

DARK ANAEROBIC OXIDATION OF NADH BY FUMARATE WITH  
CHROMATOPHORES FROM RHODOSPIRILLUM RUBRUM

by

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## ABSTRACT

A segment of the photosynthetic electron transfer complex in chromatophores from Rhodospirillum rubrum has been studied by the dark anaerobic oxidation of reduced nicotinamide adenine dinucleotide (NADH) by fumarate. The reaction is stoichiometric and entirely chromatophore-dependent. Maximal activity occurred at pH 7.2, and all activity was lost below pH 5.0, and above pH 8.0. Malonate, rotenone,  $\text{Hg}^{++}$ , and p-hydroxymercuribenzoate, but not 2N-heptyl-4-hydroxyquinoline-N-oxide, inhibited the reaction; malonate inhibition was competitive with the substrate fumarate. Catalytic quantities of quinacrine, riboflavin, flavin mononucleotide, flavin adenine dinucleotide, and a flavoprotein from Azotobacter vinelandii each enhanced the rate of NADH oxidation. Using stoichiometric amounts of riboflavin the reaction was further studied: riboflavin was first reduced by NADH, and then re-oxidized by fumarate. Some reduction of riboflavin by NADH occurred in the absence of chromatophores, but addition of chromatophores increased the rate of this reduction seven-fold. Oxidation of the reduced riboflavin by fumarate did not proceed in the absence of chromatophores. The dark enzymatic reduction and subsequent oxidation of riboflavin were similarly inhibited by

rotenone, but only the oxidation of reduced riboflavin by fumarate was inhibited by malonate. The results of these studies are in agreement with the findings of others for the photochemical reduction of NAD by succinate.

## INTRODUCTION

The photochemical apparatus of photosynthetic bacteria is located in pigmented particles called chromatophores. These organelles are responsible for the conversion of light energy to an energy form which the organism stores. Although chromatophores are similar to the organelles (mitochondria) responsible for energy conversion in mammalian systems (1), many reactions of chromatophores require light. Thus these complex particles have been used to demonstrate photophosphorylations, photoreductions, photooxidations, and, more recently, dark oxido-reductions (2).

Chromatophores from Rhodospirillum rubrum, a non-sulfur purple photosynthetic bacterium, have been studied to the greatest extent. Illumination of bacteriochlorophyll-containing chromatophores promotes electrons to a high energy state. As these excited electrons subsequently return to their ground states through a system of electron transfer components containing cytochromes, quinones, heme proteins, non-heme iron proteins, and appropriate enzymes, the released energy is stored as adenosine triphosphate (3). Exogenous electron sources (reductants) may be used to reduce added electron acceptors (oxidants). For example, in the presence of

NAD,<sup>1</sup> as an oxidant, and chromatophores, succinate may be photooxidized to fumarate simultaneously with the photo-reduction of NAD (4).

This paper describes a study of the anaerobic oxidation of NADH by fumarate, a dark oxido-reduction involving a segregated part of the photochemical apparatus contained in Rhodospirillum rubrum chromatophores. Evidence for the function of this reaction in the photochemical electron transfer sequence will be presented, and the possible involvement of riboflavin in this reaction will be discussed. Just as similar partial reactions catalyzed by mitochondria have been useful for the study of electron transfer in submitochondrial particles (5), the oxidation of NADH by fumarate should be a useful tool for similar studies with subchromatophore particles.

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1. The following abbreviations will be used in this paper:  $A_{\lambda}$ , absorbance, the subscript representing the wavelength in nm; ADP, adenosine diphosphate; ATP, adenosine triphosphate; Bchl, bacteriochlorophyll; BSA, bovine serum albumin; DPIP, 2,6-dichlorophenol indophenol; FAD, FADH<sub>2</sub>, flavin adenine dinucleotide and its reduced form; FMN, FMNH<sub>2</sub>, flavin mononucleotide and its reduced form; HOQNO, 2N-heptyl-4-hydroxyquinoline-N-oxide;  $K_m$ , Michaelis constant;  $K_I$ , inhibition constant; NAD, NADH, nicotinamide adenine dinucleotide and its reduced form; PHMB, p-hydroxymercuribenzoate; PMS, phenazine methosulfate; RF, RFH<sub>2</sub>, riboflavin and its reduced form; RHP, Rhodospirillum rubrum heme protein; SDH, succinate dehydrogenase; Tris, tris-(hydroxymethyl)aminomethane.

## LITERATURE REVIEW

The concept of the chromatophore as the locus of photochemical activity in photosynthetic bacteria was introduced in 1952 by Schachman, Pardee, and Stanier (6), who separated these pigmented particles from broken cells of light-grown Rhodospirillum rubrum by differential centrifugation. Although some disagreement with their proposal exists (7), chromatophore preparations have been used extensively to study photophosphorylations, photo-reductions, photooxidations, and dark oxido-reductions (2).

The first significant experiment utilizing chromatophore preparations was made by Frenkel in 1954 (8). Illumination of chromatophore preparations to which ADP and inorganic phosphate had been added resulted in the formation of ATP. This was the first observation of bacterial "cyclic" photophosphorylation. No net oxidation or reduction of added substrates occurs in this type of electron transfer.

A few years later Frenkel (4), and Vernon and Ash (9), demonstrated the photoreduction of NAD by succinate with chromatophores. Both antimycin A and HOQNO inhibit this reaction. Such inhibition is overcome by using an electron donor with a low oxidation potential, e.g., the DPIP-ascorbate couple (10).

Nozaki et al. (11) obtained what they called "non-cyclic" photophosphorylation. Such phosphorylation was assumed to accompany the photoreduction of NAD occurring when antimycin A was added to block the cyclic electron transfer system, and DPIP-ascorbate was used as the electron donor system. The quantity of ATP formed was proportional to the total NAD reduced. In a review (2) on bacterial photosynthesis Vernon states that their interpretation for a phosphorylation accompanying non-cyclic electron transfer appears to be valid; however, further studies are needed to completely resolve the objections raised by Böse and Gest (12), who argue that DPIP acts as a bypass for the site of antimycin A inhibition, and that the observed phosphorylation is, therefore, actually cyclic.

Further study of the photoreduction of NAD showed that malonate inhibited the reaction when succinate was employed as an electron donor (13). Hinkson (14) reported that malonate did not affect the reaction when DPIP-ascorbate or reduced ubiquinone were used as electron sources. The same paper states that quinacrine, known to inhibit flavin-containing enzymes (15), almost completely inhibited the photoreduction, regardless of which of the three electron donors was used. Other inhibitors of the photoreduction of NAD include oligomycin, gramicidin,

amytal, and carbonyl cyanide-m-chlorophenylhydrazone, commonly referred to as m-Cl-CCP (14).

Alternatives to the study of chromatophores by photoreactions have been provided by Chance and his colleagues (16, 17), who demonstrated an ATP dependent reduction of NAD by succinate with mammalian mitochondria. In chromatophores light had been used almost exclusively as the source of energy for such reactions. However, based upon the observations of Chance et al., Löw and Alm (18) used ATP to drive a dark reduction of NAD by succinate in the presence of chromatophores. Antimycin A did not affect this reaction, but rotenone and oligomycin inhibited both the dark reduction and the photoreduction of NAD by all electron donors.

About the same time another group successfully demonstrated reversal of this reaction--the dark enzymatic reduction of fumarate by NADH (19). However, reduction of fumarate in their experiments was associated with a concurrent NADH oxidase reaction; hence, the fumarate reaction was not extensively studied.

In an attempt to correlate the many investigations of electron transfer with R. rubrum chromatophores, Vernon (2) proposed an electron transfer sequence as a working model for current studies. Figure 1 shows an outline of this pathway. The components of the system are arranged according to their oxidation potentials, from the highest,

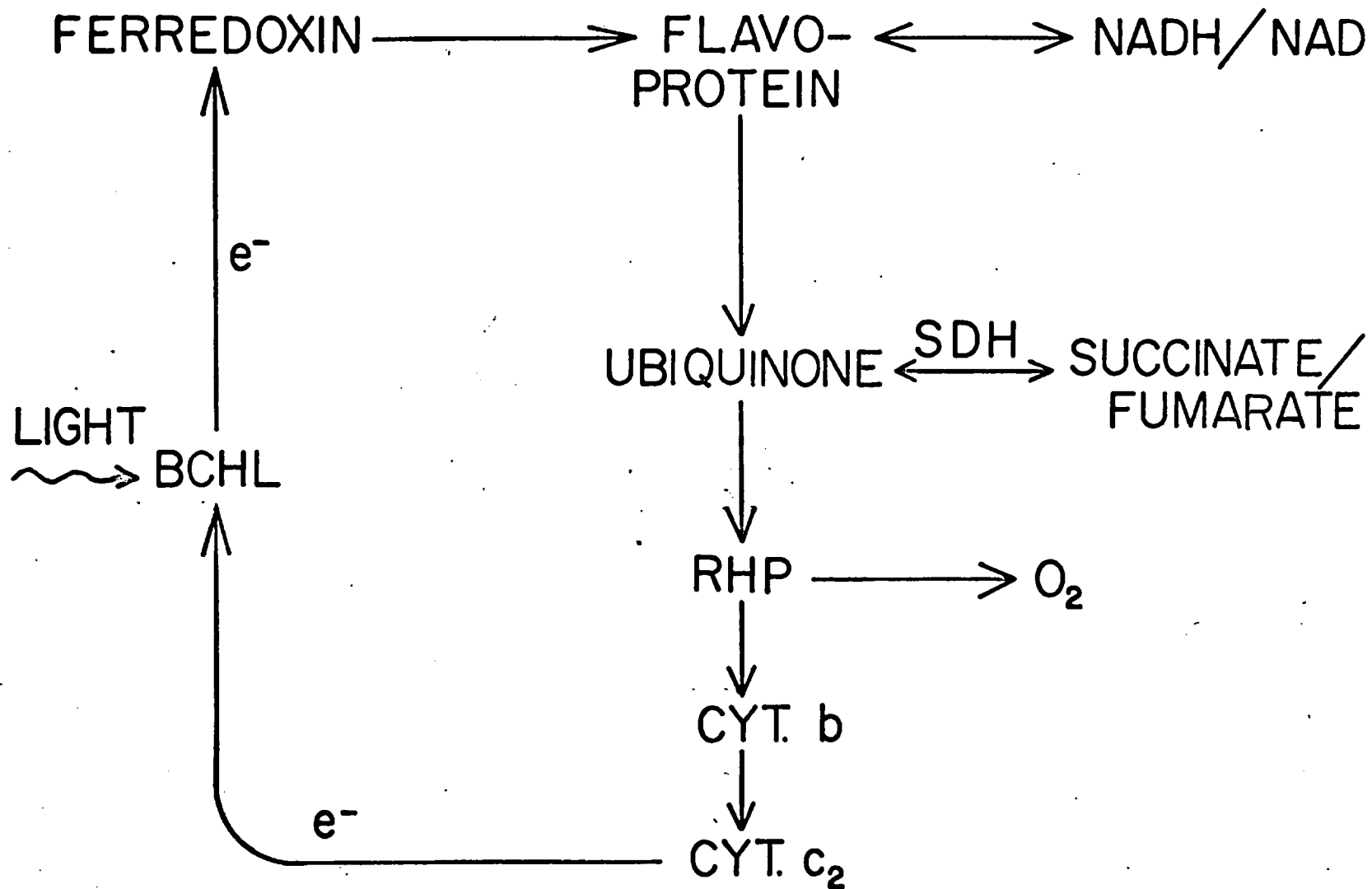


Figure 1. Proposed Electron Transfer Sequence in *R. rubrum* (2).

NAD/NADH, to the lowest, cytochrome  $c_2$ . Since HOQNO and antimycin A act between cytochromes  $b$  and  $c_2$  (20), electron donation by the DPIP-ascorbate couple, which is unaffected by these reagents, probably occurs at the cytochrome  $c_2$  level of the cycle. The site for NAD reduction is assumed to be associated with a flavoprotein, because flavoenzyme inhibitors prevent electron transfer to NAD from all electron donors studied thus far. Succinate dehydrogenase is probably near ubiquinone, as suggested from the similarity of this electron transfer system to that of the mitochondrion.

Ferredoxin, although present in R. rubrum (21), may or may not be a component of the photosynthetic pathway. Its placement arises from a comparison of its position in electron transfer processes postulated for plant photosynthesis. R. rubrum heme protein (RHP) is readily oxidized by molecular oxygen, and may be part of an NADH oxidase system. This protein is essential for phosphorylation (22), and was, therefore, given an intermediate position in the electron transfer pathway.

Although other proposed electron transfer pathways are consistent with current knowledge (23, 24, 25), the simplified pathway presented in Figure 1 will be used as a basis for discussion of the experimental results obtained in this study.

## MATERIALS AND METHODS

### Chemicals

Riboflavin, white label grade, was obtained from Eastman Organic Chemical Co., Rochester, New York. Crystallized bovine albumin was obtained from Pentex, Inc., Kankakee, Illinois. Fumaric acid, succinic acid, DL-malic acid, L(+)-glutamic acid, and iodoacetic acid, all Baker grade, were obtained from J. T. Baker Chemical Co., Phillipsburg, New Jersey. Malonic acid, tris(hydroxymethyl)-aminomethane, and 2,6-dichlorophenol indophenol, each B-grade, phenazine methosulfate and nicotinamide-adenine dinucleotide (oxidized and reduced forms), each A-grade, were obtained from Calbiochem, Los Angeles, California. Rotenone, quinacrine hydrochloride, reduced nicotinamide-adenine dinucleotide phosphate, Type I, flavin adenine dinucleotide, grade III, flavin mononucleotide, commercial grade, adenosine-5'-triphosphate, Sigma grade, snake venom (Naja-Naja cobra), and 2N-heptyl-4-hydroxyquinoline-N-oxide were obtained from Sigma Chemical Co., St. Louis, Missouri. All other chemicals were obtained from commercially available sources, and were of reagent grade. Ion-low water was prepared by filtering distilled water through a Barnstead Bantam Demineralizer which was equipped with a standard cartridge.

### Equipment

The following spectrophotometers were used for absorbance measurements: Cary Model 14; Cary Model 11; Beckman Model DB, and a Gilford Model 240. Either a Beckman ten-inch Potentiometric Linear-Log recorder, or a Heath Servo recorder, model EUW-20, equipped with a multi-speed chart drive, was used with the Beckman and Gilford spectrophotometers. Centrifugations were executed in a Sorvall Superspeed automatic refrigerated centrifuge, model RC2-B, and a Beckman Model-L Ultracentrifuge; bacteria were harvested with a Sharples Type T-1 Super-centrifuge. A Heath pH recording electrometer, model EUA-20-11, equipped with a Sargent combination electrode was used for all pH measurements. The instrument was calibrated with standard buffer, pH 7.0, made with Coleman buffer tablets. A Branson 20kc Sonifier was used for all sonic oscillations. Disposable micropipets were obtained from Drummond Scientific Co., Broomall, Pennsylvania. "Plumpers," tiny plastic reagent spoons which were used for mixing solutions in cuvettes, were obtained from Calbiochem. Thunberg tubes modified for spectrophotometric assays were obtained from Kontes Glass Co.

### Growth of Bacteria

Rhodospirillum rubrum was grown with illumination under anaerobic conditions in the medium of Cohen-Bazire

et al. (26). The components were divided into the three concentrated stock solutions whose composition is shown below, and were stored at about 5°.

Solution I

1.0 M Potassium phosphate buffer, pH 6.8

Solution II

1.0 M DL-Malic acid

5% L-Glutamic Acid

5% Sodium acetate

5% Ammonium chloride

Adjusted to pH 6.8 with sodium hydroxide

Solution III (per liter solution)

EDTA	10.0 g
MgSO <sub>4</sub>	14.5 g
CaCl <sub>2</sub> ·2H <sub>2</sub> O	3.3 g
(NH <sub>4</sub> ) <sub>6</sub> Mo <sub>7</sub> O <sub>24</sub> ·4H <sub>2</sub> O	90.0 mg
FeSO <sub>4</sub> ·7H <sub>2</sub> O	99.0 mg
Nicotinic acid	50.0 mg
Thiamine-HCl	25.0 mg
Biotin	1.0 mg
Metals "44" Solution	50.0 ml

Adjusted to pH 6.8 with sodium hydroxide

Metals "44" Solution contains, per liter:

EDTA	2.5 g
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	10.9 g
FeSO <sub>4</sub> ·7H <sub>2</sub> O	5.0 g

$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	1.5 g
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.39 g
$\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$	0.25 g
$\text{Na}_2\text{B}_4\text{O}_7 \cdot \text{H}_2\text{O}$	0.18 g

For growth medium 20.0 ml of each stock solution were mixed, and the resulting solution was diluted to one liter with distilled water. Needed quantities of this diluted solution were then poured into the appropriate pyrex tube or bottle and sterilized. Sterile medium was stored at room temperature until needed.

The illuminating apparatus consisted of a large nearly cubic wooden box, approximately 30 inches on a side, with only the front side open. Arranged around the inside, on the three vertical walls, were ten 25 watt Sylvania clear-glass T-1 bulbs. These 6-1/2-inch bulbs were mounted in a vertical position for maximal light distribution. A fan placed in an opening in the back wall kept the temperature between 27° and 35°.

The initial colony of R. rubrum was started from an agar stab culture obtained from Charles F. Kettering Research Laboratory, Yellow Springs, Ohio. A few mg of cells were removed from the agar and transferred to a 30 ml pyrex test tube filled two-thirds with sterile medium. From another tube enough sterile medium was added to completely fill the tube, which was sealed with a screwcap.

The tube was then placed in the illumination box. After about two days, when the culture had grown to give the tube a deep red color, a few ml of suspended cells were transferred to a fresh tube of sterile medium. This tube was, in turn, filled, sealed, and incubated. In order to maintain viable cells this procedure was repeated every two days. (Cells older than two days tended to settle, and, when transferred to fresh medium, grew more slowly than did two-day cultures.)

When chromatophores were needed, some of the bacteria were also transferred to fresh sterile medium in a 250 ml bottle. This bottle was then completely filled with sterile medium from another bottle, and was sealed with a sterile ground-glass stopper. After illumination for two days the contents of the bottle were transferred to 6 liters of sterile medium in a 2-1/2 gallon bottle, 7 inches in diameter, and 18 inches tall. From another bottle were added approximately 5 liters of sterile medium to completely fill the culture bottle, which was then sealed with a sterile rubber stopper. After the usual two-day illumination period the cells were ready for harvest.

#### Preparation of Chromatophores

(See Figure 2 for an outline of the procedure used.)

Cells were harvested by centrifugation with a Sharples Super-centrifuge. Usually 50-60 grams of wet packed cells

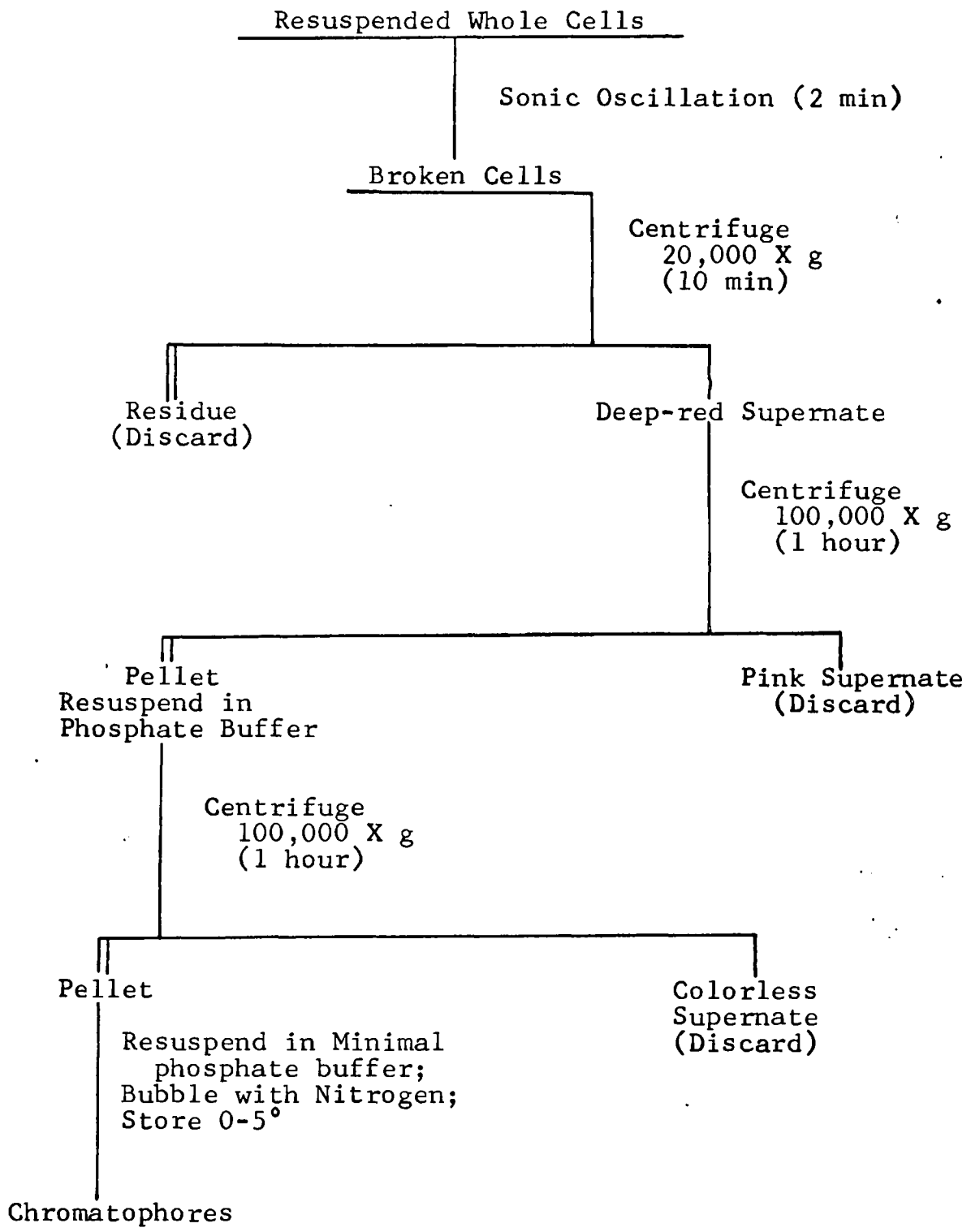


Figure 2. Outline for the Preparation of Chromatophores.

were collected per 2-1/2 gallon culture. The cell paste was resuspended in ice cold 0.1 M phosphate buffer, pH 7.5, hereafter referred to as phosphate buffer, in the ratio of 40 ml per 11 grams packed cells. Nitrogen was bubbled through the suspension to avoid a loss of enzymatic activity from air oxidation in subsequent steps. The material was also kept at zero degrees throughout preparation of the chromatophores.

Rupture of the whole cells was accomplished by sonic oscillation for about 2 minutes. The sonifier was adjusted to a power setting of 8, and a meter reading of 8.5 DC amps. As the cells were broken, the color of the suspension turned a darker red. Nitrogen was again bubbled through the mixture.

Cell wall and other debris were removed by centrifugation at 20,000 X g for ten minutes and were discarded. The chromatophore fraction was separated by centrifugation of the supernatant fluid at 100,000 X g for one hour. The resulting pink supernate was discarded, and the deep-red pellets were resuspended and were washed with phosphate buffer. After centrifugation for another hour at 100,000 X g, the colorless supernate was discarded, and the pellets were resuspended in a minimal amount of phosphate buffer to maximally concentrate the chromatophores. The suspension of material contained in the 150 ml, after cell

debris had been removed, was thereby concentrated to 30-40 ml in the final resuspension.

After nitrogen had bubbled through the concentrate for about five minutes, the chromatophores were poured into 2 or 3 polyethylene centrifuge tubes, which were then sealed with parafilm and tight-fitting caps. The preparation was stored in a refrigerator at 0-5° until needed. These chromatophores usually maintained their activity for reduction of fumarate by NADH, and also for photoreduction of NAD by succinate, for at least one month. (It should be noted that the above method results in chromatophore fragments as well as whole chromatophores.)

#### Determination of Protein Concentration

The concentration of chromatophores in terms of mg protein/ml was determined spectrophotometrically by the Biuret method of Gornall et al. (27). Biuret reagent was prepared by dissolving 1.5 g of  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  and 6.0 g of  $\text{NaKC}_4\text{H}_4\text{O}_6 \cdot 4\text{H}_2\text{O}$  in 500 ml of water. In this were added 300 ml of 10% NaOH solution. After an addition of KI (0.1%) to retard reduction of the copper, the mixture was diluted to 1.0 liter and stored in a polyethylene bottle.

Copper complexes with protein, producing an absorbance maximum at 540 nm. Therefore, a standard curve of absorbance vs protein concentration could be plotted by reacting a number of solutions of known concentrations of

protein with Biuret reagent. Bovine serum albumin (BSA) was used for this purpose. A typical standard curve is shown in Figure 3.

A known weight of BSA was carefully dissolved in phosphate buffer to give five solutions varying in concentration from 2.0 to 20.0 mg/ml. For the assay 1.5 ml of Biuret reagent was pipetted into each of 7 test tubes. To the first tube, the control which contained no protein, 1.5 ml of water were added. To the next five tubes 1.4 ml of water were added, plus 0.1 ml of the appropriate BSA solution. To the last tube 1.4 ml of water and 0.1 ml of chromatophores were added. Each tube was carefully tipped back and forth to mix the contents. A 1-hour incubation period at room temperature allowed maximal color development for all the samples.

The absorbance of each sample at 540 nm was then measured against a water blank. Because chromatophores also absorb in this region, the following procedure was used to correct for this absorption (28). After the absorbance of the chromatophore sample was determined, a few mg of KCN were added (to this sample only). Cyanide forms a colorless copper complex, and "dissolves" the copper-protein complex so that only the red color of the chromatophores remains. Cyanide added to the BSA samples or the control sample obliterated all absorbance at 540 nm. Therefore cyanide normally was added only to the unknown

Figure 3. Standard Curve for the Determination of Protein Concentration by the Biuret Method.

The curve was obtained by plotting the  $A_{450}$  (copper-protein complex) of the treated BSA samples against their concentrations (2.5, 5.0, 10.0, 15.0, and 20.0 mg/ml, respectively). The concentration of the chromatophore sample was determined by locating its  $A_{450}$  (corrected) on the curve and recording the corresponding protein concentration. The dashed lines illustrate the use of this calibration curve for protein determination. See text for other details.

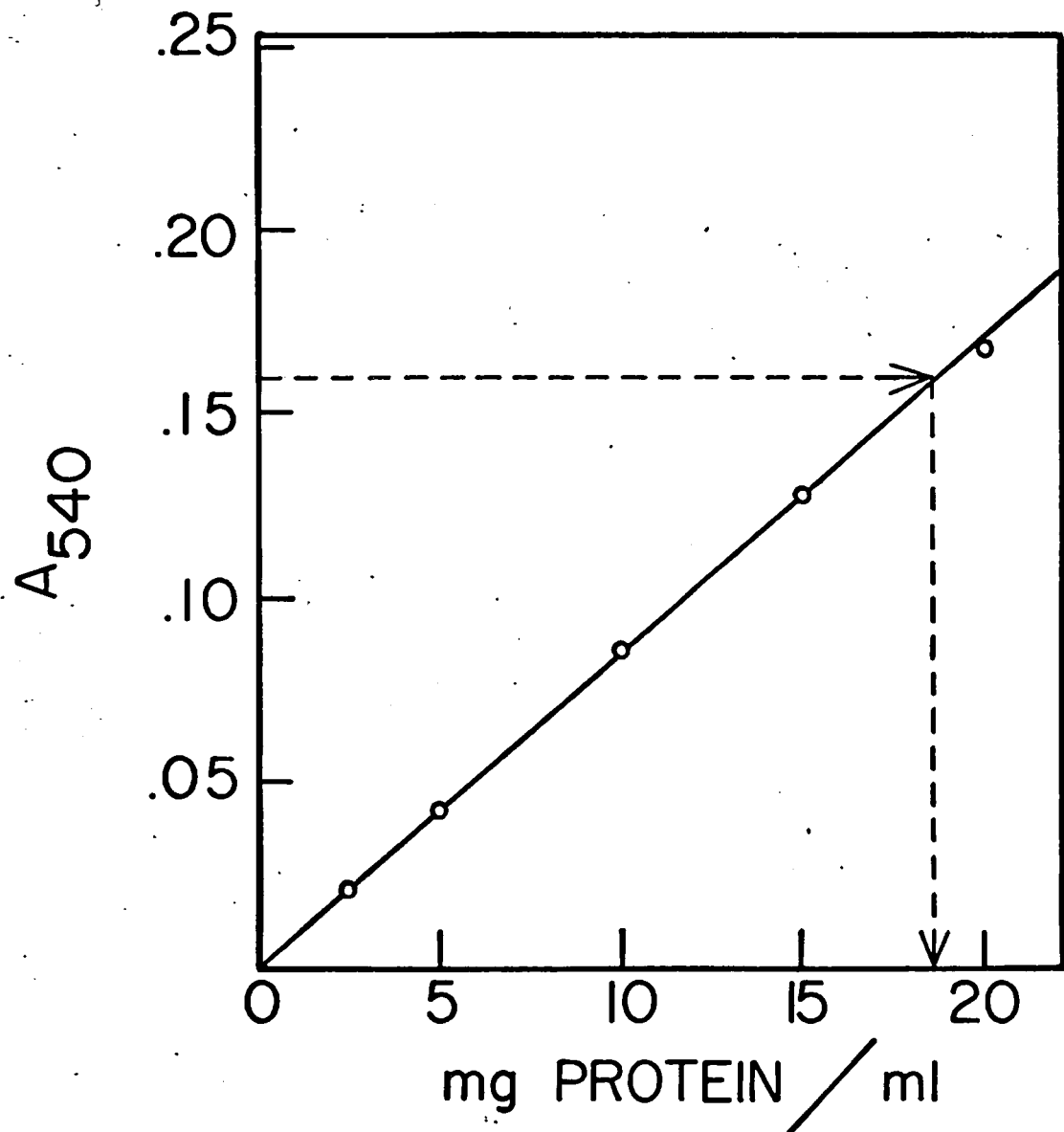


Figure 3. Standard Curve for the Determination of Protein Concentration by the Biuret Method.

sample. The absorbance due to the copper-protein complex was then calculated from the following formula:

$$A_{540}(\text{copper-protein complex}) = A_{540}(\text{before } \text{CN}^-) - A_{540}(\text{after } \text{CN}^-) - A_{540}(\text{control})$$

The standard curve was then plotted from the corrected BSA readings and from the known concentrations of the BSA solutions. The concentration of the chromatophore sample was determined by locating its corrected absorbance on the standard curve, and noting the corresponding protein concentration. Chromatophore samples usually contained 12-18 mg protein/ml.

#### Determination of Bacteriochlorophyll Concentration

Bacteriochlorophyll (Bchl) was determined spectrophotometrically according to the method of Clayton (29). After chromatophores were added to a 7:2 (v/v) acetone-methanol solution, the mixture was shaken vigorously for a few minutes. Precipitated protein was then removed by centrifugation, and the bluish-pink supernatant liquid containing the Bchl was quickly transferred to a cuvette. Absorbance of the extracted Bchl was measured at 772 nm, and the concentration was calculated by use of a millimolar absorptivity of 75 (29). During the experiment the sample was kept as dark as possible to minimize destruction of

extracted Bchl by illumination. Concentrations were expressed as  $\mu\text{moles Bchl/ml}$  of chromatophores.

#### Determination of Succinate Dehydrogenase Activity

Succinate dehydrogenase activity was measured at 600 nm with a recording spectrophotometer, as the rate of reduction of DPIP by succinate. PMS was used as an intermediate electron transferring agent to speed the reaction (30). The reaction mixture contained 2.8 ml of DPIP reagent, 10  $\mu\text{moles}$  of succinate, 1  $\mu\text{mole}$  PMS, and 0.1 ml of chromatophores in a total volume of 3.0 ml. DPIP reagent contained, per 100 ml: 14  $\mu\text{moles}$  DPIP; 20  $\mu\text{moles}$   $\text{MgCl}_2$ ; 40  $\mu\text{moles}$   $\text{K}_2\text{HPO}_4$ ; and 3.2 mmoles tris buffer, pH 8.0. All components except the succinate were mixed in a cuvette, and then were allowed to equilibrate while the recorder was running. Normally some non-succinate-dependent reduction of DPIP occurred, presumably from electron donors within the chromatophores. When this endogenous reaction ceased, succinate was added with a blowout pipet, quickly mixed into the reaction mixture with a "plumper," and the succinate-dependent reduction of DPIP was determined from the change in absorbance. The specific activity of fresh chromatophores was approximately 40 nmoles DPIP reduced/mg protein/minute; however, chromatophore activity varied considerably with age of the preparation.

Determination of the Oxidation  
of NADH by Fumarate

Anaerobic oxidation of NADH by fumarate was measured by the decrease in absorbance of NADH at 340 nm with a recording spectrophotometer. The success of this assay hinges on the maintenance of nearly anaerobic conditions. Thunberg tubes attached to pyrex cuvette cells, as shown in Figure 4, were used for this reaction. The final 3.0 ml reaction mixture contained 270  $\mu$ moles phosphate buffer, pH 7.5, 0.3  $\mu$ moles NADH, 7.5  $\mu$ moles fumarate, and 0.1 ml of chromatophores (approximately 1.5 mg of protein).

Chromatophores were mixed with phosphate buffer in the cuvette section of the tube. To aid in removal of oxygen, nitrogen was bubbled through this mixture for 90 seconds. Meanwhile, NADH was added to the side bulb, which, after nitrogen bubbling was stopped, was fitted to the cell containing the chromatophores. With the NADH still in the side bulb, the vessel was alternately evacuated and flushed with nitrogen 3 or 4 times, and finally filled with nitrogen to a slightly positive pressure. After closing the side arm the evacuation tubing was removed. Then the side arm was turned to the open position for a moment to release the internal pressure, and again closed.

The cell, having been made nearly anaerobic, was placed in the spectrophotometer. Because the cell protruded from the instrument, a black cloth was placed over

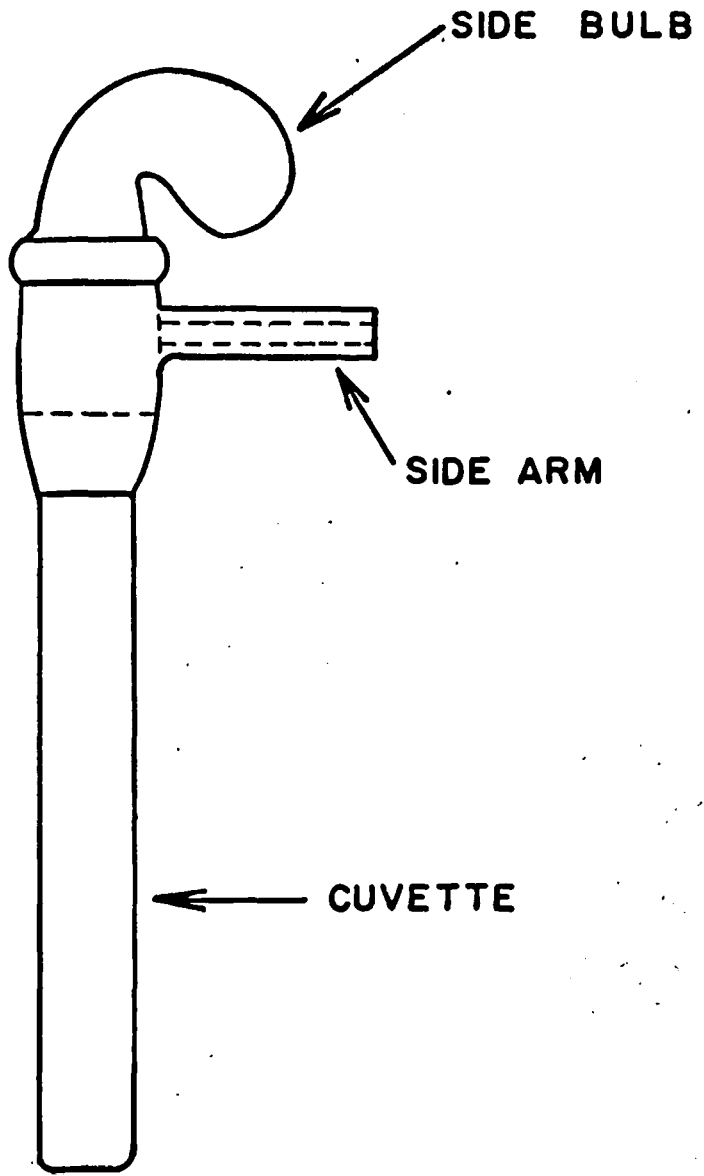


Figure 4. A Thunberg Tube Modified for Spectrophotometric Measurements.

the cell compartment to prevent stray light from interfering with the reading. After instrument adjustments were made to compensate for the absorbance of the chromatophores, NADH was added from the side bulb by removing the cell from the compartment and tipping it gently 2 or 3 times to insure complete mixing. The cell was replaced in the spectrophotometer, and the  $A_{340}$ , about 0.7 absorbance greater than that observed before NADH addition, was measured. A gradual decrease in  $A_{340}$  (undoubtedly resulting from oxidation of NADH by residual oxygen in the system) was then recorded until there was no change in  $A_{340}$  for at least 2 minutes. After this equilibrium was reached, the cell was again removed from the instrument, and the side arm was opened. Fumarate was injected into the cell with a 15  $\mu$ l disposable micropipet. Care was taken to inject a minimal amount of air with the fumarate by applying just enough pressure to the pipet to slowly eject the solution. The side arm was closed, and the fumarate was mixed by gently tipping the cell 2 or 3 times. The vessel was repositioned in the spectrophotometer, and the change in  $A_{340}$  was recorded at a chart speed appropriate for the activity of the sample. From a millimolar absorptivity for NADH of 6.22 (31) the change in  $A_{340}$ /minute was converted to specific activity, defined as nmoles NADH oxidized/mg protein/minute.

Determination of Riboflavin Reduction by NADH and  
Oxidation of Reduced Riboflavin by Fumarate

When riboflavin was used as an electron-transferring intermediate, the change in oxidation state of riboflavin was followed by the change in absorbance at 450 nm. The reaction mixture was identical with that stated for the reduction of fumarate by NADH, except that approximately 0.40  $\mu$ moles of riboflavin were added to the buffer containing chromatophores in the cuvette section of the Thunberg tube. The side bulb contained NADH, as usual, and nearly anaerobic conditions were achieved, as described earlier.

After the vessel had been made anaerobic, it was positioned in the spectrophotometer and was covered with a black cloth. As soon as the instruments had been adjusted to compensate for the absorbance of the chromatophores, NADH was tipped from the side bulb. The decrease in  $A_{450}$  from reduction of riboflavin was recorded, until little change occurred during a two-minute period, showing that all the NADH had reacted. Fumarate was then added through the side arm from a 15  $\mu$ l disposable micropipet, as described previously. After mixing, the cell was quickly replaced in the instrument, and the increase in  $A_{450}$  from oxidation of the reduced riboflavin was recorded. The quantity of riboflavin reacted was calculated from a millimolar absorptivity for oxidized riboflavin at 450 nm

of 12.2 (32). Specific activity was defined as nmoles riboflavin reacted/mg protein/minute.

Determination of the Photoreduction  
of NAD by Succinate

Photoreduction of NAD by succinate also required anaerobic conditions, and was followed by an increase in  $A_{340}$ . Two Thunberg cells were used for these determinations. Each reaction mixture contained 300  $\mu$ moles tris buffer, pH 8.0, 10  $\mu$ moles  $MgCl_2$ , 20  $\mu$ moles phosphate, 40  $\mu$ moles succinate, 4  $\mu$ moles NAD, and 0.2 ml of chromatophores, in a total volume of 3.5 ml. The control mixture contained no succinate.

Each cell was made anaerobic by nitrogen bubbling and evacuation, as described in an earlier section. After the initial  $A_{340}$  of each cell was measured, both cells were supported in a beaker filled with water at room temperature ca. 25°, and were illuminated for 10 minutes.

The illumination apparatus for photoreduction of NAD consisted of two Champion 150 watt flood lamps positioned 40 cm from the supported cuvettes. Between the lamps and the beaker was placed a rectangular pyrex jar of water, 11 cm wide, to absorb the heat produced by the lamps. This arrangement allowed the reaction vessels to remain at room temperature throughout the illumination period.

After illumination the absorbance of each cell was again measured. Any change (positive or negative) in  $A_{340}$  of the control cell was subtracted from that of the reaction cell. This procedure corrected for an increase in the control, which could be due to non-succinate-dependent photoreduction of NAD, or for a decrease in  $A_{340}$ , which possibly was due to solubilization of suspended chromatophores. The change in  $A_{340}$  was converted to specific activity defined as  $\mu\text{moles NAD reduced}/\mu\text{mole Bchl}/\text{hour}$ .

The reproducibility of the data obtained in these assays have generally been within 5% for a given experiment. Only occasionally did an experiment give results outside this limit.

## EXPERIMENTAL RESULTS

### Evidence for the Dark Enzymatic Oxidation of NADH by Fumarate

Support for a dark enzymatic oxidation of NADH by fumarate comes from evaluation of the data in Table 1. As seen from these data normal reduction of fumarate by NADH requires chromatophores, and is not merely a spontaneous reaction. NADH is required, since no change in  $A_{340}$  occurs in its absence. Lastly, under anaerobic conditions NADH oxidation requires fumarate.

When an injection of an identical volume of phosphate buffer replaced the usual addition of fumarate, no oxidation of NADH occurred. Thus the observed rate of NADH oxidation in a normal NADH-fumarate assay is due entirely to the reduction of fumarate, and not to reduction of molecular oxygen remaining in solution. Since removal of dissolved oxygen to the same low concentration is not always, but usually (about 90% of the time) reproducible, all experiments were performed at least twice. This procedure was used to check the possibility that the observed rate of NADH oxidation was due to insufficient removal of oxygen.

To eliminate the possibility that light from the spectrophotometer may have stimulated the reduction of

Table 1. Requirements of the NADH-Fumarate Assay

Experiment	Reaction Conditions	Per Cent Relative Activity
1	Control	100
2	-Chromatophores	0
3	-NADH	0
4	-Fumarate; +Phosphate	0
5	-NADH; +NADPH	0
6	"Dark" Assay	100

The normal NADH-fumarate assay, as described in the section on materials and methods, served as a control reaction. In each of the other assays the specified components were omitted from or added to the reaction mixture. Chromatophore concentration was 1.37 mg protein/3.0 ml reaction mixture. Control activity was:  $\Delta A_{340}/10 \text{ min} = 0.370$ .

fumarate by NADH, an NADH-fumarate assay was carried out as described in Figure 5. The normal reaction occurred even while the reaction vessel was wrapped in a black cloth. Thus a truly dark anaerobic oxidation of NADH by fumarate has been shown.

Table 1 also shows the specificity of the reaction for NADH. Replacement of NADH with NADPH (same millimolar absorptivity at 340 nm as NADH) resulted in no change in

$A_{340}$ .

Figure 5. Dark Anaerobic Oxidation of NADH by Fumarate.

A typical time course of the NADH-fumarate assay is shown. In this particular experiment, after fumarate had been added, and the linear decrease in  $A_{340}$  was evident, the Thunberg tube was removed from the spectrophotometer and was wrapped in a black cloth for about 10 minutes. After the tube had been replaced in the instrument, the change in  $A_{340}$  was again recorded. The dashed line through the dark period shows that the reaction rate remained constant throughout the experiment. Chromatophore concentration was 1.37 mg protein/3.0 ml reaction mixture. Relative activity was:  $\Delta A_{340}/10 \text{ min} = 0.37$ . Other experimental details may be found in Materials and Methods.

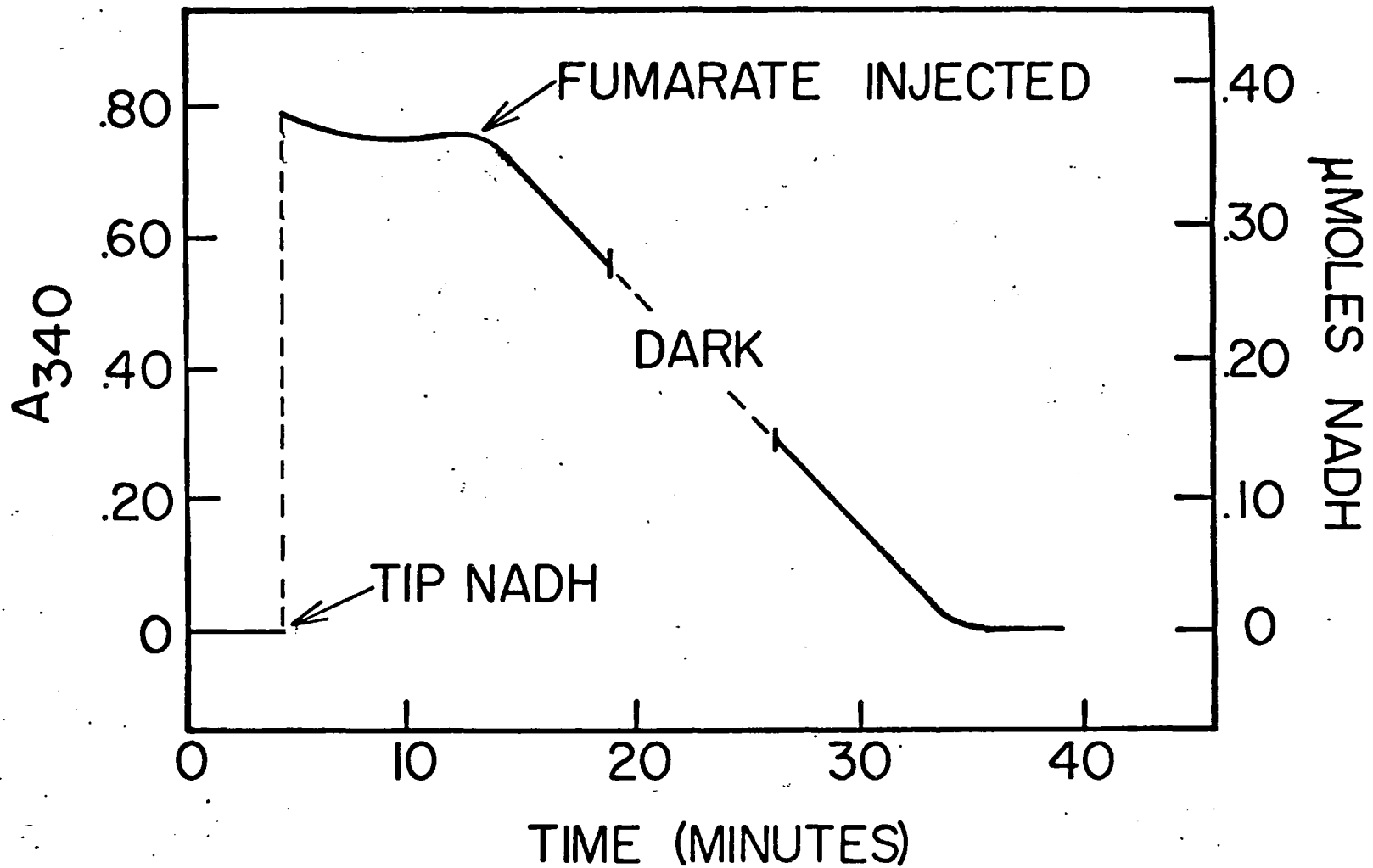


Figure 5. Dark Anaerobic Oxidation of NADH by Fumarate.

In these studies both the dark NADH-fumarate assay and the photoreaction of NAD gradually lost activity as the chromatophore preparations aged. Activity for the dark reaction remains nearly constant for about one week after preparation, and then declines. The effect of age of chromatophore preparations upon the velocity of the NADH-fumarate reaction is shown in Table 2. In an attempt to stabilize the high initial activity, chromatophores were suspended in phosphate buffer containing 10% sucrose. This procedure had no effect upon the stability of the particles, but chromatophore separation from the suspending medium by centrifugation was more difficult because of the increased viscosity of the suspension. Moreover, addition of 10% sucrose to the reaction mixture for the NADH-fumarate assay slightly decreased (about 10%) the rate of NADH oxidation.

As chromatophores aged and NADH-fumarate activity declined, an increase in succinate dehydrogenase activity was noted. The latter enzyme may be solubilized by treating chromatophores with snake venom phospholipase, and may be separated from the chromatophore remnants by centrifugation (J. M. Quirk and J. W. Hinkson, unpublished observations). Therefore the NADH-fumarate activity of phospholipase treated chromatophores was examined.

One mg of phospholipase was added to 10 ml of chromatophores, and this solution was incubated at room

Table 2. Effect of Chromatophore Age upon NADH-Fumarate Activity

Sample Age (Days)	Activity-- $\Delta A_{340}/10 \text{ min}$		
	Prep A	Prep B	Prep C
0	0.690	0.650	0.420
10	0.650	0.610	0.350
20	0.350	0.280	0.220
30	0.300	---	0.180

Chromatophore protein concentrations for 3.0 ml reaction mixtures were as follows: Prep A, 1.88 mg; Prep B, 1.72 mg; Prep C, 1.37 mg. Each preparation was stored at 0-5° as described in Materials and Methods.

temperature (ca. 25°) in a nitrogen-filled dessicator. After its activity had been checked, a control containing no snake venom was also incubated. After 6 hours incubation the control maintained 100% activity for the NADH-fumarate reaction, while the treated sample lost all activity.

#### Stoichiometry of the NADH-Fumarate Reaction

In each of several assays the number of moles of NADH oxidized were compared with the molar quantity of fumarate added. Table 3 and Figure 6 summarize the results of these experiments. Fumarate additions of 0.30  $\mu$ moles or less were used. Assuming that fumarate and NADH reacted

Table 3. Stoichiometry of NADH Oxidation by Fumarate

Experiment	NADH Oxidized ( $\mu$ moles)	Fumarate Added ( $\mu$ moles)	Ratio NADH Oxidized/Fumarate Added
1	0.28	0.30	0.94
2	0.14	0.15	0.94
3	0.070	0.075	0.94
4	0.035	0.0375	0.94

Each reaction mixture contained 0.35  $\mu$ moles of NADH, determined as stated in the text. The chromatophore concentration was 1.72 mg/3.0 ml reaction mixture. See Materials and Methods for other details.

with a 1:1 molar ratio, this addition insured the presence of excess NADH. The quantity of NADH oxidized was calculated from the total change in  $A_{340}$ , while the moles of fumarate injected were calculated from the concentration of the fumarate stock solution. However, stock solutions of fumarate had to be filtered to remove insoluble material. Therefore the listed concentration of fumarate was probably slightly higher than the actual value.

Each addition of fumarate oxidized 94% of the quantity of NADH which should have reacted, if a 1:1 molar ratio were assumed (see Table 3). To determine whether the observed ratio was due to the error in the fumarate concentration, or to the establishment of an equilibrium between reactants and products, two control experiments

Figure 6. Stoichiometry of Fumarate Reduction by NADH.

Data for this plot may be found in Table 3.

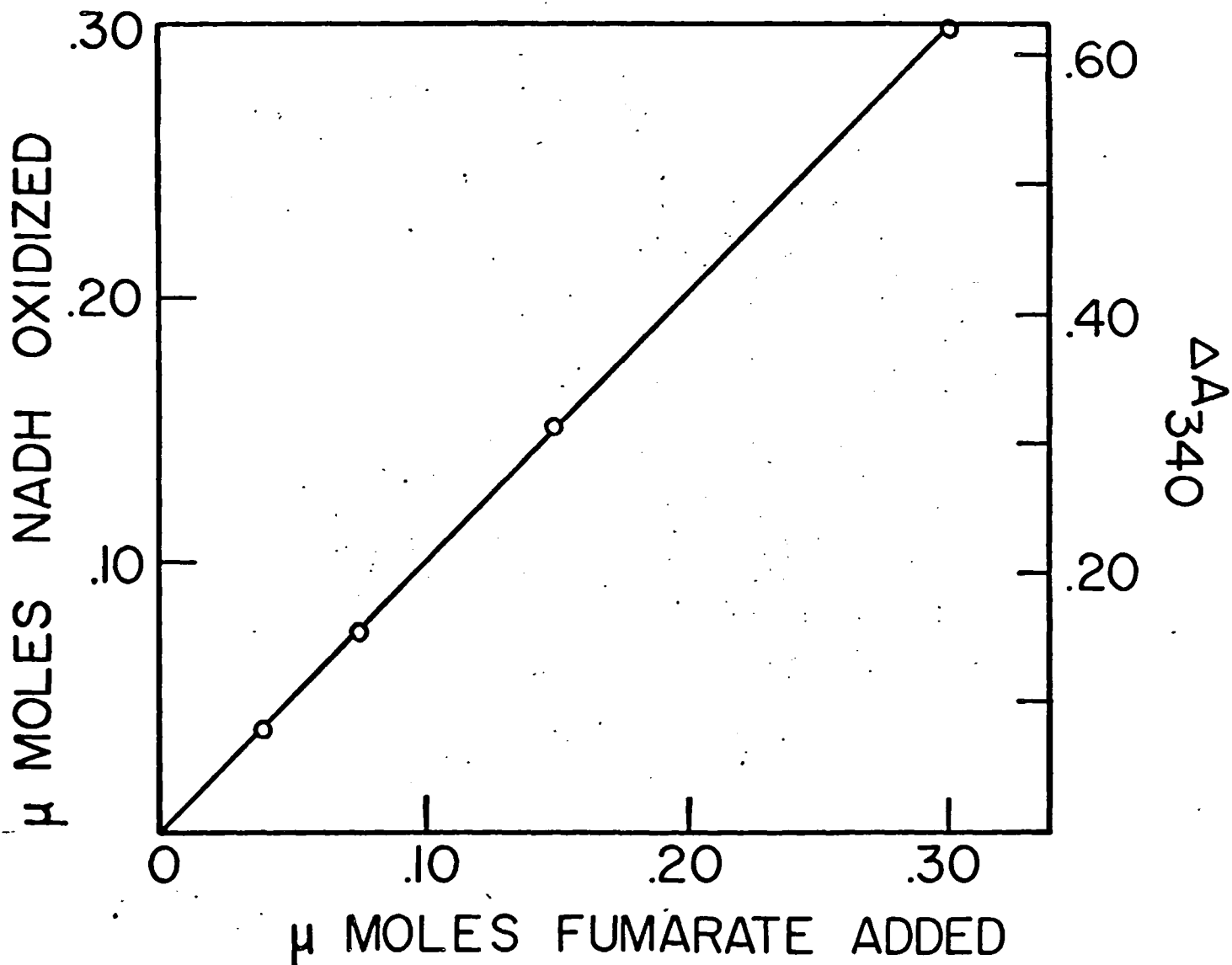


Figure 6. Stoichiometry of Fumarate Reduction by NADH.

were performed. Addition of 10  $\mu$ moles of the expected products (NAD and succinate) to separate NADH-fumarate assay mixtures, caused no change in the rate of NADH oxidation. The ratio of NADH oxidized to fumarate added remained at 0.94.

#### Effect of Chromatophore Concentration upon the Velocity of the NADH-Fumarate Reaction

The effect of the concentration of chromatophores upon the velocity of NADH oxidation by fumarate is shown in Figure 7. Analysis of the effects of chromatophore concentrations higher than those listed was impossible because the high absorbance of the particles in the 340 nm region interfered with the assay. Within the limits tested, however, the velocity increased with an increase in chromatophore concentration.

#### Effect of Fumarate Concentration upon the Velocity of the NADH-Fumarate Reaction

Data for Figure 8 were obtained from a series of NADH-fumarate assays in which the concentration of fumarate was varied. The velocity of NADH oxidation increased until a saturating fumarate concentration of 50  $\mu$ M was reached.

The Michaelis constant for fumarate was determined according to the graphical method of Hanes (33), from the data given in Figure 8. From a plot of substrate concentration versus substrate concentration/velocity,  $K_m$  may be evaluated from the negative intercept of the substrate

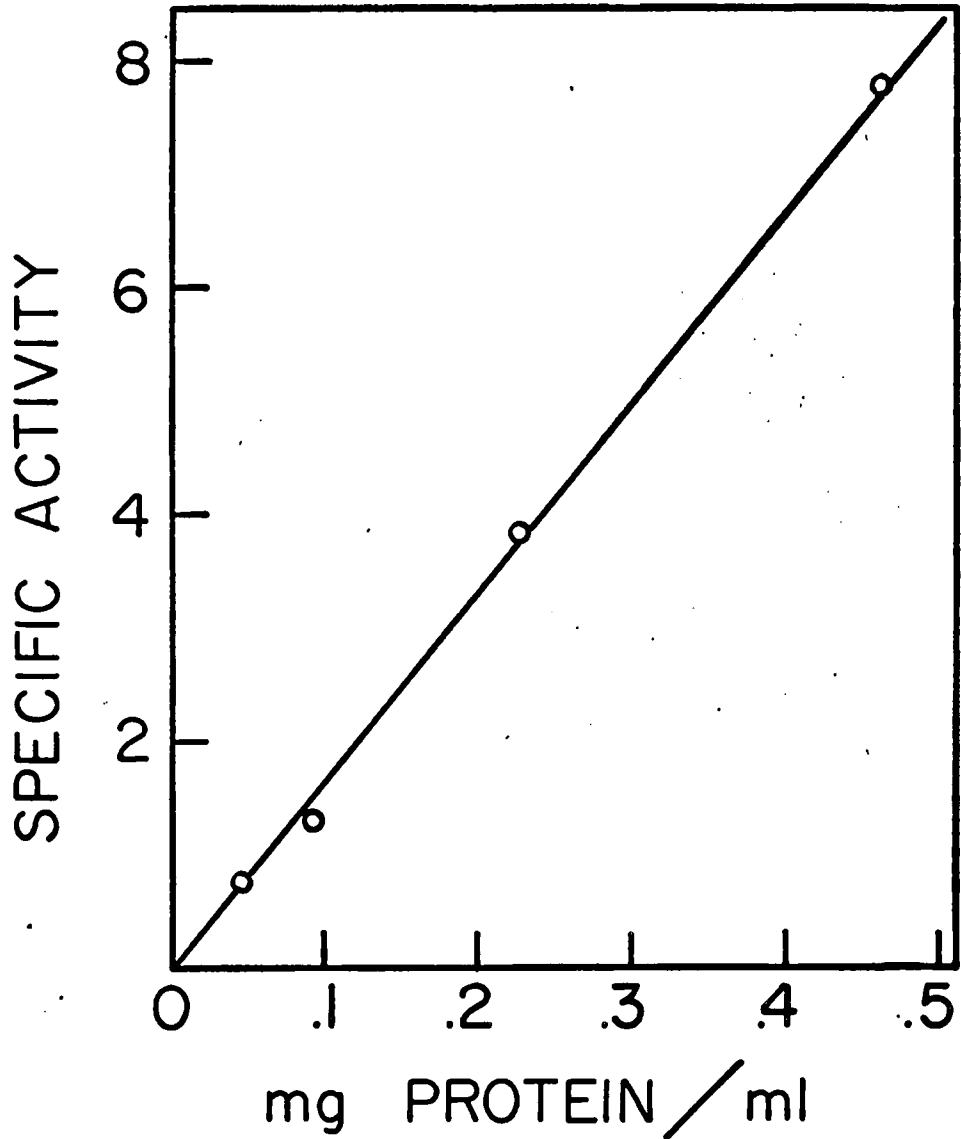


Figure 7. Effect of Chromatophore Concentration upon the Velocity of NADH Oxidation by Fumarate.

Chromatophore concentration of the preparation was determined by the Biuret method as described in Materials and Methods. Specific activity for this experiment is defined as nmoles NADH oxidized/min. Other experimental details may be found in the section on methods and materials.

Figure 8. Effect of Fumarate Concentration upon the Velocity of NADH Oxidation by Fumarate.

Maximal velocity occurred with 50  $\mu\text{M}$  fumarate. Each 3.0 ml reaction mixture contained 1.88 mg protein from chromatophores. Specific activity is defined as nmoles NADH oxidized/mg protein/min. See Materials and Methods for other experimental details.

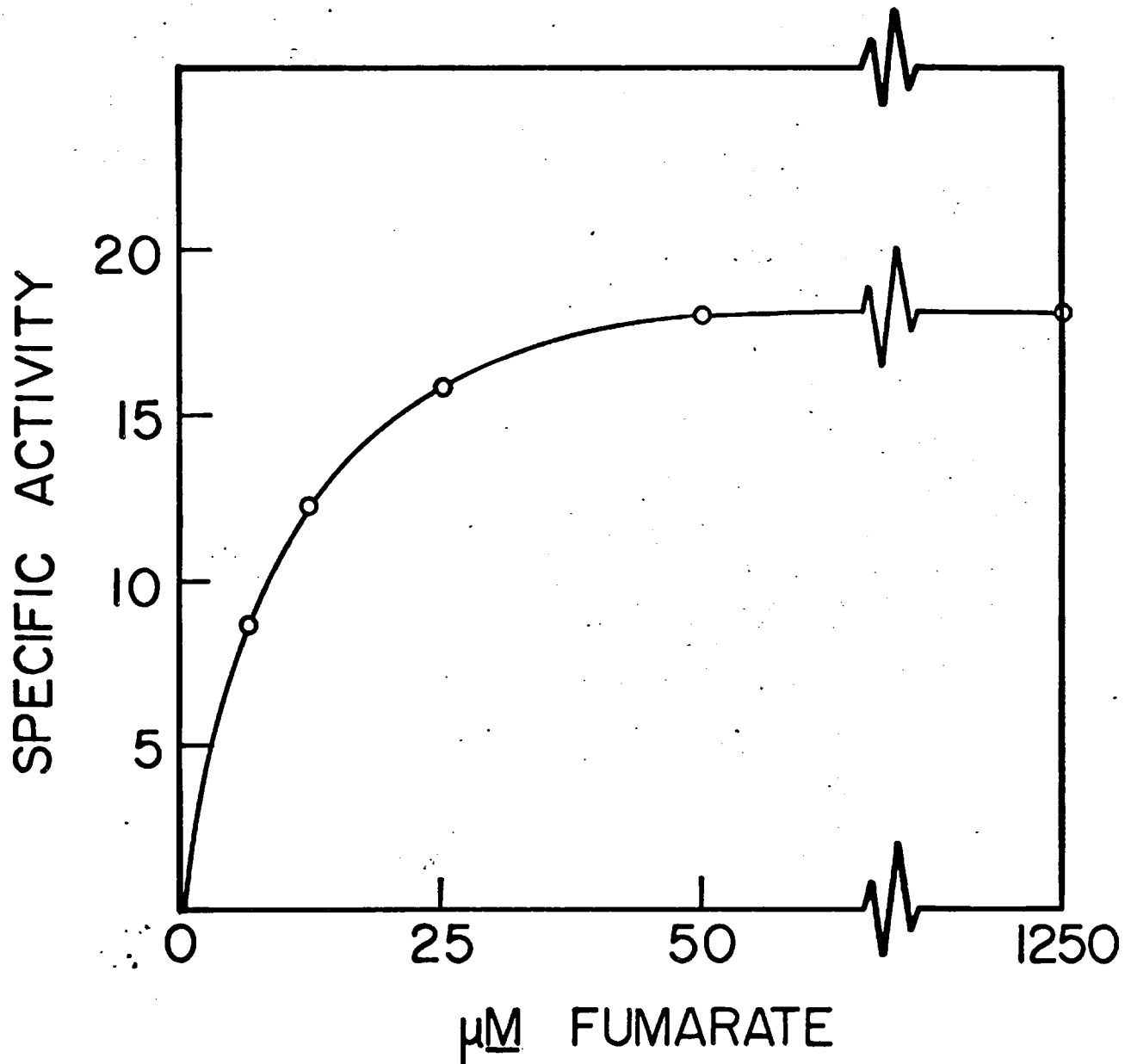


Figure 8. Effect of Fumarate Concentration upon the Velocity of NADH Oxidation by Fumarate.

axis. For the data shown in Figure 8  $K_m = 9.06 \mu\text{M}$ . Figure 9 shows a Hanes plot for this experiment, and also for the data which were obtained from experiments with several other chromatophore preparations. Variation in these plots indicates that chromatophore activity varies considerably with age, as well as from one preparation to another. Table 4 lists the  $K_m$  values which were obtained. The values for  $K_m$  varied by ten-fold even when these results were normalized to the high activity of preparation A (so that all preparations would have the same maximal velocity). Because of the variable nature of chromatophore activity, the calculated Michaelis constant for fumarate is only approximate.

#### Effect of NADH Concentration upon the Velocity of the NADH-Fumarate Reaction

As shown in Figure 10, the velocity of NADH oxidation by fumarate increased until a saturating NADH concentration of  $12 \mu\text{M}$  was reached. Concentrations of NADH were calculated from the  $A_{340}$  increase after NADH was added to the reaction mixture from the side bulb. A Michaelis constant of approximately  $4.3 \mu\text{M}$  for NADH was determined from the data in Figure 10.

#### Effect of pH upon the Velocity of the NADH-Fumarate Reaction

As shown in Figure 11, a pH of approximately 7.2 is necessary for maximal velocity of NADH oxidation by

Figure 9. Variation of the Michaelis Constant for Fumarate.

A Hanes plot shows the variation of the Michaelis constant for fumarate. Curve A represents the data obtained from a 30-day old chromatophore preparation; Curve B, fresh chromatophores from another preparation; Curves C and D, a third preparation, 30 and 10 days old, respectively.  $K_m$  values were obtained from the negative intercepts of the curve with the Fumarate axis. Other experimental details are described in the text and in Table 4.

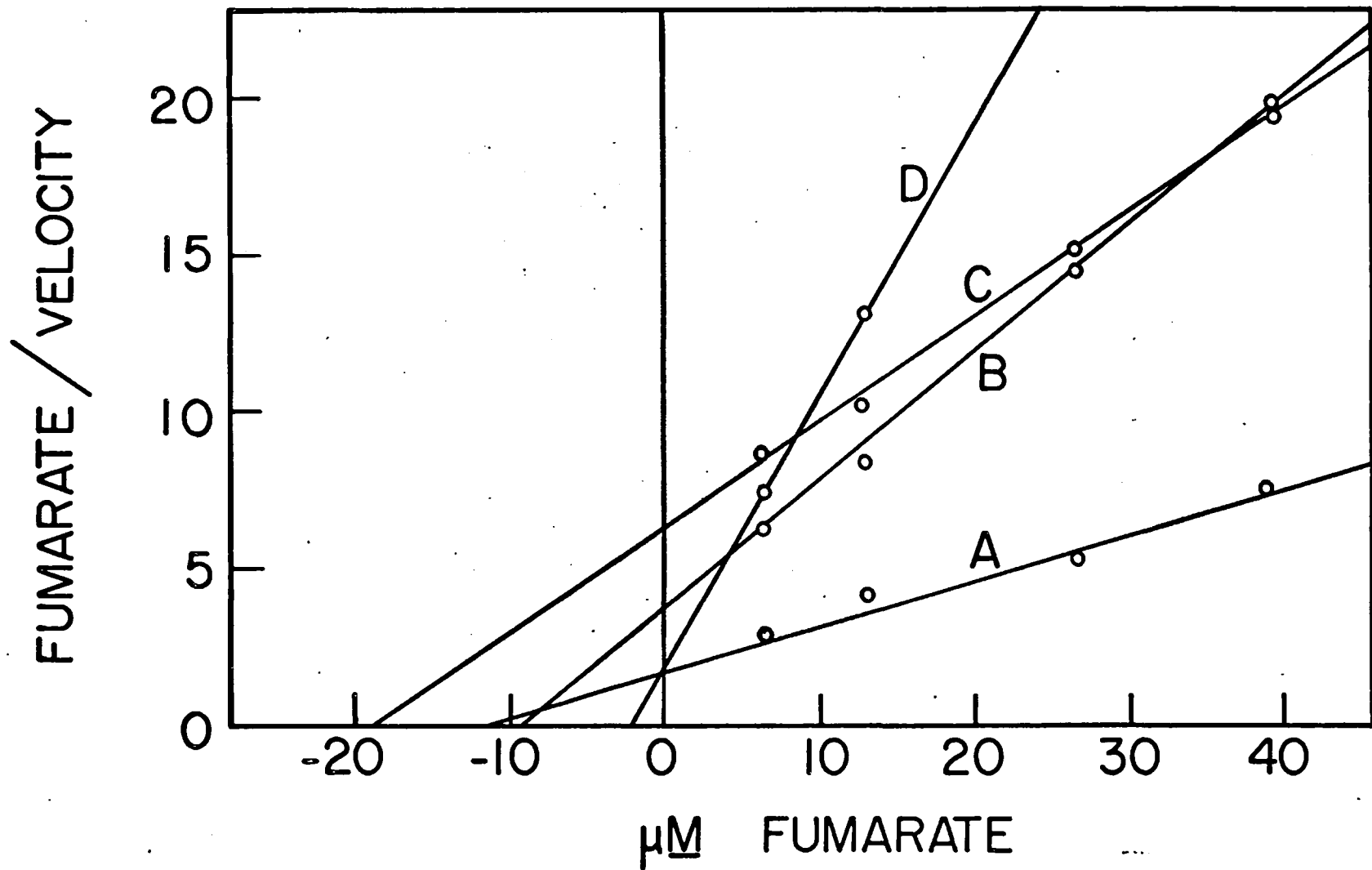


Figure 9. Variation of the Michaelis Constant for Fumarate.

Table 4. Variation of the Michaelis Constant for Fumarate

Preparation	Maximal Velocity	$K_m$ ( $\mu M$ )	$K_m'$ ( $\mu M$ )
1 (0 Days)	0.368	9.06	9.06
2 (30 Days)	0.123	10.3	3.02
3 (10 Days)	0.153	2.03	0.85
3 (30 Days)	0.138	18.75	7.20

$K_m$  values were obtained from the Hanes plot of Figure 9.  $K_m'$  is defined as  $K_m$  normalized to the high maximal velocity of preparation A by adjusting all data with the proper conversion factor. Maximal velocity is:  $\Delta A_{340}/\text{mg protein}/10 \text{ min}$ . Chromatophore concentrations for the reaction mixtures were: Prep 1, 1.88 mg; Prep 2, 1.72 mg; Prep 3, 1.37 mg. The age of each preparation is given in parentheses.

Figure 10. Effect of NADH Concentration upon the Velocity of NADH Oxidation by Fumarate.

Maximal velocity occurred with 12  $\mu\text{M}$  NADH. Chromatophore concentration was 1.88 mg protein/3.0 ml reaction mixture. Specific activity is defined as nmoles NADH oxidized/mg protein/min. A Michaelis constant, 4.3  $\mu\text{M}$ , was determined for NADH as the concentration of NADH at half the maximal velocity shown. The value given, however, is only approximate, because of the variable nature of chromatophore activity. All other experimental details may be found in the section on materials and methods.

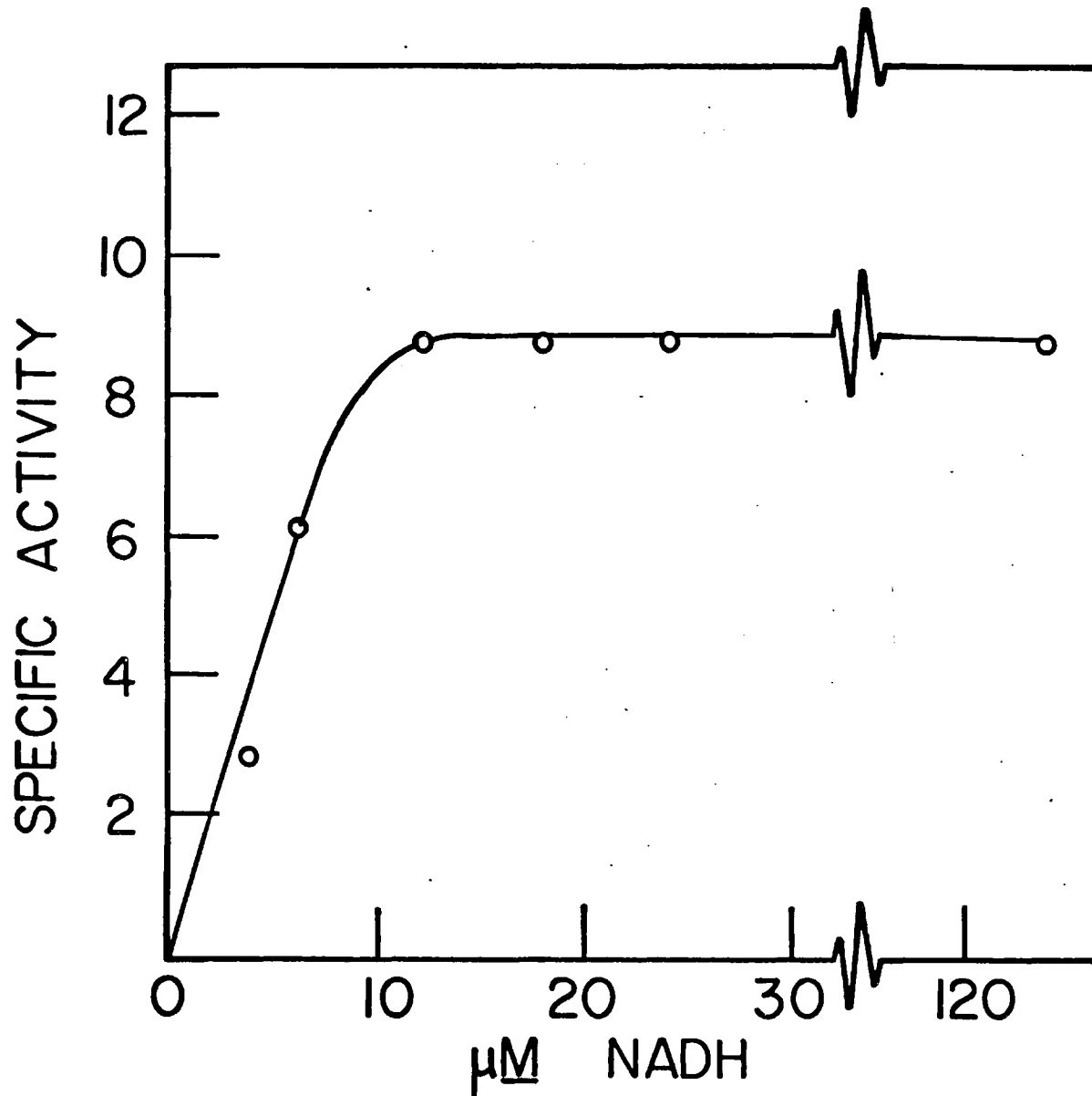


Figure 10. Effect of NADH Concentration upon the Velocity of NADH Oxidation by Fumarate.

Figure 11. Effect of pH upon the Velocity  
of NADH Oxidation by Fumarate.

Phosphate buffer (270  $\mu$ moles) adjusted to the appropriate pH with KOH or HCl were used for these determinations. Tris buffer (270  $\mu$ moles) gave the same activities at the pH values tested which were: 8.5, 8.0, and 7.5. Chromatophore concentration was 1.72 mg protein/3.0 ml reaction mixture. Specific activity is defined as nmoles NADH oxidized/mg protein/min. See Materials and Methods for all other experimental details.

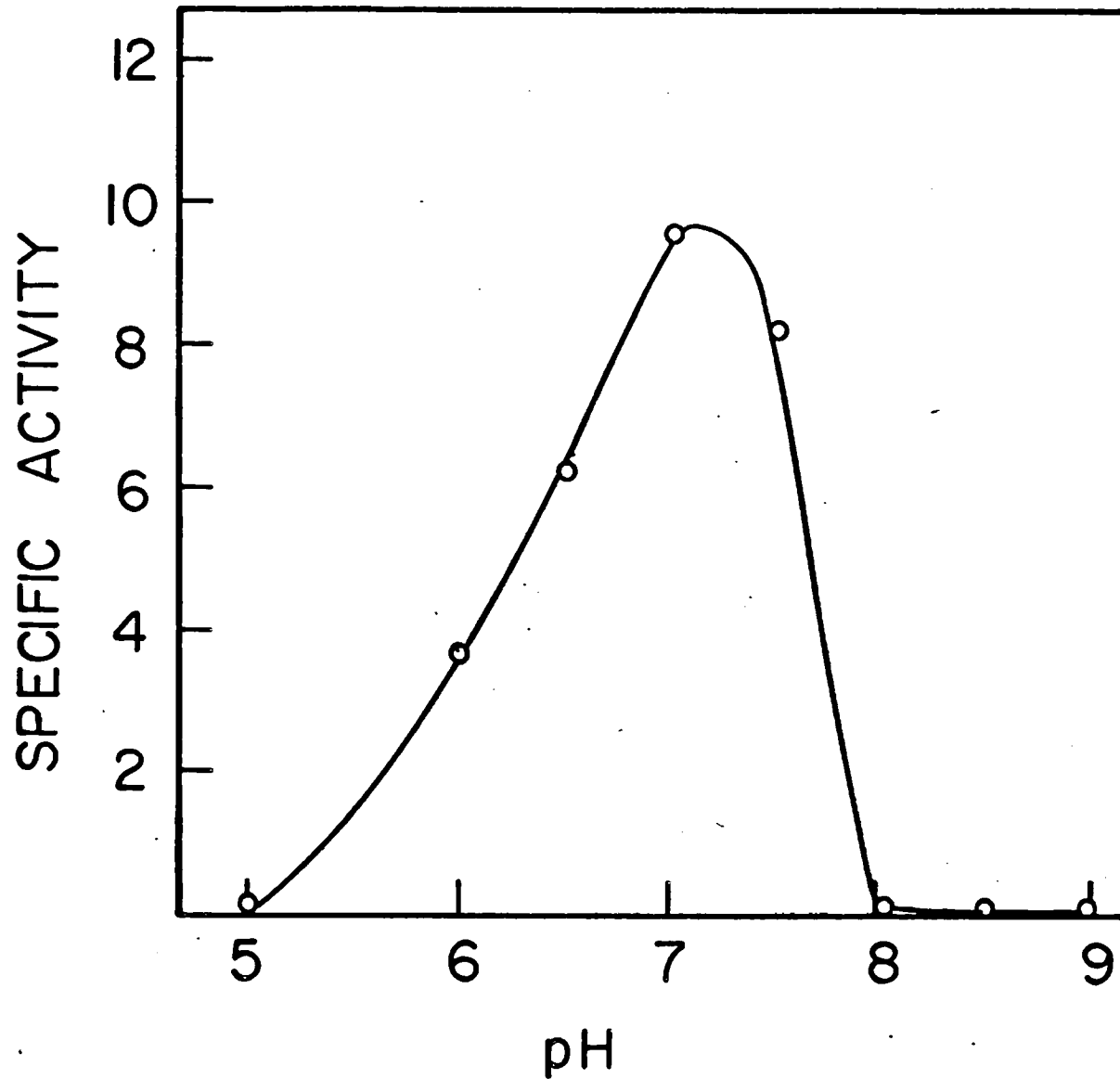


Figure 11. Effect of pH upon the Velocity of NADH Oxidation by Fumarate.

fumarate. On the acid side of the curve the velocity decreases from the maximum to zero within 2.5 pH units. In contrast, with more-basic buffer solutions all activity decreased to zero only 0.8 pH units above that for maximal velocity.

#### Malonate Inhibition of Fumarate Reduction by NADH

A Hanes plot of the data obtained from a study of the effect of malonate upon the NADH-fumarate reaction is shown in Figure 12. Each line in this graph represents a different concentration of malonate, and was obtained from a series of assays in which the concentration of fumarate was varied. The parallel lines indicate competitive inhibition (33), which would be expected of malonate, if succinate dehydrogenase participates in the reaction (34).

The effect of fumarate concentration upon malonate inhibition is shown by a Dixon plot (Figure 13) (35). The extrapolated curves appear to converge at  $-12 \mu\text{M}$  malonate.

#### Effect of Sulfhydryl Reagents upon NADH Oxidation by Fumarate

Iodoacetate and *p*-hydroxymercuribenzoate were added to separate assay mixtures to determine their effect upon the reduction of fumarate by NADH. The normal procedure for the NADH-fumarate assay was followed, except that the reagents to be tested were added to the cuvette section of the Thunberg vessel, and were allowed to incubate with the chromatophores while the vessels were made anaerobic (a

Figure 12. Malonate Inhibition of Fumarate  
Reduction by NADH.

This is a Hanes plot of data obtained from a study of the effect of malonate. Each curve represents a different concentration of malonate: A, no malonate; B, 30  $\mu\text{M}$ ; C, 0.15  $\text{mM}$ ; D, 0.3  $\text{mM}$ ; E, 1.5  $\text{mM}$ . Chromatophore concentration was 1.37 mg protein/3.0 ml reaction mixture. The vertical scale is plotted in arbitrary units. Curve A is the same as Curve A shown in Figure 9 ( $K_m = 10.3$ ). Fumarate and Malonate were mixed, and injected as one solution in these experiments. Other details may be found in Materials and Methods.

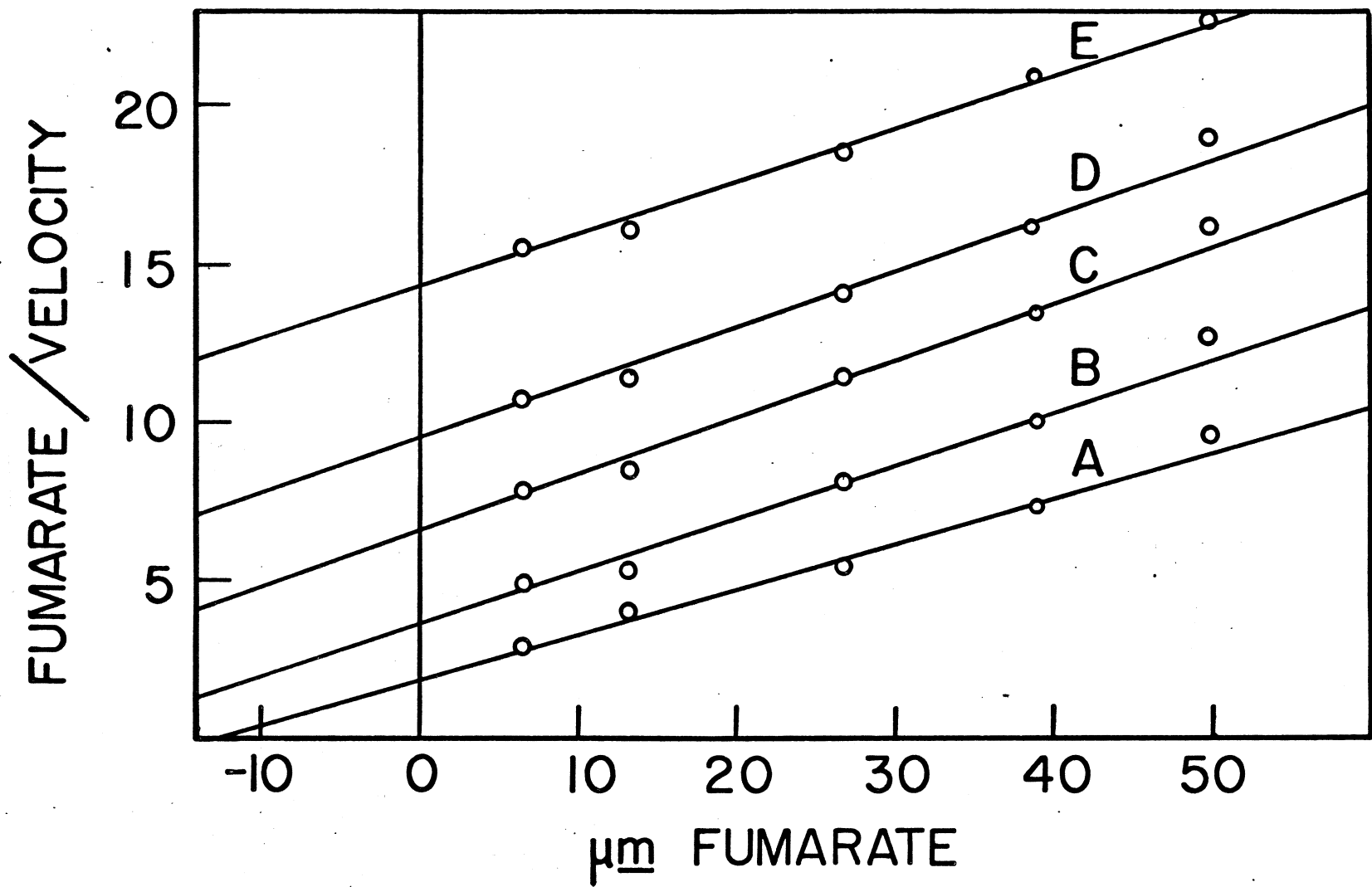


Figure 12. Malonate Inhibition of Fumarate Reduction by NADH.

Figure 13. Effect of Fumarate Concentration upon Malonate Inhibition.

This is a Dixon plot of the data obtained from a study of the effect of malonate upon the NADH-fumarate reaction. Each curve represents a different concentration of fumarate: A, 50  $\mu\text{M}$ ; B, 25  $\mu\text{M}$ ; C, 12.5  $\mu\text{M}$ ; D, 6.25  $\mu\text{M}$ . Other experimental details may be found under Figure 12, and in Materials and Methods.

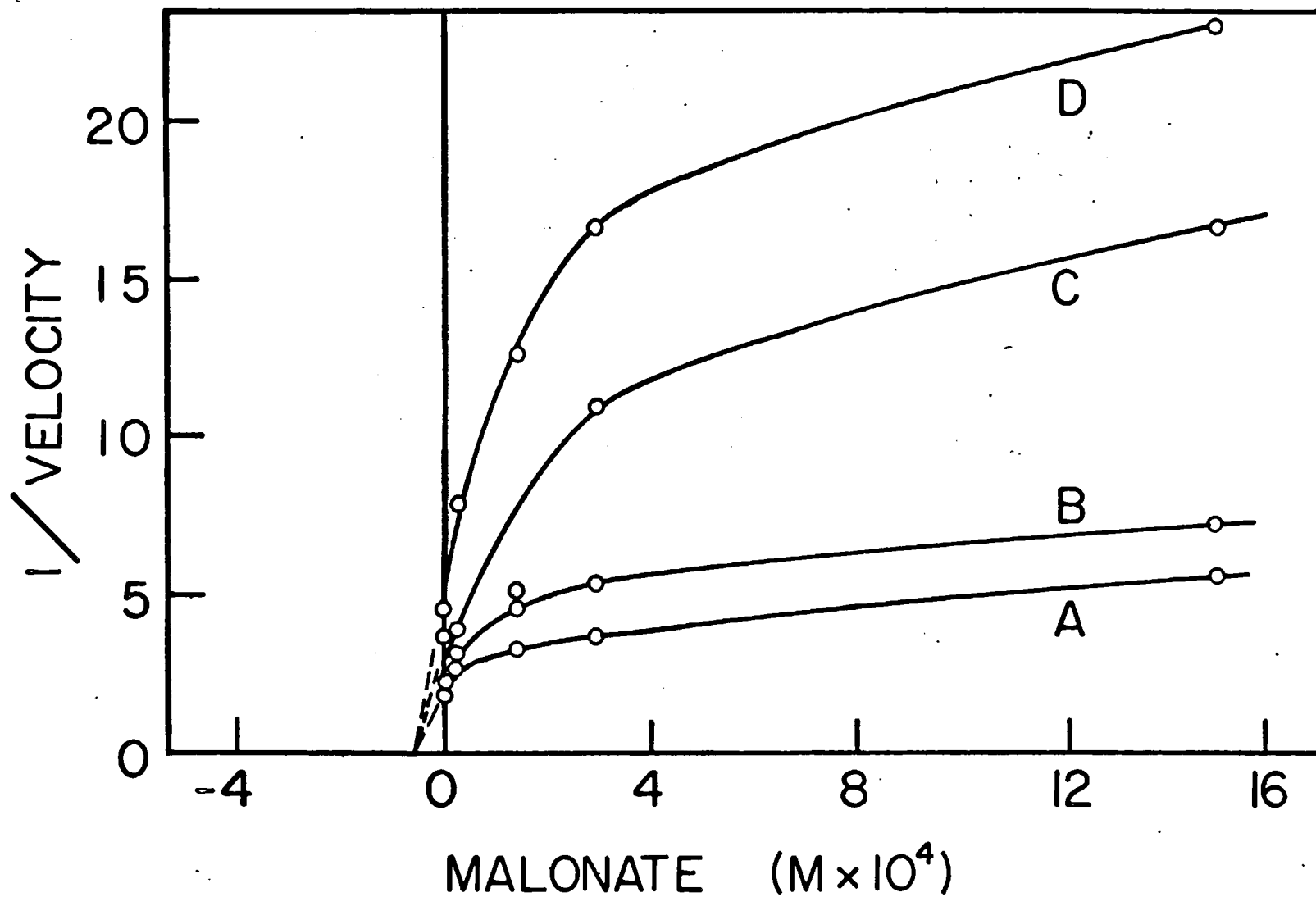


Figure 13. Effect of Fumarate Concentration upon Malonate Inhibition.

10-minute procedure). Table 5 shows the results of this analysis. Although iodoacetate slightly increased the rate of NADH oxidation, PHMB caused substantial inhibition.

#### Effect of Metals upon NADH Oxidation by Fumarate

Because the metal ions used for these studies form precipitate with phosphate, Tris buffer was used to determine the effect of various metals upon the NADH-fumarate reaction. The results are summarized in Table 6. Mercuric ion exhibited the same marked inhibition which resulted from PHMB treatment, thereby strengthening the possibility that sulfhydryl groups participate in the reaction. Ferrous iron was added to check the possibility that non-heme iron was involved in fumarate reduction by NADH. Although the expected enhancement of activity was not observed, there are insufficient data to allow a conclusion at this time.

Because others have reported that magnesium ion sometimes enhanced the photoreduction of NAD by succinate (36), the effects of this metal were also tested. No change in the rate of NADH oxidation by fumarate was observed.

#### Treatments for Comparison of NADH Oxidation by Fumarate to the Photoreduction of NAD by Succinate

The photoreduction of NAD by succinate and the dark oxidation of NADH by fumarate were compared on the basis of

Table 5. Effect of Sulfhydryl Reagents upon NADH Oxidation by Fumarate

Experiment	Reaction Conditions	Per Cent Relative Activity
1	Control	100
2	Iodoacetate (33 $\mu\text{M}$ )	106
3	PHMB (33 $\mu\text{M}$ )	56

Chromatophore concentration was 1.37 mg protein/3.0 ml reaction mixture. The final concentrations of the added reagents are given in parentheses. Control activity was:  $\Delta A_{340}/10 \text{ min} = 0.22$ . Other experimental details are given in the section on materials and methods.

Table 6. Effect of Metals upon NADH Oxidation by Fumarate

Experiment	Reaction Conditions	Per Cent Relative Activity
1	Control	100
2	Hg <sup>++</sup> (3.3 $\mu\text{M}$ )	59
3	Fe <sup>++</sup> (3.3 $\mu\text{M}$ )	100
4	Mg <sup>++</sup> (3.3 mM)	100

Tris buffer (270  $\mu\text{moles}$ , pH 7.5) replaced phosphate buffer in these experiments. Chromatophore concentration was 1.37 mg protein/3.0 ml reaction mixture. Control activity was:  $\Delta A_{340}/10 \text{ min} = 0.23$ . Other experimental details are given in Materials and Methods.

the effects of rotenone, HOQNO, and KCN upon the activities of their respective assay reactions. Table 7 shows the results of this study.

Because the position of the flavoprotein which has been proposed for the electron transfer pathway (2) is common to pyridine nucleotide oxidation and reduction, the effect of rotenone which affects electron transfer in this region (17), was analyzed. The effects of methanol, used to dissolve rotenone, were also studied. Rotenone substantially inhibited both reactions, while methanol had a greater effect upon the photoreduction than upon the dark reaction.

HOQNO, which affects electron transfer at the cytochrome level (20), had no effect upon the NADH-fumarate reaction, but significantly inhibited the photoreduction of NAD by succinate.

Cyanide had no effect upon either reaction, although others have reported that KCN enhanced the photoreduction of NAD by succinate (36).

#### Effect of Flavins upon NADH Oxidation by Fumarate

The possibility for involvement of a flavoprotein in the reaction pathway led to a series of experiments in which flavins were added to NADH-fumarate reaction mixtures. Those tested for possible enhancement of the reaction were riboflavin, FMN, and FAD. Quinacrine, a flavin analogue

Table 7. Treatments for Comparison of the Dark Oxidation of NADH by Fumarate to the Photoreduction of NAD by Succinate.

Experiment	Reaction Conditions	Per Cent Relative Activity	
		Photo-reduction of NAD	Dark Oxidation of NADH
1	Control	100	100
2	Rotenone 33 $\mu\text{M}$ / Methanol (3.3%)	19	22
3	Methanol (3.3%)	55	84
4	HOQNO (15 $\mu\text{M}$ )	28	100
5	KCN (0.33 $\text{mM}$ )	100	100

Control activities were: Photoreduction of NAD,  $\Delta A_{340}/\mu\text{mole Bchl}/\text{hour} = 0.30$  (equivalent to  $\Delta A_{340}/\text{mg protein}/\text{hour} = 0.22$ ); Dark oxidation of NADH,  $\Delta A_{340}/\text{mg protein}/10 \text{ min} = 0.20$ . Final concentrations are shown in parentheses. Chromatophore concentrations were: photo-reduction, 2.74 mg protein/3.7 ml reaction mixture; dark reaction, 1.37 mg protein/3.0 ml reaction mixture. All other experimental details are described in Materials and Methods.

which inhibits the photoreduction of NAD (15), was also used in the study.

The normal NADH-fumarate reaction mixtures were prepared, and the flavins were added to the cuvette section of the Thunberg tube, and allowed to incubate with the chromatophores while the vessels were made anaerobic. Because oxidized flavins, as well as NADH, absorb at 340 nm (37), all reactions were repeated and followed at 450 nm to determine if reduced flavins were products in the reaction. (Flavin reduction would be accompanied by a decrease in  $A_{450}$ ; NADH/NAD does not absorb in this region.) The results of these experiments are shown in Table 8.

All three flavins and, unexpectedly, quinacrine enhanced the rate of NADH oxidation by fumarate, but no change in  $A_{450}$  was observed.

The effect of the addition of a flavoprotein which had been isolated from sodium benzoate-grown cells of Azotobacter vinelandii was also studied. (Azotobacter flavoprotein was kindly supplied by D. E. Edmondson.) This flavoprotein also enhanced the reaction.

#### Riboflavin Reduction by NADH and the Oxidation of Reduced Riboflavin by Fumarate

Since a flavin probably participates in the oxidation of NADH by fumarate (see previous results), an attempt was made to reduce flavin with NADH, and then to oxidize the reduced flavin with fumarate. The procedure for these

Table 8. Effect of Flavins upon NADH Oxidation by Fumarate

Experi- ment	Reaction Conditions	Per Cent Relative Activity
1	Control	100
2	Riboflavin (1.65 $\mu\text{M}$ )	141
3	FMN (3.3 $\mu\text{M}$ )	123
4	FMN (33 $\mu\text{M}$ )	127
5	FAD (0.31 $\mu\text{M}$ )	118
6	FAD (3.1 $\mu\text{M}$ )	118
7	Quinacrine (18 $\mu\text{M}$ )	122
8	Quinacrine (0.18 $\text{mM}$ )	136
9	Flavoprotein (0.072 mg)	109
10	Flavoprotein (0.720 mg)	118

Concentrations of added reagents are shown in parentheses. Chromatophore concentration was 1.37 mg protein/3.0 ml reaction mixture. Control activity was:  $\Delta A_{340}/10 \text{ min} = 0.23$ . No change at 450 nm was observed during any of the above experiments. See Materials and Methods for other details.

experiments is described in the section on materials and methods. A typical time course for this reduction and subsequent oxidation of riboflavin is shown in Figure 14.

Several control experiments were needed to demonstrate the dependence of the overall reaction upon NADH and fumarate. Tables 9 and 10 summarize the results of these experiments. As seen from these data (Table 9) NADH spontaneously reduces riboflavin, but at a rate much slower than the chromatophore-catalyzed reduction. To determine the effect of illumination from the light source of the spectrophotometer upon riboflavin reduction, the reaction vessel was removed from the instrument and placed in the dark, as described in Figure 5 for an NADH-fumarate assay. When the tube was replaced in the instrument, the  $A_{450}$  had decreased at a rate identical to that observed when the reaction was followed continuously with a recorder.

As seen from Table 10, the oxidation of reduced riboflavin is dependent upon the presence of both fumarate and chromatophores.

Malonate and rotenone were used to study these two reactions further. The results are shown in Table 11. As expected, if succinate dehydrogenase were participating, malonate inhibited only the oxidation of reduced riboflavin by fumarate. However, rotenone affected the oxidation and reduction of riboflavin nearly identically:

Figure 14. Riboflavin Reduction by NADH and the  
Oxidation of Reduced Riboflavin by Fumarate.

A time course for the reduction and oxidation of riboflavin is shown. The experimental procedure is described in Materials and Methods. Chromatophore concentration was 1.37 mg protein/3.0 ml reaction mixture. Relative activities were: reduction of riboflavin by NADH,  $\Delta A_{450}/10 \text{ min} = 0.36$ ; oxidation of reduced riboflavin by fumarate,  $\Delta A_{450}/2 \text{ min} = 0.35$ .

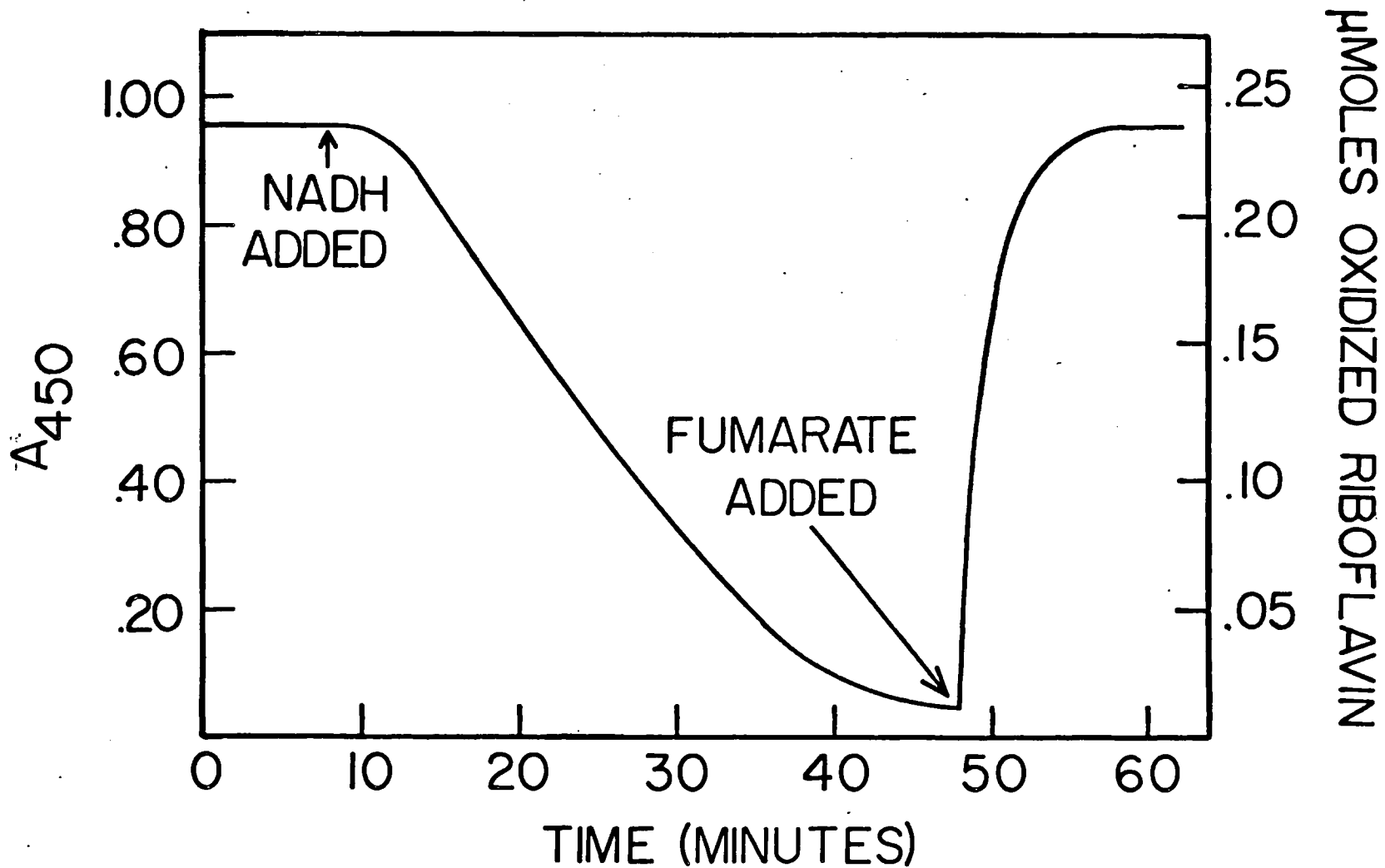


Figure 14. Riboflavin Reduction by NADH and the Oxidation of Reduced Riboflavin by Fumarate.

Table 9. Requirements for Reduction of Riboflavin by NADH

Experiment	Reaction Conditions	Per Cent Relative Activity
1	Control	100
2	"Dark" Assay	100
3	-NADH	0
4	-Riboflavin	0
5	-Chromatophores	14

Chromatophore concentration was 1.37 mg protein/3.0 ml reaction mixture. Control activity was:  $\Delta A_{450}/10 \text{ min} = 0.36$ . Other details may be found in the section on materials and methods.

Table 10. Requirements for Oxidation of Reduced Riboflavin by Fumarate

Experiment	Reaction Conditions	Per Cent Relative Activity
1	Control	100
2	-Fumarate	0
3	-Riboflavin (Reduced)	0
4	-Chromatophores	0

Chromatophore concentration was 1.37 mg protein/3.0 ml reaction mixture. Control activity was:  $\Delta A_{450}/10 \text{ min} = 0.35$ . See Materials and Methods for other details.

Table 11. Treatments Affecting the Riboflavin-Mediated Oxidation of NADH by Fumarate

Experiment	Reaction Conditions	Per Cent Relative Activity	
		Riboflavin Reduction by NADH	Riboflavin-H <sub>2</sub> Oxidation by Fumarate
1	Control	100	100
2	Malonate	100	26
3	Rotenone/ Methanol	80	62
4	Methanol	100	84

Final concentrations: malonate, 2.5 mM; rotenone, 33  $\mu$ M; methanol, 3.3%. Other details may be found under Tables 9 and 10.

$$\text{Effect of rotenone} = \frac{\% \text{ Activity Rotenone/Methanol}}{\% \text{ Activity Methanol}}$$

Thus, the rate of reduction of riboflavin by NADH was  $80/100 = 80\%$  of the control velocity when rotenone was present, while the rate of oxidation of reduced riboflavin by fumarate was  $62/84 = 74\%$  of the control velocity under the same conditions.

## DISCUSSION

Several experimental results suggest that the dark anaerobic oxidation of NADH by fumarate with chromatophores from Rhodospirillum rubrum is a reality and not an artifact. The reaction is not spontaneous, but requires chromatophores. Fumarate is also required since no oxidation of NADH occurred when an addition of phosphate buffer replaced the usual fumarate injection during the assay. The absence of NADH oxidation upon buffer addition also demonstrates that a negligible amount of oxygen is introduced into the reaction vessel with the 15  $\mu$ l addition of fumarate in a normal NADH-fumarate assay; however, when larger volumes of buffer, such as 100  $\mu$ l, which contain proportionally greater quantities of dissolved oxygen are injected NADH oxidation is observed until endogenous oxygen is consumed. Finally, NADH oxidation by fumarate is not light-induced since the velocity of the reaction is unaffected when light is excluded.

Maximal activity for the NADH-fumarate reaction requires a pH of approximately 7.2, but activity as a function of pH is unsymmetrical and drops sharply above pH 7.5. Such a sudden loss of activity could indicate denaturation of the electron transfer complex. However, since the photoreduction of NAD by succinate readily occurs

at pH 8.0 (14) denaturation of a unique component involved in the dark oxidation of NADH by fumarate seems unlikely but not impossible. A shift in equilibrium in the reaction:



caused by variation of pH qualitatively explains why the photochemical reduction of NAD by succinate proceeds more readily at pH 8.0 than the dark NADH-fumarate reaction. But this shift does not explain the sudden loss of NADH-fumarate activity above pH 7.5. The activity of the NADH-fumarate reaction in the alkaline range should be investigated further to explain this rather abrupt loss of activity.

Equimolar quantities of fumarate and NADH likely react in this reaction since the molar ratio of NADH oxidized to fumarate added approached 1.0, even when succinate and NAD (the likely products) were added to the reaction mixture. Combined with observations that fumarate substantially inhibits R. rubrum succinate dehydrogenase (unpublished observations) the data in Table 3 suggest that equilibrium favors the products of the NADH-fumarate reaction. The value of less than one for the observed ratio (0.94) probably results from experimental error in the determination of fumarate caused

by filtering the fumarate solutions when they were prepared to remove insoluble foreign material.

The increased succinate dehydrogenase activity concurrently appearing with decreased NADH-fumarate activity as each chromatophore preparation aged has interesting implications. Apparently as the electron transfer complex slowly disintegrates SDH availability for the DPIP-PMS assay increases while those reactions requiring intact units, the NADH-fumarate reaction and the photoreduction of NAD, decrease. Treating chromatophores with snake venom phospholipase, which speeds disintegration of the complex by hydrolyzing the lipid matrix, solubilizes SDH and causes a complete loss of NADH-fumarate activity. Thus the velocities of these reactions catalyzed by chromatophores are especially dependent upon the integrity of the electron transfer complex.

The variable values of  $K_m$ 's obtained for different preparations suggest that  $K_m$  has no physical significance for this complex electron transport system. The data for this parameter have been included to emphasize the effect of age and different preparations upon NADH-fumarate activity.

Competitive inhibition of malonate with fumarate for the oxidation of NADH indicated by the Hanes plot (Figure 12) suggests that succinate dehydrogenase participates in the reaction pathway. When an attempt was made

to determine the inhibition constant for malonate, the Dixon plot (Figure 13) yielded a series of non-linear curves which extrapolated to approximately  $-12 \mu\text{M}$  malonate. Such plots are inconsistent with the existence of simple competitive inhibition. However, Dixon plots are obtained from studies of malonate inhibition of solubilized SDH from R. rubrum (J. M. Quirk and J. W. Hinkson, unpublished observations) and therefore may be characteristic for this enzyme.

Sulfhydryl reagents (PHMB,  $\text{Hg}^{++}$ ) affect the activity of both solubilized SDH and the NADH-fumarate reaction. Other sulfhydryl groups besides those of SDH may be involved in transfer of electrons from NADH to fumarate since these reagents may react with other components of the electron transfer chain.

#### Comparison of the Dark NADH-Fumarate Reaction to the Photoreduction of NAD by Succinate

Figure 15 shows the proposed electron transfer sequence (2), modified to include the results of this study. As chromatophore preparations age the activity of the photoreduction of NAD by succinate decreases, just as the activity of the NADH-fumarate reaction does. The photoreduction is also inhibited by malonate when succinate is employed as the electron donor (14).

Figure 15. Probable Positions of Interaction of Reagents Affecting the Electron Transfer System.

The electron transfer sequence proposed by Vernon (2), including the probable reaction sites of HOQNO, malonate, and rotenone, is shown. Flavin and its reduced form is represented by F/FH<sub>2</sub>.

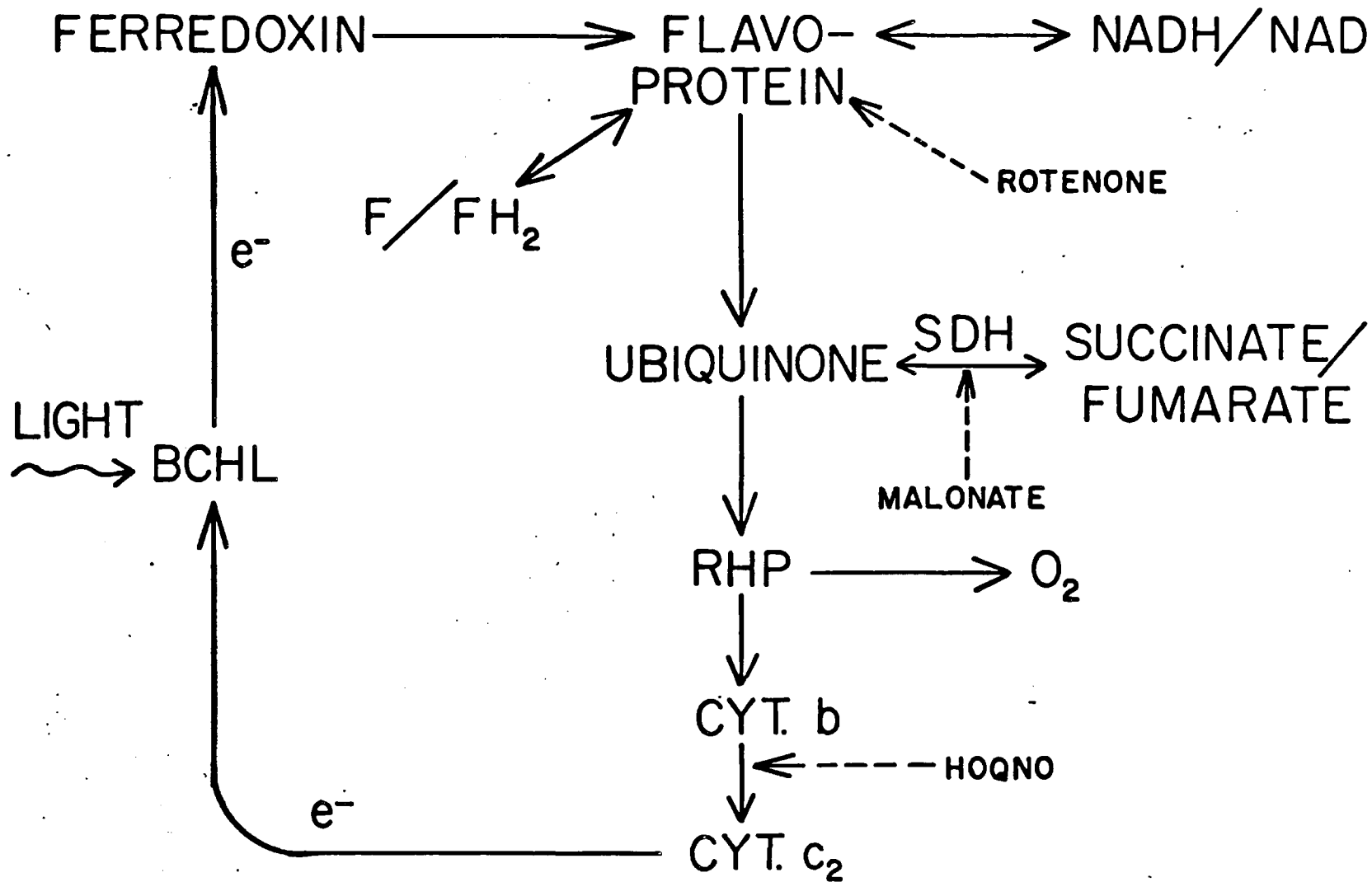


Figure 15. Probable Positions of Interaction of Reagents Affecting the Electron Transfer System.

Both reactions are specific for NAD(H). NADPH could not be oxidized by fumarate, and NADP could not be photoreduced by succinate (unpublished observations). Although others have reported that  $Mg^{++}$  and  $CN^-$  enhance the photoreduction of NAD (36), in this investigation the presence of these ions had no effect upon either reaction.

HOQNO, which inhibits electron transfer from cytochrome b to cytochrome c<sub>2</sub> (11), was used to distinguish between the two reactions. Dark oxidation of NADH by fumarate was unaffected by the presence of HOQNO, while photoreduction of NAD by succinate was almost entirely inhibited. Thus the NADH-fumarate reaction allows investigation of an isolated portion of the photosynthetic electron transfer chain (see Figure 15).

Rotenone, assumed to act upon the electron transfer chain in mitochondrial systems at the flavoprotein position (38), probably acts at a flavoprotein in the chromatophore since it inhibits both the photoreduction of NAD and the dark oxidation of NADH. The site of rotenone inhibition is not SDH since rotenone has no effect upon SDH activity (unpublished observations).

Methanol, used to dissolve rotenone, had a greater effect upon the photoreaction than upon the dark reaction. Assuming the validity of the scheme in Figure 15 these results could be explained if Bchl, readily extracted from chromatophores with an acetone-methanol mixture (29), is

partially dislodged from the chromatophore by methanol. Under this circumstance the effect upon the photochemical reaction would be expected to be greater than that upon the dark reaction.

Quinacrine, which acts upon flavoenzymes (15), and inhibits the photoreduction of NAD, enhances the dark NADH-fumarate reaction. Perhaps two flavoproteins are involved in the photoreduction of NAD, while only one participates in the dark reaction. Then, if the site of quinacrine inhibition is at the flavoprotein nearest Bchl, possibly the binding of quinacrine somehow affects the reactivity of the other flavoprotein, with a resultant enhancement of the dark reaction. However, a definite explanation of the effects of quinacrine requires further investigation.

#### Possible Involvement of Flavin

Flavins added to the NADH-fumarate reaction mixture enhanced the activity in all cases, strongly suggesting that a flavin participates in the electron transfer sequence. Flavin concentrations (ca. 3  $\mu\text{M}$ ) were much lower than the concentration of NADH (ca. 0.2  $\text{mM}$ ). Therefore, the observed increase (20-40%) in the rate of NADH oxidation probably results from cycling of flavin between its oxidized and reduced forms, which, in turn, speeds the transfer of electrons from NADH to fumarate.

Because riboflavin showed the greatest enhancement of the NADH-fumarate reaction, it was chosen for further studies in which large quantities of riboflavin, nearly equal to the concentration of NADH were used. In these investigations the NADH-fumarate reaction was divided into two parts: in the absence of fumarate riboflavin was reduced by NADH; then fumarate was added and oxidation of the reduced riboflavin ensued. (Experiments with FMN yielded similar results, but the reaction rates were slightly lower.) Some spontaneous reduction of riboflavin by NADH occurred at a slow rate, but maximal activity for both reactions required chromatophores. Rotenone inhibited riboflavin reduction and re-oxidation nearly identically, suggesting that both reactions proceed through the same flavoprotein. Malonate affected only the transfer of electrons from reduced riboflavin to fumarate, as would be expected if SDH participates in the reaction.

The velocities of the reduction and oxidation of riboflavin are compared in Table 12 to that of the NADH-fumarate reaction. The rates of NADH oxidation by fumarate and by riboflavin are nearly identical, and are substantially slower than the oxidation of reduced riboflavin by fumarate. Evaluation of these data suggests that the reduction of the flavoprotein by NADH is the rate-limiting step in the transfer of electrons from NADH to fumarate.

Table 12. Comparison of the Velocities for the Reduction and Oxidation of Riboflavin with that for the NADH-Fumarate Reaction

Reaction	Specific Activity (nmoles/mg protein/min)
NADH-Fumarate	7.04
NADH-Riboflavin	6.10
Riboflavin-Fumarate	32.2

Chromatophore concentration was 1.37 mg protein/3.0 ml reaction mixture. All reactions were performed on the same day with the same chromatophore preparation. See Materials and Methods for other experimental details.

All three reactions listed in Table 12 should be valuable for future study of the photosynthetic electron transfer system. At present, the phosphorylation sites in the pathway are unknown; however, the NADH-fumarate reaction can be used to investigate the possibility that phosphorylation occurs between NADH and ubiquinone, as suggested by Vernon (2). Nishimura (19) has reported that o-phenanthroline inhibits the dark oxidation of NADH by fumarate; thus the riboflavin reactions might elicit information as to the site of such inhibition. Furthermore, if chromatophores are able to be subdivided into smaller fragments (subchromatophore particles) similar to submitochondrial particles, reactions which occur in isolated segments of the electron transfer complex--such

as those reported in this paper--will be essential for such investigations.

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