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PURIFICATION, CHARACTERIZATION, AND X-IRRADIATION STUDIES OF NADH-DEHYDROGENASE FROM PHOTOBACTERIUM FISCHERI

by

Dennis Franklin Brendel

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Environmental Health Sciences)
in The University of Michigan
1972

Doctoral Committee:

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DEDICATION

TO MY SON, BRIAN

ACKNOWLEDGEMENTS

The largest single contribution to this endeavor has come from a person whom I have discovered has no equal, my wife, Judy. Without her expert help in the laboratory during periods of frustration and exhaustion, suggestions on experiments, critical review of the results, and uncountable hours of her time, this manuscript would not exist.

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INTRODUCTION

The effect of radiation on enzymes has been studied extensively by Dale (1) and many others, whose approach invariably has been to irradiate an enzyme either *in vivo* or *in vitro*, extract the enzyme in question, assay the enzyme, and determine the percent of enzyme inactivated by radiation. Through this type of study researchers have concluded that enzymes are fairly radioresistant, requiring several thousand rads to even partially inactivate the enzyme in question. The only deviation from this conclusion has been the data of Barron (2) who states that enzymes which depend on sulfhydryl groups for their biological activity are as much as one hundred times more sensitive to radiation than enzymes not requiring such sulfhydryl groups. Recently Lange (3) failed to confirm Barron's conclusion that sulfhydryl enzymes are more radiosensitive than non-sulfhydryl enzymes, which leaves the conclusion that enzymes are fairly radioresistant to ionizing radiation. To date, the effects on enzymes *in vitro* during irradiation have not been studied.

In 1955 Hug and Wolf (4) studied the effect of radiation on the luminescence of *Photobacterium fischeri* growing on agar plates. Their results showed that during irradiation of *P. fischeri* at 25 to 100 kR/min, luminescence was immediately

suppressed. Within five minutes after exposure, luminescence recovered to 85% of the initial luminescence. The non-recoverable portion of luminescence was attributed by the authors to enzyme inactivation.

Burns, Nevill, and Whipple (5) sought to determine whether this radiation-induced depression of bacterial luminescence observed by Hug and Wolf resulted from *in vivo* inactivation of the enzyme system or from damage to supporting systems. A suspension of *P. fischeri* in buffered saline was irradiated with doses up to 24.6 krads. The cells were then lysed, substrates added, and luminescence measured. Luminescence in the irradiated sample was equal to that of the non-irradiated control. These results showed that the luminescent enzyme system was not inactivated. Thus, the luminescent enzyme system of *P. fischeri* appears to be as radioresistant *in vivo* as most other enzyme systems.

Subsequent studies by Burns, Jacobson, and Whipple (6) indicated that a suspension of *P. fischeri* in buffered saline produced the same phenomena during irradiation as those on agar plates observed by Hug and Wolf. However, in Burns' study, reversible depression of luminescence was produced at a much lower dose rate, one krad/min, with complete recovery of luminescence 30 seconds after irradiation. Additionally, they found that the same phenomena occurred during irradiation of cell-free extracts of *P. fischeri*. In the cell-free extract, luminescence was depressed by irradiation, but completely recovered 10 seconds after irradiation. Burns also

showed that depression of luminescence in cell-suspensions and cell-free extracts was directly proportional to the dose rate. These results indicated that the substrates or enzyme-substrate complexes necessary for bioluminescence were affected by radiation.

Research Proposal

This investigation was undertaken to study the biochemical mechanism by which X-irradiation temporarily depresses luminescence in P. fischeri. Since X-irradiation most likely degrades or interferes with one or more of the substrates or intermediates of the bioluminescent reaction, temporary depression of luminescence during irradiation may result from: 1) interference with the enzyme-substrate complex, 2) interference by free radicals produced during irradiation, 3) temporary blocking of the active site on the enzyme, and/or 4) temporary alteration of the conformation of the enzyme. Each of the above would alter the kinetics of the bioluminescent reaction.

In the cell-free extract experiments by Burns, luminescence was initiated by the addition of NADH and FMN, the required substrates for the reaction of NADH-dehydrogenase to produce $FMNH_2$. A hypothetical reaction scheme of bioluminescence appears in Figure 1, page 9. $FMNH_2$ is the substrate for luciferase, the enzyme responsible for luminescence in P. fischeri. Other substrates required for the luciferase reaction are a long chain aldehyde and molecular oxygen. To

produce luminescence the luciferase reaction is coupled *in vitro* to the $FMNH_2$-producing reaction of NADH-dehydrogenase:

$$NADH + H^+ + FMN \xrightleftharpoons{\text{NADH-dehydrogenase}} NAD^+ + FMNH_2$$

Before this study NADH-dehydrogenase had not been isolated from *P. fischeri* for *in vitro* studies. Therefore, the first objective of this research was to purify and characterize NADH-dehydrogenase from *P. fischeri*. The kinetics of NADH-dehydrogenase were then studied before, during, and after irradiation. Thus, the contribution of the NADH-dehydrogenase reaction to the depression of luminescence during irradiation was determined. Finally, NADH-dehydrogenase was coupled to luciferase to determine the contribution of the luciferase reaction to the depression of luminescence during irradiation.

Background Material

Bacterial Luminescence of P. fischeri

It has been observed that bioluminescence is a special form of chemiluminescence. In principle, the only requirements for chemiluminescence are a sufficiently exergonic reaction and a suitable emitter, which is then formed into an excited electronic state or which can be raised to an excited state by the absorption of energy. The requirements for bioluminescence are, therefore, two-fold:

1. Sufficient energy in a chemical oxidation reaction must be available in a single step to leave the product or intermediate molecule in a state corresponding to the energy of the photon of light emitted (7).
2. The product or intermediate molecule must be a reasonably fluorescent molecule so that de-excitation by fluorescence is a probable event (7).

The components necessary for luminescence of P. fischeri have been determined by a number of researchers over a period of many years. In 1951 Strehler and Shoup found that an acetone precipitate from P. fischeri would luminescence for a few minutes after being suspended in water. Later Strehler and Cormier (8,9) began a systematic study to restore luminescence to cell-free extracts by adding agents to the concentrated lysate. A wide variety of biochemical reagents, too numerous to list here, were tested. Examples of the reagents are NAD, TPN, NADH, FMN, lipoic acid, ATP, ADP, AMP, ethanol, acetaldehyde, Vitamin A, cysteine, numerous steroid hormones, and various combinations of the above. Of all the compounds examined only eight restored luminescence. Of these, NADH increased luminescence to a greater extent than any others. NADH was also the factor which first became limiting for luminescence in crude extracts of P. fischeri. In testing combinations of different biochemical agents, it was found that the addition of NADH and FMN further

increased luminescence by three-fold, which suggested that the luminescent system of P. fischeri is a NADH-FMN coupled respiratory pathway.

Later McElroy (10) lysed freshly grown P. fischeri cells in distilled water and removed the cellular debris by centrifugation. The active fraction, which emitted light upon the addition of NADH, was precipitated with HCl at pH 4.5 and was further purified by ammonium sulfate fractionation. The resulting fraction after dialysis would not catalyze the emission of light upon the addition of NADH. However, when FMN was added, luminescence immediately commenced. The authors therefore concluded that FMN was necessary for luminescence of P. fischeri.

The effect of a long chain aldehyde in the bioluminescent reactions of P. fischeri was discovered by Cormier and Strehler (11). In an attempt to protect the luminescing system in an extract of P. fischeri from heat denaturation, they added another source of protein, kidney cortex powders, and promptly obtained a large increase in luminescence. They termed this component the kidney cortex factor (KCF). Later determinations of the KCF revealed that the stimulating compound was palmitaldehyde (12). This led to additional research into the effect of other aldehydes on luminescence. Hastings was able to show that the straight chain aldehydes of six carbon atoms or more were effective (13). What has not been determined is whether there exists an aldehyde of six carbons or more in P. fischeri.

Although many studies have been concerned with the function of aldehyde, its role in the bioluminescent reaction has not been proved. Strehler contends that the aldehyde is not used in a stoichiometric manner, but that its role is catalytic in nature (14). However, McElroy and Green have presented evidence indicating that aldehyde was converted to the corresponding fatty acid, possibly by peroxidation (15). In that manner the necessary energy needed to produce an excited state would be available. None of the experiments performed by McElroy and Green has shown the utilization of aldehyde or has succeeded in the isolation of its corresponding acid.

When a glowing suspension of luminous P. fischeri is allowed to stand, the light produced begins to dim in a few seconds. Shortly thereafter the light has practically disappeared except for a thin layer at the surface which continues to glow. If air is bubbled through the suspension, the light reappears. Harvey and Morrison demonstrated the absolute requirement of oxygen for luminescence by varying the concentration of oxygen with hydrogen in a gaseous mixture and passing it through the bacterial suspension (16).

Luciferase has been isolated from P. fischeri and was shown to be an absolute requirement for luminescence (17). Purified luciferase produced luminescence by the addition of reduced FMN ($FMNH_2$) to a solution which contained purified luciferase, oxygen, and the aldehyde, decanal.

These studies have indicated that in cell-free extracts of P. fischeri the following components are necessary for the

production of luminescence: NADH, FMN, NADH-dehydrogenase, luciferase, oxygen, and an aldehyde. NADH-dehydrogenase has been postulated to be present in the cell-free extract of P. fischeri because of the continual production of $FMNH_2$ by the addition of NADH. However, this enzyme had not yet been isolated from the bacteria. This study, in part, describes that isolation.

After determining the reactants for luminescence in P. fischeri, Strehler and Cormier (12) studied the kinetics of the bioluminescent system to determine the order in which the substrates react to produce luminescence. In their experiments all components necessary for luminescence (NADH, FMN, O_2, and aldehyde) except one were added to the cell-free extract. At zero time the missing component was added. The light produced was measured as a function of time, until luminescence reached a steady-state. The data were plotted as the time required to reach half-maximal luminescence, the half-rise time. It was found that the half-rise time was greatest for the component which reacted first. NADH had the greatest half-rise time; aldehyde had the least. These conclusions were substantiated in a study by Hastings (18) who later proposed the following reaction scheme for bioluminescence in P. fischeri (Figure 1) (19).

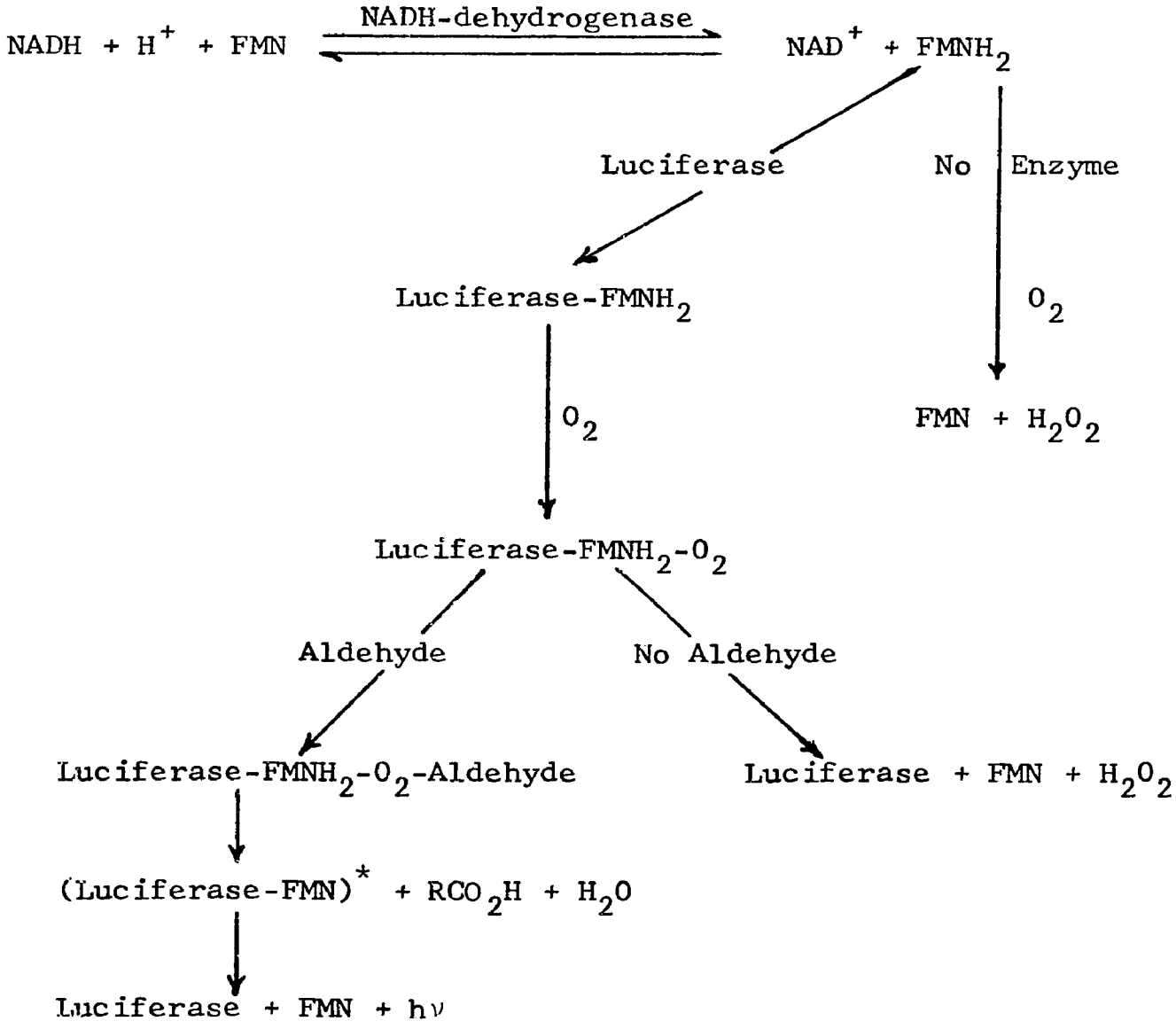


Figure 1. Hypothetical Reaction Scheme of Bioluminescence in P. fischeri (19).

Radiation Effects on Enzymes in vitro

Life depends on a complex network of chemical reactions mediated by specific enzymes. Any modifications of the enzyme systems may have far-reaching consequences for the living organism. If the effects of ionizing radiation on enzymes and their activity are thoroughly understood, one may have greater insight into the radiobiological action of radiation. The alteration of enzymes or their substrates by radiation may disrupt, distort, or cause complete disappearance of a vital function which may result in physiological injury.

Radiobiological phenomena, exhibited by two modes of action, are initiated by the interaction of radiation with molecules. Direct action by irradiation results when a molecule undergoes change itself by being excited or ionized by radiation (20). When a molecule does not absorb the energy itself, but receives energy by transfer from another molecule, indirect action by radiation results (20). The indirect effect has been shown to be the primary means by which radiation interacts with enzymes in solutions (21).

The principle action of X-irradiation on water was shown by Allen and others (22,23,24) to be the formation of hydrogen (H·) and hydroxyl (OH·) free radicals:

$$H_2O \longrightarrow H\cdot + OH\cdot$$

These highly reactive free radicals are likely to react with other molecules in solutions. It has been observed that

solutions which contain oxygen, as most biological systems do, are more sensitive to radiation. Oxygen amplifies the initial reaction by free radicals formed in water by converting the H· and OH· radicals into the more reactive forms of perhydroxyl free radical (HO_2·) and hydrogen peroxide (H_2O_2) (25).

Dale was one of the first to study inactivation of enzymes by radiation. His in vitro studies on poly phenol oxidase, D-amino-acid-oxidase, and carboxypeptidase showed that these enzymes in solution could be inactivated at high doses of radiation (1). Dale also showed that inactivation could be prevented if irradiation of the enzyme occurred in the presence of its substrate. D-amino-acid-oxidase was completely protected in the presence of its coenzyme, flavin adenine dinucleotide. Additionally, Sutton (26) has shown that H_2O_2, the substrate of catalase, protects that enzyme during irradiation. Since the research of Dale and Sutton, it has been shown that enzymes in solution can be protected from radiation by substances which act as protective agents, although their effectiveness is difficult to predict. Two of the more effective protective agents are cysteine and cysteamine (27). It therefore appears that protection of enzymes from radiation can be achieved by addition of free radical scavengers or by masking the active site with substrate.

Radiation effects on proteins irradiated in the dry state or in an aqueous environment have been extensively studied. The most complete study on proteins was done by Alexander (28,29) with bovine serum albumin (BSA). When BSA

was irradiated in the solid state, the sedimentation constant was increased, but without a corresponding alteration in the molecular weight. This observation reflects a change in the conformation of the protein. The disulfide bonds were broken and reduced to sulfhydryl groups. The solubility of BSA in buffered aqueous medium was decreased, formation of peroxy groups bound to the protein were formed, and amine groups were lost. There was a change in the absorbance of BSA at 280 nm which the authors felt indicated an alteration of the amino acids tyrosine and tryptophan. There were also changes in viscosity, light scattering, and electrophoretic mobility, all of which indicated conformational changes of the protein.

When enzymes in solution have been irradiated under different environmental conditions, the degree of inactivation has been shown to be affected by enzyme concentration, pH, ionic strength, temperature, and presence of activators, inhibitors, or substrates. When enzymes are irradiated in solution the molecules are acted upon by both direct and indirect mechanisms, but the effect is primarily indirect. Enzyme solutions of ribonuclease, trypsin, and carboxypeptidase have been shown to be inactivated to the same extent in the presence or absence of oxygen (30,31,32), which supports the view that hydroxyl radicals, $OH\cdot$, are responsible for the inactivation.

The effects of radiation on enzymes indicates that irradiation damage is irreversible. However, one area of radiation effects which indicates a possible reversibility is that of disruption of hydrogen bonding within the enzyme

structure. Hydrogen bonds between peptide chains are an important part of the three-dimensional structure of enzyme molecules. Disruption of one hydrogen bond requires less than two eV of energy (21). Although a hydrogen bond is easily disrupted, it is also easily reformed. Because of the number of hydrogen bonds involved, considerable doses of penetrating radiation are required to alter the protein structure. The disruption of hydrogen bonds may alter the secondary and tertiary configuration of the enzyme, thereby either permanently or temporarily altering the activity of the enzyme.

In summary, the radiation effects on enzymes *in vitro* are greatly varied by the conditions in which the enzyme is irradiated. Generally enzymes are most sensitive to radiation in dilute solution. They become increasingly resistant when substrates, other proteins, and other metabolites are added to the solution. *In vivo*, enzymes are rendered even more resistant to radiation. Thus, the more complex the system, the greater the degree of protection afforded to the enzyme from radiation.

MATERIALS AND METHODS

Growth of Bacteria

A lyophilized culture of Photobacterium fischeri, American Type Culture Collection No. 7744, was rehydrated, aseptically, by adding 0.4 ml of liquid medium (Table I) to the freeze-dried material. After being transferred to a test tube containing six ml of liquid medium, the mixture was incubated at 27°C for several days. A luminescing strain of P. fischeri was obtained by streaking the culture onto agar plates (Table III) and selecting only isolated, luminescing colonies. The strain was maintained on agar plates by weekly transfers. The optimum temperature and pH ranges for growth and luminescence are 25-28°C and pH 6.5-7.8 (33). Media were autoclaved for 15 minutes at 15 psig.

Growth Curve of P. fischeri

One ml of a 16-hour-old, glowing culture of P. fischeri was inoculated into a one-liter flask containing 500 ml of sterile medium. The culture was incubated at 27°C with aeration. Foaming was controlled by the addition of Dow-Corning Antifoam. Aliquots of three ml each were removed

Table I

Composition of Liquid Growth Medium (33, 34)

Reagent	Per Liter Distilled Water
Sodium Chloride (NaCl)	30.0 g
Sodium Dibasic Phosphate ($Na_2HPO_4 \cdot 7H_2O$)	14.0 g
Potassium Monobasic Phosphate (KH_2PO_4)	2.0 g
Ammonium Dibasic Phosphate ($[NH_4]_2HPO_4$)	0.5 g
Magnesium Sulfate ($MgSO_4 \cdot 7H_2O$)	0.2 g
Glycerol	5.0 ml
Difco Bacto Tryptone	5.0 g
Difco Bacto Yeast Extract	5.0 g
Trace Elements Solution (Table II)	0.05 ml
Sodium Hydroxide (NaOH) to adjust pH to 7.0	

Table II

Trace Elements Solution (35)

Reagent	Per Liter Distilled Water
Calcium Chloride ($CaCl_2$)	2.7 g
Ferric Chloride ($FeCl_3 \cdot 6H_2O$)	0.96 g
Manganous Sulfate ($MnSO_4 \cdot 7H_2O$)	0.045 g
Cupric Sulfate ($CuSO_4 \cdot 5H_2O$)	0.039 g

Table III

Composition of Agar Medium (35)

Reagent	Per Liter Distilled Water
Difco Nutrient Agar	23.0 g
Glycerol	5.0 g
Methionine	4.6 g
Sodium Chloride (NaCl)	30.0 g
Calcium Carbonate ($CaCO_3$)	5.0 g
Trace Elements Solution (Table II)	0.05 ml
Sodium Hydroxide (NaOH) to adjust pH to 7.0	

hourly from the growth culture in order to monitor growth as a function of absorbance at 350 nm (Figure 8).

Luminescence Curve of P. fischeri

A luminescence curve of P. fischeri was obtained by measuring light intensity as a function of time (Figure 8). Light emission of aliquots of culture was measured with a photometric apparatus described by Burns (36), in which the output current from the photomultiplier tube was directly proportional to the light intensity. To maintain constant luminescence during measurement, air was continuously bubbled into the culture aliquots.

Calibration of the Photomultiplier Tube

To verify that the photomultiplier tube current was directly proportional to light intensity, the photomultiplier tube was calibrated by the Inverse Square Law (Figure 9). The photomultiplier tube was removed from its housing and placed in a vertical position on the floor in a light-tight room which gave a background reading of 2.8×10^{-8} amps. The voltage applied to the photomultiplier tube was 1080. The light source consisted of a three-volt flashlight. The reflecting surface of the flashlight was covered with black photographic paper in which a hole of approximately 0.2 mm diameter had been made. The flashlight was then suspended from the ceiling. The height of the flashlight above the

photomultiplier tube was varied and the corresponding light intensity measured. After calibration the range of the photometer was increased by adding neutral density filters. A light source consisting of a strontium-90 activated phosphor in a solid lucite cylinder (37) was used to check the instrument for constant current before each use.

Preparation of Large Quantities of Bacterial Cells

The following procedure was developed to grow large quantities of P. fischeri from which NADH-dehydrogenase could be purified. Five hundred ml of sterile medium in a one-liter, round, flat-bottom flask was inoculated with one ml of a glowing, 14-hour-old culture of P. fischeri. The flask was aerated through a gas dispersion tube and incubated at 27°C for 12 hours or until the absorbance at 350 nm reached 2.1. During the initial growth of the culture, 45 liters of liquid medium were prepared, autoclaved for 45 minutes at 15 psig, cooled to 27°C, and placed in a 60-liter polyethylene carboy. After the initial culture had reached the stationary phase of growth and peak luminescence, 450 ml were inoculated into the 45 liters of sterile medium and incubated at 27°C with agitation by aeration. The 45-liter culture was aerated at a rate of 50 liters/min. Foaming during growth was controlled by adding Dow-Corning Antifoam. The culture was incubated for eight hours or until maximum growth and luminescence were reached. The temperature of the culture was then reduced to 4°C in an ice bath. The cells were harvested by

centrifugation with a Sharple's Continuous Centrifuge at 50,000 rpm at a rate of eight liters/hr. The cells were then frozen in an ethanol-dry ice bath and stored at -25°C.

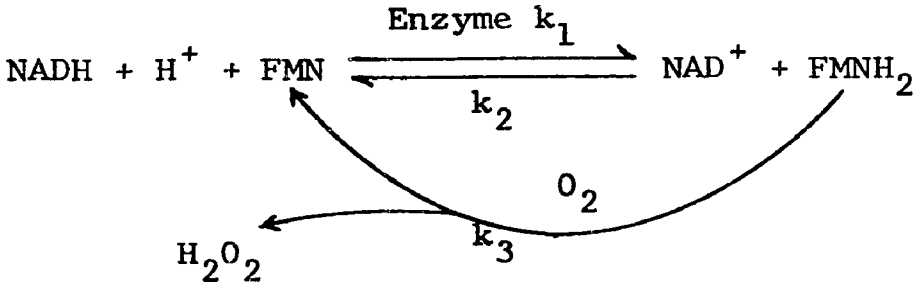
Protein Determination

The Biuret (38) and Lowry (39) methods were used for protein determination. Bovine Serum Albumin (BSA), acquired from Sigma Chemical Co., was used for calibration and as a standard. Protein elution from chromatography columns was monitored by absorbance at 280 nm.

NADH-dehydrogenase Enzyme Assay

Aerobic Assay

The activity of NADH-dehydrogenase was followed spectrophotometrically with a recording Gilford Spectrophotometer, Model 240. The NADH-dehydrogenase reaction is:



Reduced flavin mononucleotide ($FMNH_2$) is autoxidized by oxygen to FMN. Hastings and Gibson have shown that a fully reduced, 2.5×10^{-5} M FMN solution was reoxidized in

200 milliseconds when rapidly mixed with water in equilibrium with air (40). Since $k_3 >> k_1$ the overall reaction rate proceeds according to k_1 at low enzyme concentrations. NADH and FMN have spectra which overlap at 340 nm, Figures 10 and 11. Since $k_3 >> k_1$ the rate of change of absorbance at 340 nm is attributed to NADH because the absorbance due to FMN remains constant. Therefore, the oxidation of NADH to NAD^+ by NADH-dehydrogenase can be followed spectrophotometrically at 340 nm. The concentrations of the solutions used in the NADH-dehydrogenase assay appear in Table IV. A 10 mg/ml solution of BSA in 0.1 M potassium phosphate buffer, pH 7.5 was prepared. Ten mg of NADH were dissolved in five ml BSA (10 mg/ml) and then diluted to 50 ml with 0.1 M potassium phosphate buffer, pH 7.5. The NADH-dehydrogenase reaction mixture consisted of 3.0 ml NADH-BSA solution, 0.1 ml FMN, 0.3 ml 0.1 M potassium phosphate buffer, pH 7.5, and 0.1 ml enzyme for a total volume of 3.5 ml. The reaction of NADH-dehydrogenase was followed spectrophotometrically at 340 nm at 25°C.

Table IV

Solutions for Aerobic NADH-dehydrogenase Assay

Reagent	Concentration of Stock Solution
NADH	2.56×10^{-4} moles/l
BSA	10.0 g/l
FMN	7.50×10^{-5} moles/l
Potassium Phosphate Buffer, pH 7.5	0.10 moles/l

Anaerobic Assay

To observe the rate of enzymatic reduction of FMN, the assay must be performed anaerobically because of the rapidity with which $FMNH_2$ is autoxidized by oxygen. In an anaerobic environment, the reaction of NADH-dehydrogenase is:

$$NADH + H^+ + FMN \underset{k_2}{\overset{\text{Enzyme } k_1}{\rightleftharpoons}} NAD^+ + FMNH_2$$

A schematic of the apparatus and procedure developed to obtain an anaerobic environment for the NADH-dehydrogenase assay appears in Figure 2. Apiezon grease was used to provide a vacuum seal for all ground glass joints. The apparatus was first flushed with pre-purified (>95% oxygen-free) nitrogen gas, Matheson Chemical Co. The nitrogen gas flowed into the first impinger which contained Fieser's solution. Fieser's solution, composed of anthroquinone-β-sulfonate and sodium hyposulfite (41), absorbs oxygen. From the first impinger the gas flowed into the second impinger which also contained Fieser's solution. Upon leaving the last impinger the nitrogen gas was free of oxygen. The oxygen-free nitrogen gas then entered a specially designed dual side-arm cuvette, Figure 2, through a double stopcock. The other side of the double stopcock was attached to a vacuum. The vacuum was used to change the atmosphere in the cuvette to pure nitrogen in the following manner. The NADH-BSA substrate solution was placed in the base of the cuvette. FMN substrate was added

to one arm of the cuvette and the enzyme placed in the other arm. A vacuum was slowly applied to remove the atmospheric gases within the solutions at 25°C. As the temperature of the cuvette was reduced to 0°C with an ice bath, nitrogen gas was admitted to the cuvette. Again a vacuum was slowly applied to the cuvette, this time at 0°C. The temperature was slowly raised to 25°C to avoid bumping which could result in premature mixing of enzyme and substrate solutions. To insure an oxygen-free atmosphere in the cuvette, the above steps were repeated four times. Contaminating oxygen could

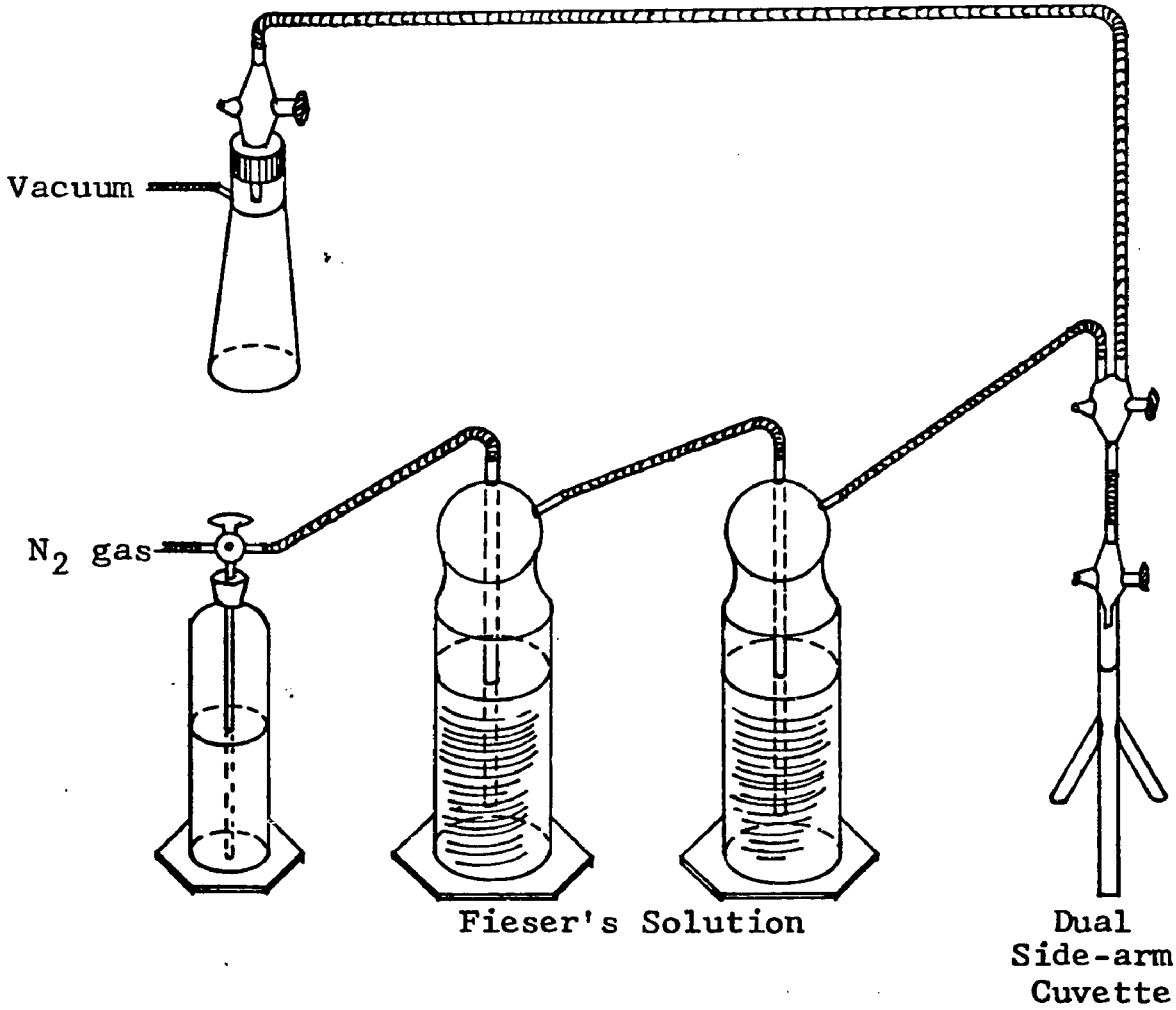


Figure 2. Schematic of Apparatus for Anaerobic NADH-dehydrogenase Assay.

easily be detected because the absorbance of FMN at 450 nm would remain constant. The concentrations of the components of the NADH-dehydrogenase anaerobic assay mixture appear in Table V. Ten mg of NADH were dissolved in two ml BSA

Table V

Solutions for Anaerobic NADH-dehydrogenase Assay

Reagent	Concentration of Stock Solution
NADH	1.28×10^{-3} moles/l
BSA	10.0 g/l
FMN	7.50×10^{-5} moles/l
Potassium Phosphate Buffer, pH 7.5	0.10 moles/l

(10 mg/ml) and then diluted to 10 ml with 0.1 M potassium phosphate buffer, pH 7.5. The NADH-dehydrogenase reaction mixture consisted of 0.5 ml NADH-BSA solution, 0.5 ml FMN, 0.2 ml 0.1 M potassium phosphate buffer, pH 7.5, and 0.1 ml enzyme for a total volume of 1.3 ml. The reaction was followed spectrophotometrically at 450 nm and 25°C.

Purification of NADH-dehydrogenase from P. fischeri

The following procedure, which was repeated several times, gives the specific details of a typical purification of NADH-dehydrogenase from 280 g wet P. fischeri cells.

Lysis

Johnson and Harvey have shown that P. fischeri, a marine bacteria, is lysed by osmotic pressure when suspended in distilled water (42). P. fischeri cells were lysed in five ml of cold, deionized, distilled water per gram of cells. All solutions used in the purification procedure contained 0.01 M 2-mercaptoethanol. All steps of the purification procedure were performed at 4°C. Deoxyribonuclease (DNAase) and ribonuclease (RNAase) were each added to the crude extract to a final concentration of one mg/liter. DNAase and RNAase were obtained from Sigma Chemical Company. After stirring for 30 minutes the cell debris was removed by centrifugation at 23,300 g at 4°C for 90 minutes. The supernatant was decanted and the pellet re-extracted. The supernatants from both extractions were pooled; the pellets were discarded.

Ammonium Sulfate Precipitation

An experiment to determine at which ammonium sulfate concentrations NADH-dehydrogenase was selectively precipitated was performed. The amount of solid ammonium sulfate to be added to obtain a particular percent saturation was obtained from the nomogram by Dixon (43). Reagent grade ammonium sulfate was added to the nuclease treated crude lysate to 30% saturation; the pH was maintained at 7.5 by the addition of 3.0 M potassium hydroxide (KOH). The 30% precipitate was removed by centrifugation at 23,300 g for 15 minutes and saved. The 30% supernatant was increased to 40% ammonium sulfate

saturation, and the newly formed precipitate was removed as above and saved. In an analogous manner precipitates at 50%, 60%, 70%, 80% and 90% ammonium sulfate saturation were obtained. The precipitates were dissolved in 0.05 M potassium phosphate buffer, pH 7.5. The solutions were dialyzed in 5-liter changes of 0.05 M potassium phosphate buffer, pH 7.5 until no barium sulfate ($BaSO_4$) precipitate was formed with the addition of barium chloride ($BaCl_2$) to the dialysate. After dialysis the solutions were diluted to equal volumes, after which the enzyme assays were performed, Figure 12. NADH-dehydrogenase activity was found in the precipitates obtained at 50%, 60%, 70% and 80% saturation with respect to ammonium sulfate. By an additional assay no NADH dehydrogenase activity was found above 75% ammonium sulfate saturation. Therefore, in the NADH-dehydrogenase purification procedure 0-40% and 40-75% ammonium sulfate cuts were made. The 40% ammonium sulfate precipitate was removed by centrifugation at 23,300 g for 15 minutes and discarded. The supernatant was then saturated to 75% with ammonium sulfate and the precipitate was removed by centrifugation and dissolved in 0.05 M potassium phosphate buffer, pH 7.5. The solution was dialyzed over a 14-hour period against four changes of 20 liters of 0.05 M potassium phosphate buffer, pH 7.5.

DEAE-Sephadex Column Chromatography

A 4.0 x 46 cm DEAE-Sephadex (Pharmacia) column was equilibrated with 0.05 M potassium phosphate buffer, pH 7.5. The void volume of the DEAE-Sephadex column bed was

determined with Blue Dextran 2000. The dialyzed solution of the 40-75% ammonium sulfate precipitate was applied to the column at the rate of 60 ml/hr and then rinsed with buffer until the eluate had no absorbance at 280 nm. The enzyme was then eluted with a four-liter linear gradient from 0.05 to 0.5 M potassium phosphate buffer, pH 7.5. Fractions containing 22 ml of eluate were collected at a flow rate of 75 ml/hr. The first fraction collected after the void volume was considered to be the first fraction of the linear gradient. Fractions were assayed for absorbance at 280 nm and for NADH-dehydrogenase activity. Fractions containing NADH-dehydrogenase activity were pooled. The elution pattern of the DEAE-Sephadex column appears in Figure 13.

Concentration by Diaflo

The pooled fractions of NADH-dehydrogenase from the DEAE-Sephadex column were concentrated by using a Model 402 Diaflo with a PM-10 ultrafiltration membrane at a nitrogen gas pressure of 50 psig. The 1045 ml of pooled enzyme solution from the DEAE-Sephadex column was reduced to 90 ml by this method. The concentrated enzyme solution was aliquoted into three 30-ml fractions. An enzyme assay on the membrane effluent indicated that no NADH-dehydrogenase had penetrated the membrane.

G-75 Sephadex Column Chromatography

A 5.0 x 90 cm column of G-75 Sephadex (Pharmacia, dry pore size 40-120 microns) was equilibrated with 0.1 M

potassium phosphate buffer, pH 7.5. One 30 ml aliquot of concentrated enzyme solution from the DEAE-Sephadex column was applied to the G-75 Sephadex column. The column was eluted at a rate of 36 ml/hr with 0.10 M potassium phosphate buffer. Fractions of 12 ml were collected, analyzed for absorbance at 280 nm, and assayed for NADH-dehydrogenase activity; the elution pattern is shown in Figure 14. Fractions containing NADH-dehydrogenase were pooled. This fractionation procedure was repeated twice with the two remaining 30-ml aliquots from the DEAE-Sephadex column. The pooled NADH-dehydrogenase solutions from all three columns were combined. The solution was concentrated by ultrafiltration using a PM-10 membrane in a Model 52 Diaflo Cell.

Superfine G-75 Column Chromatography

Two columns 2.5 cm in diameter of Superfine G-75 Sephadex (Pharmacia) with a combined length of 186 cm, were equilibrated with 0.1 M potassium phosphate buffer, pH 7.5. The pooled, concentrated NADH-dehydrogenase solution from the column of Sephadex G-75 was loaded onto the column of Superfine G-75 Sephadex. The column was eluted with 0.1 M potassium phosphate buffer, pH 7.5. Fractions of three ml were collected at the rate of eight ml/hr and were analyzed for absorbance at 280 nm and NADH-dehydrogenase activity; the elution pattern appears in Figure 15. Fractions containing NADH-dehydrogenase were pooled and concentrated by Diaflo. The concentrated, purified enzyme solution was aliquoted into one-ml fractions, frozen in an ethanol-dry ice bath, and

stored at -25°C until used for characterization and X-irradiation studies.

Purity of NADH-dehydrogenase

Disc Gel Electrophoresis

Disc gel electrophoresis of NADH-dehydrogenase eluted from Superfine G-75 was run as described by Brewer and Ashworth (44) at a pH of 8.9 with a lower gel concentration of 7.5% acrylamide. Samples of NADH-dehydrogenase varying in concentration from 50-250 μg in 10% sucrose were applied to 5 x 60 mm gels. The gels were electrophoresed at 1.5 milliamps/gel for approximately two hours or until the tracking dye was 0.5 mm from the bottom of the gel. The gels were stained for protein with Coomassie brilliant blue by the method of Chrambach (45). The gels were scanned at 550 nm on a linear transport coupled to a Gilford Spectrophotometer, Model 240. Gel scans appear in Figures 16 and 17.

NADH-dehydrogenase Extraction

To determine which protein band or bands on the gel scan contained NADH-dehydrogenase, an unstained gel was sliced into 0.25 cm sections. Each section was homogenized in two ml 0.1 M potassium phosphate buffer, pH 7.5 with a tissue homogenizer; the debris was removed by centrifugation. The extracted protein associated with each band was assayed for NADH-dehydrogenase activity.

Dehydrogenase Stain

A dehydrogenase stain, developed by Brewer and Ashworth (44), was used to locate NADH-dehydrogenase on the gel. Table VI lists the required reagents for the dehydrogenase stain.

Table VI

Dehydrogenase Stain (44)

Reagent	Concentration of Stock Solution		Volume
Tris(hydroxymethyl)aminomethane pH 9.0	1.0	moles/l	0.5 ml
Nicotinamide adenine dinucleotide (NAD^+)	0.01	moles/l	0.1 ml
Nitro blue tetrazolium (NBT)	10.0	g/l	0.1 ml
Phenazine methosulfate (PMS)	0.4	mg/l	0.1 ml
Substrate (NADH)	0.01	moles/l	0.1 ml
Water, distilled			1.1 ml

Luciferase as an Impurity

To determine the presence of luciferase as an impurity, a luciferase assay was performed after each step of the NADH-dehydrogenase purification procedure, Table XII. To assay for luciferase, the luciferase reaction was coupled to the NADH-dehydrogenase reaction and the resultant luminescence measured. Luminescence was measured with a photomultiplier tube, the high voltage for which was supplied by a Hamner Model N401 high voltage supply. The photomultiplier tube

current was measured by a Keithley Model 410 picoammeter. The luminescence produced by the coupled enzyme reaction was a measure of luciferase activity. The concentrations of the solutions used in the luciferase assay appear in Table VII.

Table VII

Solutions for Luciferase Assay

Reagent	Concentration of Stock Solution
NADH	1.28×10^{-3} moles/l
BSA	10.0 g/l
FMN	7.5×10^{-5} moles/l
Decyl aldehyde	1.2×10^{-3} moles/l
Potassium Phosphate Buffer, pH 7.5	0.10 moles/l

Luciferase activity was determined as follows. The cuvette was placed in the photomultiplier apparatus. Into the cuvette was placed 0.5 ml of NADH-dehydrogenase solution from one of the steps of the enzyme preparation. Ten mg of NADH were dissolved in two ml BSA (10 mg/ml) and then diluted to 10 ml with 0.1 M potassium phosphate buffer, pH 7.5. NADH-BSA solution (0.5 ml) and 0.1 ml decyl aldehyde were added to the enzyme. Oxygen, a required substrate for the luciferase reaction, was provided by allowing the assay solutions to be in equilibrium with air. The 0.1 ml of FMN solution was added last because it initiates the coupled reaction. NADH was depleted in approximately one minute during which time the maximum current was recorded. The dark current of

the photomultiplier tube was 5.6×10^{-10} amps; this lower limit of detectibility was approximately equal to one μg of luciferase.

Visible and Ultraviolet Absorption Spectra

Visible and ultraviolet absorption spectra of NADH, FMN, and NADH-dehydrogenase were determined at 25°C in 0.1 M potassium phosphate buffer, pH 7.5 with a Beckman DK-2 recording spectrophotometer.

NADH

Reduced and oxidized spectra of NADH (Sigma Chemical Co.) were run at a concentration of 4.80×10^{-5} M, Figure 10. NADH was oxidized by treatment with 0.1 M potassium phosphate buffer, pH 5.0 for 24 hours after which the pH was adjusted to 7.5 with potassium hydroxide (KOH). Fawcett has shown that NADH is relatively stable for a few hours in base solutions of pH greater than 7.5, whereas, NADH in acid solution is slowly oxidized to NAD^+ (46).

FMN

Reduced and oxidized spectra of FMN (Sigma Chemical Co.) were run at a concentration of 2.5×10^{-5} M, Figure 11. FMN was anaerobically reduced with sodium borohydrate ($NaBH_4$); the spectra for $FMNH_2$ were run from 340 to 500 nm.

NADH-dehydrogenase

Reduced and oxidized spectra of purified NADH-dehydrogenase were run at 150 μg/ml, Figure 23. The enzyme was reduced with sodium dithionite ($Na_2S_2O_4$).

Molecular Weight Estimation

The molecular weight of NADH-dehydrogenase was estimated by column chromatography on Superfine G-75 Sephadex (Pharmacia, dry pore size 10-40 microns) (47). The void volume of the 2.5 x 96 cm column was determined with Blue Dextran 2000. Proteins of known molecular weight, Table VIII, which were used to calibrate the column were acquired from Pharmacia.

Table VIII

Standards for Chromatography Column Calibration

Standard	Source	Molecular Weight
Blue Dextran 2000	Sigma Chemical Co.	2,000,000
Bovine Serum Albumin	Bovine Serum	67,000
Ovalbumin	Egg White	45,000
Chymotrypsinogen A	Bovine Pancreas	25,000
Ribonuclease A	Bovine Pancreas	13,700

Two ml of purified NADH-dehydrogenase was applied to the column along with Blue Dextran and chymotrypsinogen A which served to check column stability. The column was eluted with 0.1 M potassium phosphate buffer, pH 7.5 at a flow rate of nine ml/hr. Fractions of 2.1 ml were collected and analyzed.

Figure 24 shows the elution pattern of protein from the column as determined by absorption at 280 nm; NADH-dehydrogenase was located on the column by assaying for enzyme activity. The apparent molecular weight of NADH-dehydrogenase was determined from the chromatography column molecular weight calibration curve, Figure 25.

Irradiation Studies

X-ray Generator

The irradiation studies of the NADH-dehydrogenase and luciferase reactions were done with a General Electric Maxitron 300 X-ray unit. The unit had a beryllium window 4.75 mm thick. The only filtration used throughout the study was the inherent beryllium filter. The X-ray generator was operated at 250 kVp with a tube current of 20 milliamps throughout the study except where otherwise indicated. Using ferrous sulfate dosimetry, the half value layer was determined to be 4.2 mm of aluminum.

X-ray Beam Collimation

Collimation of the X-ray beam was achieved with five cm of lead plates. Each plate, 15 x 15 x 0.63 cm, had a central, rectangular opening of 1.5 x 1.2 cm.

Irradiation Chamber

An irradiation chamber was designed to meet the following specifications:

1. To allow ultraviolet and visible absorption spectrophotometry during irradiation,
2. To shield the monochromator from the collimated or scattered X-rays,
3. To shield the photometer from the collimated or scattered X-rays,
4. To allow coupling to a Gilford Spectrophotometer Model 240,
5. To allow enzyme assays of small sample volume,
6. To allow use of standard size cuvettes, and
7. To allow intersection of the X-ray beam and monochromator beam.

A cell to meet the above specifications was made of lead encased in 0.63 cm thick aluminum. The cell was easily installed in the Gilford spectrophotometer by replacing the sample handling system of the instrument. The distance from the tungsten target to the center of the cuvette was 14 cm. The maximum dose from scattered X-rays at 250 kVp and 20 milliamps delivered to the monochromator and the photometer of the spectrophotometer was determined to be 30 rads/min and 0.2 rads/min, respectively. A schematic drawing of the chamber appears in Figure 3.

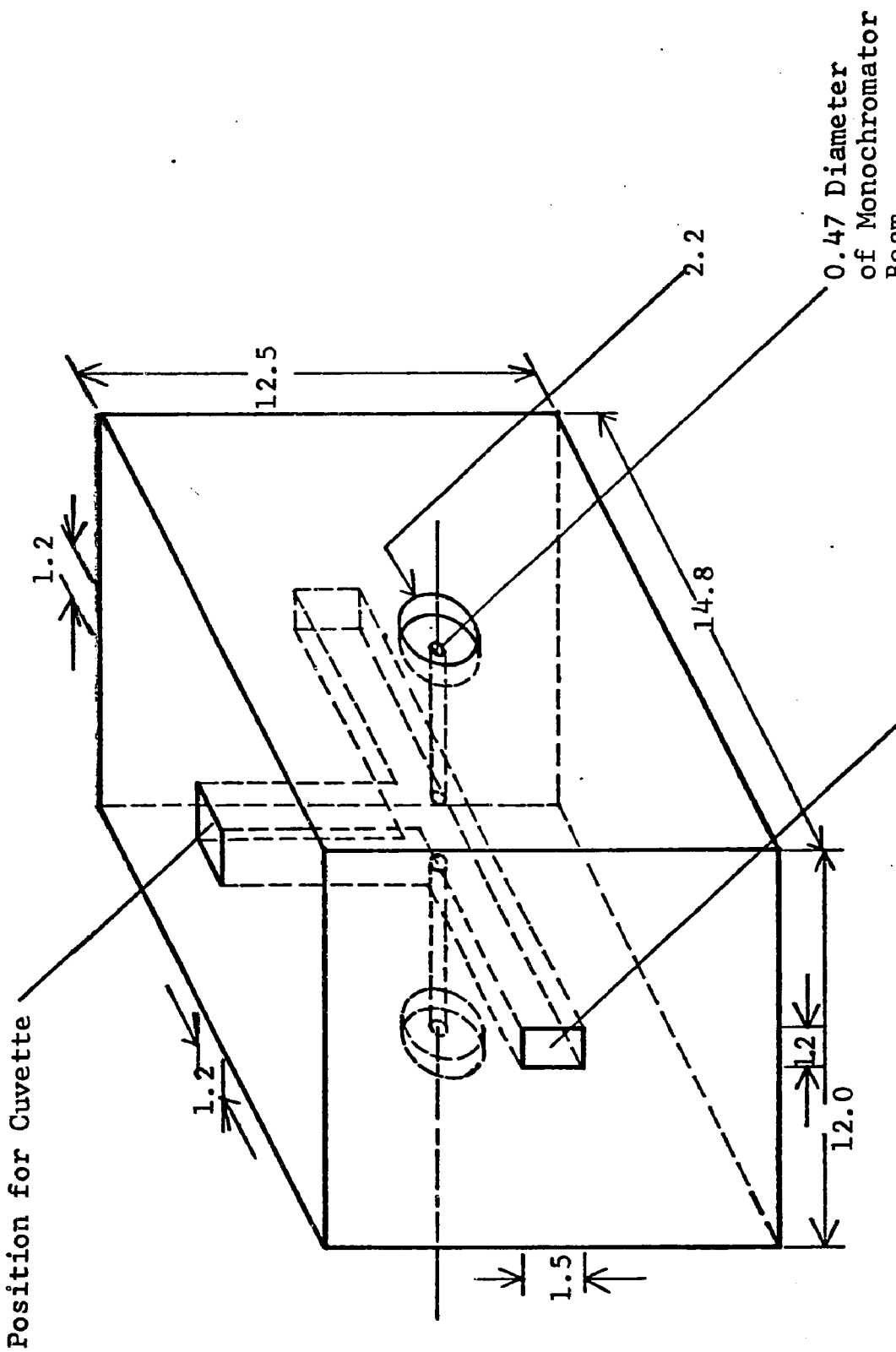


Figure 3. Schematic of Irradiation Chamber. All dimensions are in centimeters.

Cuvettes

The enzyme studies during irradiation were performed in cuvettes made of T-20 Supracil II. The Supracil material was selected because the material is practically fluorescent free and no darkening of the material occurs even after prolonged exposure to ultraviolet, X-ray, or gamma radiation. The Supracil material was produced by Engelhard Industries, Inc., Amersil Quartz Division, 685 Ramsey Avenue, Hillside, New Jersey. The cuvettes were manufactured by Smyth Precision Glass Mfg. Co., 3127 Central Avenue, Union City, New Jersey. Only the two polished surfaces of the cuvettes were made of Supracil; the other two surfaces were made of quartz. The finished cuvettes were 1.4 x 1.4 x 3.8 cm with a 1.0 cm path length. The Supracil cuvettes were tested for darkening by exposing them to X-rays for 100,000 rads, after which the absorbance at 340, 450, and 550 nm was measured. After irradiation there was no discoloration or change of absorption of the Supracil cuvettes.

Dose Rate Determination

Ferrous sulfate dosimetry was used to determine the dose rate within the irradiation chamber. The molar extinction coefficient of the ferric ion (Fe^{+++}) was determined by the method of the National Bureau of Standards (48). The value obtained in 0.8 N sulfuric acid (H_2SO_4) at 20°C was 2090 $\pm$ 30 liter $moles^{-1}cm^{-1}$. The density of the solution was

1.024 ± 0.001 (49). A G-value of 14.9 ± 0.8 was used in the dose determination (50). The dose rate was calculated to be 1480 ± 87 rads/min.

Apparatus for Measuring NADH-dehydrogenase Activity During Irradiation

The NADH-dehydrogenase reaction was studied before, during and after X-irradiation by coupling a recording spectrophotometer to an X-ray generator. The sample handling system of the Gilford Spectrophotometer, Model 240 was replaced with the irradiation chamber. A block diagram and a picture of the apparatus appear in Figures 4 and 5. After assembling the apparatus, the recording spectrophotometer was used in the usual manner to record the kinetics of the NADH-dehydrogenase reaction.

Apparatus for Measuring Luminescence

The coupled enzyme reaction between NADH-dehydrogenase and luciferase was monitored during irradiation by utilizing the luminescence produced by the luciferase reaction. The luciferase reaction produces a spectrum of light with a peak at 480 nm which can be followed with a photomultiplier tube. The apparatus consisted of coupling the irradiation chamber to a Dumont No. 6292 photomultiplier tube which was powered by a Hamner Model N401 high voltage supply. The voltage applied to the photomultiplier tube was 1080 volts. The photomultiplier current was measured by a Keithley Model 410 picoammeter. The current was recorded with a Varian Model

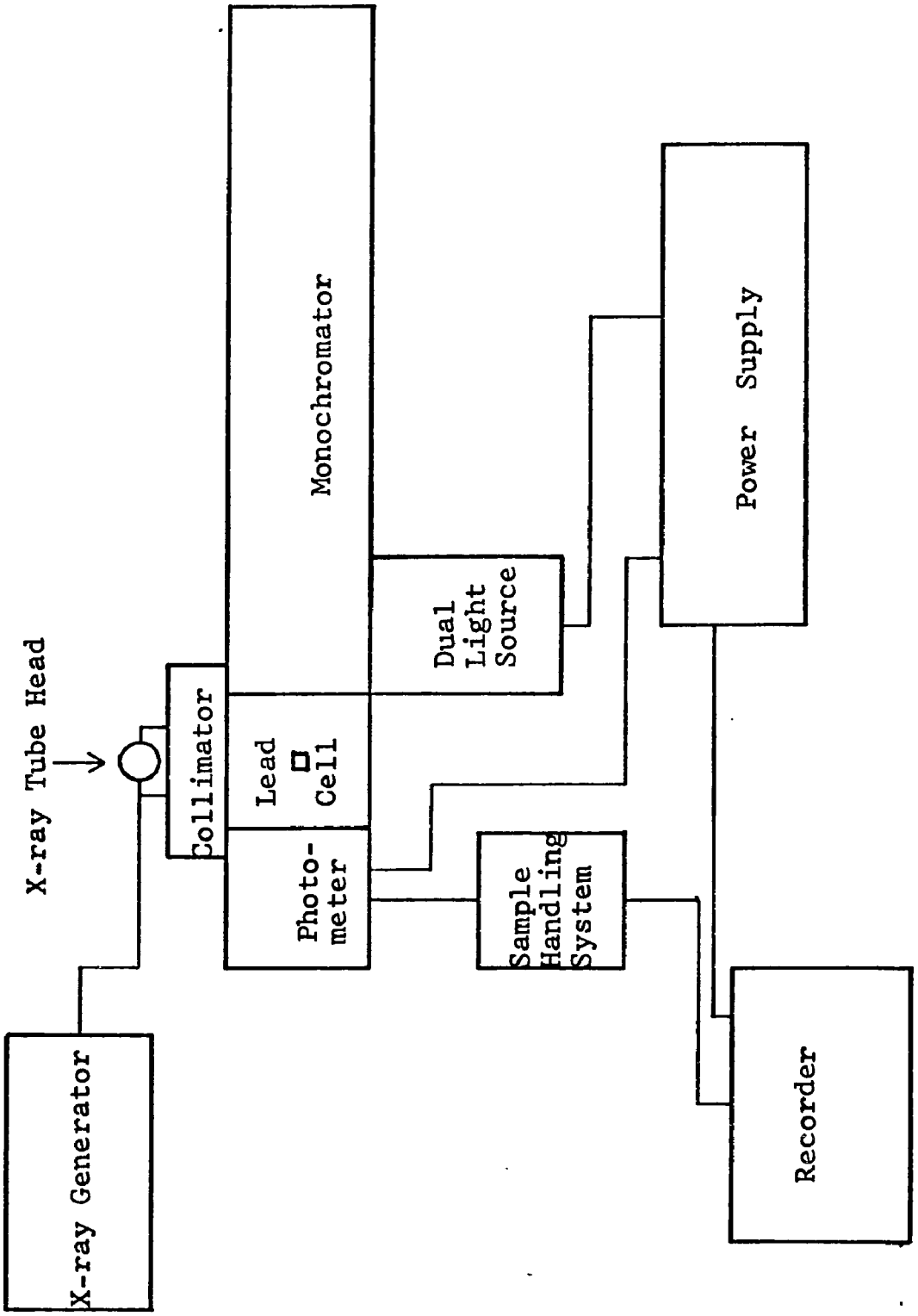


Figure 4. Block Diagram of Experimental Apparatus for Measuring the Reaction of NADH-dehydrogenase before, during, and after Irradiation.



Figure 5. View of Apparatus for Measuring the NADH-dehydrogenase Reaction during Irradiation.

G-14 strip chart recorder. Because the photomultiplier tube was light sensitive, the irradiation chamber and the photomultiplier tube were enclosed with black plastic polyethylene sheeting; the background radiation was lowered to 5.6×10^{-10} amps. A schematic diagram of the apparatus is shown in Figure 6; a picture of the irradiation chamber and photomultiplier tube is shown in Figure 7. The concentrations of solutions used in the NADH-dehydrogenase-luciferase reaction mixture appear in Table IX.

Table IX

Solutions for the NADH-dehydrogenase-Luciferase Reaction

Reagent	Concentration of Stock Solution	
Luciferase	2.5	mg/ml
NADH	1.28×10^{-3}	moles/l
BSA	10.0	g/l
FMN	7.5×10^{-6}	moles/l
Decyl aldehyde	1.2×10^{-3}	moles/l

Ten mg of NADH were dissolved in five ml BSA (10 mg/ml) and diluted to ten ml with 0.1 M potassium phosphate buffer, pH 7.5. Luciferase (Sigma Chemical Co.), FMN, and decyl aldehyde solutions were made up in 0.1 M potassium phosphate buffer. All solutions were in equilibrium with the air. The reaction mixture consisted of 0.50 ml luciferase, 0.25 ml NADH-BSA, 0.35 ml FMN, 0.10 ml aldehyde, and 0.10 ml purified NADH-dehydrogenase. Luminescence of the coupled enzyme reaction was measured before, during, and after X-irradiation.

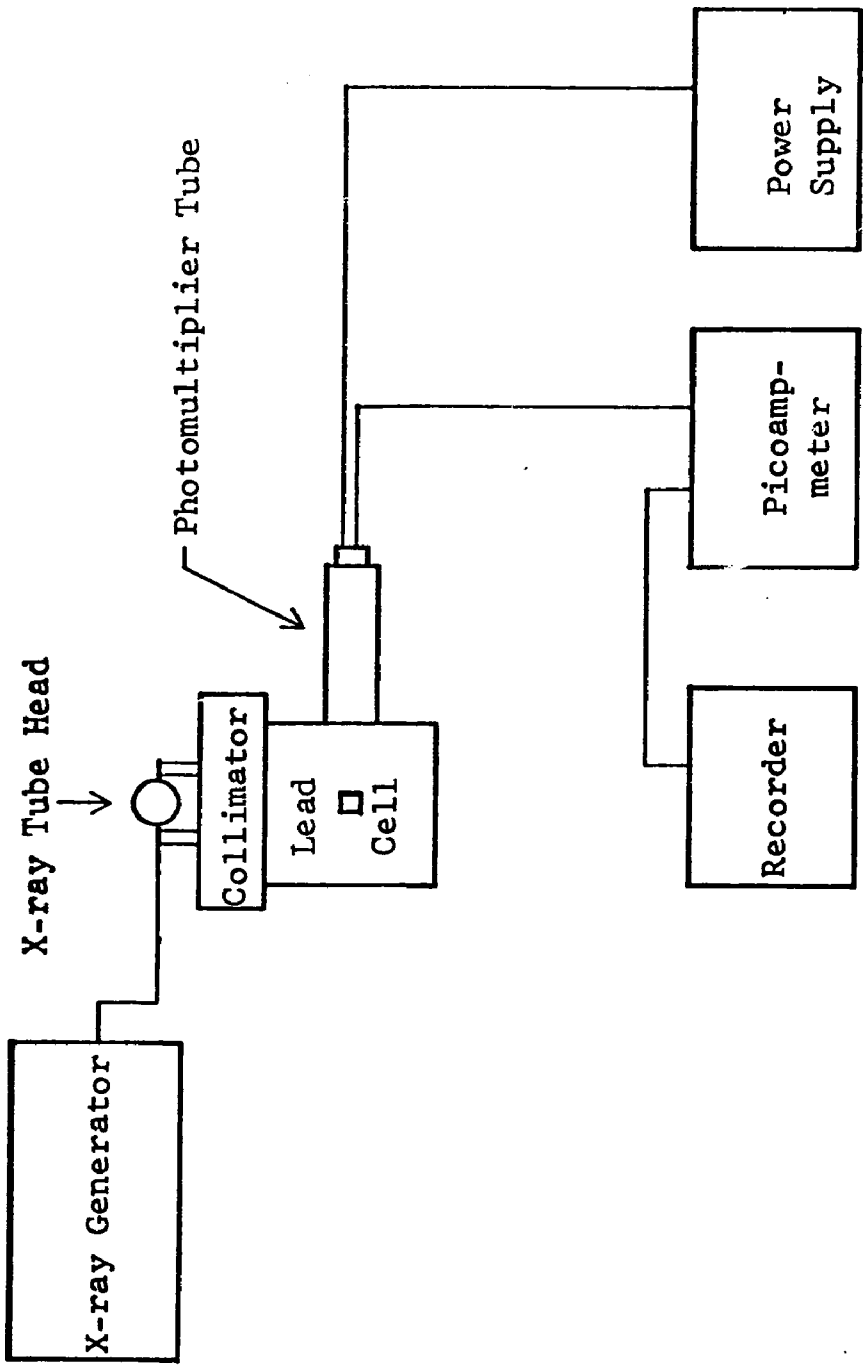


Figure 6. Block Diagram of Experimental Apparatus for Measuring Luminescence Produced by the Coupled Enzyme Reaction of NADH-dehydrogenase and Luciferase.

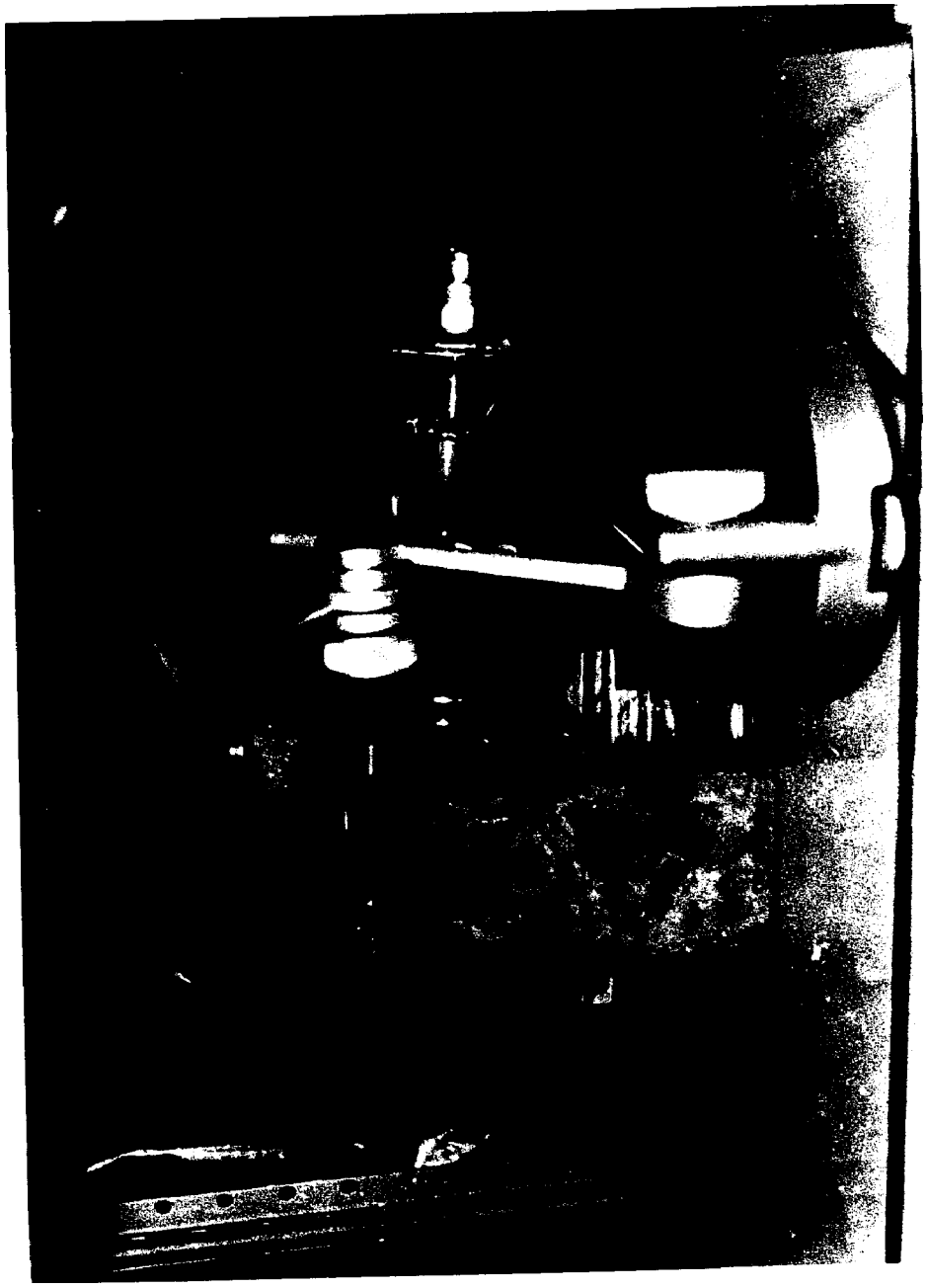


Figure 7. View of Apparatus for Measuring Luminescence during Irradiation.

RESULTS

Purification of NADH-dehydrogenase

Large quantities of Photobacterium fischeri were grown for a source of NADH-dehydrogenase. As shown in Table X, several batches were grown, the average yield for which was 6.34 g of wet bacterial cells/liter of culture.

Table X

Yield of Forty-five-liter Batches of P. fischeri

Yield	g/liter of culture
290	6.44
293	6.51
273	6.07
280	6.22
291	6.47

The point in the growth of P. fischeri at which the cells were harvested was determined from the growth and luminescence curves of P. fischeri, Figure 8. Peak luminescence occurred 12 hours after inoculation; peak luminescence was 6.5×10^{-5} amps and corresponding absorbance at 350 nm was 2.1. The

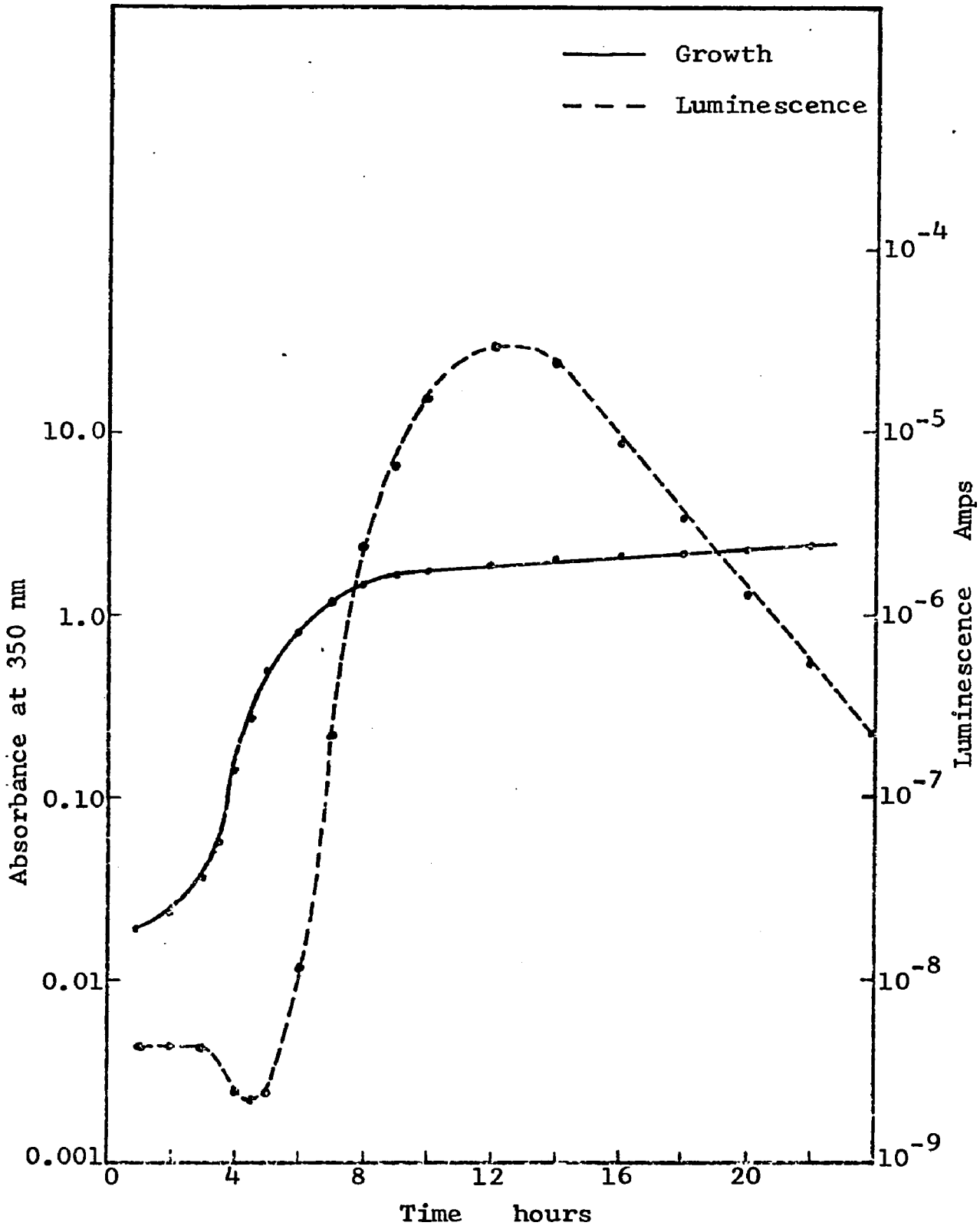


Figure 8. Growth Curve and Luminescence Curve of Photobacterium fischeri.

proportionality of the photomultiplier tube was determined by the Inverse Square Law, Figure 9; the photomultiplier tube was shown to be linear over at least three orders of magnitude.

In the enzyme reaction of NADH-dehydrogenase, NADH is oxidized to NAD^+; the loss of the peak at 340 nm during oxidation of NADH provides a reliable measure of the reaction rate. Spectra of NADH and NAD^+, run at a concentration of 4.80×10^{-5} M, appear in Figure 10. NADH has absorption maxima at 260 and 340 nm; NAD^+ has one maximum at 265 nm. The molar extinction coefficient of NADH at 340 nm and pH 7.5 was calculated to be 6.2×10^3 liter $mole^{-1}cm^{-1}$. Spectra of FMN and $FMNH_2$, run at a concentration of 2.5×10^{-5} M appear in Figure 11. FMN has absorption maxima at 260, 370, and 450 nm; $FMNH_2$ does not exhibit a maximum at 450 nm allowing the anaerobic reduction of FMN to be followed at 450 nm. The molar extinction coefficient of FMN at 450 nm was calculated to be 1.2×10^4 liter $moles^{-1}cm^{-1}$.

The supernatant from lysis of *P. fischeri* cells was a clear, amber-colored fluid in which NADH-dehydrogenase was soluble. NADH-dehydrogenase was selectively precipitated by ammonium sulfate as shown in Figure 12; in the purification procedure a 40-75% ammonium sulfate cut was made. After dialysis the enzyme solution was applied to a DEAE-Sephadex column, the elution pattern of which appears in Figure 13. Fractions 76-123 which contained NADH-dehydrogenase were pooled and concentrated from 1045 to 90 ml by Diaflo. Figure 14 shows the elution pattern of NADH-dehydrogenase from the G-75 Sephadex column; fractions 118-138 contained

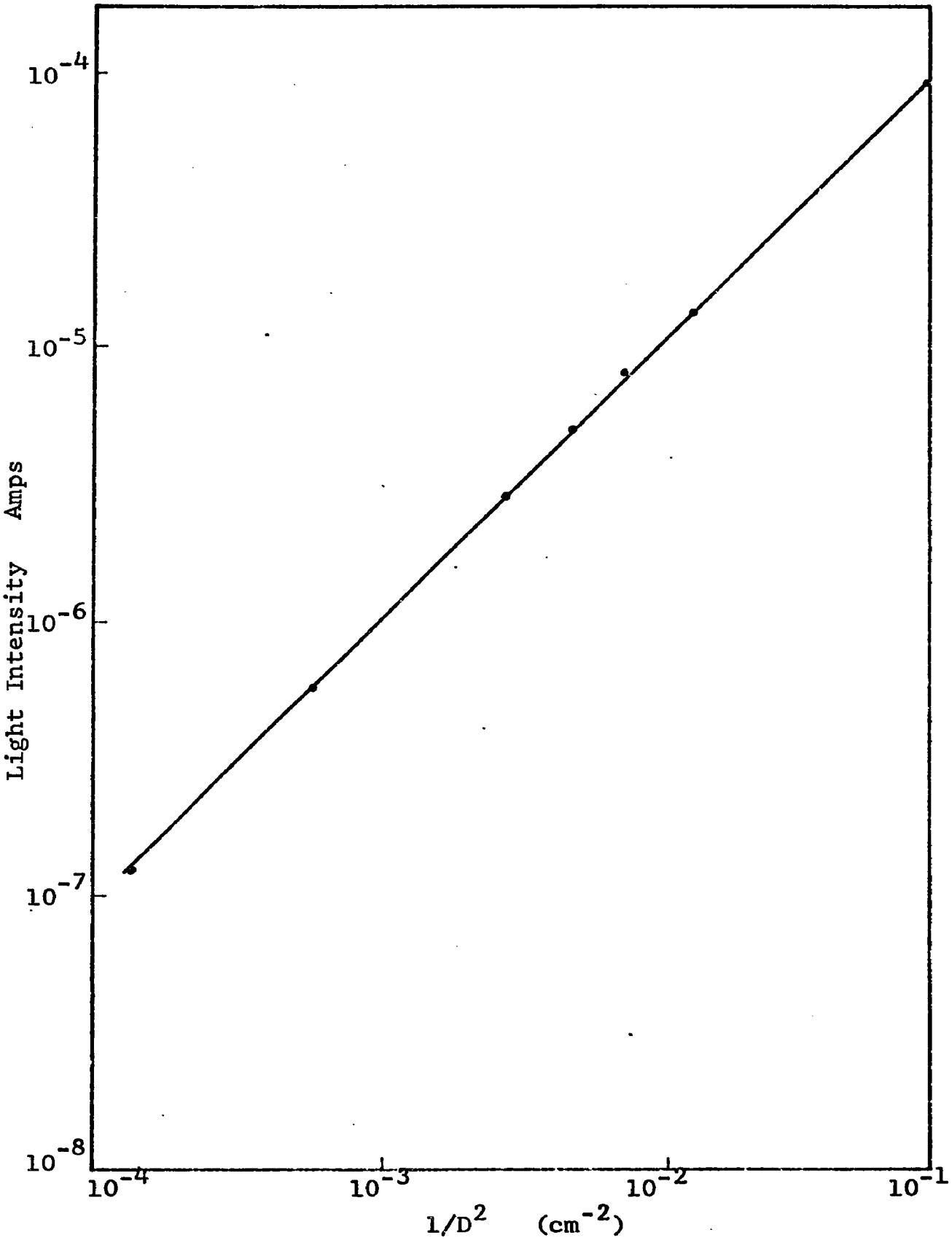


Figure 9. Calibration of the Photomultiplier Tube by Inverse Square Law.

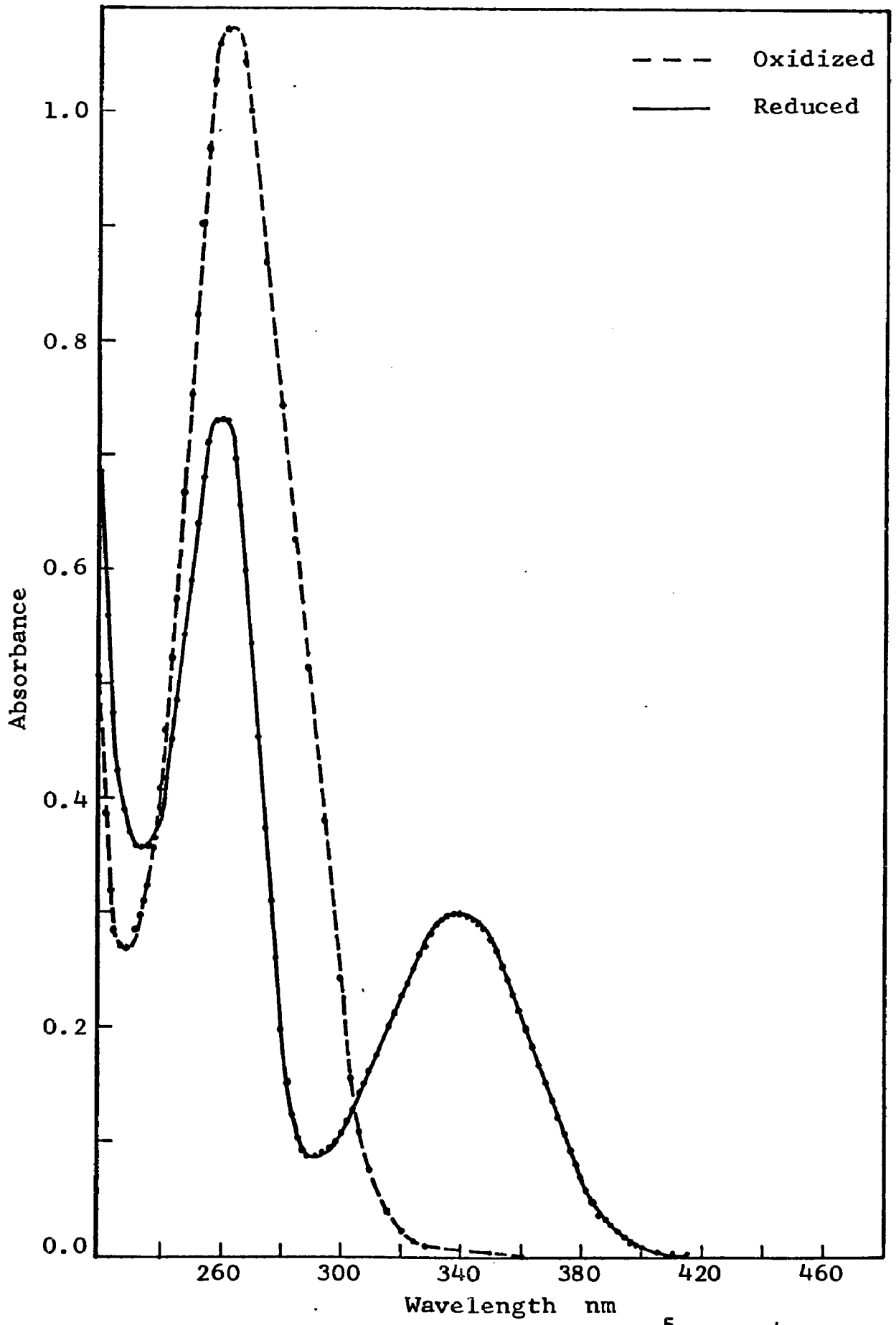


Figure 10. Absorption Spectra of 4.80×10^{-5} M NAD^+ and NADH.

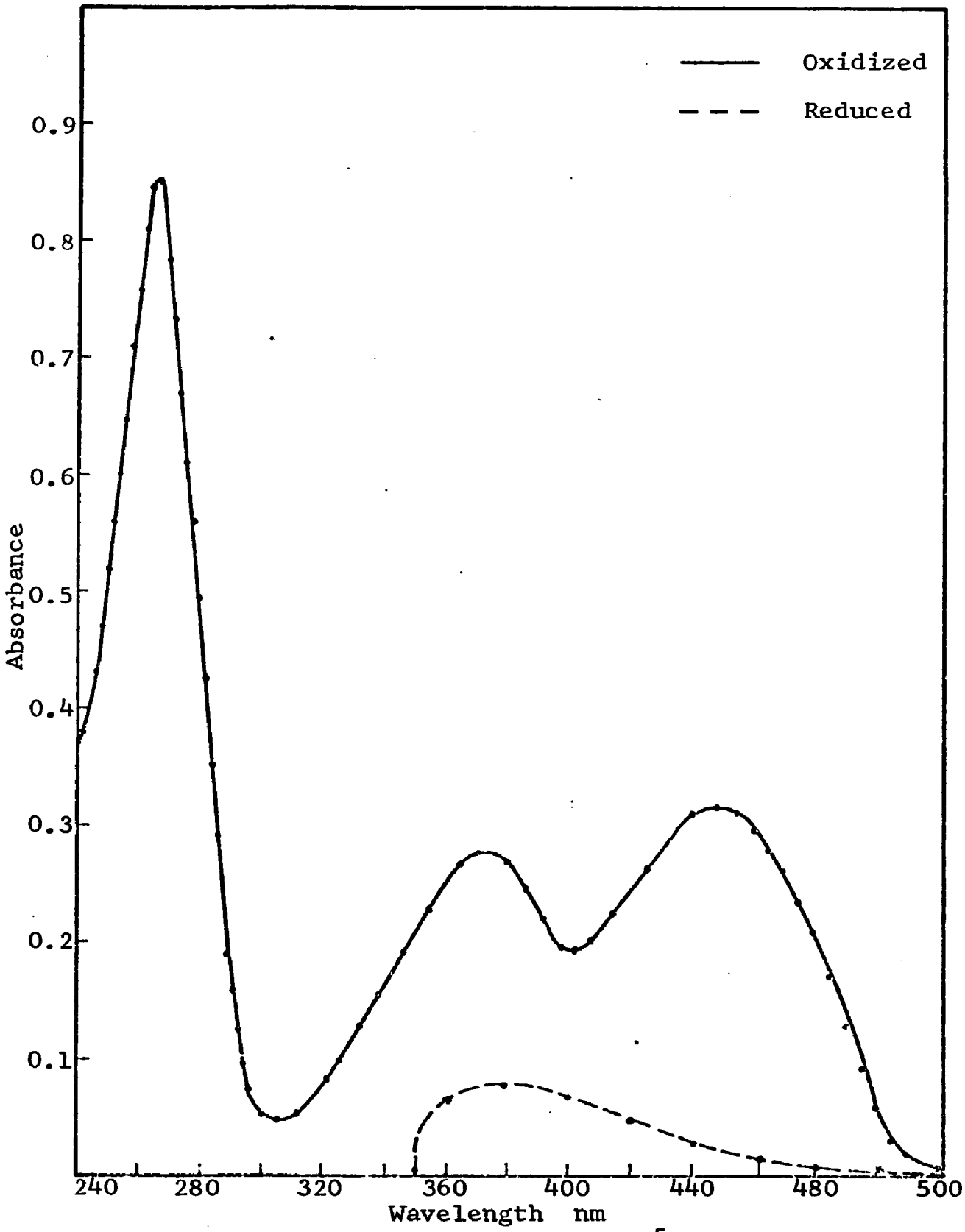


Figure 11. Absorption Spectra of 2.5×10^{-5} M FMN and $FMNH_2$.

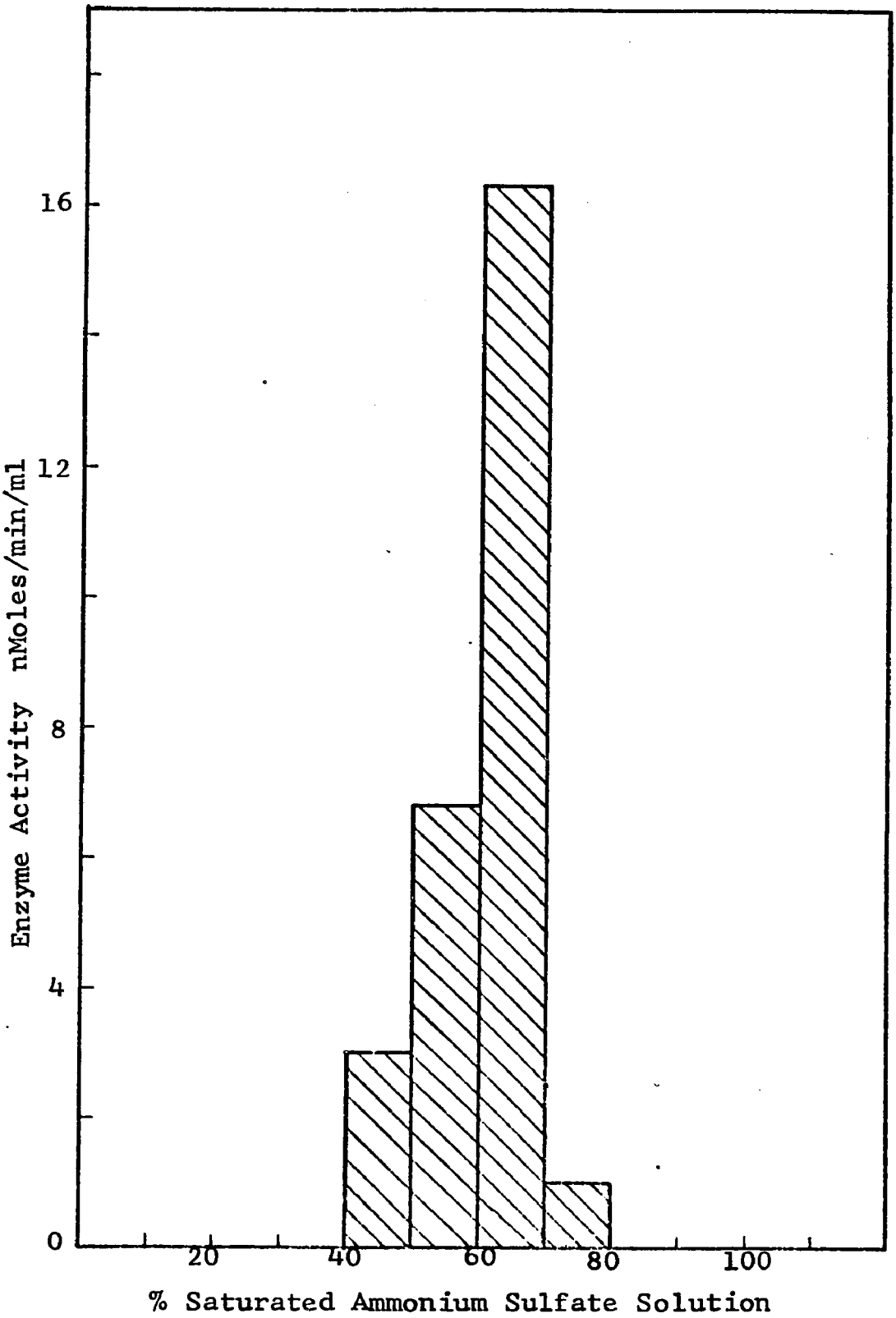


Figure 12. Selective Protein Precipitation by Ammonium Sulfate.

Figure 13. DEAE-Sephadex Column Chromatography of NADH-dehydrogenase.

A 4.0 x 46 cm column was eluted with a four-liter linear gradient of 0.05 to 0.5 M potassium phosphate buffer, pH 7.5 at the rate of 75 ml/hr. Fractions of 22 ml were collected.

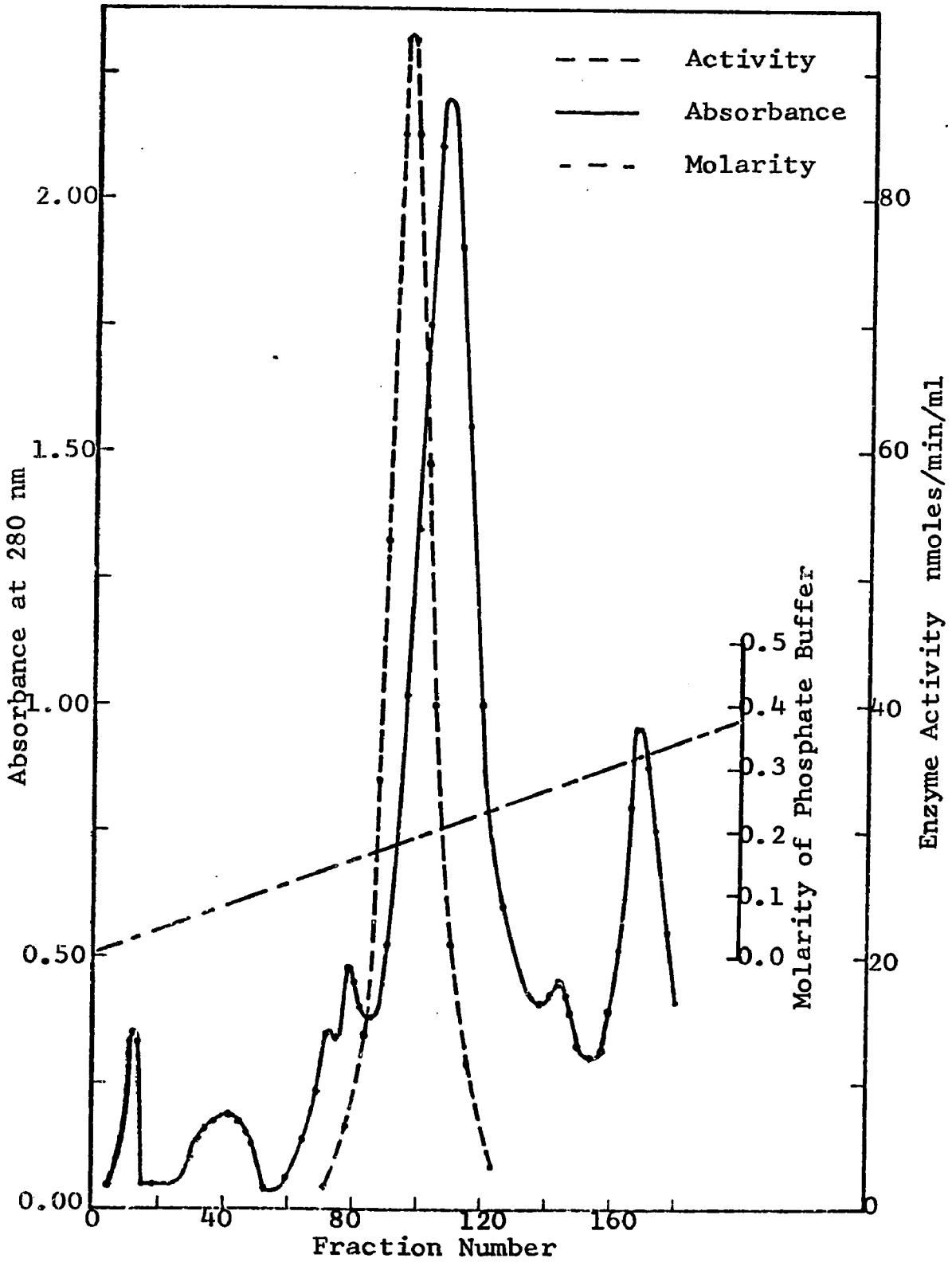


Figure 13. DEAE-Sephadex Column Chromatography of NADH-dehydrogenase.

Figure 14. G-75 Sephadex Column Chromatography of NADH-dehydrogenase.

A 5.0 x 90 cm G-75 Sephadex column was eluted with 0.1 M potassium phosphate buffer, pH 7.5. Fractions of 12 ml were collected at a flow rate of 36 ml/hr.

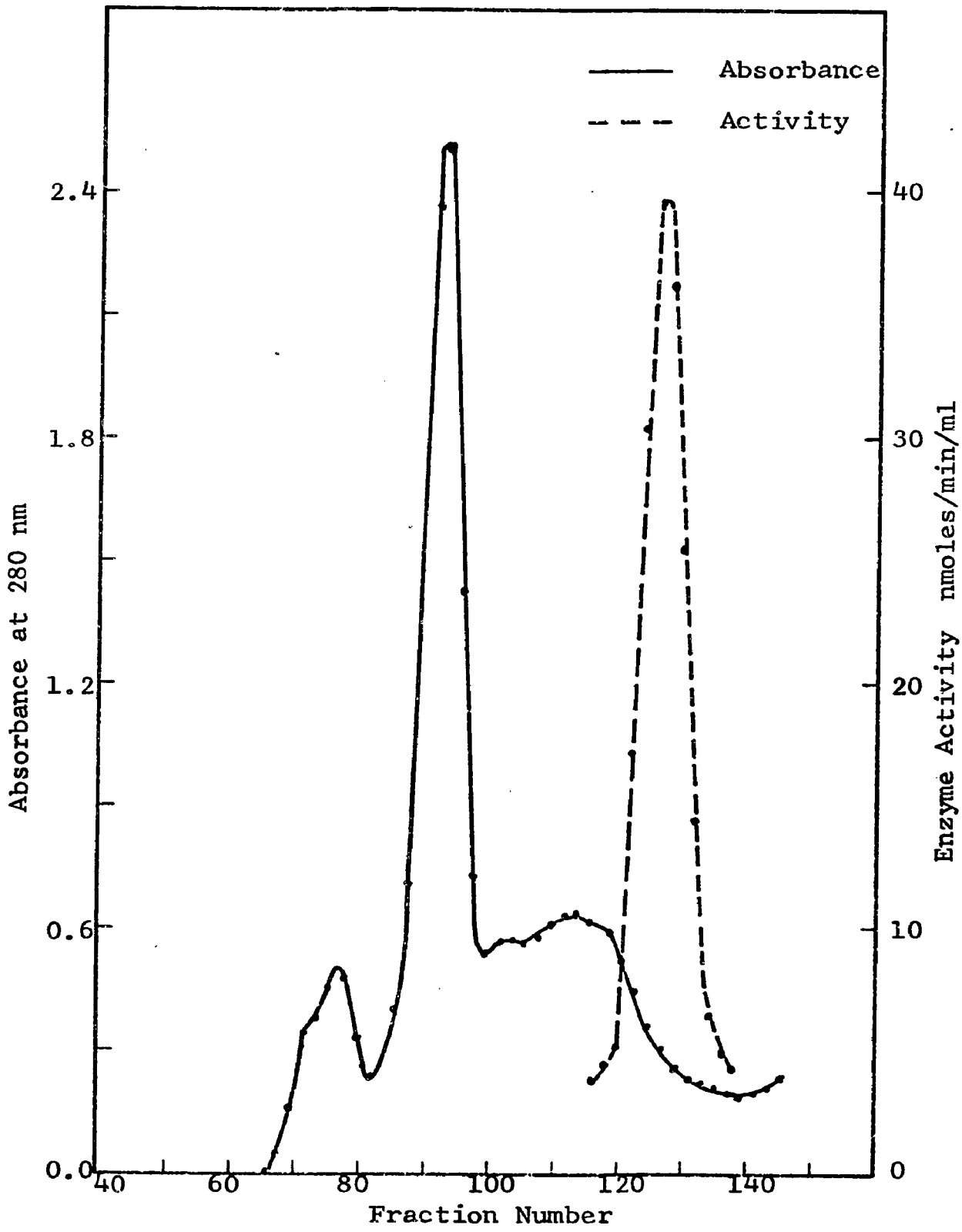


Figure 14. G-75 Sephadex Column Chromatography of NADH-dehydrogenase.

enzyme. The elution pattern of the Superfine G-75 column appears in Figure 15; fractions 119-126 contained NADH-dehydrogenase. A typical purification of NADH-dehydrogenase from 280 g of cells is summarized in Table XI Parts A and B. A NADH-dehydrogenase yield of 34% with a fold purification of 145 was obtained. An aliquot of the pooled NADH-dehydrogenase from the G-75 Sephadex and Superfine G-75 Sephadex columns were run on disc gel electrophoresis, the scans of which appear in Figures 16 and 17. NADH-dehydrogenase, the striped band, was located on the gel by staining and by extraction of the enzyme from the gel. The gel scan in Figure 16 shows one major band at 2.7 cm and five additional minor bands. Only the major band was identified as NADH-dehydrogenase. The scan in Figure 17 shows one major band and two minor bands. Only the major band was identified as NADH-dehydrogenase. A luciferase assay was performed after each step of the purification procedure; the results appear in Table XII. One μg of luciferase (Sigma Chemical Co.) produced a current of 7.5×10^{-10} amps. No luciferase could be detected in the purified NADH-dehydrogenase.

Figure 15. Superfine G-75 Sephadex Column Chromatography of NADH-dehydrogenase.

Two 2.5 cm columns with a combined length of 186 cm were eluted with 0.1 M potassium phosphate buffer, pH 7.5. Fractions of three ml were collected at a flow rate of eight ml/hr.

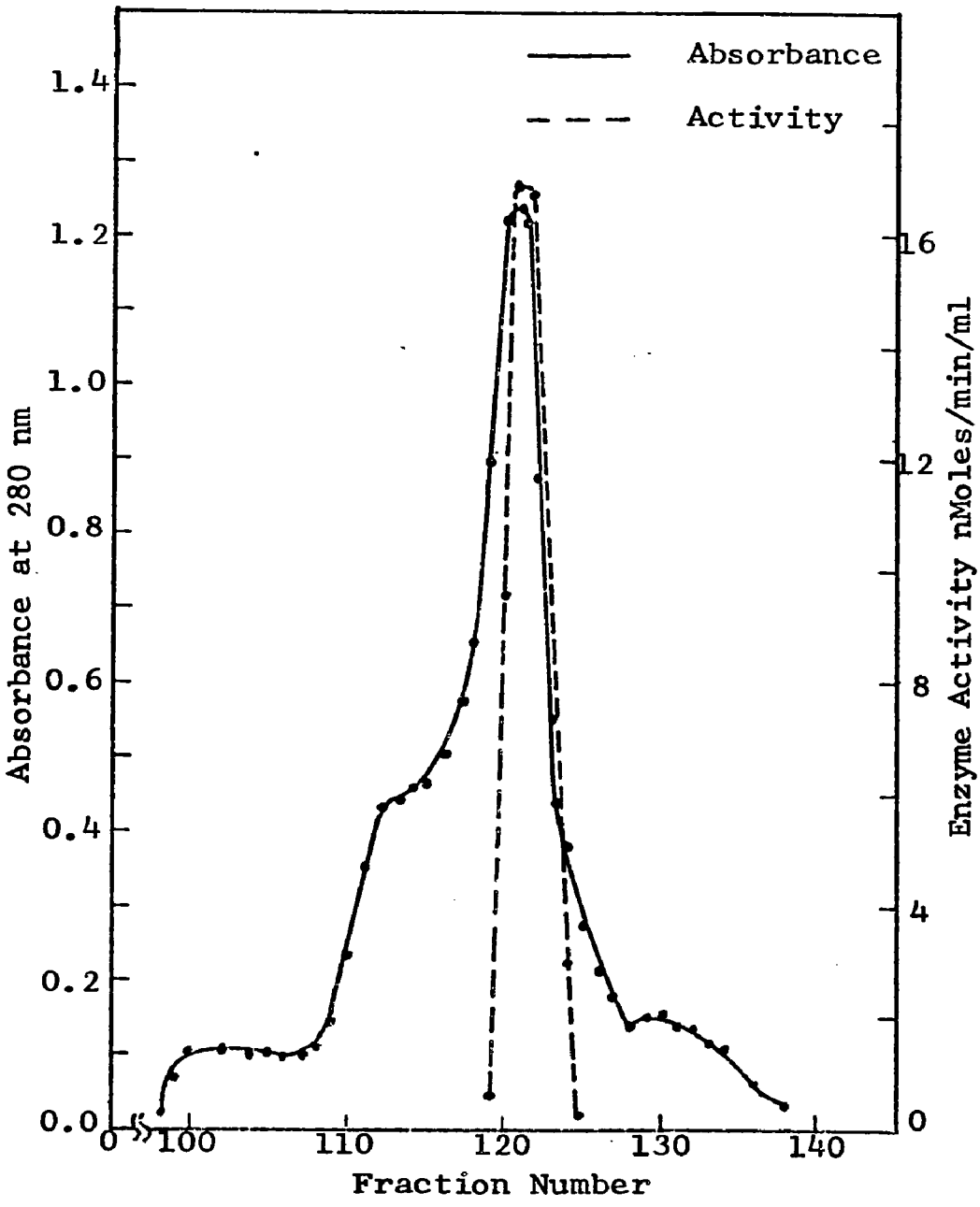


Figure 15. Superfine G-75 Sephadex Column Chromatography of NADH-dehydrogenase.

Table XI. Part A

Summary of Purification of NADH-dehydrogenase from P. fischeri

Step	Volume ml	Protein mg/ml	Activity nMoles/min/ml	Specific Activity nMoles/min/ml	Fold Purification
Lysate (Crude Extract)	1255.0	11.13	10.62	0.95	1.00
40-75% Ammonium Sulfate Precipitate after Dialysis	1040.0	7.60	9.74	1.28	1.35
DEAE-Sephadex Column after Diaflo	90.0	17.53	106.20	6.06	6.38
G-75 Sephadex Column after Diaflo	60.0	2.20	127.26	57.86	60.91
Superfine G-75 Sephadex Column after Diaflo	11.0	3.00	411.91	137.30	144.53

Table XI. Part B

Summary of Purification of NADH-dehydrogenase from *P. fischeri*

Step	Volume ml	Total Protein ml x mg/ml	Total Activity ml x nMoles/min/ml	Percent Yield
Lysate (Crude Extract)	1255.0	13,968.15	13,328	100
40-75% Ammonium Sulfate Precipitate after Dialysis	1040.0	7,904.00	10,130	76
DEAE-Sephadex Column after Diaflo	90.0	1,577.70	9,558	72
G-75 Sephadex Column after Diaflo	60.0	132.00	7,636	57
Superfine G-75 Sephadex Column after Diaflo	11.0	33.00	4,531	34

Figure 16. Disc Gel Electrophoresis of NADH-dehydrogenase from G-75 Sephadex Column.

Triplicate polyacrylamide gels were loaded with 150 μg of NADH-dehydrogenase from the G-75 Sephadex Column. A current of 1.5 milliamps/gel was applied for two hours. One gel was stained for protein and scanned at 550 nm; the gel scan appears in Figure 16. The absorbance peak at 0.0 cm corresponds to the end of the gel; the peak at 0.5 cm is the interface between the stacking and running gels. The unstained gels were used to locate NADH-dehydrogenase on the gel. One gel was sectioned into 0.25 cm slices which were homogenized and assayed for NADH-dehydrogenase activity. The remaining gel was stained with a dehydrogenase stain. The striped area on the gel scan represents NADH-dehydrogenase.

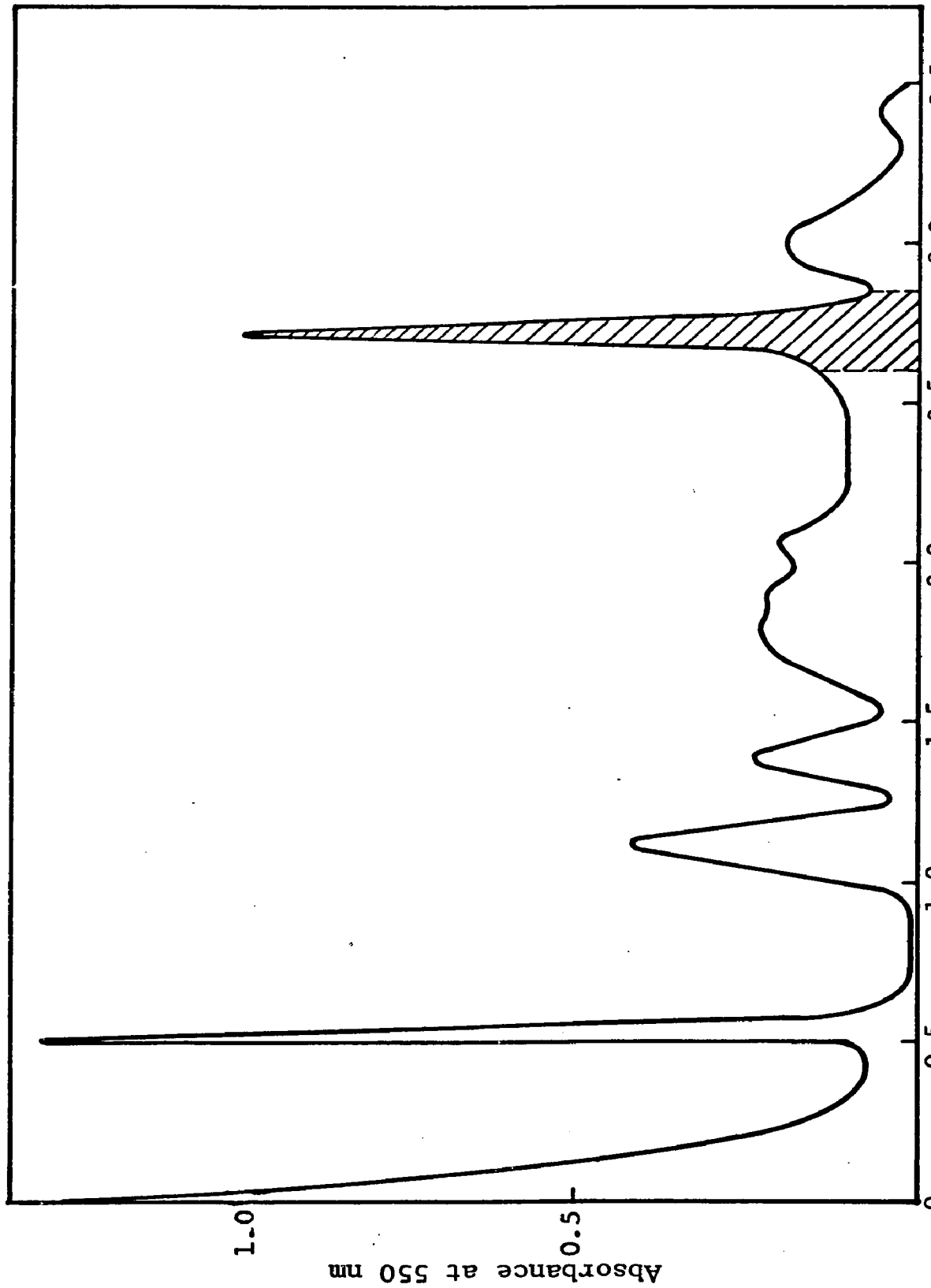


Figure 16. Disc Gel Electrophoresis of NADH-dehydrogenase from G-75 Sephadex Column.

Figure 17. Disc Gel Electrophoresis of NADH-dehydrogenase from Superfine G-75 Sephadex Column.

Triplicate polyacrylamide gels were loaded with 200 μg of purified NADH-dehydrogenase. A current of 1.5 milliamps/gel was applied for two hours. One gel was stained for protein and scanned at 550 nm; the gel scan appears in Figure 17. The absorbance peak at 0.0 cm corresponds to the end of the gel, the peak at 0.5 cm is the interface between the stacking and running gels, and the off-scale peak at 3.7 cm is the tracking dye. The remaining gels were used to locate NADH-dehydrogenase on the gel. One gel was sectioned into 0.25 cm slices which were homogenized and assayed for NADH-dehydrogenase activity. The remaining gel was stained with a dehydrogenase stain. The striped area on the gel scan represents NADH-dehydrogenase.

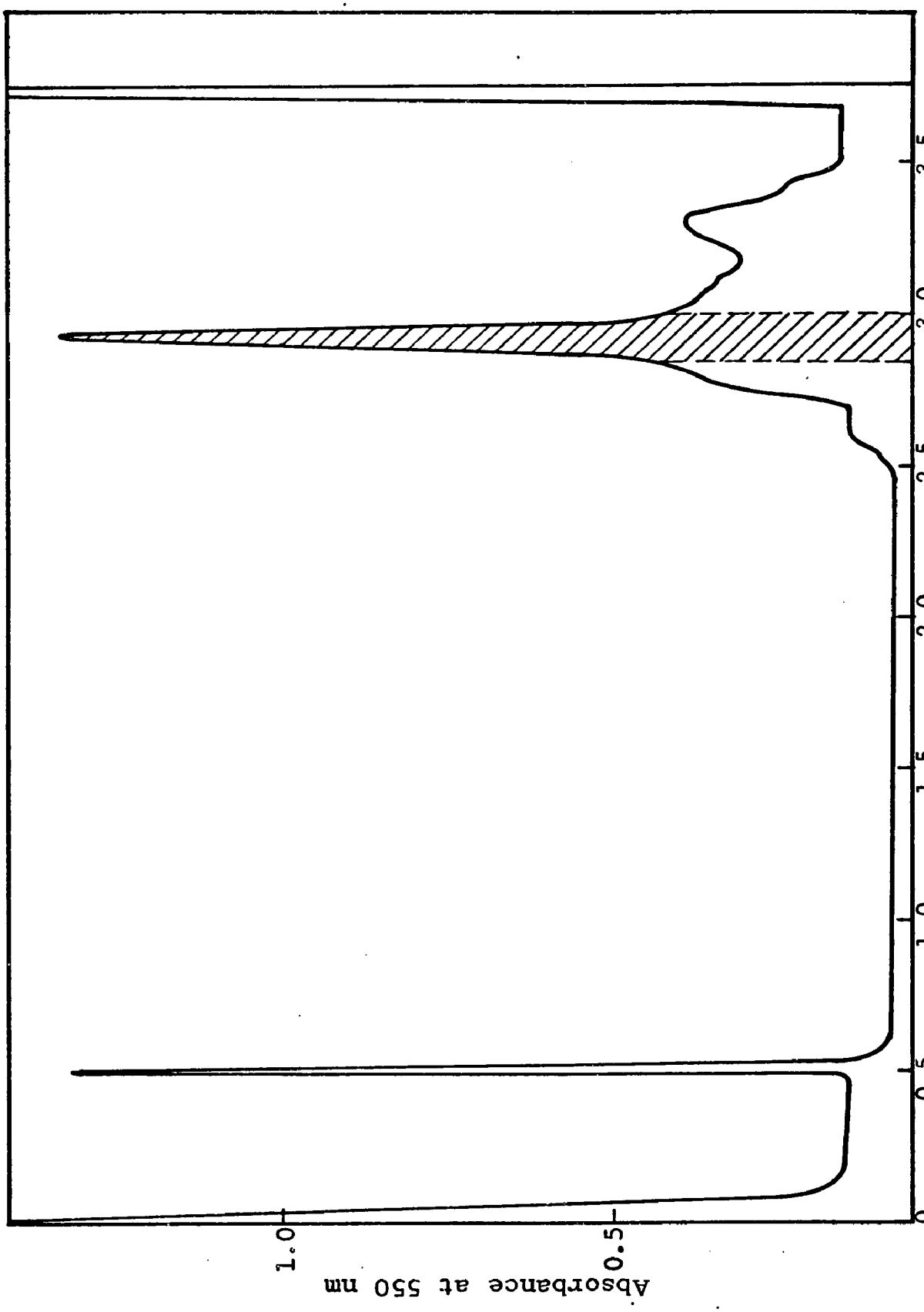


Figure 17. Disc Gel Electrophoresis of NADH-dehydrogenase from Superfine G-75 Sephadex Column.

Table XII

Loss of Luciferase Activity During Purification Procedure

Step	Amps
Lysate (Crude Extract)	8.6×10^{-6}
40-75% Ammonium Sulfate Precipitate after Dialysis	6.4×10^{-6}
DEAE-Sephadex Column after Diaflo	2.8×10^{-6}
G-75 Sephadex Column after Diaflo	1.9×10^{-8}
Superfine G-75 Sephadex Column after Diaflo	*

* Luminescence was below 5.6×10^{-10} amps, the lower limit of detectability of the luciferase assay.

Characterization of NADH-dehydrogenase

The characterization studies were performed with purified NADH-dehydrogenase from the Superfine G-75 Sephadex column which, by disc gel electrophoresis, contained two minor protein bands.

Studies of Optimal NADH-dehydrogenase Activity

The initial velocity of the NADH-dehydrogenase reaction was determined as a function of enzyme concentration, substrate concentration, pH, ionic strength, and addition of BSA.

Enzyme Concentration

The initial velocity of the NADH-dehydrogenase reaction was measured as a function of concentration of purified NADH-dehydrogenase. The results of this experiment, which appear in Figure 18, show that the reaction rate is directly proportional to enzyme concentration.

Substrate Concentration

The initial velocity of the NADH-dehydrogenase reaction was measured as a function of NADH-concentration. A Lineweaver-Burk plot (51) was used to present the data. The initial velocity and substrate concentration were plotted as $1/V$ and $1/S^2$, a slight variation of Michaelis theory, as shown in Figure 27. The apparent Michaelis-Menton constant, K_M, for NADH oxidation was calculated to be 2.8×10^{-5} M and the maximum velocity, V_{max}, was 7.4×10^{-5} moles/liter/min. To check for possible inhibition of NADH-dehydrogenase by potassium cyanide (KCN), cyanide was added to the reaction mixture. The results, shown in Figure 27, indicate that KCN has no effect on the NADH-dehydrogenase enzyme reaction.

pH

The initial velocity of NADH-dehydrogenase was measured as a function of pH as shown in Figure 19. The curve indicates optimal velocity from pH 7.0 to pH 9.0.

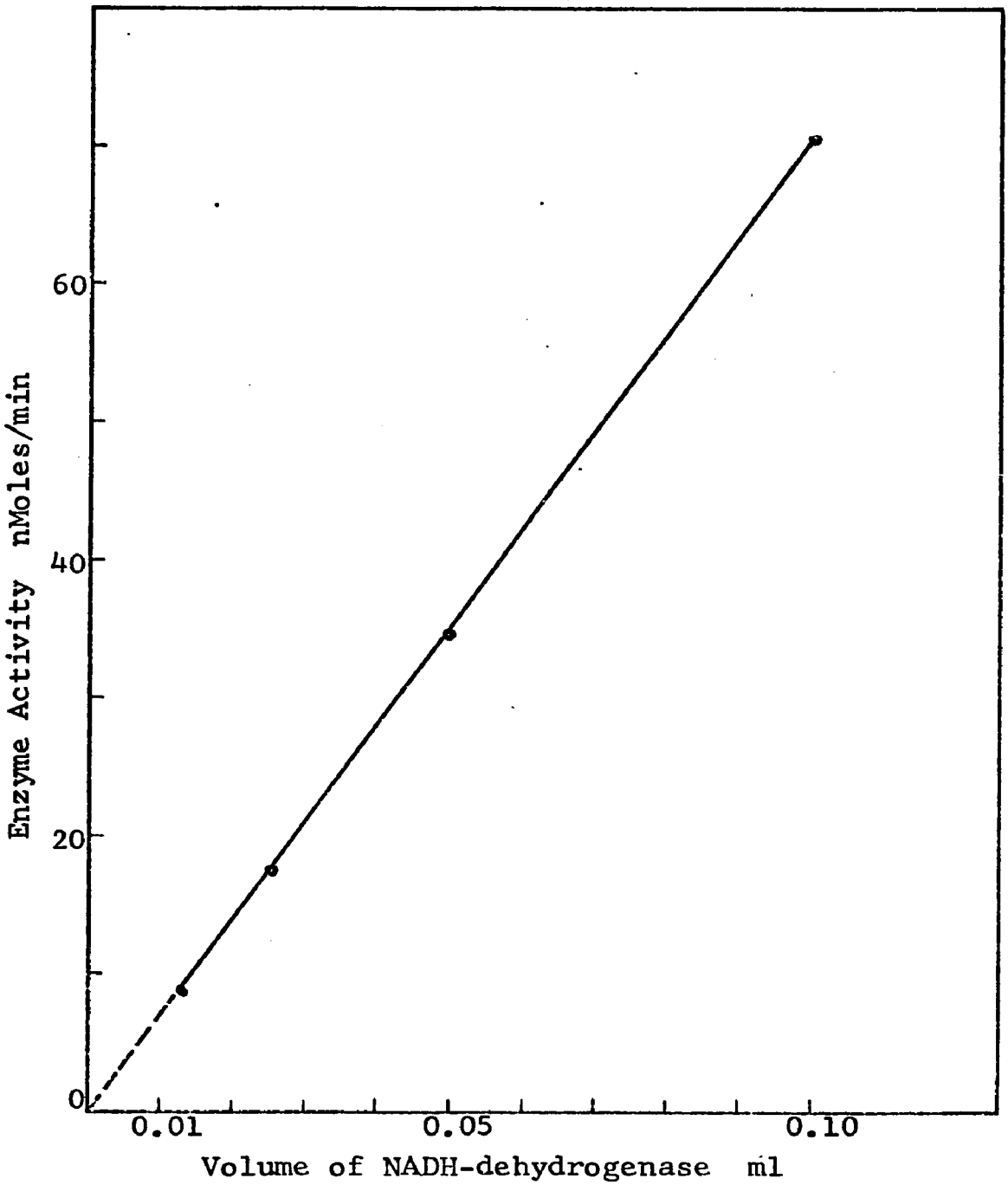


Figure 18. Effect of Enzyme Concentration on Initial Velocity of NADH-dehydrogenase.

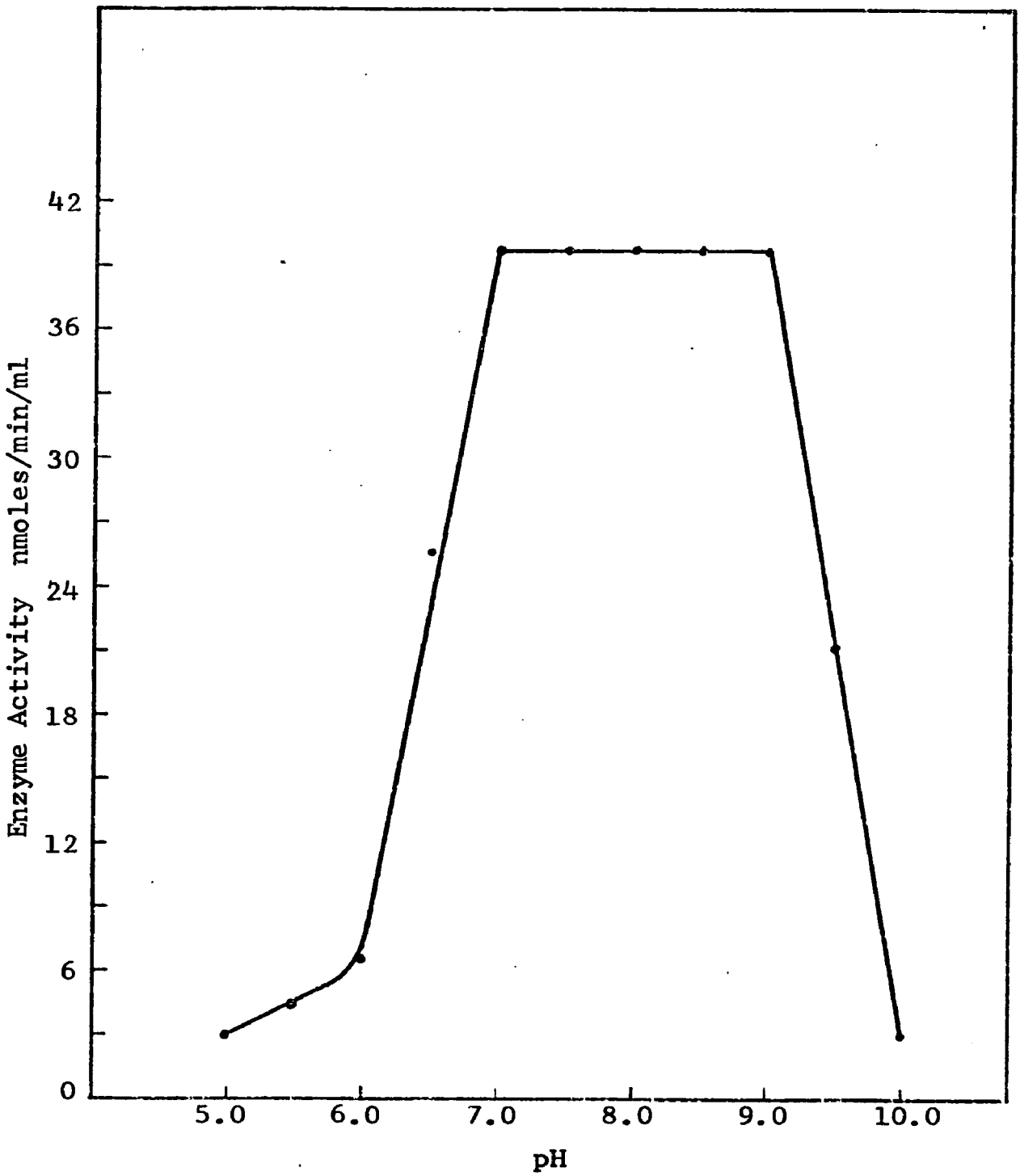


Figure 19. Effect of pH on the Initial Velocity of NADH-dehydrogenase.

Ionic Strength

The initial velocity of the enzyme reaction was measured as a function of the molarity of potassium phosphate buffer at pH 7.5. The molarity, plotted on the abscissa in Figure 20, is related to ionic strength by the equation:

$$\text{Ionic Strength} = 1/2\Sigma c_i z_i^2$$

where c_i = molar concentration of the solution and z_i = charge of each ion in the solution. Therefore, ionic strength is proportional to molarity. A one molar solution of potassium phosphate buffer, pH 7.5 is equal to 2.64 ionic strength. In Figure 20 the maximum velocity occurs from 0.1 M to 1.0 M potassium phosphate buffer, pH 7.5.

Addition of BSA

Because Hastings (52) had shown an increased enzyme rate with the addition of BSA to the assay mixture of luciferase from *P. fischeri*, the effect of the addition of BSA to the assay mixture of NADH-dehydrogenase from *P. fischeri* was studied. To determine the effect of BSA, the initial velocity of the NADH-dehydrogenase reaction was measured as a function of increasing BSA concentration. The addition of BSA to a final concentration of 0.7 mg/ml to 1.3 mg/ml increased the reaction rate by 48% as can be seen in Figure 21.

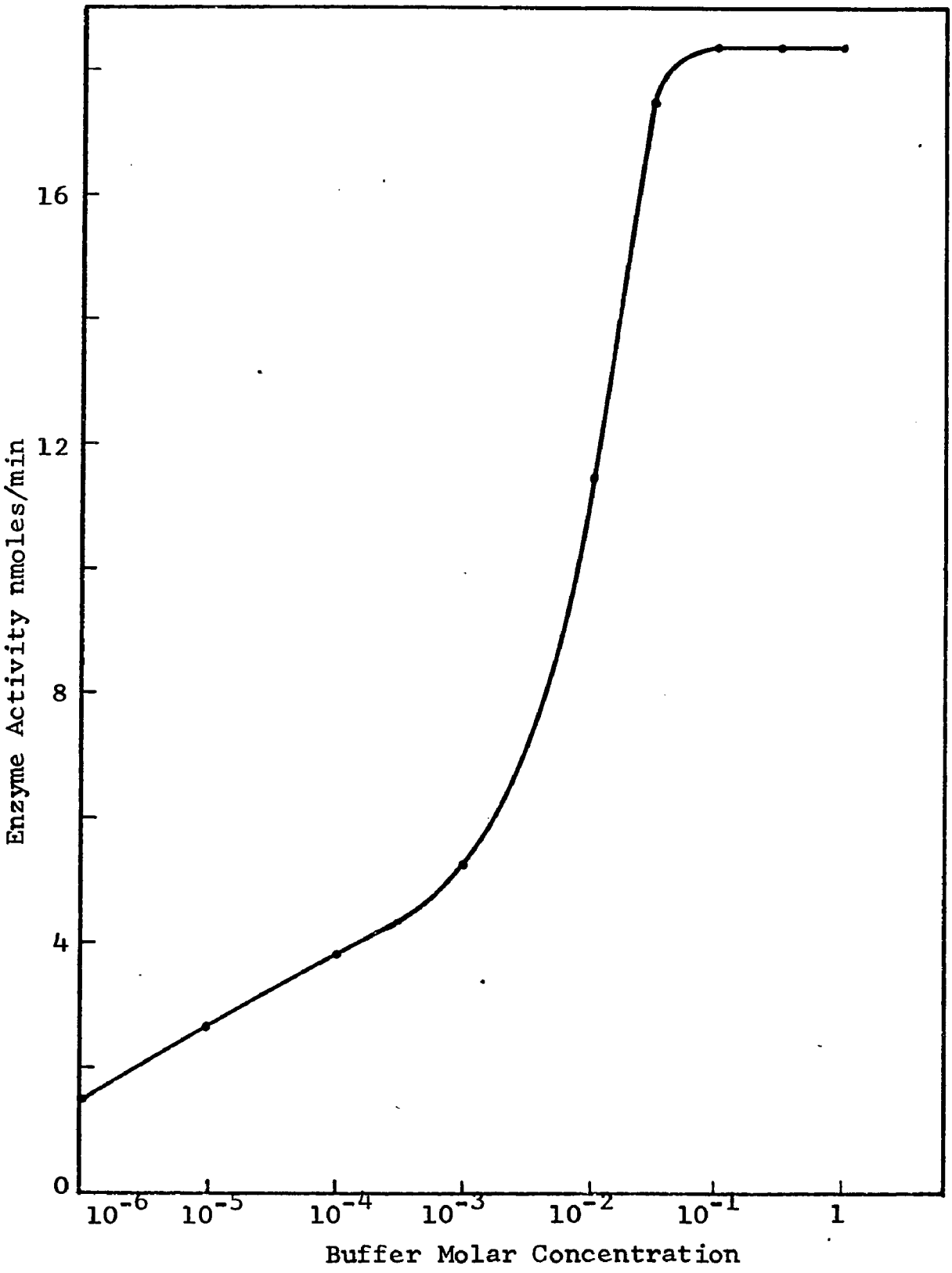


Figure 20. Effect of Ionic Strength on the Initial Velocity of NADH-dehydrogenase.

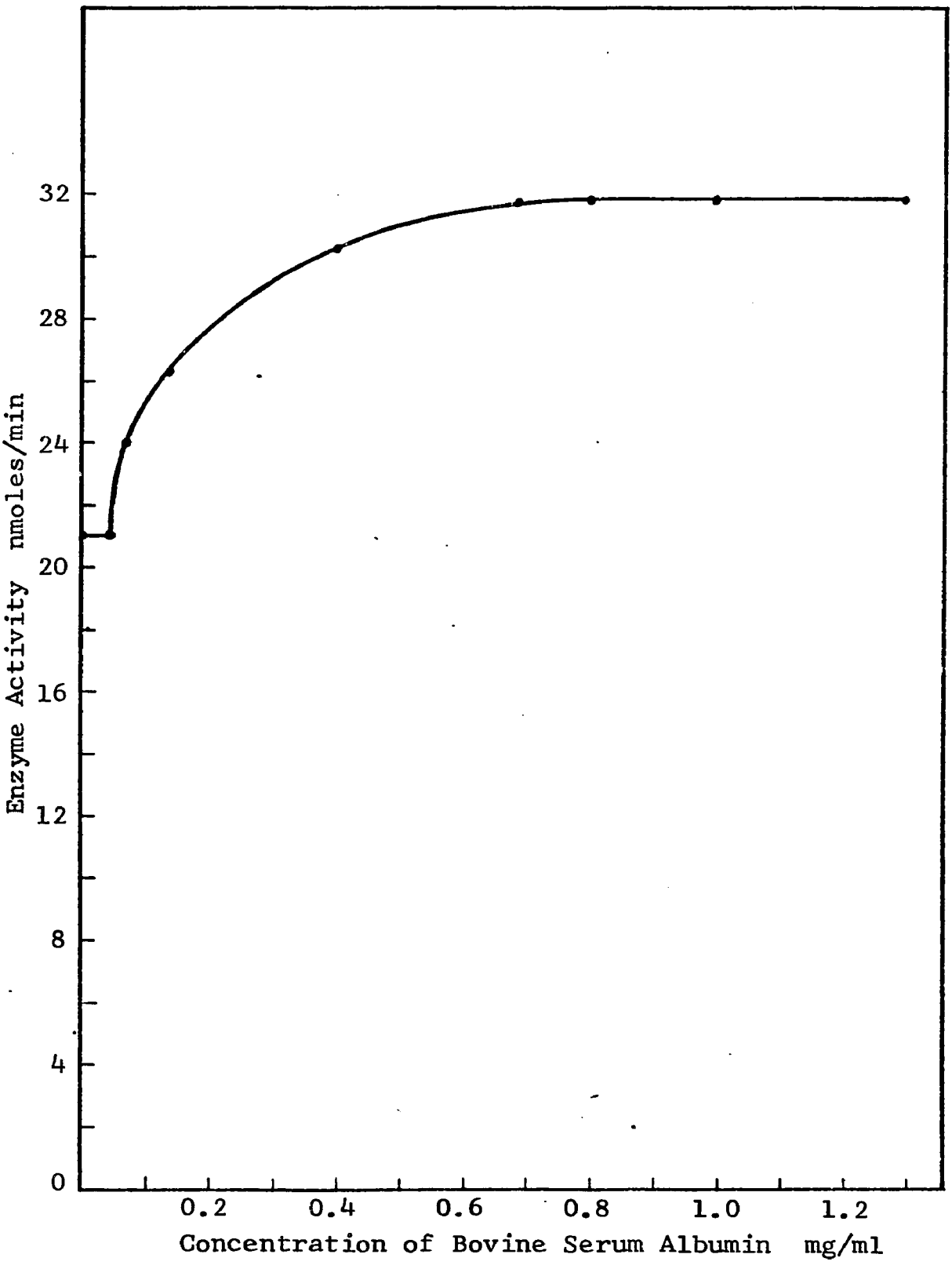


Figure 21. Effect of Bovine Serum Albumin on the Initial Velocity of NADH-dehydrogenase.

Absorption Spectra of NADH-dehydrogenase

The oxidized and reduced visible absorption spectra of NADH-dehydrogenase, run at a concentration of 150 μg/ml, appear in Figure 22. The oxidized enzyme exhibits a maximum at 410 nm and a broad peak at 530 nm; the reduced enzyme showed maxima at 423, 527, and 557 nm. The difference spectrum between reduced and oxidized NADH-dehydrogenase, shown in Figure 23, exhibits maxima at 423, 527, and 557 nm.

Molecular Weight Estimation

The molecular weight of NADH-dehydrogenase was estimated by column chromatography. Figure 24 shows the elution pattern of protein from the column as determined by absorption at 280 nm; NADH-dehydrogenase was located on the column by assaying for enzyme activity. The average apparent molecular weight of NADH-dehydrogenase is 53,000 as determined from the chromatography column molecular weight calibration curve in Figure 25.

Radiation Studies

The NADH-dehydrogenase reaction was studied before, during, and after X-irradiation. These studies were facilitated by coupling a recording spectrophotometer to an X-ray generator. The effective energy and dose rate were measured and calculated to be 35 keV and 1480 $\pm$ 87 rad/min, respectively. X-irradiation was limited to one- or two-minute exposures to avoid significant damage to the enzyme.

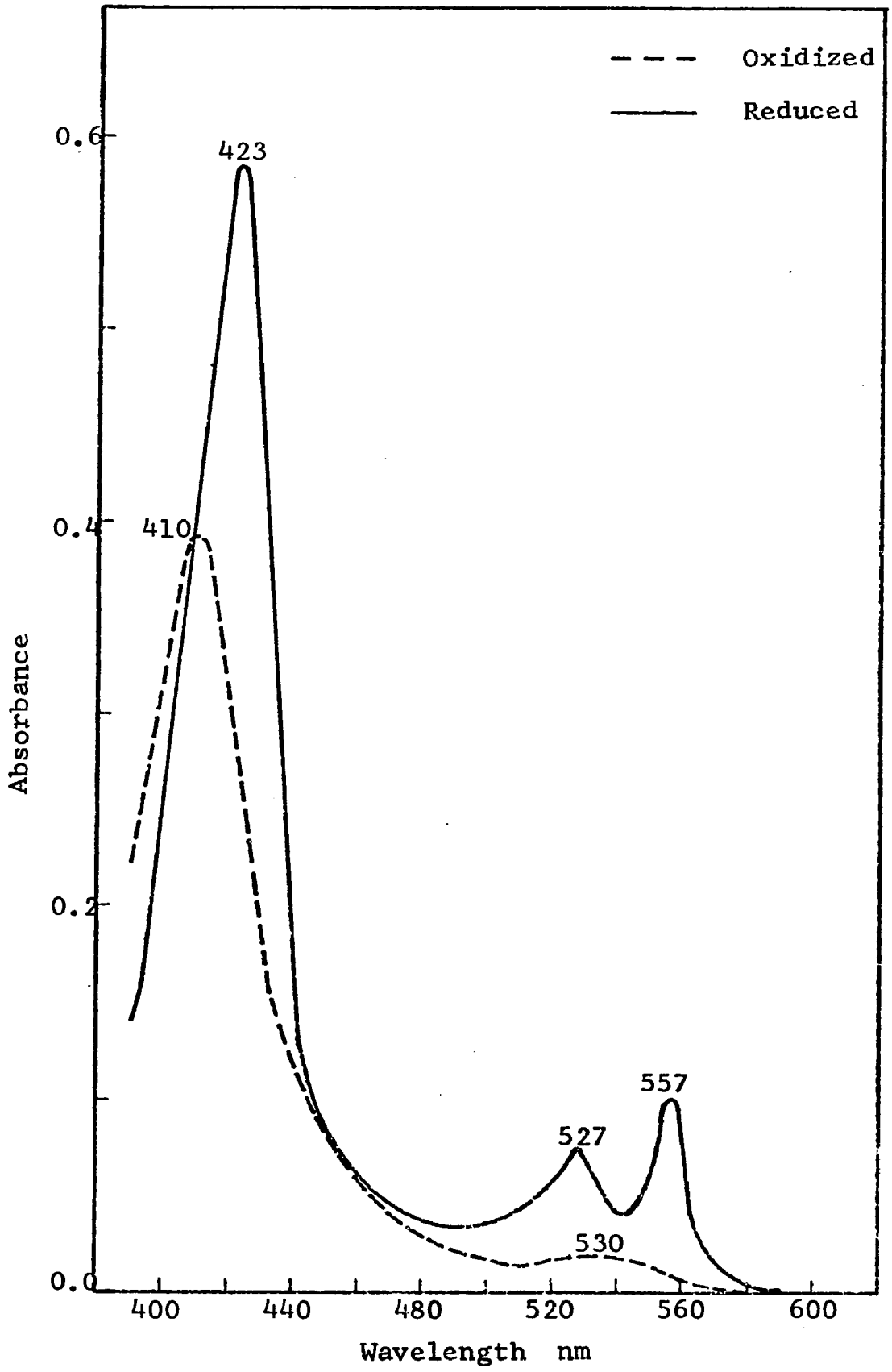


Figure 22. Visible Absorption Spectra of Oxidized and Reduced NADH-dehydrogenase.

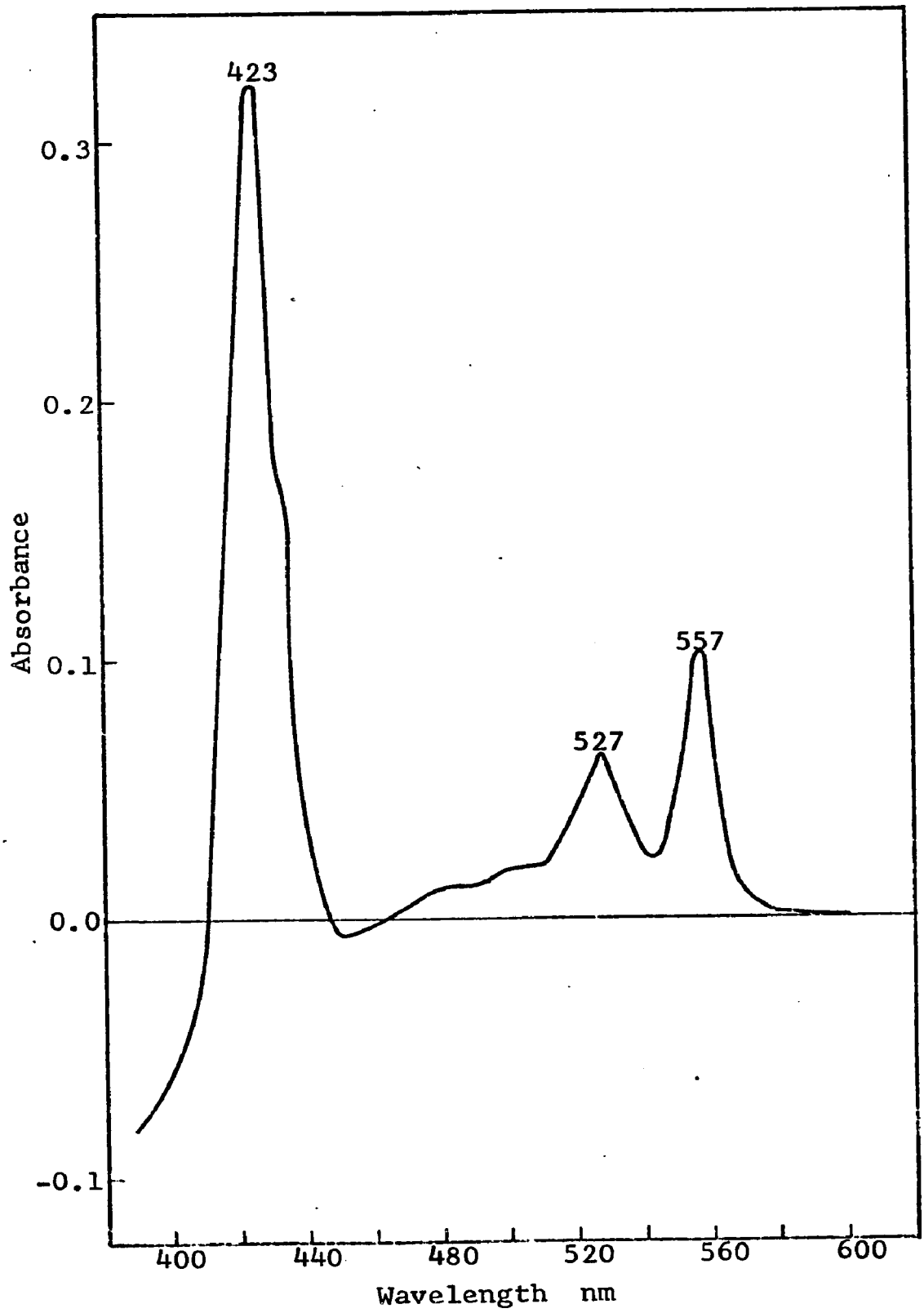


Figure 23. Difference Spectrum Between Reduced and Oxidized NADH-dehydrogenase.

Figure 24. Superfine G-75 Sephadex Column Chromatography of Proteins for Column Calibration and Molecular Weight Determination of NADH-dehydrogenase.

A 2.5 x 96 cm column was calibrated with the following standards:

	Standard	Molecular Weight
A	Blue Dextran 2000	2,000,000
B	Bovine Serum Albumin	67,000
C	Ovalbumin	45,000
D	Chymotrypsinogen A	25,000
E	Ribonuclease A	13,700

A two-ml sample containing all of the calibration proteins was applied to the column and eluted with 0.1 M potassium phosphate buffer, pH 7.5. Fractions of 2.1 ml were collected at the rate of nine ml/hr. After column calibration, a two-ml sample containing Blue Dextran (A), Ribonuclease A (E), and purified NADH-dehydrogenase were applied to the column. The column was eluted as before and assayed for absorbance at 280 nm and NADH-dehydrogenase activity.

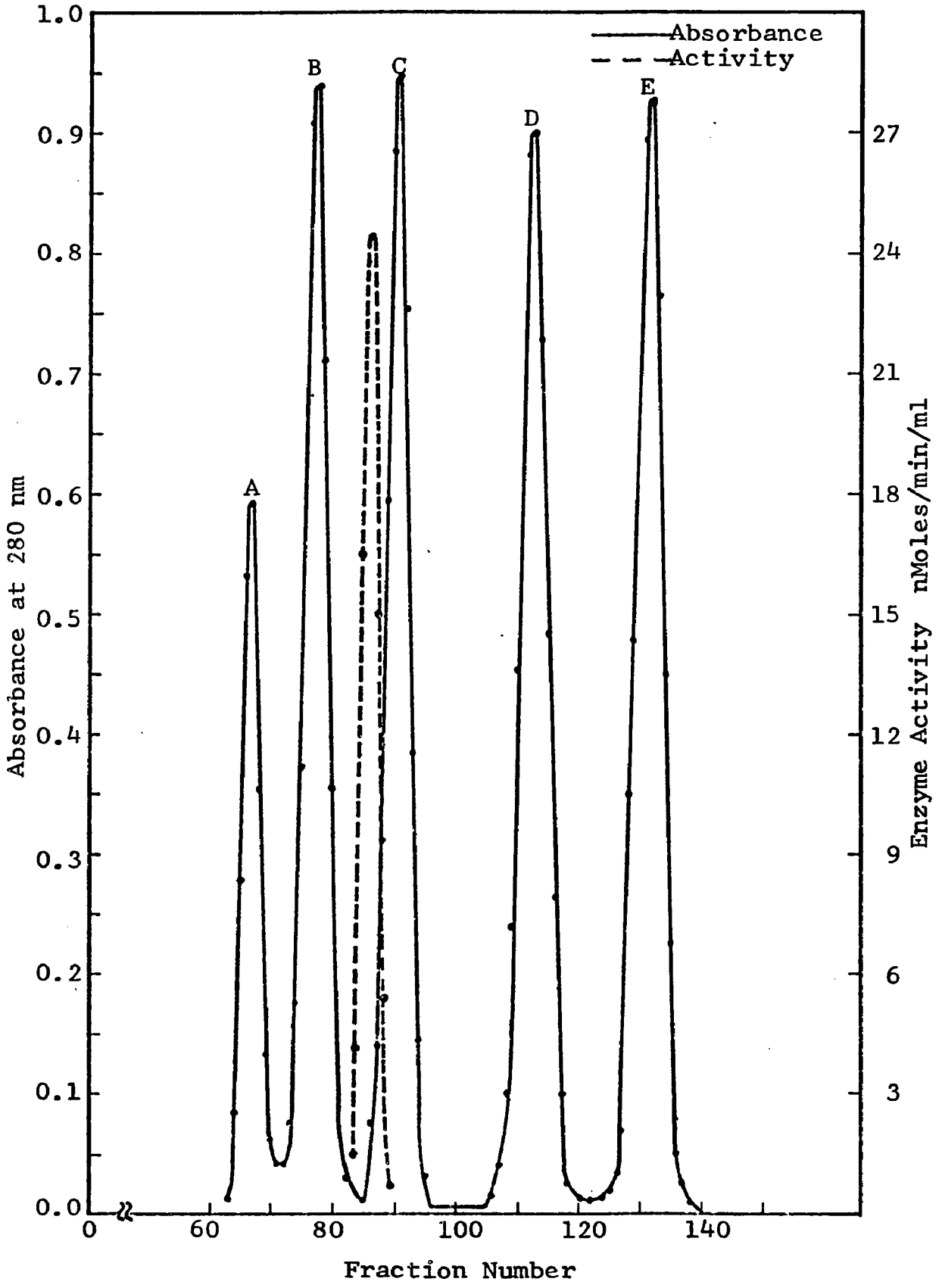


Figure 24. Superfine G-75 Sephadex Column Chromatography of Proteins for Column Calibration and Molecular Weight Determination of NADH-dehydrogenase.

Figure 25. Superfine G-75 Sephadex Chromatography Column Molecular Weight Calibration Curve.

From Figure 24 the elution constant, K, for each calibration standard and NADH-dehydrogenase was calculated using the following equation:

$$K = \frac{V_e - V_o}{V_f - V_o}$$

where K = elution constant, V_e = elution volume for a specific protein, V_o = elution time for Blue Dextran 2000, and V_f = total bed volume. The elution constants, plotted in Figure 25, are tabulated below.

	Protein	Molecular Weight	K
B	Bovine Serum Albumin	67,000	0.071
C	Ovalbumin	45,000	0.159
D	Chymotrypsinogen A	25,000	0.306
E	Ribonuclease A	13,700	0.463
	NADH-dehydrogenase		0.128

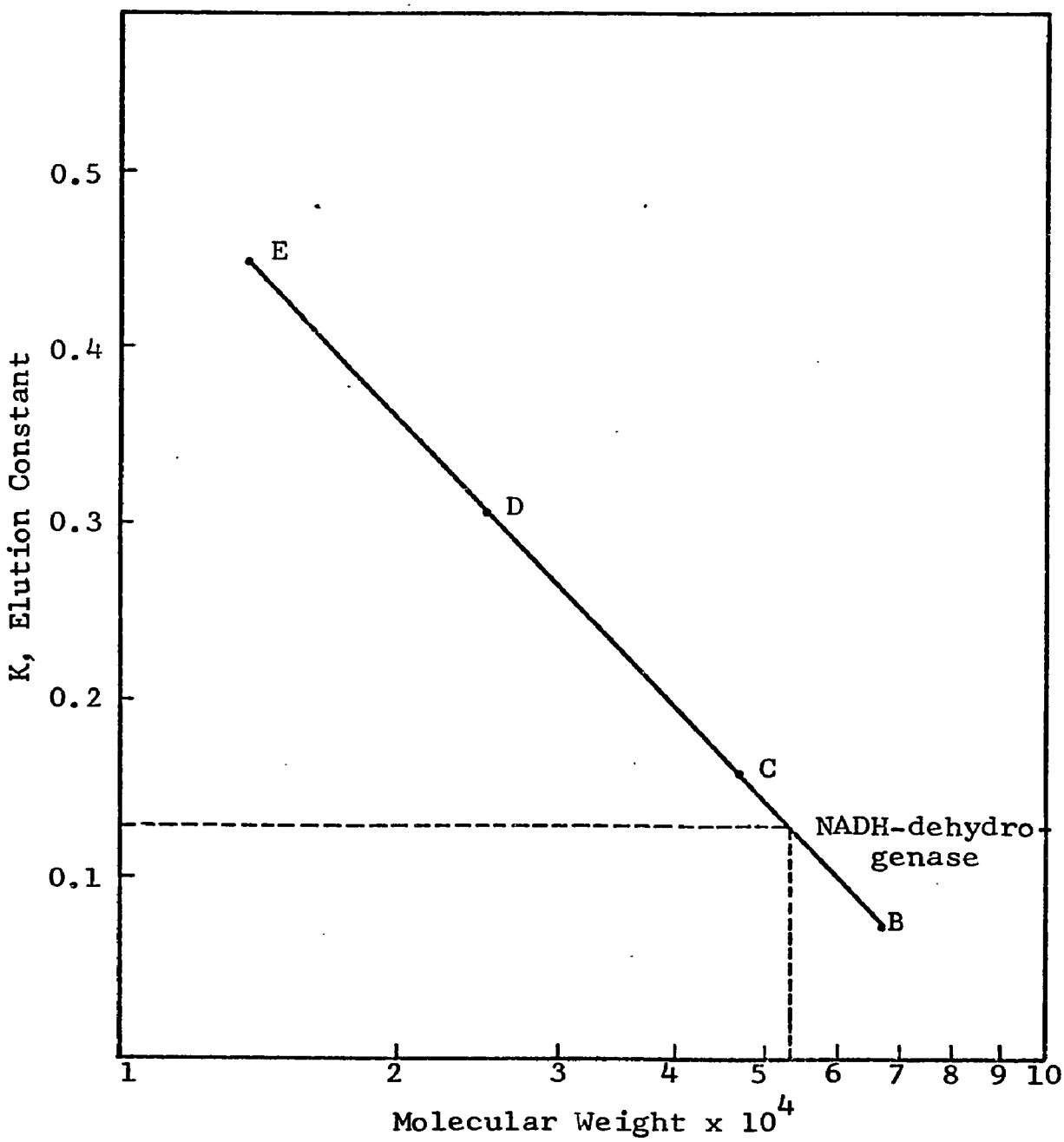


Figure 25. Superfine G-75 Sephadex Chromatography Column Molecular Weight Calibration Curve.

Enzymatic and Nonenzymatic Oxidation of NADH during X-irradiation

Figure 26 shows the enzymatic and nonenzymatic oxidation of NADH before, during, and after irradiation. The enzymatic sample in Figure 26-A contained NADH-dehydrogenase, NADH, and FMN; the sample in Figure 26-B contained only substrates. The enzymatic reaction, Figure 26-A, shows a change in absorbance before irradiation which is a measure of the enzymatic reaction rate (k_1). During irradiation the rate of the reaction (k_3) is increased. After an irradiation of two minutes, the rate of the reaction returns to its previous rate. In the nonenzymatic reaction, Figure 26-B, there is no change in the absorbance of NADH until X-irradiation at four minutes. During irradiation NADH is oxidized (k_2) until X-irradiation is terminated at six minutes, after which there is no further oxidation of NADH. The enzymatic and nonenzymatic reaction rates were measured at various concentrations of NADH, the results of which were the same as those shown in Figure 26.

Effect of X-irradiation on Substrate Dependence of NADH-dehydrogenase

The initial velocity of the NADH-dehydrogenase reaction during irradiation was measured as a function of NADH concentration. The nonenzymatic reaction rate was subtracted from the enzymatic rate. The resulting rate was plotted as a function of the initial velocity and substrate concentration as shown in Figure 27. From the results it can be seen that

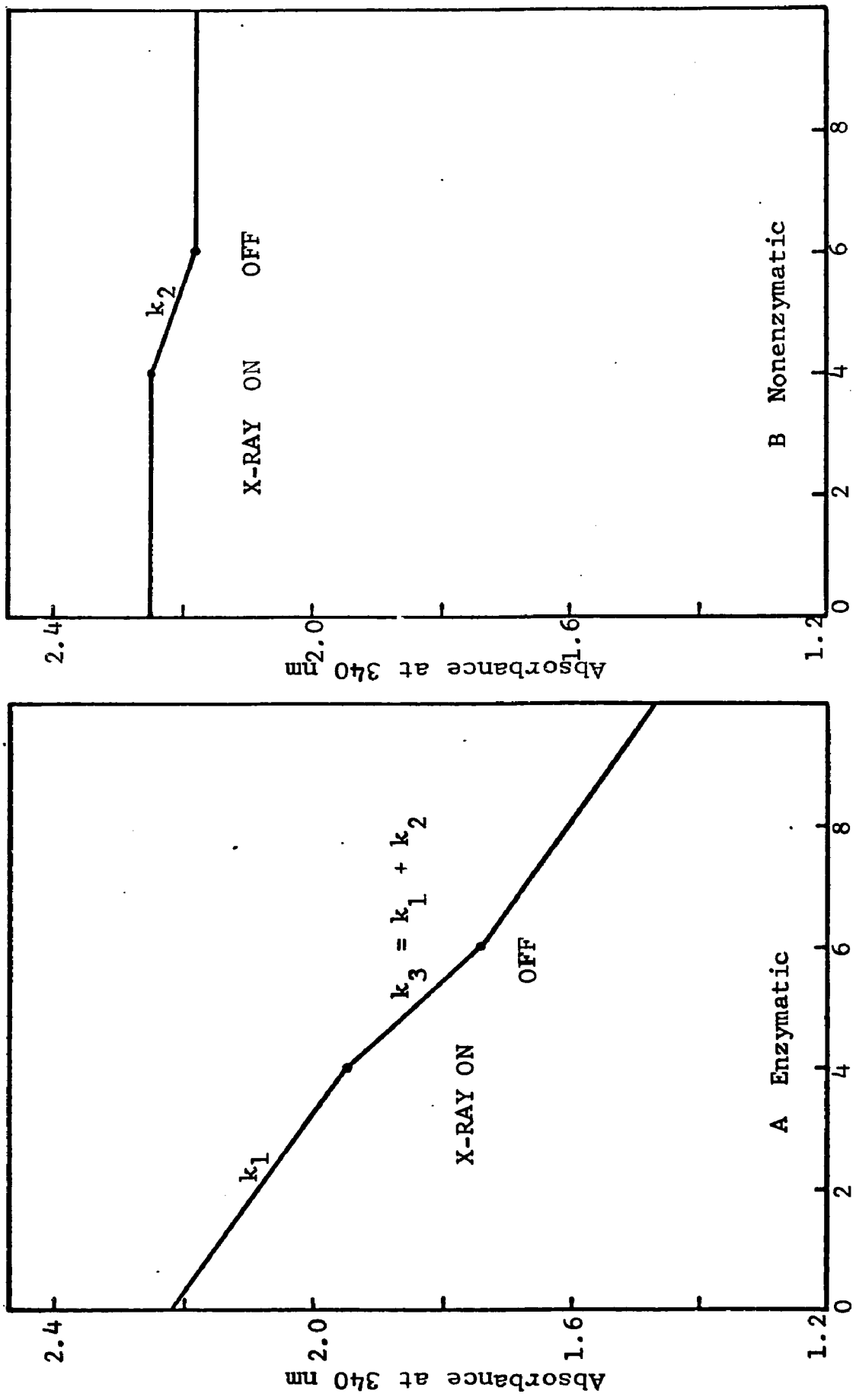


Figure 26. Enzymatic and Nonenzymatic Oxidation of NADH during X-irradiation.

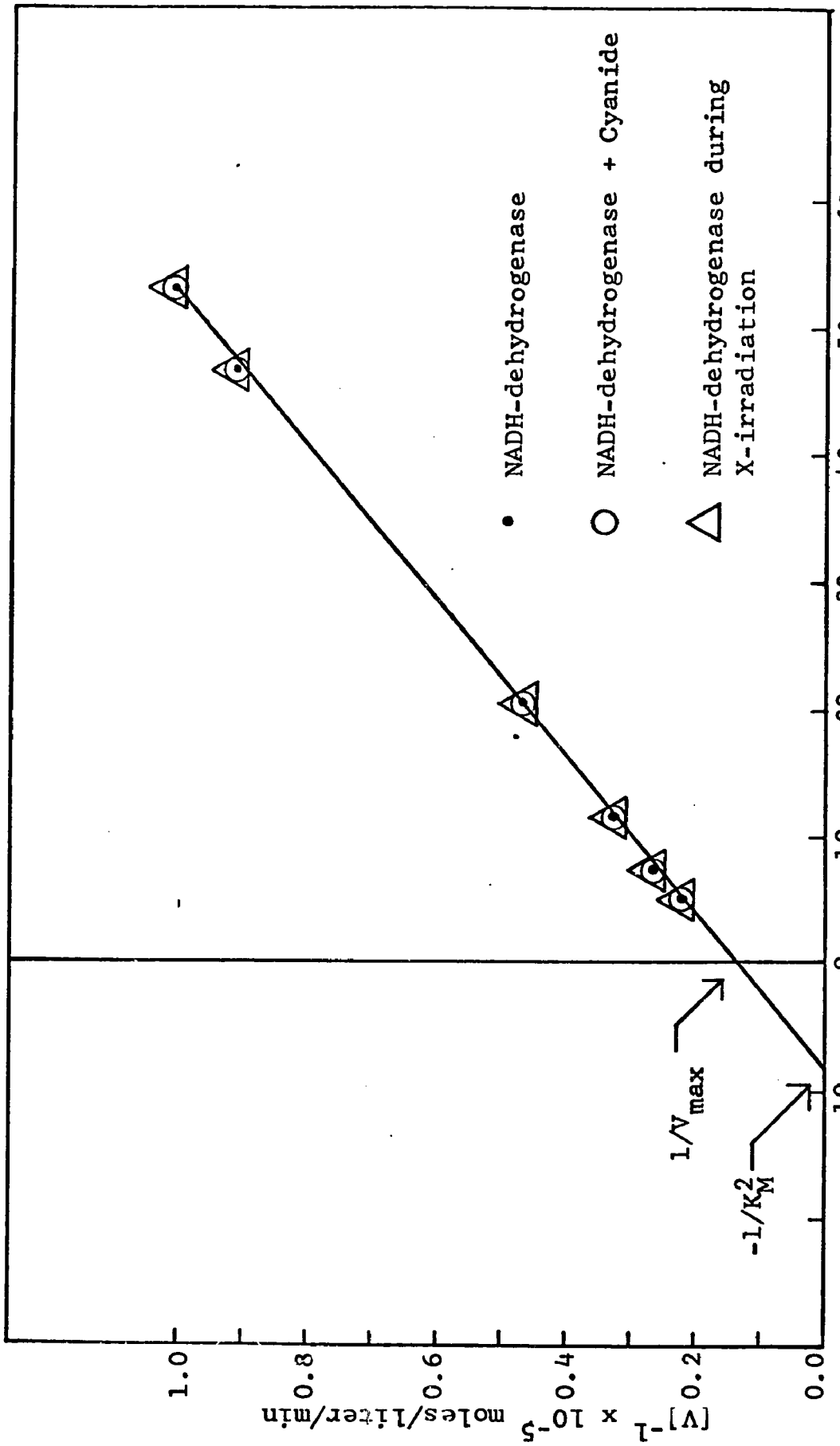


Figure 27. A Lineweaver-Burk Plot of the Effect of Substrate Concentration Alone, with Cyanide, and during X-irradiation on the Initial Velocity of NADH-dehydrogenase.

radiation did not affect the maximum velocity, V_{max}, of the reaction or the apparent affinity of the enzyme for its substrate, K_M.

Oxidation of NADH during Irradiation as a Function of Dose Rate

The nonenzymatic oxidation rate of NADH in the presence of FMN during irradiation was measured at several dose rates. The dose rate was varied by changing the X-ray tube current from 5 to 20 milliamps. The results, which can be seen in Figure 28, indicate that the rate of oxidation is directly proportional to the dose rate between 300 and 1500 rads/min.

Dilution of NADH

The nonenzymatic oxidation of NADH during irradiation was measured as a function of NADH concentration with and without FMN, as seen in Figure 29. Curve B represents the rate of oxidation of NADH at various concentrations without FMN. Curve A indicates the rate of oxidation of NADH at various concentrations with 2.1×10^{-6} M FMN. From the figure, it is readily observed that the presence of FMN enhances the oxidation of NADH by radiation.

Enzymatic and Nonenzymatic Anaerobic Reduction of FMN during X-irradiation

The enzymatic and nonenzymatic reduction of FMN was measured anaerobically before, during, and after irradiation. The results are shown in Figure 30 in which the sample in

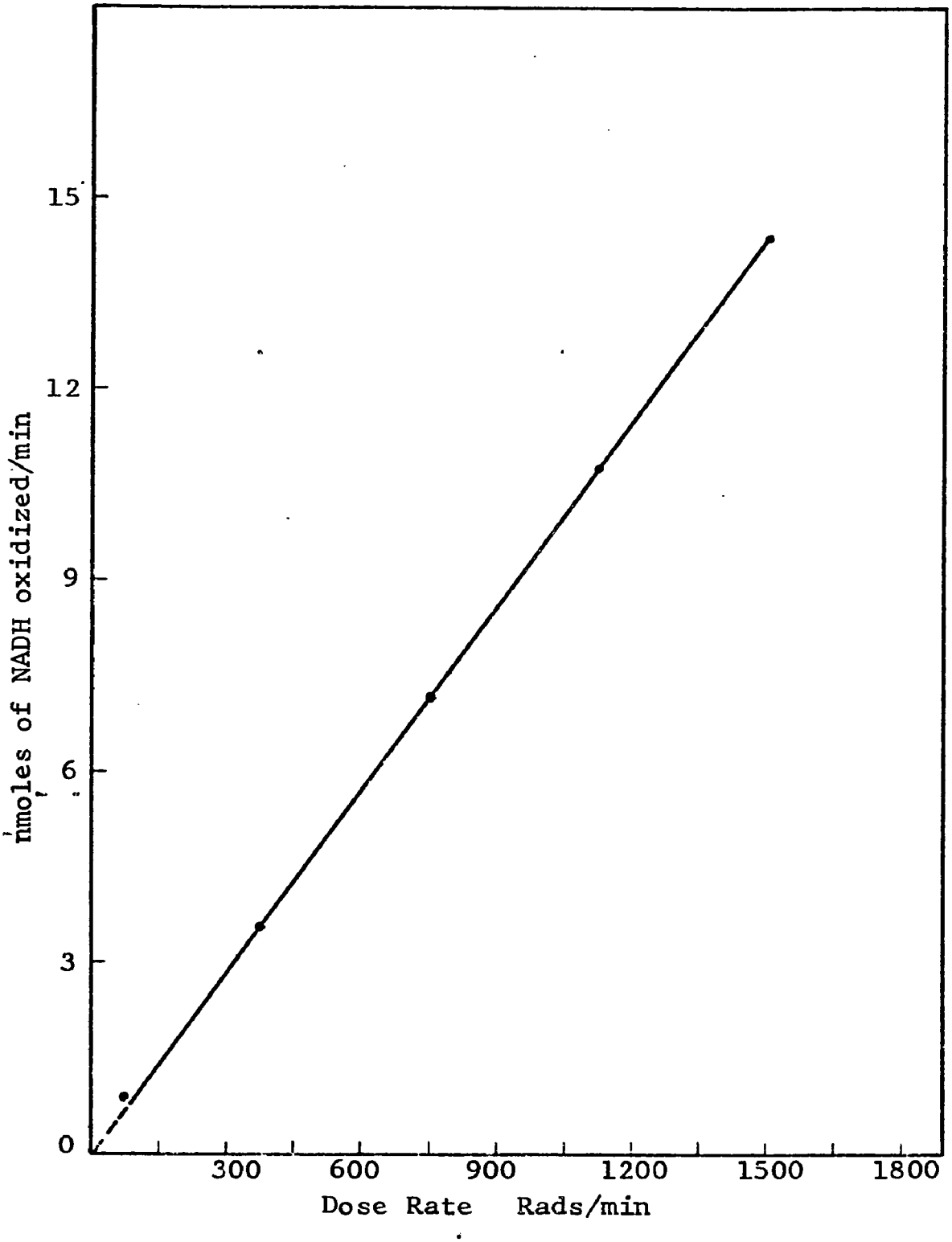


Figure 28. Nonenzymatic Oxidation of NADH during X-irradiation as a Function of Dose Rate.

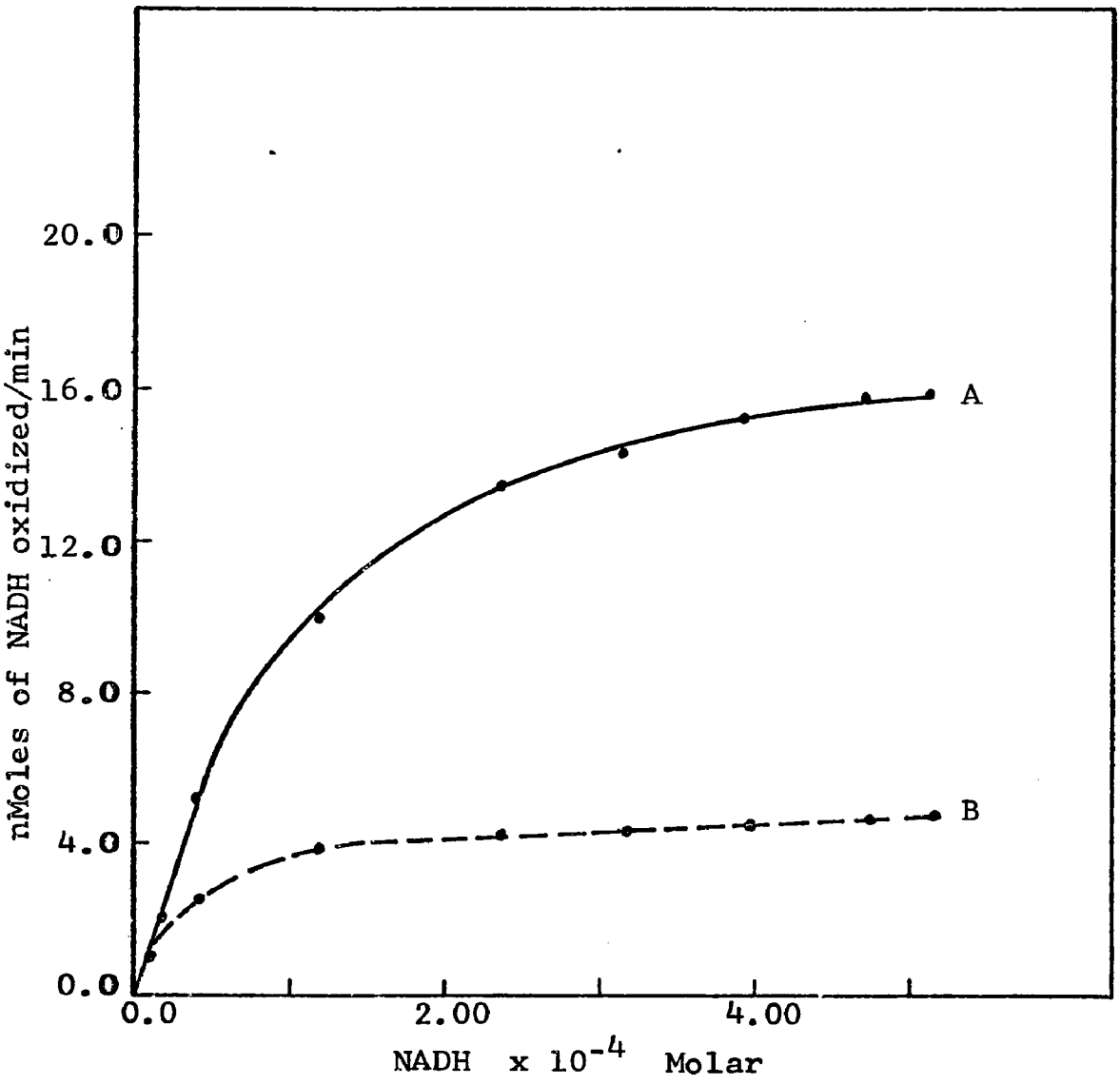


Figure 29. Oxidation of NADH during X-irradiation as a Function of NADH Concentration with and without FMN. Curve A is the oxidation of NADH with 2.1×10^{-6} M FMN. Curve B is the oxidation of NADH without FMN.

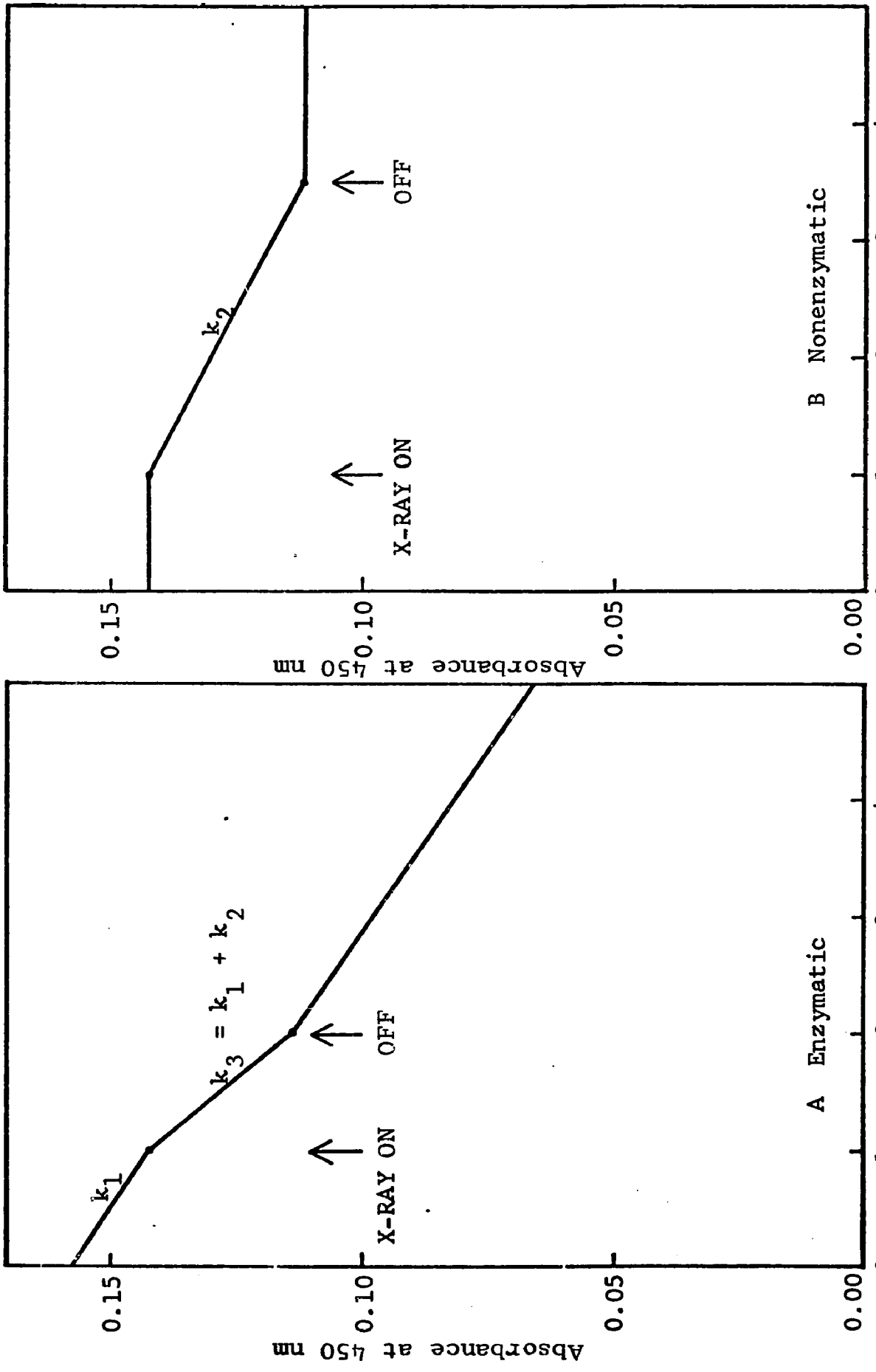


Figure 30. Enzymatic and Nonenzymatic Anaerobic Reduction of FMN during X-irradiation.

Figure 30-A contained enzyme, NADH, and FMN; the sample in Figure 30-B contained only substrates. In Figure 30-A, the enzymatic reduction of FMN, the initial rate (k_1) is a measure of enzymatic reduction of FMN. With X-irradiation the rate of reduction (k_3) increases until X-irradiation is terminated, after which the reaction rate returns to its initial rate. In Figure 30-B it can be seen that there is no reduction of FMN until X-irradiation. During irradiation FMN is reduced (k_2) until X-irradiation is terminated, after which there is no reduction of FMN. The enzymatic and nonenzymatic reaction rates were measured at various concentrations of NADH, the results of which were the same as those in Figure 30.

Dilution of FMN

The nonenzymatic anaerobic reduction of FMN was measured as a function of FMN concentration, with and without NADH, as seen in Figure 31. Curve B represents the reduction of FMN without NADH. Curve A represents the rate of reduction of FMN with 9.5×10^{-4} M NADH. The results indicate that FMN is not appreciably reduced by X-irradiation without NADH.

Luminescence During X-irradiation

To observe *in vitro* luminescence during X-irradiation, the NADH-dehydrogenase reaction was coupled to the luciferase enzyme reaction. The experimental results appear in Figure 32, a typical recording of luminescence before, during and

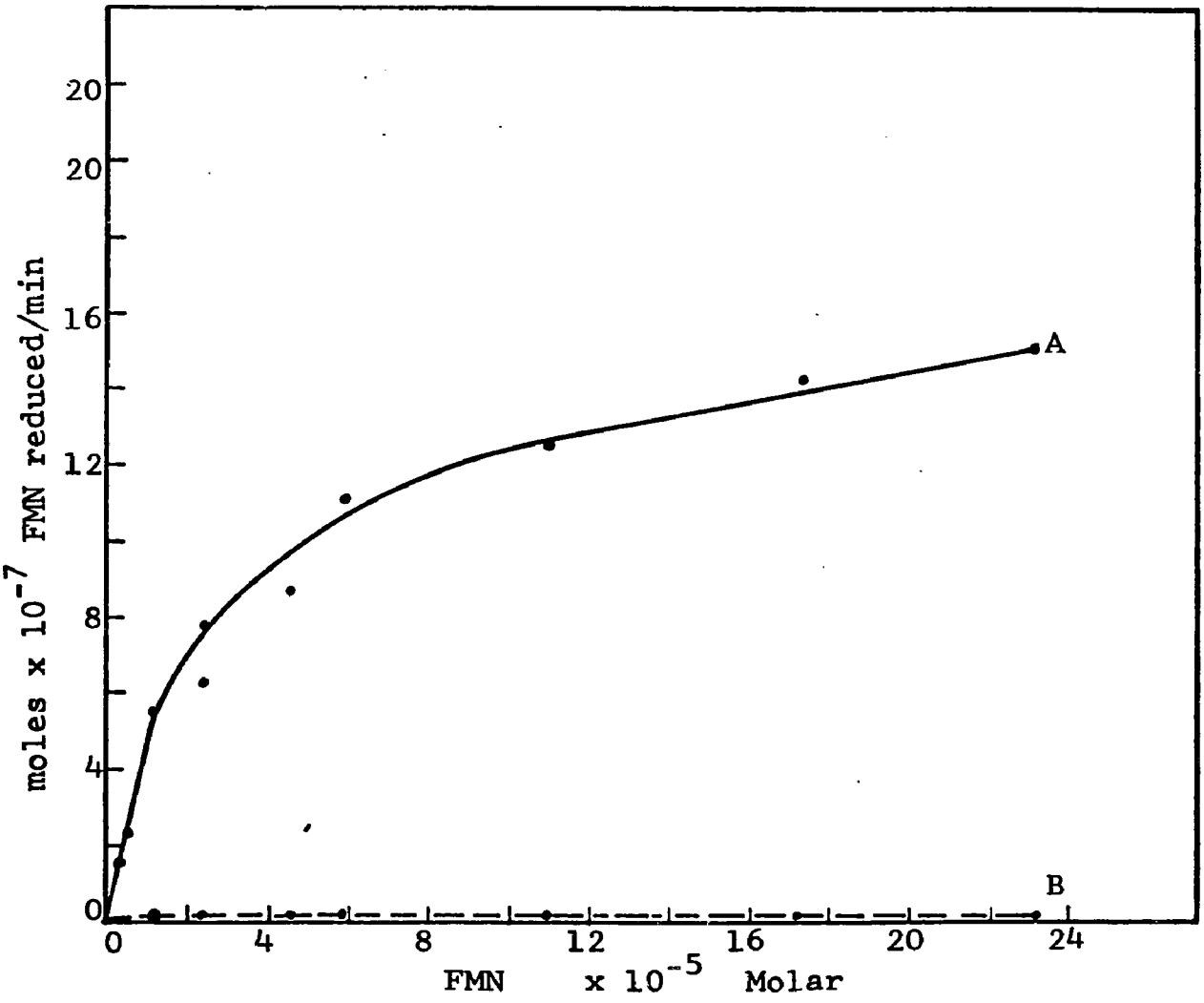


Figure 31. Anaerobic Reduction of FMN during X-irradiation as a Function of FMN Concentration with and without NADH. Curve A is the reduction of FMN with 9.5×10^{-4} M NADH. Curve B is the reduction of FMN without NADH.

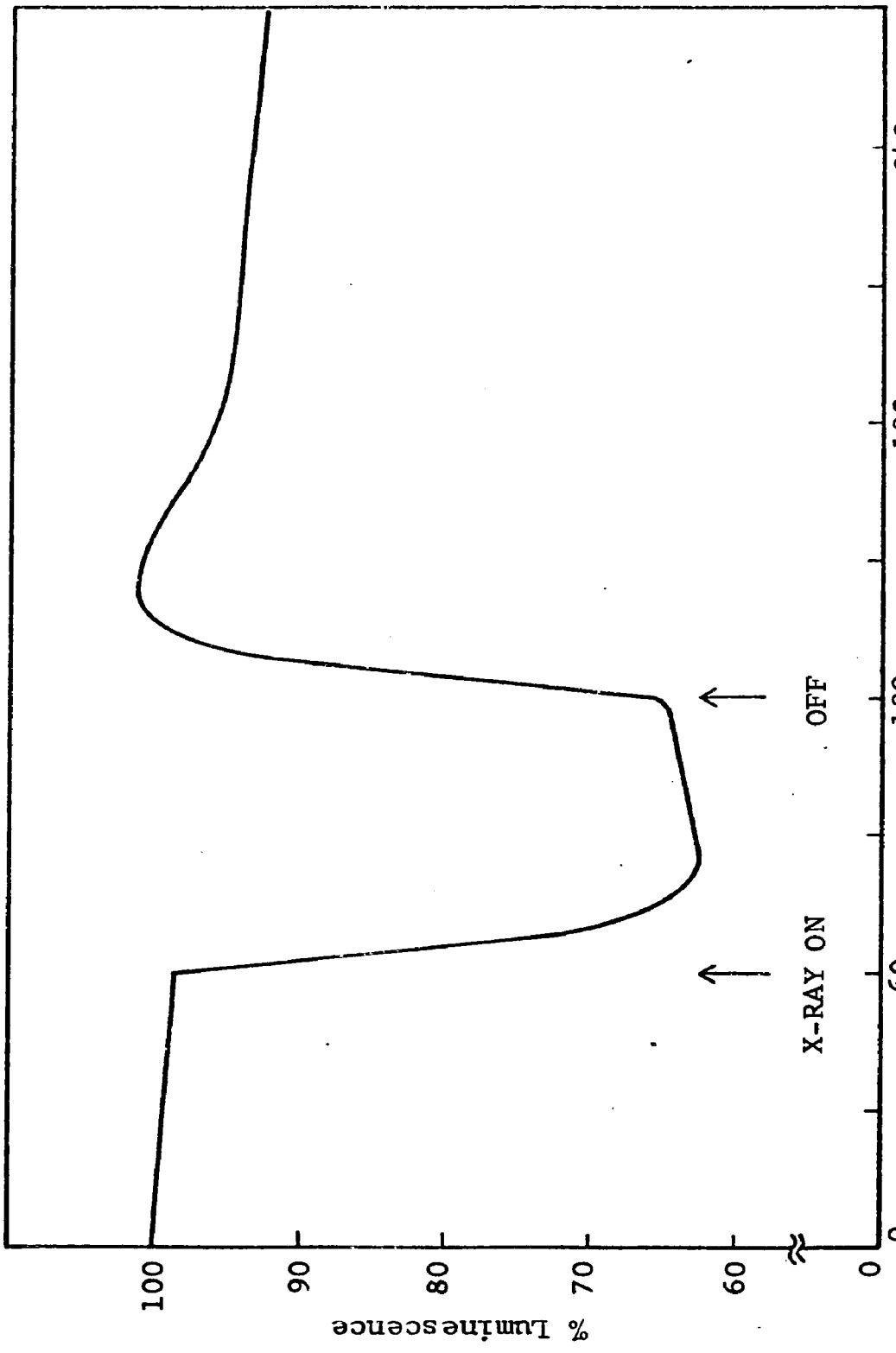


Figure 32. Effect of X-irradiation on Luminescence Produced by the Coupled Reaction of NADH-dehydrogenase and Luciferase.

after X-irradiation. Luminescence can be held fairly constant during the few minutes necessary for the experiment. Upon exposure to X-irradiation, luminescence is promptly depressed to 63% of the initial luminescence. After reaching the lowest point, luminescence begins to increase during irradiation. After irradiation, the luminous intensity promptly recovers. Approximately 10 seconds after exposure, the luminous intensity exceeds that of the initial luminescence for a period of one minute before returning to the control level of luminescence.

DISCUSSION

Purification

A number of researchers, Green and McElroy (51), Hastings, et al. (17), Riley (34), and Kuwabara, et al. (52), have purified luciferase from P. fischeri. However, until this study NADH-dehydrogenase from P. fischeri had not been purified. A 145 fold purification of NADH-dehydrogenase was achieved through the use of selective salt precipitation, ion-exchange and molecular seive column chromatography.

Several batches of P. fischeri cells were grown for the purification of NADH-dehydrogenase; the average yield was 6.34 g wet cells/liter of culture. Using the same growth medium (Table I) Farghaly (33) and Riley (34) obtained a yield of 10 g/liter. The cells in this study and those of Riley were both harvested at peak growth and luminescence. The cells were harvested at peak growth for a maximum yield of bacteria and at peak luminescence for maximum yield of enzyme. The difference between the yield values could possibly be attributed to the efficiency of removal of the cells from the medium by different continuous centrifuges.

The substrates for the NADH-dehydrogenase reaction are NADH and FMN. The spectra of oxidized and reduced NADH and

FMN were run to show the feasibility of assaying NADH-dehydrogenase spectrophotometrically. Either the disappearance of the peak at 340 nm due to the oxidation of NADH or the disappearance of the FMN peak at 450 nm upon anaerobic reduction could be utilized. Although FMN does exhibit absorbance at 340 nm, its contribution to the total absorbance of the assay solution is minimal and constant because: 1) its molar extinction coefficient at 340 is lower than that of NADH, 2) its concentration is 100 fold less than that of NADH, and 3) the rapidity with which $FMNH_2$ is autoxidized by oxygen insures a constant contribution to absorbance. The molar extinction coefficients of NADH at 340 nm and FMN at 450 nm were calculated to be 6.2×10^3 liter $mole^{-1}cm^{-1}$ and 1.2×10^4 liter $mole^{-1}cm^{-1}$, respectively; these values agree with those found in the literature.

In the purification of NADH-dehydrogenase it was necessary to remove nucleic acids before selective salt precipitation with ammonium sulfate could be used. The highly viscous nature of nucleic acids in the cell lysate made selective salt precipitation an ineffective means of purification. Removal of nucleic acids by manganous chloride resulted in extremely large losses of NADH-dehydrogenase, probably from co-precipitation or inactivation. Precipitation of nucleoproteins by protamine sulfate was also attempted; again NADH-dehydrogenase appeared to be precipitated or inactivated. Therefore, the removal of nucleic acids in the crude extract of P. fischeri was achieved by addition of specific nucleases,

DNAase and RNAase, to the crude extract. Nucleases lowered the viscosity of the lysate and permitted an efficient precipitation with ammonium sulfate. When ammonium sulfate was used to selectively precipitate NADH-dehydrogenase from other cellular substances, the fold purification for this step was only 1.35; however 50% of the total protein was removed by this step as can be seen in Table XI Parts A and B.

One of the difficulties that had to be solved in the purification procedure was the inactivation of NADH-dehydrogenase apparently by being in solutions of low ionic strength and/or without a sulfhydryl protector. In the ammonium sulfate precipitation step only slight inactivation was noted, possibly because the step is a rapid one performed in solutions of high ionic strength. A great amount of inactivation of NADH-dehydrogenase was noted on the first DEAE-Sephadex column that was run. The inactivation may have resulted from the low ionic strength buffer of 0.002 M potassium phosphate with which the linear gradient began. On subsequent DEAE-Sephadex chromatography the lower salt concentration was increased to 0.05 M. Hastings (17) and Kuwabara (34) found that 2-mercaptoethanol stabilized luciferase from P. fischeri; they also observed that 2-mercaptoethanol partially stabilized NADH-dehydrogenase, but not to the degree that it had stabilized luciferase. A possible explanation of the protective effect of 2-mercaptoethanol is that its sulfhydryl groups compete with sulfhydryl groups on the enzyme for oxidative reactions which could easily alter the conformation and/or

activity of the enzyme. 2-mercaptoethanol was used in all purification procedure solutions. The results of Hastings and Kuwabara that 2-mercaptoethanol did not fully stabilize NADH-dehydrogenase were substantiated. Further attempts to stabilize the enzyme in solutions of low ionic strength were unsuccessful. NADH-dehydrogenase appeared to be fairly stable in solutions of high ionic strength and/or solutions with a sulfhydryl protector.

During purification of luciferase from P. fischeri Hastings (17) also assayed for NADH-dehydrogenase, in an attempt to completely separate the two enzymes; he was only able to remove 87% of NADH-dehydrogenase activity from luciferase. The elution pattern from DEAE chromatography obtained by Hastings and the pattern in Figure 13 are quite similar. Both patterns show a large protein peak at about the same buffer concentration and the NADH-dehydrogenase peak fits within the left-hand side of the large protein peak. Separation of NADH-dehydrogenase from luciferase on DEAE-Sephadex was incomplete.

Ultrafiltration by Diaflo proved an excellent method for re-concentrating large volumes of dilute enzyme solutions which were invariably obtained when pooling active fractions from column chromatography. There was no loss of NADH-dehydrogenase through the membrane filter.

The G-75 Sephadex column, Figure 14, successfully separated the major protein peak from the active, NADH-dehydrogenase peak and gave a fold purification of 10. A luciferase

assay on the pooled enzyme from the G-75 Sephadex column indicated the presence of some luciferase in the NADH-dehydrogenase preparation, Table XII. A polyacrylamide gel of pooled NADH-dehydrogenase from the G-75 Sephadex column, Figure 16, indicated the presence of one major protein band and five minor bands. The pink, NADH-dehydrogenase band was well-separated from the other bands. Thus, at this point the investigator was encouraged to try preparative disc gel electrophoresis as the final step in the purification procedure. The preparative gel technique consisted of electrophoresis of 50 mg of the NADH-dehydrogenase preparation from the G-75 Sephadex column on a 2.5 cm x 10 cm polyacrylamide gel. The pink band was sliced from the gel and the enzyme extracted. The yield of active enzyme was extremely low, too low to merit using this technique to obtain the large amount of purified enzyme that was needed for characterization and irradiation studies. NADH-dehydrogenase was either inactivated or, more likely, trapped in the gel matrix. The small amount of enzyme that was extracted was used to run the reduced and oxidized absorption spectra of NADH-dehydrogenase, Figure 22. Thus, to further purify NADH-dehydrogenase and to separate it from luciferase, the Superfine G-75 Sephadex column was run, Figure 15. After this column the presence of luciferase in the preparation of NADH-dehydrogenase could not be detected, Table XII. NADH-dehydrogenase from the Superfine G-75 column was pooled and was aliquoted for use in characterization and irradiation studies.

The overall yield of the purification procedure is very high, 34%. One reason for the high yield is that this purification scheme primarily utilizes column chromatography from which loss of enzyme is due to inactivation. Purification schemes utilizing precipitation procedures usually exhibit a lower yield. The purification scheme successfully removed 99.8% of the total protein and gave a fold purification of 145 for NADH-dehydrogenase.

Disc gel electrophoresis was run with NADH-dehydrogenase preparation pooled from the Superfine G-75 Sephadex column. The gel scan, Figure 17, shows one major enzyme band and two minor protein bands. The major protein band was NADH-dehydrogenase. By comparing electrophoresis of the enzyme after G-75 Sephadex, Figure 16, and after Superfine G-75 Sephadex, Figure 17, it can be seen that two proteins were eliminated from the preparation by Superfine G-75 Sephadex chromatography. The NADH-dehydrogenase preparation represented in Figure 17 was used for the irradiation studies.

A luciferase assay was performed after each step of the purification procedure to assure separation of NADH-dehydrogenase from luciferase. It was essential to remove luciferase from the NADH-dehydrogenase preparation, so that during irradiation only the effect of radiation on the NADH-dehydrogenase reaction would be observed. As seen in Table XII NADH-dehydrogenase was, indeed, separated from luciferase. At any rate, the amount of luciferase in the NADH-dehydrogenase preparation was below the lower limit of detectability of the

luciferase assay. It should be noted that the luciferase assay was qualitative, rather than quantitative, in nature. The velocity of the luciferase reaction depends on the available substrate for the luciferase reaction, $FMNH_2$. Because the enzyme solutions from each purification step contained different amounts of NADH-dehydrogenase, the amount of $FMNH_2$ produced would vary. Thus, the test would be qualitative rather than quantitative.

Characterization

Physical factors affecting the NADH-dehydrogenase reaction were optimized, so that during irradiation studies the enzyme would be operating at its optimal activity. In the enzyme concentration curve, Figure 18, the slope is linear and intersects the origin. Thus, the enzyme preparation contains no inhibitors; additionally, the enzyme does not require an activator for its activity. NADH-dehydrogenase which showed optimal activity at pH 7.0 to 9.0, Figure 19, showed decreased activity at pH below 7.0 and above 9.0. Decreased activity probably resulted from a change of ionization of the enzyme. No attempt was made to determine whether the affinity of the enzyme for its substrate, K_M, or whether the maximal velocity, V_{max}, was affected by pH. Just as Hastings (17) and Riley (34) found that BSA enhanced the activity of luciferase, it was found that the addition of BSA increased NADH-dehydrogenase activity by 48%, Figure 21.

The reduced spectrum of NADH-dehydrogenase exhibits peaks at 423, 527, and 557 nm, the characteristic spectrum of a cytochrome enzyme. More specifically, the spectrum of reduced NADH-dehydrogenase is identical to that of cytochrome b_5 reductase from human erythrocytes as determined by Passon and Hultquist (53).

The estimation of molecular weight by column chromatography is based on the assumption that the calibration proteins and the enzyme in question are of similar conformation. The apparent molecular weight of NADH-dehydrogenase determined by this method is 53,000, Figure 25. Hastings (17) and Kuwabara (52) have shown the molecular weight of luciferase to be 58,000. From DEAE chromatography by Hastings (17) and in this study, it is indicated that the net charge on NADH-dehydrogenase and luciferase are similar. Now it appears that their molecular weights are also very close. These two factors may well account for the fact that no purification of luciferase has, as of yet, been free of NADH-dehydrogenase. This study reports the separation of NADH-dehydrogenase from luciferase.

Radiation Studies

The reaction of NADH-dehydrogenase from *P. fischeri* was measured before, during, and after irradiation in the presence of its substrates (NADH, FMN). As seen in Figure 26-A, the rate of the reaction increases during irradiation

and returns to the original rate immediately after irradiation. Since the rate of oxidation of NADH before and after irradiation exposure are the same, NADH-dehydrogenase was not permanently altered by X-irradiation. Within the first two seconds of exposure, the increased oxidation rate of NADH was evident. The time lag resulted from response time of the recorder and instrument. Although the increased rate of oxidation could not be detected during the very first second of exposure, it appears that the increased reaction rate is an immediate effect commencing with the start of X-irradiation.

To study whether this increased reaction rate was due to an increased rate of the enzymatic reaction or to another means of NADH-oxidation, a nonenzymatic reaction was run using the same substrate concentrations. In Figure 26-B no reaction rate is observed before irradiation; during irradiation NADH is nonenzymatically oxidized, and after exposure this oxidation stops. Nonenzymatic oxidation of NADH occurs only during irradiation.

The enzymatic reaction, as shown in Figure 26-A, is a first order reaction where k_1 is the rate constant before irradiation. In Figure 26-B the rate of oxidation of NADH appears to be linear, and is, therefore, a reaction of the first order. The nonenzymatic rate constant during exposure will be designated k_2. Considering the fact that the oxidation of NADH occurs enzymatically in the absence of radiation and nonenzymatically in the presence of X-irradiation, one would conclude that during irradiation NADH-dehydrogenase and

the products of radiolysis compete for NADH. The results indicate a parallel first order reaction in which the rate constant of the enzymatic reaction in the absence of X-irradiation, k_1, plus the nonenzymatic reaction during irradiation, k_2, are equal to the rate constant of the enzymatic oxidation of NADH during irradiation, k_3. As can be seen from Figures 26-A and 26-B, when rate constants k_1 and k_2 are added, the sum equals the rate constant k_3 of the enzymatic reaction during radiation. The enzymatic and non-enzymatic oxidation of NADH during irradiation was run at various concentrations of NADH from which identical results were obtained. It can therefore be concluded that the increased rate of oxidation of NADH enzymatically during irradiation is due to the non-enzymatic oxidation of NADH by radiation.

After examining the enzymatic oxidation of NADH during irradiation, the effect of X-radiation on the substrate dependence of NADH-dehydrogenase was determined. As the concentration of NADH was varied, the NADH dehydrogenase reaction was measured with and without exposure to X-irradiation. The nonenzymatic oxidation of NADH was subtracted from the enzymatic oxidation. The resulting data were plotted by the method of Lineweaver and Burk, Figure 27. Neither the apparent affinity of the substrate for its enzyme, K_M, nor the apparent maximum velocity, V_{max}, were altered during irradiation which indicates that the NADH-dehydrogenase enzyme intermediates are not affected by radiation.

Bacq (32) has stated that if the product of the radiation reaction does not react again with free radicals, then the number of molecules changed will be directly proportional to dose rate. This relationship has been observed in the oxidation of ferrous ions to ferric ions (54). Because the nonenzymatic oxidation of NADH in the presence of FMN during irradiation in Figure 28 is directly proportional to the dose rate, it is implied that NADH is oxidized by indirect action. One cause for doubt that NADH is oxidized by indirect action as shown in Figure 28 is when the proportion of the total molecules affected is small then the semi-logarithmic response is indistinguishable from a linear response. Also if the dose does not cover a large range, the curve is effectively a straight line.

To effectively distinguish between direct and indirect action on molecules in solution, the dilution test as described by Bacq and Alexander (32) is decisive. For a given dose, the effect is by direct action if the number of molecules affected in the irradiation solution is directly proportional to the concentration. However, the action is indirect if the number of molecules affected is independent of the concentration because a fixed number of free radicals are produced by a constant dose. Because of free radical recombination, the dilution test does not hold for solutions of low concentrations. When the dilution test was applied to the rate of oxidation of NADH the results in Figure 29 indicate that the rate of oxidation during irradiation in the presence

of FMN, Curve A, is greater than the rate of oxidation of NADH at identical concentration in the absence of FMN, Curve B. Barron (53) has also shown that NADH is oxidized by X-irradiation in an aqueous solution of pH 7.0. Curve B demonstrates that the rate of oxidation of NADH is constant and therefore must result from indirect action. From Curve A it is observed that the presence of FMN dramatically increases the rate of oxidation of NADH. Thus, FMN must be participating in some manner to increase the rate of oxidation of NADH. Additionally, since Curve A appears to be approaching a maximum at the higher concentrations of NADH, an indirect radiation effect is indicated.

Burns, et al. (6) postulated that the radiation-sensitive component of the in vitro luminescent system is reduced flavin mononucleotide ($FMNH_2$). $FMNH_2$, a product of the NADH-dehydrogenase reaction, is also a substrate for the luciferase reaction. If $FMNH_2$ were oxidized during irradiation, and if this oxidation ceased after irradiation, it would account for the depression of luminescence demonstrated by Burns.

The effect of X-rays on the production of $FMNH_2$ by the anaerobic NADH-dehydrogenase reaction is shown in Figure 30-A. X-irradiation is seen to increase, rather than to decrease or reverse, the rate of production of $FMNH_2$. The nonenzymatic rate of FMN reduction by irradiation is seen in Figure 30-B; identical results were obtained with various concentrations of FMN. As with the increased enzymatic rate of oxidation of NADH during irradiation, the increased production of $FMNH_2$

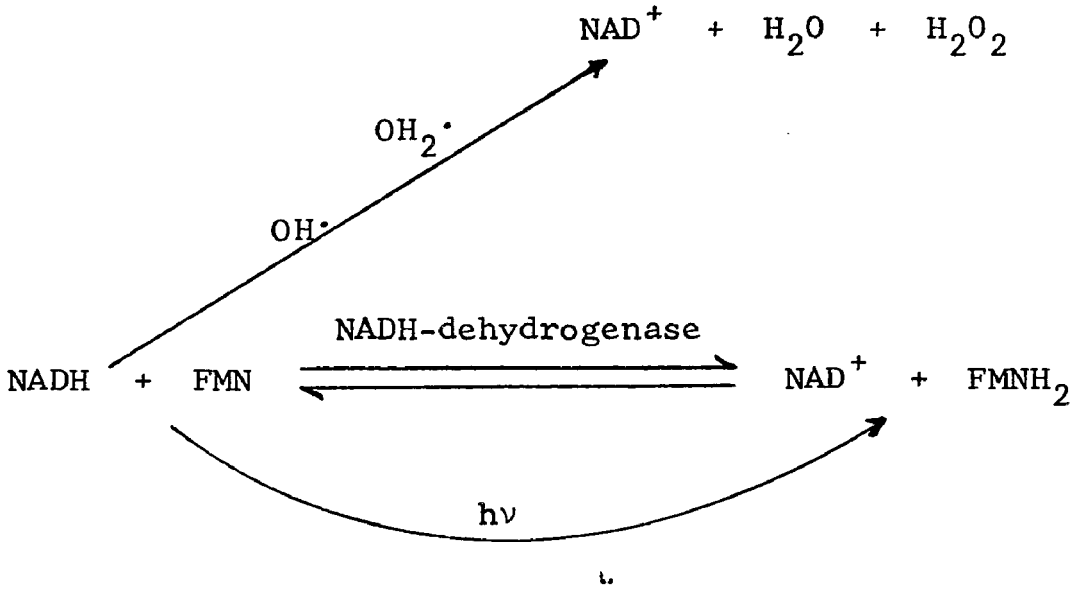
during irradiation is due to the nonenzymatic reduction of FMN. As before, the reaction is a parallel first order reaction where the enzymatic pre-irradiation rate, k_1, plus the non-enzymatic reduction rate during irradiation, k_2, is equal to the enzymatic reduction rate during irradiation, k_3. Thus, the increased rate of production of $FMNH_2$ is due to radiation-induced reduction of FMN. Because $FMNH_2$ in the presence of NADH is produced by irradiation and not oxidized by radiation, luminescence in vitro is not depressed by the mechanism suggested by Burns.

The dilution test was also used to determine the action by which anaerobic reduction of FMN occurs during irradiation, Figure 31. The results, instead of indicating whether the effect of radiation was direct or indirect, indicated a phenomenon of a completely different nature. The anaerobic irradiation of FMN in the presence of NADH as shown in Figure 31 Curve A indicates that FMN is reduced to $FMNH_2$, whereas Curve B shows that FMN is not reduced in the absence of NADH during irradiation. This indicates that NADH not only reduced FMN, but also that the X-rays act as a catalyst for the reaction. When Schachinger (56,57) irradiated the alcohol dehydrogenase reaction in the presence of its substrates, he observed that the reverse reaction, namely

$$NADH + CH_3CHO \rightleftharpoons NAD^+ + C_2H_5OH,$$

takes place during irradiation in the absence of enzyme. These observations imply that radiation may supply the necessary activation energy for these reactions to occur.

From the previous reaction results, one can postulate a possible mechanism for the increased rate of the NADH-dehydrogenase reaction during irradiation.



This mechanism indicates that NADH is oxidized by free radicals produced by radiation. Both NADH and FMN are activated by radiation to increase the rate of the reaction during irradiation. From the results obtained by this investigator there is no evidence that NADH-dehydrogenase reaction plays a role in the depression of luminescence during irradiation.

NADH-dehydrogenase was coupled to the luciferase reaction. Luminescence, produced after the addition of the substrates, is initially steady-state, Figure 32. Upon exposure to X-irradiation the luminescence decreases to a lower state of

luminescence and returns to the initial level after passing through a peak that exceeds the pre-exposure luminescence. These radiation effects on the NADH-dehydrogenase-luciferase reactions during irradiation are identical to those observed by Burns (6) and Jacobson (58) in their experiments with cell-free extracts. During irradiation luminescence, after reaching a minimum value, increases. Since luciferase is in excess, this increase may be attributed to the increased rate of the reduction of FMN from the dehydrogenase reaction as indicated in the previous experiments. After irradiation luminescence exceeds the initial luminescence, indicating a burn-off of a component that has accumulated during irradiation. The identity of the accumulated intermediate might be reduced luciferase-$FMNH_2$. As shown in Figure 1, the reduced luciferase-$FMNH_2$ reacts with oxygen to form the luciferase-$FMNH_2$-O_2 complex as postulated by Hastings and Eberhard (19). Jacobson (58) has shown that radiation depression of luminescence is increased by decreased dissolved oxygen in the enzyme solution. During radiation of aqueous solutions, hydrogen free radicals and e^- (aqueous electrons) are formed, which react with oxygen in the following manner:

$$H^{\cdot} + O_2 \longrightarrow HO_2^{\cdot}$$

$$e^- + H^+ \longrightarrow H^{\cdot} + O_2 \longrightarrow HO_2^{\cdot}$$

$$e^- + O_2 \longrightarrow O_2^-$$

As seen from the reactions these species would compete for oxygen in the solution, thereby depressing luminescence. These species are short-lived species which only exist during irradiation and could account for the depression during irradiation.

SUMMARY

During this investigation NADH-dehydrogenase was purified from Photobacterium fischeri. The purified enzyme was used to study the characteristics of the enzyme. Additionally, the enzyme was used to study the kinetics of the reaction before, during, and after irradiation. This will aid in explaining the depression of bioluminescence. After studying the contribution of the NADH-dehydrogenase reaction to the depression of luminescence, the reaction was coupled to the luciferase reaction to determine its contribution to the depression of bioluminescence.

1. NADH-dehydrogenase was purified from P. fischeri with a fold purification of 145 and a yield of 34%.
2. Disc gel electrophoresis of purified NADH-dehydrogenase indicated one major enzyme band and two minor protein bands.
3. The apparent molecular weight of NADH-dehydrogenase was 53,000 as determined by column chromatography.
4. The visible absorption spectra of purified NADH-dehydrogenase, with oxidized peaks at 410 and 530 nm and reduced peaks at 423, 527, and 557 nm, exhibited a characteristic cytochrome spectrum.

5. NADH-dehydrogenase activity was unaltered by radiation; neither the apparent V_{max} nor the apparent K_M were altered.
6. Oxidation of NADH and reduction of FMN occur during irradiation in the absence of NADH-dehydrogenase.
7. Under the conditions described $FMNH_2$ is not oxidized by radiation.
8. The NADH-dehydrogenase reaction does not participate in the depression of luminescence.
9. When NADH-dehydrogenase is coupled to the luciferase reaction, luminescence is depressed by radiation. Therefore, depression of luminescence must occur in the luciferase reaction.

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