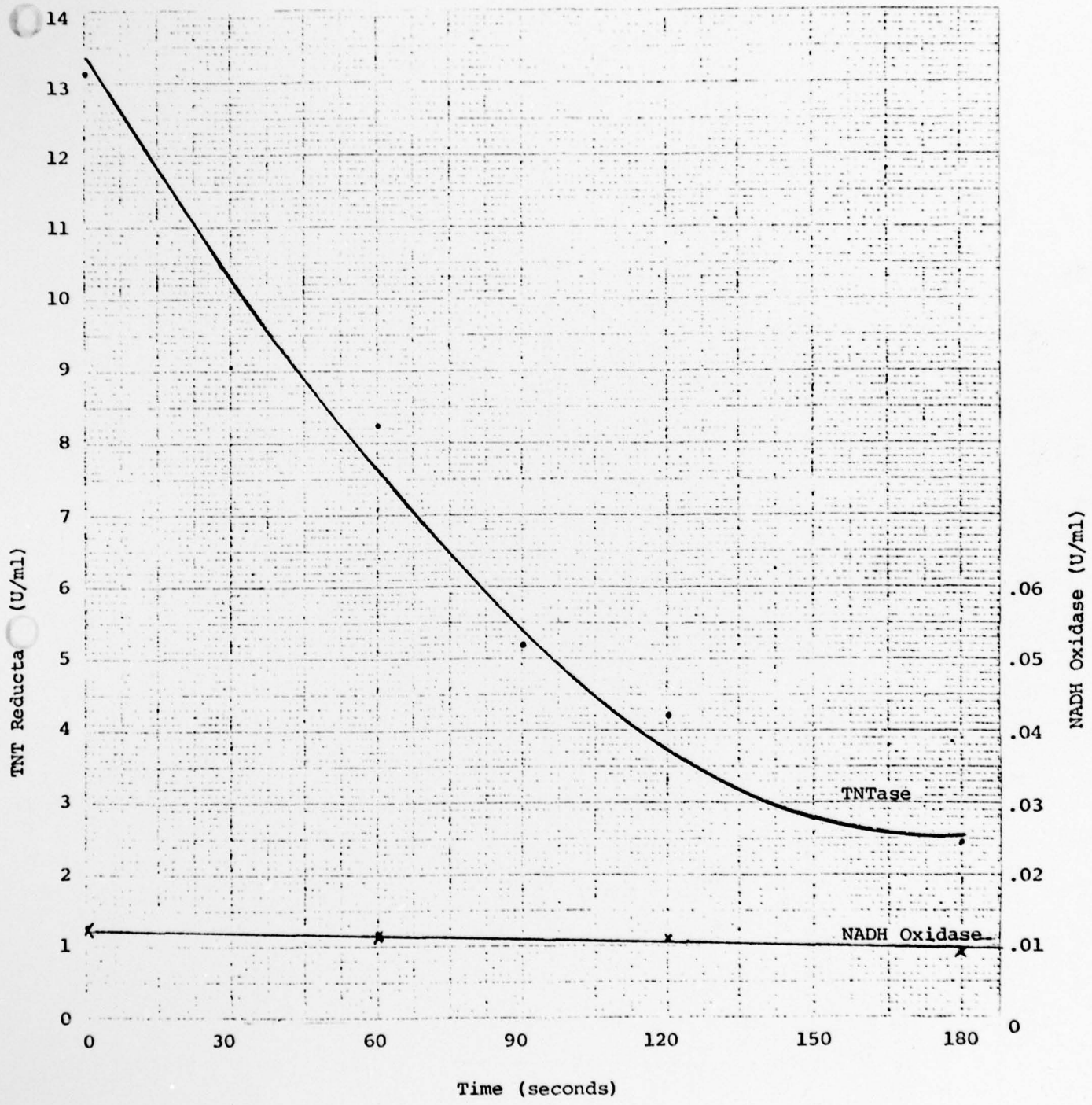

FIGURE 3-6: Effect of Preincubation with 10^{-4} M NADH on TNTase and NADH Oxidase Activities

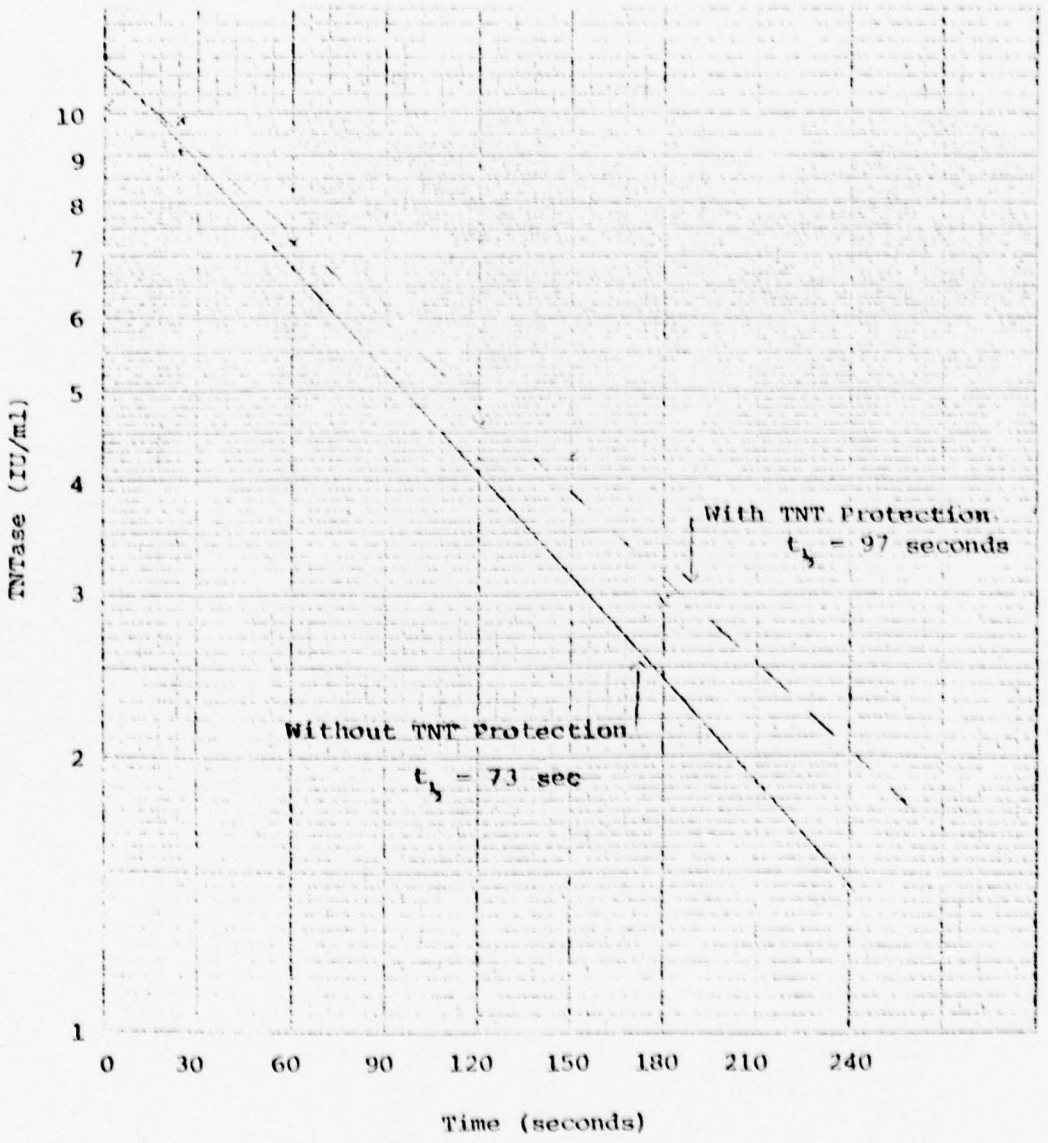

FIGURE 3-7: Kinetic Analysis of TNTase Destruction by 10^{-4} M NADH and the Protective Effect of 4.45×10^{-5} M TNT.

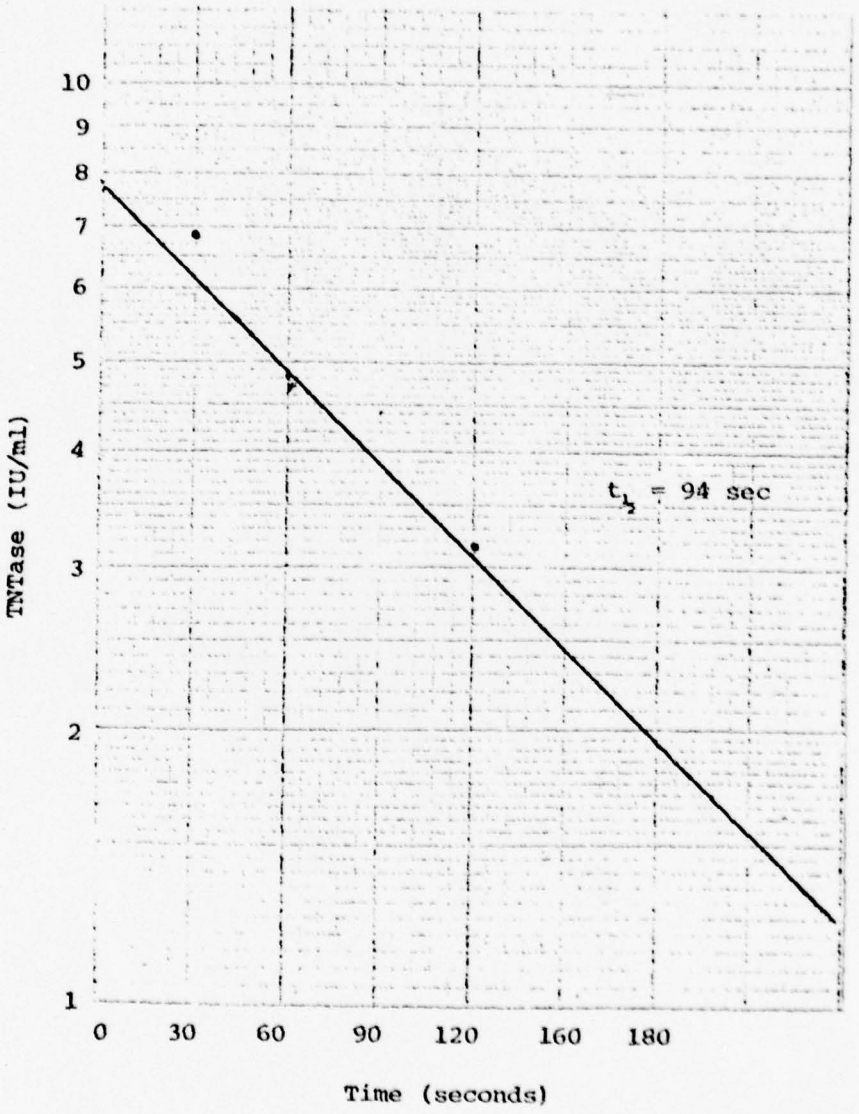

FIGURE 3-8: Kinetic Analysis of TNTase Destruction by 10^{-6} M NADH

TABLE 3-6: Kinetics of TNTase Destruction in NADH

NADH (M)	$t_{\frac{1}{2}}$ (Seconds)
1.0×10^{-4}	73
1.0×10^{-6}	94
5.0×10^{-7}	110
1.0×10^{-7}	114
0	1240

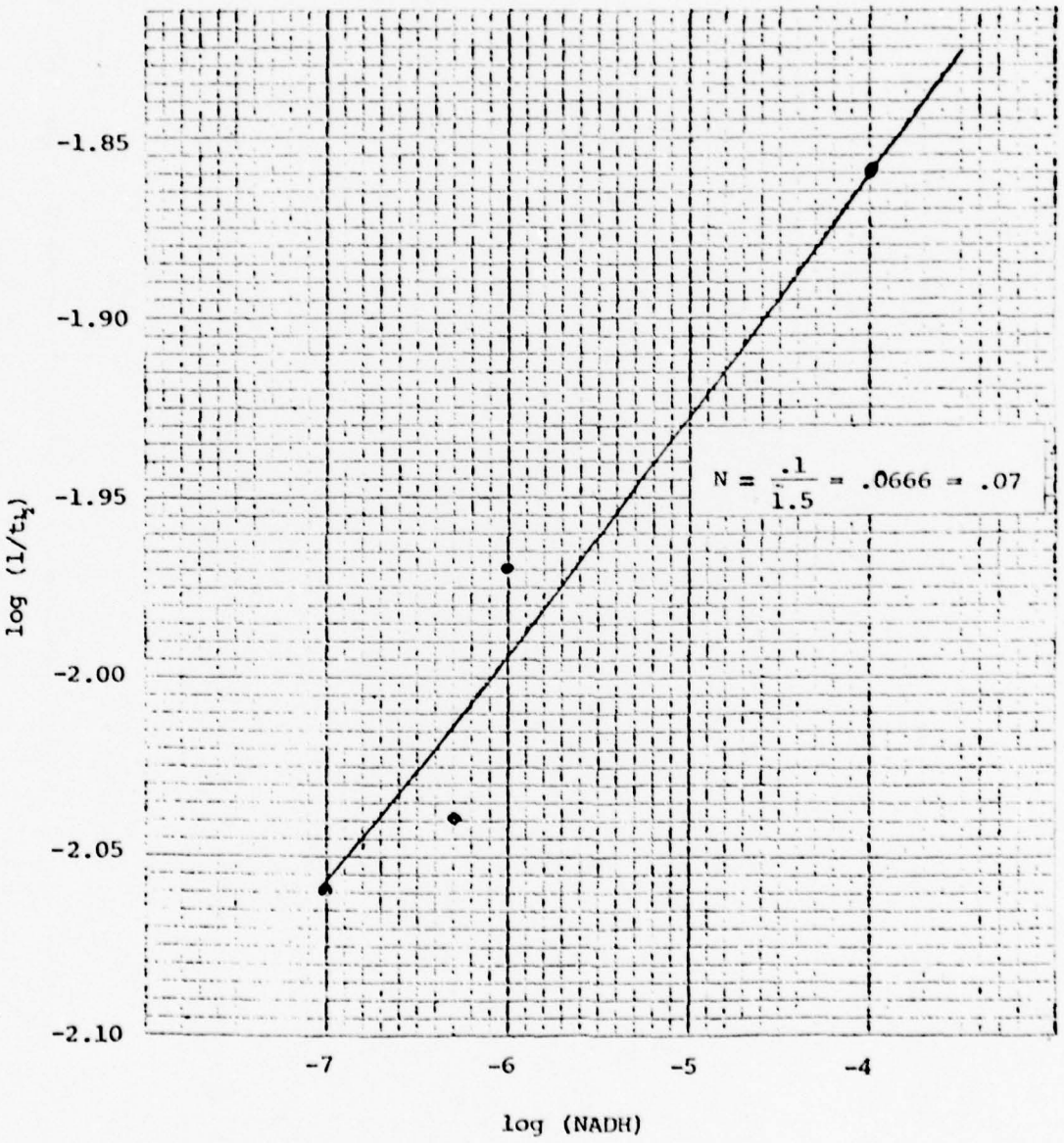

FIGURE 3-9: Effect of NADH Concentration on the Rate of TNTase Inactivation

3.3.1.4 Protection Effect: Pre-incubation with 4.45×10^{-6}M TNT

The pre-incubation procedure was as described in Section 3.3.1.1 except that 4.45×10^{-6}M TNT was included. The final concentration of TNT in the assay cuvette was 4.9×10^{-5}M. The results are shown graphically in Figure 3-7.

Inclusion of TNT in pre-incubation solutions containing 10^{-4}M NADH does protect TNT Reductase activity. The $t_{\frac{1}{2}}$ for enzyme destruction in the presence of 4.45×10^{-6}M TNT was 97 seconds, compared with a $t_{\frac{1}{2}}$ of 73 seconds obtained in the absence of TNT.

3.3.1.5. Effect of Increased TNTase Concentration on the Kinetics of NADH - Induced Inhibition

TNTase was pre-incubated at 100 X assay concentration in 10^{-4}M NADH in .05M potassium phosphate buffer, pH 7.0, for various times. Aliquots were then added to pre-warmed TNTase assay mix and activity was recorded.

A 100-fold increase in TNTase concentration did not significantly alter the rate of TNTase inactivation. The $t_{\frac{1}{2}}$ was 85 seconds in 10^{-4}M NADH compared with the previously observed value of 73 seconds obtained at low TNTase concentration.

3.3.2. Kinetics of TNTase Inactivation in NADH Analogs

3.3.2.1. NADH Analogs: Enzymatic Assays

Three NADH analogs (α-NADH, Nicotinamide Hypoxanthine Dinucleotide, and 3-Acetylpyridine - DPNH) were utilized in an attempt to further characterize the physical properties of TNT Reductase and NADH Oxidase. 10^{-4}M concentrations of each of the NADH analogs were used. TNT Reductase and NADH Oxidase activities were assayed spectrophotometrically at 340 nm. The data appear in Table 3-7.

The three NADH analogs used appear to have similar effects on the activity of TNT Reductase and NADH Oxidase, except that α-NADH appears to be a poor substrate for TNTase.

3.3.2.2. Kinetics of TNTase Inactivation in 10^{-4}M NADH Analogs

The rate of TNTase inactivation in 10^{-4}M NADH analogs appears to be somewhat more rapid than in 10^{-4}M NADH. The experimental procedure used was as described in Section 3.3.1.1 except that 10^{-4}M Nicotinamide Hypoxanthine Dinucleotide and 10^{-4}M 3-Acetylpyridine-DPNH were substituted for 10^{-4}M NADH. The data is shown graphically in Figure 3-10.

The kinetic analysis of TNT Reductase inactivation in 10^{-4}M Nicotinamide Hypoxanthine Dinucleotide indicated that $t_{\frac{1}{2}}$ was approximately 63 seconds. The $t_{\frac{1}{2}}$ for 10^{-4}M 3-Acetylpyridine - DPNH was approximately 57 seconds.

TABLE 3-7: Effect Using NADH Analogs on TNT Reductase and NADH Oxidase Assays

	TNTase (IU/ml)	NADH Oxidase (IU/ml)	% Contaminant
(1) 10^{-4} M Nicotinamide Hypoxanthine Dinucleotide, Reduced Form	11.024	.0057	.0509
(2) 10^{-4} M α-Nicotinamide Adenine Dinucleotide, Reduced form	0.812	.0057	.7020
(3) 10^{-4} M 3-Acetylpyridine - DPNH	7.957	.0034	.0427
(4) 10^{-4} M β-NADH (Positive Control)	15.426	.0061	.0395

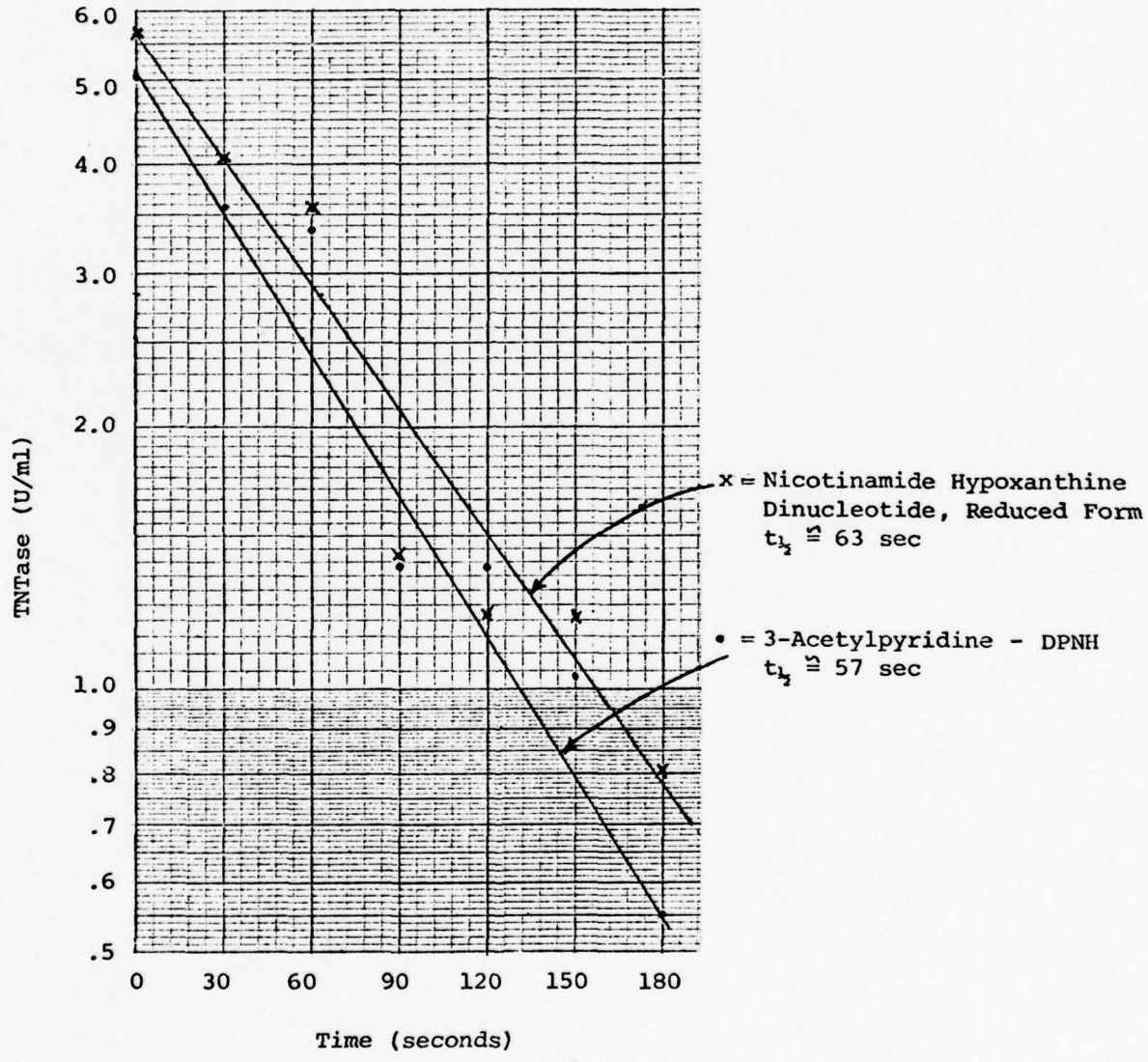

FIGURE 3-10: Kinetics of TNTase Destruction in 10^{-4} M NADH Analogs

3.3.2.3. α - NADH: Inactivation of TNT Reductase Activity

Inclusion of 10^{-4}M α - NADH with 10^{-4}M β - NADH and 10 µg/ml TNT (no enzymatic pre-incubation) yields results equivalent to a standard TNT Reductase assay containing no α - NADH. However, pre-incubation of TNT Reductase in 10^{-4}M α - NADH for 3 minutes causes approximately a 75% inactivation of TNT Reductase activity. Remaining enzymatic activity was assayed using 10 µg/ml TNT and 10^{-4}M β - NADH in .05M potassium phosphate buffer, pH 7.0.

3.3.2.4. Comparison of Different Dilutions of α - and β - NADH

TNTase was added to different concentrations of α- and β-NADH in .05 M potassium phosphate buffer, pH 7.0, and assayed for NADH Oxidase activity. The respective concentrations of α- and β-NADH used and the assay results appear in Table 3-8. The results indicate that equivalent concentrations of α- and β-NADH appear to exhibit similar activities for NADH Oxidase.

3.3.2.5. α-NADH as Substrate for TNT Reductase

TNTase was preincubated in both 10^{-4}M α-NADH and 10^{-4}M β-NADH in .05 M potassium phosphate buffer, pH 7.0, for various time intervals and assayed for respective TNT Reductase activity. The results are shown graphically in Figure 3-11. It appears that α-NADH is not a substrate for TNT Reductase. Note that the observed activity using α-NADH possibly can be attributed to the presence of less than 3% of β-NADH in the α-NADH preparation.

3.3.2.6. Kinetics: 10^{-4}M α-NADH vs. 10^{-4} M β-NADH

The experimental procedure used was the same as that described in Section 3.3.2.5 with the exception that the preparations containing 10^{-4} M α-NADH were assayed in the presence of 10^{-4} M β-NADH. The data is presented graphically in Figure 3-12. 10^{-4} M α-NADH appears to inactivate TNT Reductase activity at the same rate as 10^{-4} M β-NADH. The $t_{\frac{1}{2}}$ for both α- and β-NADH was approximately 70 seconds.

3.3.3. Studies Using NADPH

Substitution of 10^{-4} M NADPH for 10^{-4} M NADH in TNT Reductase and NADH Oxidase assays gave similar increases in both enzyme activities. The data are presented in Table 3-9.

TNTase was preincubated in a pre-warmed (30°C) assay cuvette containing 10^{-4} M NADPH in .05 M potassium phosphate buffer, pH 7.0. At appropriate intervals, the preparations were assayed for TNT Reductase activity. The data is shown graphically in Figure 3-13. Kinetic analysis of TNT Reductase inactivation in 10^{-4} M NADPH gave $t_{\frac{1}{2}}$ of 50 seconds compared with the $t_{\frac{1}{2}}$ of 73 seconds in 10^{-4} M NADH.

3.3.4. Effect of Additives on Inactivation of TNTase

The experimental procedure is described in Section 3.3.1.1. with the exception that either 50 IU/ml catalase or 10^{-3} M N-methyl-L-tryptophan was included in the preincubation. TNT Reductase activity was measured after 3 minutes. The data appears in Table 3-10.

TABLE 3-8: The Effect of Different Dilutions of α- and β-NADH on NADH Oxidase Activity.

NADH (M)	α-NADH (IU/ml)	β-NADH (IU/ml)
2.0×10^{-4}	.0084	.0068
1.0×10^{-4}	.0068	.0084
5.0×10^{-5}	.0047	-
2.5×10^{-5}	.0034	.0037
1.0×10^{-5}	.0027	.0034

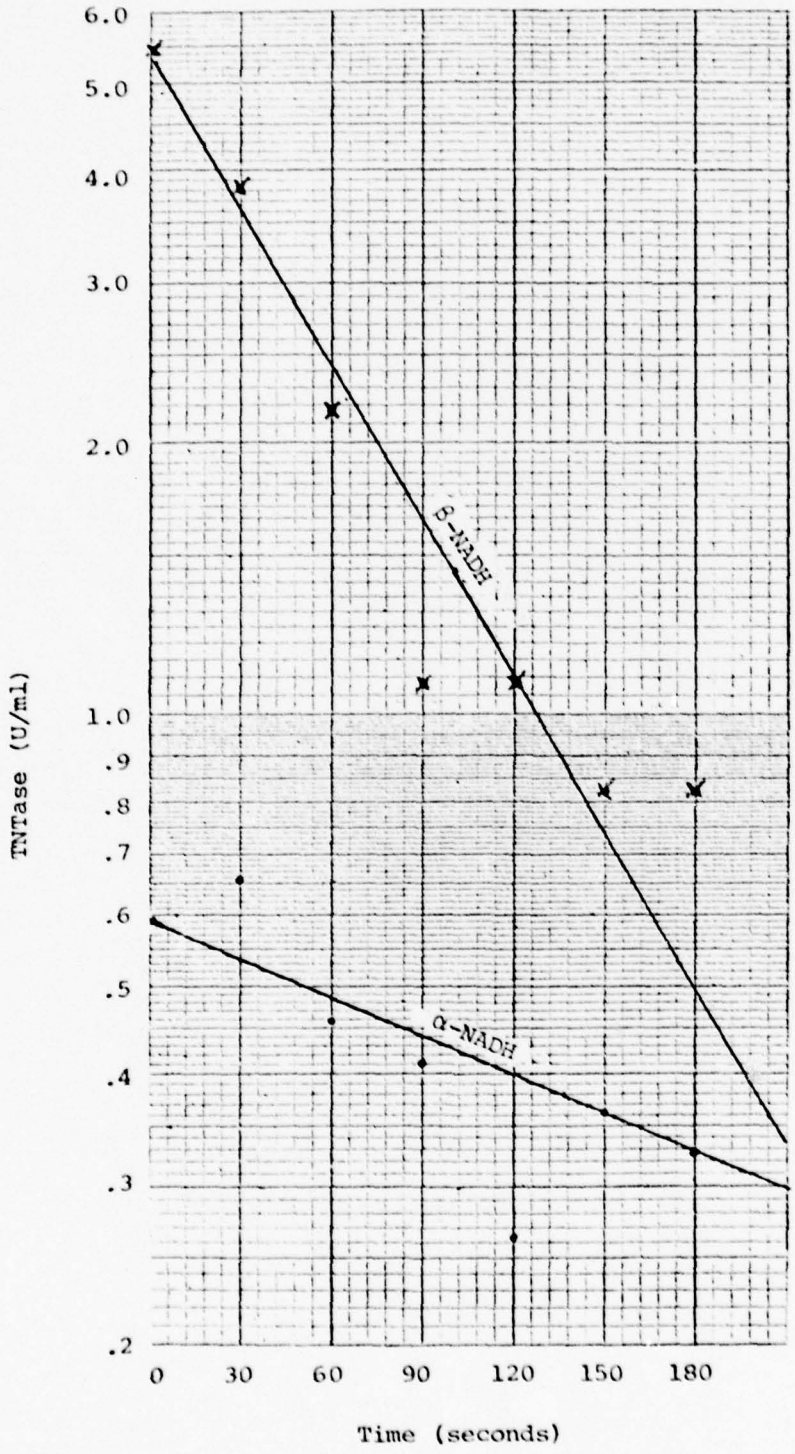

FIGURE 3-11: α-NADH as Substrate for TNT Reductase

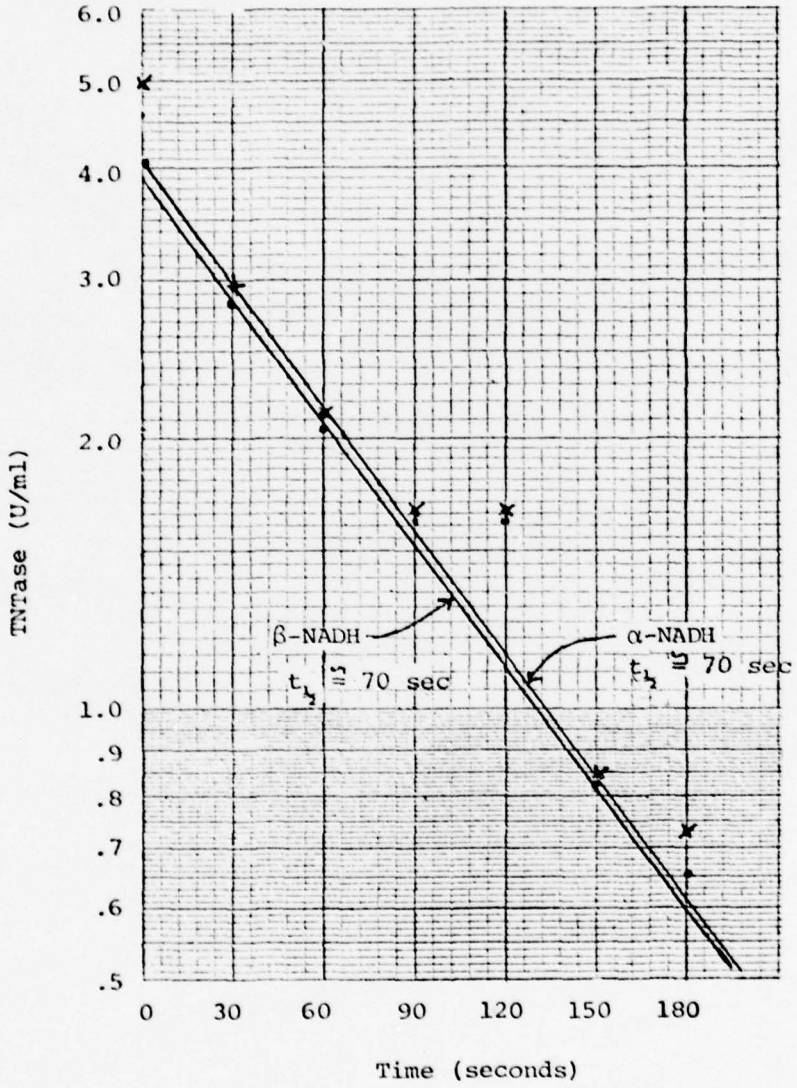

FIGURE 3-12: Kinetic Analysis of TNTase Destruction in 10^{-4} M α-NADH vs. 10^{-4} M β-NADH.

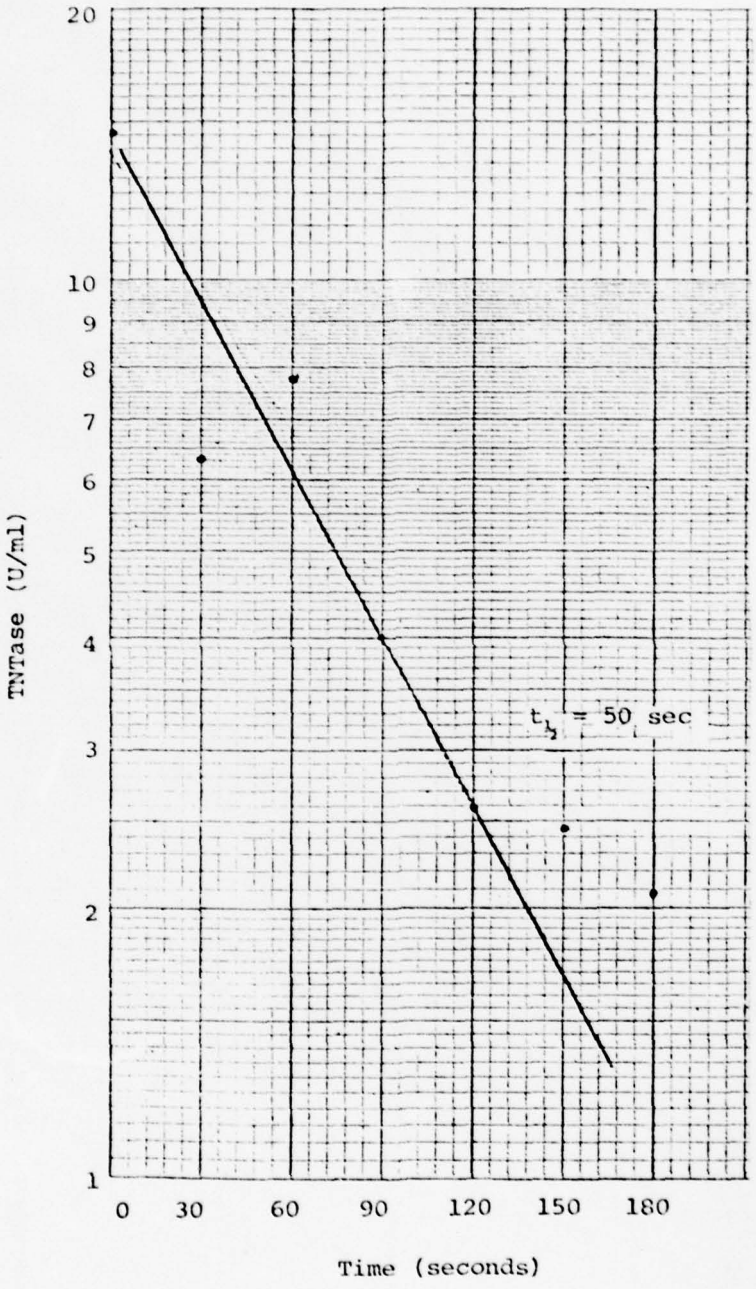

FIGURE 3-13: Kinetic Analysis of TNTase Destruction in 10^{-4} M NADPH

TABLE 3-9: Substitution of NADPH for NADH in Enzymatic Assays

Cofactor	TNTase Activity (U/ml)	NADH Oxidase Activity (U/ml)
NADH	17.2	.016
NADPH	21.2	.024

TABLE 3-10: Effect of Additives on Inactivation of TNTase

Addition to Preincubation Mixture	TNTase Activity
None, NADH omitted (positive control)	18.1
None	3.3
50 IU/ml catalase	3.5
10^{-3} M N-methyl-L-tryptophan	1.5

TABLE 3-11: Effect of Pre-incubation in Hydrogen Peroxide on TNTase Activity

Preincubation Addition	TNTase Activity
None (positive control)	14.5
10^{-7} M Hydrogen Peroxide	13.7

Addition of catalase, which destroys hydrogen peroxide, to NADH pre-incubations did not protect TNTase activity against NADH-induced inactivation nor did N-methyl-L-tryptophan, which can react rapidly with highly reactive forms of oxygen which might be produced in this system.

3.3.5. Effect of Pre-Incubation in 10^{-7} M Hydrogen Peroxide on TNTase Activity

TNTase was pre-incubated in .05 M potassium phosphate buffer, pH 7.0, containing 10^{-7} M hydrogen peroxide for 3 minutes. TNT Reductase activity was then measured. The data appears in Table 3-11.

Pre-incubation of TNTase preparations with hydrogen peroxide, a possible product of NADH oxidation, does not produce inhibition of TNTase activity.

3.4. Effects of Group Specific Reactive Compounds on TNTase and NADH Oxidase

N-ethylmaleide (NEM), p-chloromercuribenzoate (PMB), phenylmethyl sulfonyl-fluoride (PMSF), acetic anhydride, iodoacetic acid, 3-bromopyruvic acid and N-bromosuccinimide (NBS) were used as group specific reagents in an attempt to physically characterize the structure of the enzymatic active site(s).

Approximately 10^{-3} M concentrations of NEM, PMB, PMSF, iodoacetic acid and 3-bromopyruvic acid were used. Each respective compound was added to 0.2 ml TNTase and allowed to pre-incubate on ice for 30 minutes. Samples were then assayed for respective TNT Reductase and NADH Oxidase activities. The results appear in Table 3-12.

NEM, PMB, PMSF, iodoacetic acid and 3-bromopyruvic acid did not appear to affect either TNT Reductase or NADH Oxidase activities. This possibly suggests that neither free sulfhydryl groups nor serine residues exist in the enzymatic active site(s).

Aliquots of various concentrations of N-bromosuccinimide in .05M potassium phosphate buffer, pH 7.0, were added to 0.2 ml TNTase and allowed to pre-incubate on ice for 60 minutes. Samples were then assayed for respective TNTase and NADH Oxidase activity.

N-bromosuccinimide inhibits both TNT Reductase and NADH Oxidase activity proportionately depending on the concentration of NBS used. The data appears in Table 3-13. This indicates that possibly a tryptophan, tyrosyl and/or histidyl residue contribute(s) to enzymatic activity in the active site(s).

TNTase was treated with acetic anhydride as follows: 10 mg total protein was suspended in 100 µl .10 M sodium acetate, .10 M sodium pyrophosphate buffer, pH 8.5, and cooled on ice. 10 µl acetic anhydride/10 mg total protein was added to the suspension and allowed to incubate for approximately 1.5 hours. An aliquot of the suspension was then assayed for TNT Reductase and NADH Oxidase activity. The remaining treated enzyme was dialyzed overnight vs. 30 volumes .05 M potassium phosphate buffer, pH 7.0, and assayed for respective enzymatic activities. Positive controls verified that the assay mix minus acetic anhydride was not the cause of loss of respective activities. The data appear in Table 3-14.

TABLE 3-12: Effects of Group Specific Reactive Compounds on TNTase and NADH Oxidase

Group Specific Reagent	TNTase (IU/ml)	NADH Oxidase (IU/ml)	% Contamination
First Experiment			
(1) NEM	8.593	0.0240	0.279
(2) PMSF	9.478	0.0246	0.260
(3) PMB	10.495	0.0226	0.215
(4) Positive Control	8.527	0.0226	0.265
Second Experiment			
(1) Iodoacetic acid	7.871	0.0135	0.172
(2) 3-bromopyruvic acid	8.757	0.0203	0.232
(3) Positive Control	7.511	0.0145	0.193

TABLE 3-13: Effect of N-bromosuccinimide on TNTase and NADH Oxidase

			TNT Reductase			NADH Oxidase				
(NBS)M	Step	Volume(ml)	u/ml	total u	%recovery	u/ml	total u	%recovery	% cont.	Note
0	non dil. enz (P.C.)	0.20	10.266	2.053	100	.0219	.00438	100	.213	
0	NBS control (no NBS)	0.18 enz. +.02 buffer 0.20	6.789	1.358	66.1	.0182	.00364	83.1	.268	Actual % Recovery TNTase: 77.3 N. Ox.: 78.5
10^{-2}	NBS	0.18 enz. +.02 NBS 0.20	4.460	0.892	43.4	.0135	.00270	61.6	.303	
10^{-3}	NBS	0.20 enz. +.002 NBS 0.202	9.118	1.842	89.7	.0211	.00426	97.3	.231	
10^{-4}	NBS	0.20 enz. +.0002 NBS 0.2002	9.478	1.897	92.4	.0211	.00422	96.3	.223	

TABLE 3-14: Effect of Acetic Anhydride on TNTase and NADH Oxidase

	TNTase (IU/ml)	NADH Oxidase (IU/ml)	% Contamination
Positive Control (No acetic anhydride)	11.479	0.0270	0.2352
Experimental	6.002	0.0169	0.2818
Experimental (post dialysis)	6.002	0.0098	0.1633

Acetic anhydride inhibits both TNT Reductase and NADH Oxidase activity by approximately 50%. This possibly suggests that lysine and/or tyrosine residues contribute to the respective enzymatic activities observed in the active site(s).

3.5. Attempts to Differentially Denature TNTase and NADH Oxidase Using Physical Methods

3.5.1. Storage Effect

TNTase is quite stable when subjected to prolonged frozen storage, while a significant fraction of the NADH Oxidase activity is lost. After approximately one year of frozen storage, the TNTase lost 8.0% of its original activity whereas the NADH Oxidase lost 34.8% of its original activity.

3.5.2. Heat

Aliquots of TNTase were placed in thermostated water baths of appropriate temperature. At selected time intervals, samples were withdrawn and assayed for TNT Reductase and NADH Oxidase activity. The data appears in Table 3-15.

Thermal denaturation experiments indicate that loss of TNT Reductase activity, produced by storage at elevated temperatures, is paralleled closely by loss of NADH Oxidase activity. A linear regression analysis showed that the correlation coefficient for residual TNT Reductase and NADH Oxidase activities was 0.714.

3.6. Luciferase Glow Effect: Removal of DTE

Four Luciferase reagent vials were combined to yield one standard Luciferase reagent to be used in this experiment. 2.5 ml Luciferase reagent was dialyzed for 4 hours vs. 180 volumes .02 M potassium phosphate buffer, pH 7.0, with 5.0 mM FMN to remove the DTE from the reagent. 0.9 mg DTE was added to another 3.0 ml Luciferase reagent to increase the DTE concentration to 2 mM DTE (the standard Luciferase reagent used contains .5 mM DTE). A total of 3 separate Luciferase reagent samples were tested containing no DTE,0.5 mM DTE and 2 mM DTE.

The Luciferase reagent samples were tested using the luminescent photometer assay system. The Beckman luminescent photometer was blanked with 1.0 ml .05 M potassium phosphate buffer, pH 7.0. 50 µl of the desired Luciferase reagent and 10 µl tetradecanal in ethanol were added to the assay cuvette. The Luciferase glow effect was examined over a several minute time-period. 20 µl injections of 2.0×10^{-7} M NADH into various assay cuvettes containing different concentrations of DTE were then examined for relative % light output. The data appears in Table 3-16.

Removal of DTE by dialysis from the Luciferase reagent does not eliminate the Luciferase glow effect. It, therefore, appears that DTE is not related to the cause of the Luciferase glow effect.

TABLE 3-15: Thermal Denaturation of TNTase and NADH Oxidase

Temperature (°C)	Incubation Time (Hr.)	TNTase Activity (U/ml)	NADH Oxidase Activity (U/ml)
0 (control)	0	18.0	.012
30	1	12.0	.007
	2	12.3	.008
	20	8.3	.009
35	1	12.7	.011
	2	11.0	.011
	20	5.5	.007
40	1	6.2	.010
	2	4.3	.006

TABLE 3-16: Removal of DTE from Luciferase Reagent

(DTE) in Luciferase Reagent	Glow Effect Luciferase (% Light Output)	Signal : Noise	Mean % Light Output	Standard Deviation
(1) Standard Reagent (.5 mM DTE)	.24	127.2	30.5	3.1
(2) Without DTE (post-dialysis)	.24	53.2	12.8	0.3
(3) 2 mM DTE	High Glow	-	45.2	1.4

4.0 CONCLUSIONS

From the results discussed in Sections 2.0 and 3.0, the following conclusions may be drawn:

The accumulated data appear to support the hypothesis that both TNT Reductase and NADH Oxidase activities reside on a single macromolecule or macromolecular complex and that the two activities may be inseparable. Isoelectric focusing experiments indicate that the isoelectric points are identical. Gel filtration data suggests that the stokes radii are essentially identical. Considering this result, gel electrophoresis data indicate that the net charges associated with these enzyme activities are identical. These data, taken in concert, suggest that the two activities cannot be separated using classical methods. Further evidence for close association of TNT Reductase and NADH Oxidase was obtained from denaturation and chemical modification experiments which showed concurrent loss of both activities.

Molecular oxygen does not appear to be the electron acceptor in the NADH Oxidase reaction. Data obtained using a radioisotope tracer suggests that this reaction may involve reduction of NADH (or NAD^+). That is, NADH may serve as both electron donor and acceptor in the NADH Oxidase reaction.

Dithioerythritol (DTE) does not appear to be related to the cause of the Luciferase glow effect. Elimination of DTE from this system reduces light output but does not reduce glow. Very high levels of DTE do produce enhanced Luciferase glow.

5.0 RECOMMENDATIONS

Further work should be directed toward the identification of the actual mechanisms of NADH Oxidase activity and NADH-produced TNTase destruction. A useful step in understanding these processes is determination of the fate of NADH. This would be especially useful in the case of NADH Oxidase activity, where the electron acceptor is unknown. Preliminary data, obtained using radioisotape labeled NADH(T) indicate that NADH itself may serve as electron acceptor in the NADH Oxidase reaction. Direct identification of the reaction products may prove useful.

An immunochemical approach to the separation of TNT Reductase and NADH Oxidase activities, might also be explored. Immune methods can be extremely powerful since they make it possible to separate proteins based on their individual surface structure. TNT Reductase activity could be eliminated by incubation with NADH, leaving only NADH Oxidase activity. This preparation could be used to produce an antibody preparation specific for the NADH Oxidase active site. After obtaining the antibody, it is expected that two types of experiments could be performed. The first is direct titration of the TNT Reductase-NADH Oxidase preparations with the anti-serum. The variables to be measured are the activities of both enzymes. This procedure might provide a method for reducing the NADH Oxidase activity in TNTase preparations. The second procedure to be attempted using the antibody preparation might be immuno-affinity chromatography. This procedure might also be successful in eliminating NADH Oxidase activity from TNT Reductase preparations, or, alternatively, might demonstrate the association of these activities.

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7.0 GLOSSARY OF SPECIAL TERMS

The following are definitions of special terms and abbreviations used throughout this report:

- TNT: α-2,4,6-trinitrotoluene
- NAD and NADH: oxidized and reduced forms, respectively, of nicotinamide adenine dinucleotide
- NADP and NADPH: oxidized and reduced forms, respectively, of nicotinamide adenine dinucleotide phosphate
- FMN and $FMNH_2$: oxidized and reduced forms, respectively, of flavin mononucleotide
- TNTase: *TNT reductase*, the enzyme which catalyzes reduction of TNT and DNT in the presence of NAD(P)H
- DTE: dithioerythritol

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13. ABSTRACT

The Final Report covers work performed during the period August, 1977 to June, 1979. The work presented in this report deals primarily with the physical characterization and attempted separation of TNT Reductase and NADH Oxidase activities. The data accumulated using isoelectric focusing, cellulose-based ion exchange chromatography and polyacrylamide gel electrophoresis in conjunction with denaturation and chemical modification techniques appear to support the hypothesis that with TNT Reductase and NADH Oxidase activities reside on a single macromolecule complex and that the two activities may be inseparable. Oxygen elimination experiments as well as radioisotope trace studies indicate that molecular oxygen is not the electron acceptor in the NADH Oxidase reaction. Dithioerythritol (DTE) does not appear to be related to the cause of the Luciferase glow effect.

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	ROLE	WT	ROLE	WT	ROLE	
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