THE OXIDATION OF REDUCED NICOTINAMIDE ADENINE DINUCLEOTIDE BY ENZYMES FROM *Lactobacillus casei*

Thesis for the Degree of Ph. D.

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Glenn A. Walker

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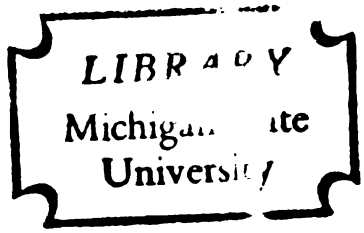

MICHIGAN STATE UNIVERSITY

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ABSTRACT

THE OXIDATION OF REDUCED NICOTINAMIDE ADENINE DINUCLEOTIDE BY ENZYMES FROM *LACTOBACILLUS CASEI*

by Glenn A. Walker

Crude sonicates of *Lactobacillus casei* have been shown to exhibit NADH oxidase, peroxidase and diaphorase activity. By means of ammonium sulfate fractionation, Sephadex filtration, and DEAE-cellulose chromatography, we have been able to purify the diaphorase some ninety-fold. At this purity, it is completely free of NADH oxidase and NADH peroxidase activity.

The properties of this purified enzyme have been studied. The major findings may be summarized as follows: FMN is necessary for maximal activity; benzoquinone, 2,6-dichlorophenolindophenol, and ferricyanide act as electron acceptors while methylene blue does not; the enzymatic rate is not appreciably affected by N-ethylmaleimide, cyanide, amytal or EDTA.

Attempts to separate the oxidase and peroxidase from one another by a variety of methods were without success. However, it was possible to study each of these enzymes

separately in the semi-purified state.

The NADH oxidase was found to require FAD as a co-factor; the enzyme declined in activity during isolation but could be reactivated by incubation with FAD and cysteine. In addition to oxygen, substrate-level concentrations of FAD or methylene blue could be utilized as electron acceptors. Using methylene blue as acceptor, the enzymatic rate was not affected significantly by p-hydroxymercuribenzoate, N-ethylmaleimide, cyanide, amytal, or EDTA.

The NADH peroxidase was found to bind its flavin component very tightly. Conditions sufficient to remove the flavin resulted in denaturation of the enzyme activity. The native enzyme was unaffected by added FAD or FMN, and was not found to catalyze reaction with any electron acceptor other than peroxide. It is inhibited rather strongly by p-hydroxymercuribenzoate and by N-ethylmaleimide, but not at all by the other inhibitors tested.

As was the case with the other two enzymes, the peroxidase was found to be specific for NADH. A maximum activity of seven percent of the NADH rate was found with NADPH.

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By

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INTRODUCTION

Variability in strength of binding of flavin coenzymes to various enzyme proteins is well established. The relative ease of dissociation of D-amino acid oxidase (1) or of NADPH-cytochrome c reductase (2) contrasts with the covalent bonding of flavin and protein as found in dihydroorotic dehydrogenase (3) and in succinic dehydrogenase (4). However, aside from the reports of Huennekens and Kilgour (5) and of DeLuca and Kaplan (6) that several analogs of FAD[1] would not replace FAD as cofactors for hog kidney D-amino acid oxidase, there has been little done in any systematic way to study the different binding characteristics among various flavoproteins and the consequence of these differences.

In 1950 Snell and Strong (7) introduced a standard microbiological assay for riboflavin using the organism Lactobillus casei. It was shown that L. casei was specific for riboflavin.

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[1]The following abbreviations are used: NAD^+ and NADH for oxidized and reduced nicotinamide adenine dinucleotide; $NADP^+$ and NADPH for oxidized and reduced nicotinamide adenine dinucleotide phosphate; indophenol for 2,6-dichlorophenolindophenol; FAD for flavin adenine dinucleotide; FMN for flavin

In 1953, Snell et al. (8) found that the closely-related organism Lactobacillus lactis was capable of growing on lyxoflavin without converting it to riboflavin.

Heunnekens et al. (9) then demonstrated that L. lactis actually incorporated the lyxoflavin into the corresponding mono- and dinucleotides, designated LMN and LAD respectively. They also showed that these nucleotides functioned as flavin coenzymes in the cell and could act as coenzymes for NADPH cytochrome c reductase and D-amino acid oxidase, although with reduced efficiency.

These differences in ability to utilize lyxoflavin for growth suggest either that the flavin-nucleotide-synthesizing enzymes of L. casei are incapable of acting on lyxoflavin, or that the flavin enzymes of this organism differ markedly from those of L. lactis.

Preliminary experiments have shown that the majority of the flavin content of an L. casei sonicate is precipitated out in the 40-80% ammonium sulfate fraction, whereas a much smaller percent of the flavins of L. lactis are precipitated with the protein under the same conditions,

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mono-nucleotide; LAD for lyxoflavin adenine dinucleotide; LMN for lyxoflavin mononucleotide; DEAE cellulose for diethylamino-ethyl-cellulose; and EDTA for ethylenediamine-tetraacetate.

the rest appearing in the supernatant solution.

In view of these differences, the isolation and purification of the major flavoproteins of both organisms were undertaken. This report will describe the work on the L. casei enzymes.

Strittmatter (10) has shown that a major portion of the flavin-linked oxidative activity of L. casei is due to enzymes oxidizing reduced pyridine nucleotides.

Before beginning the Historical Section, there are several problems in the area of reduced-pyridine-nucleotide-oxidizing enzymes which should be discussed at the beginning of this thesis.

There has been much confusion concerning the terminology of flavoproteins which catalyze the oxidation of reduced pyridine nucleotides. The terms NADPH or NADH oxidase, diaphorase, NADH dehydrogenase, and NADH-cytochrome c reductase do not seem to have an exact meaning. As a result they are often used by different workers to denote quite different enzymatic activities.

In order to avoid most of this ambiguity, the following terminology will be used in this paper: (The basic definitions are those of Dolin (11).)

1. NADPH or NADH Oxidase: Flavin enzymes that oxidize reduced pyridine nucleotides using molecular oxygen

as the hydrogen acceptor and producing water or hydrogen peroxide as by-products.

2. Diaphorase: Originally this term designated the flavoprotein which Straub isolated in 1939, but today it has come more generally to mean any flavin enzyme which catalyzes the oxidation of reduced pyridine nucleotides by artificial oxidants, such as ferricyanide, methylene blue, etc. In many cases the physiological hydrogen acceptors, if any, are not known. Huennekens et al. (12) have made one distinction in regard to the term diaphorase. They pointed out that if the dye is autooxidizable, as in the case of methylene blue, then the diaphorase can be classified as an oxidase, with the dye serving as an electron carrier.

3. Cytochrome c Reductase: Flavoproteins which catalyze the oxidation of reduced pyridine dinucleotide using cytochrome c as acceptor. Many of these enzymes show some "diaphorase" activity to artificial oxidants.

4. Dehydrogenases: These are flavoproteins that couple the oxidation of reduced pyridine nucleotides to physiological substrates other than

cytochromes or molecular oxygen, i.e., non-terminal oxidation. A typical physiological substrate might be Coenzyme Q.

5. Direct Flavoprotein Oxidases: These are flavoprotein-enzymes that couple the oxidation of "non-coenzyme" substrates to the reduction of molecular oxygen without involving pyridine nucleotides as intermediates.

6. Flavoprotein Peroxidase: These are flavoproteins which couple the oxidation of reduced pyridine nucleotide to hydrogen peroxide reduction.

The above enzymes are illustrated in Figure 1.

Another problem encountered in this area is what will be referred to as "multiple activities."

As was mentioned under cytochrome c reductases, these enzymes exhibit diaphorase activity toward some artificial oxidants. These are not the only enzymes which exhibit this activity. Other flavoproteins which exhibit diaphorase activity in addition to the classic diaphorase are: NADH oxidase from beef heart (12); NADPH-cytochrome c reductase from pig liver (13); xanthine oxidase from milk (14); and NADH-cytochrome b reductase from liver microsomes (15).

This duality of activities has presented several

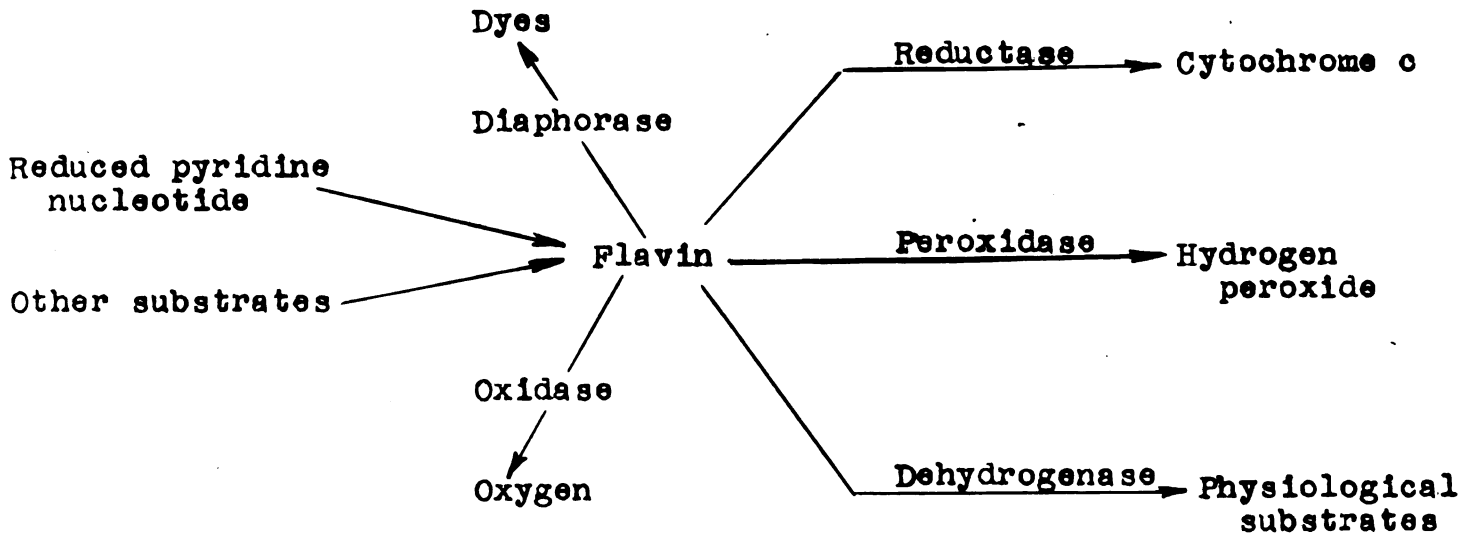

Figure 1 Summary of Definitions

interesting questions. First, is there actually one enzyme with two activities, or are there two closely related enzymes? Second, if there is only one enzyme with two activities, then is one activity real, the true physiological one, and the other an artifact, a product of the isolation procedure? For example, are the cytochrome c reductases one enzyme with two activities--cytochrome c reductase and diaphorase--or are they composed of two separate but relatively inseparable enzymes? There has been much speculation concerning the diaphorases, since the physiological hydrogen acceptor, if any, is not known. Mahler (16) has suggested that diaphorase is a degraded form of cytochrome reductase. On the other hand, Massey (17) has found that Straub's diaphorase is a potent lipoyl dehydrogenase. This was demonstrated by a constant ratio of the two activities during purification, by inhibition studies and by demonstration of the rapid reoxidization of reduced diaphorase by lipoic acid. Therefore, he suggests that this could be the true physiological role of diaphorase.

In 1939, Straub (18) isolated from pig heart muscle a soluble flavoprotein which coupled the oxidation of NADH to methylene blue or indophenol. The enzyme was named diaphorase; it showed no cytochrome c reductase activity. The flavin prosthetic group was shown to be FAD.

In 1952 Mahler, et al. (19) isolated from pig heart muscle a soluble flavoprotein which coupled the oxidation of NADH to cytochrome c reduction. The enzyme, as stated earlier, exhibited diaphorase activity. The flavin prosthetic group was identified as a flavin dinucleotide not identical with FAD.

In 1957 deBernard (20), using the same method of extraction as Mahler, obtained a NADH dehydrogenase from the electron transport particle of heart mitochrondria. The solubilized flavoprotein catalyzed the oxidation of NADH by both cytochrome c and ferricyanide. Again, the flavin portion of this flavoprotein was reported to differ from FAD.

In 1959 Green et al. (21) isolated, from beef heart mitochrondria, a lipoprotein having NADH dehydrogenase activity using ferricyanide as oxidant. The prosthetic group of this enzyme was FAD. The enzyme was found to be essentially inactive with cytochrome c as an electron acceptor.

Green's group found that under appropriate conditions the lipoflavoprotein could be converted to a flavoprotein with properties indistinguishable from those of Straub's flavoprotein. The conversion involved the loss of the bound lipid.

They were also able to show that under different conditions their lipoflavoprotein could be converted to the deBernard or Mahler enzyme. This conversion involved not only loss of lipid but also chemical modification of the prosthetic group.

Therefore, it seems that the properties of some of these solubilized flavoproteins depend upon isolation procedures. The answers to such problems, particularly with regard to mitochondrial enzymes, are still some distance away. It is as well, however, to bear some of the possibilities in mind when discussing any flavoprotein electron-transfer system.

HISTORICAL

To the present time there have been very few reports concerning purified NADH oxidizing enzymes as such. In 1955, Dolin reported (22) that *Streptococcus faecalis* contained several oxidizing enzymes for NADH. These enzymes utilized oxygen, cytochrome c, peroxide and indophenol or fericyanide as oxidants. In 1957, Dolin reported (23) the isolation of one of these enzymes, the peroxidase. In 1960, Dolin and Wood reported (24) the purification of the diaphorase. Both of these enzymes were flavoproteins, the first requiring FAD as the prosthetic group; the second, FMN.

In 1959 Strittmatter (25), using a crude sonicate of *Lactobacillus casei*, reported the presence of a NADH oxidase, NADH peroxidase, and NADH diaphorase.

In 1959 Lightbown and Kogut (26, 27) reported the presence of NADH oxidase, NADH peroxidase, and NADH diaphorase activity in lysed cells of *Bacillus subtilis*. They were able to isolate and purify the diaphorase; the oxidase and peroxidase could not be separated from one another.

Since we are dealing with three enzymatic activities, the remainder of this historical review will be divided into

three corresponding parts.

Diaphorase

The first diaphorase was isolated and purified by Straub (18) as mentioned previously.

In 1951 Robinson and Mills (28) reported the separation from a sonicate of Pasteurella tularensis of four diaphorases. A NADH-specific diaphorase was obtained from the particulate fraction. It was stimulated by menadione. From the supernatant or soluble fraction three other diaphorases were obtained:

1. A NADPH-specific diaphorase
2. A NADPH-spceific diaphorase
3. A non-specific diaphorase

In 1958, Stein and Kaplan (29) studied the distribution[1] of diaphorases in rat-liver cytoplasm. They found that both the particulate and soluble fractions contained high levels of NADPH- and NADH-diaphorase activity. Similar results were reported by Erster (30).

In 1961 Giuditta and Strecker (31) isolated and purified a water-extractable diaphorase from ox brain.

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[1]There have been numerous reports in the literature concerning histochemical studies on the distribution of diaphorase. These are beyond the scope of this review.

This enzyme catalyzed the oxidation of NADPH and NADH by methylene blue, ferricyanide, 2,6-dicholorphenolindophenol, menadione and vitamin K_1. It would not react with cytochromes, lipoic acid or coenzyme Q_{10}. The prosthetic group was FAD.

Peroxidase

Most of the peroxidases which have been isolated belong to the class of heme-proteins. They are conjugated proteins which have an iron-protoporphyrin structure as a prosthetic group, usually with one iron-protoporphyrin per molecule.

This type of peroxidase is present in most plant tissues; for example, Theorell (32) isolated and crystallized a peroxidase from horse radish, while Hosoya (33, 34, 35, 36) has carried out extensive studies on a peroxidase from turnips.

Peroxidases have been reported (37) to be able to use some fifty-four compounds as electron donors. The latter can generally be classified as aromatic amines, phenol compounds, aromatic acids and other miscellaneous substances such as ascorbic acid, NADPH, ferrocytochrome c, NADH, etc.

The NADH peroxidase present in L. casei does not seem to belong to this general class. In 1959, Strittmatter (38), working with a crude preparation, found no heme

component. The prosthetic group for the peroxidase was a flavin compound, which was not further identified.

The NADH peroxidases studied by Lightbown, et al. (26) and Dolin (23) were shown to have FAD as a prothetic group. Neither group reported the presence of any heme component in their preparations. These appear to be the only examples of non-heme flavin-requiring peroxidases reported in the literature.

Oxidase

The first reduced pyridine nucleotide oxidase was isolated from yeast in 1932 by Warburg and Christian (39). They called the enzyme simply "yellow enzyme." The prosthetic group of the "yellow enzyme" was determined by Theorell (40) to be FMN.[1] It is of historical significance that this "yellow enzyme" was the first flavoprotein to be isolated.

Warburg's oxidase catalyzed the oxidation of NADPH by either molecular oxygen or methylene blue.

In 1938 Haas (41) found in yeast a specific FAD-requiring protein that catalyzed the oxidation of NADPH by

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[1]It should be noted here that Warburg and Christian demonstrated that the apoenzyme of their enzyme could be combined with FAD to form a flavoprotein which catalyzed the oxidation of NADPH by molecular oxygen.

molecular oxygen. This protein was named "new yellow enzyme" in order to distinguish it from the FMN-containing enzyme of Warburg.

In 1952, Conn et al. (42) reported that wheat germ contained an enzyme system, NADPH oxidase, which catalyzed the oxidation of NADPH by molecular oxygen. They found that the system contained at least two proteins, one of which was a peroxidase.

There have been several reports (43, 44) that NADH and NADPH could be oxidized by peroxidases in the presence of manganous ions and oxygen.

Several flavoproteins have been reported in the literature which possess NADH oxidase activity as a "secondary" activity. For example, in 1930's xanthine oxidase was isolated from milk (45). This metalloflavoprotein catalyzed the oxidation of hypoxathine to xanthine. It has been found (46) that highly purified preparations of this enzyme catalyze the oxidation of DPNH.

Also, Friedmann and Vennesland (3) found that their crystalline dihydroortic dehydrogenase possessed NADH oxidase activity.

In 1955, Huennekens et al. (12) purified a NADH oxidase[1] from pig heart. This enzyme catalyzed the oxidation

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[1]This enzyme could also be classified as a diaphorase on the basis of the definitions given in the Introduction.

of DPNH by molecular oxygen in the presence of small amounts of methylene blue. A naturally occurring cofactor was demonstrated but not identified.

Reports of the presence of NADH oxidases in S. faecalis, B. subtilis and L. casei have been discussed earlier, so details will not be given here.

In 1962 Mackler et al. (47) reported a fifty-fold purification of the NADH oxidase from S. faecalis. The enzyme was found to be a FAD-requiring enzyme.

In 1961 Fujui (48) reported crystallization of a NADH oxidase from L. plantarum. No details are available at present concerning this crystalline oxidase.

EXPERIMENTAL

Enzyme Assay Procedures

All assays were carried out either in 1.5 milliliters silica cells or standard three milliliter silica cells fused into a standard Thunberg tube (both supplied by Pyrocell Manufacturing Corporation). Both cells had a light path of 1.0 centimeters.

Changes in optical absorbance were measured by using a Beckman DU spectrophotometer equipped with a log converter (Ledland Instrumental Engineering) and recorder.

The standard assay for NADH oxidase consisted of the following: 40 micromoles of phosphate buffer, pH 7.0; 0.13 micromoles of NADH; 0.05 micromoles of FAD; and enzyme, in a final volume of one milliliter. The reactions were started by the addition of the enzyme.

The standard assay for diaphorase activity consisted of the following: 40 micromoles of phosphate buffer, pH 7.0; 0.13 micromoles of NADH; 0.05 micromoles of FMN; 0.5 micromoles ferricyanide; and enzyme, in a total volume of one milliliter. The reaction was started either by addition of enzyme or ferricyanide.

The standard assay for peroxidase activity consisted of the following: 40 micromoles of acetate buffer, pH 5.4; 0.13 micromoles of NADH; 0.9 micromoles of hydrogen peroxide; and enzyme, in a final volume of one milliliter. The reaction was started by addition of enzyme.

The oxidation of NADH in each case was followed by the measurement of the decrease in absorbancy at 340 mμ. For all calculations, a value of 6.22×10^6 cm^2/mole was used for the molar extinction coefficient for NADH (49).

One unit of enzyme activity was defined as that which brings about a change in absorbance at 340 mμ of 0.010 per minute. Specific activity was then defined as the number of activity units present per milligram of protein.

Since we are dealing with three NADH oxidizing activities it was necessary to assay the diaphorase and peroxidase activity anaerobically during the early stages of purification. The anaerobic assays were the same as the standard, except that three times the amount of each component was used in a final volume of three milliliters. All of the components except NADH were mixed in the main compartment of the cell. The NADH was placed in the side arm. The reaction was started by tipping the NADH into the main compartment.

Fractions obtained during various column separations

were routinely assayed aerobically for peroxidase activity at pH 5.4 and for diaphorase activity at pH 7.0. Oxidase determinations were then made at both pH values and the peroxidase and diaphorase values corrected for oxidation due to oxidase activity.

Oxidase activity of cell extracts was found to decrease slowly with time during the processes of isolation of the enzymes. A considerable portion of the original activity could be restored by incubation of the inactive enzyme with cysteine and FAD.

Enzyme preparations were reactivated by incubating 0.05 to 0.2 milliliters of enzyme for ten minutes at 37° with 0.1 milliliters of FAD (1×10^{-3}M) and 0.1 milliliters of cysteine (0.242 grams of free base dissolved in 50 milliliters of 0.5 M potassium phosphate buffer at pH 7.5).

The assay for the reconstituted oxidase consisted of the following: 40 micromoles of phosphate buffer pH 7.0; 0.13 micromoles of NADH; and reconstituted enzyme in a final volume of one milliliter.

Meterials

The following materials were used: FAD, FMN cytochrome c and NADH, Sigma Chemical Company; catalase, alcohol dehydrogenase, ribonuclease and deoxyribonuclease, Worthington;

Sephadex G-25, G-75, G-100 and G-200, Pharmacia Fine Chemicals Inc.; methylene blue, National Aniline Division, Allied Chemicals; acriflavin, Mann Research Laboratory; amytal sodium, Eli Lilly; menadione, National Biochemical Corporation; 2,6-dichlorophenolindophenol, Eastman Organic Chemicals; riboflavin, General Biochemicals, Inc.; DEAE-cellulose, Eastman Chemical Company; N-ethylmaleimide, Sigma Chemical Company; p-hydroxymercuribenzoate, sodium, California Corporation for Biochemical Research.

The potassium ferricyanide was recrystallized from boiling water. The solutions were stored in brown bottles.

The p-benzoquinone and 1,4-naphthoquinone were recrystallized from light petroleum ether. The p-benzoquinone was dissolved in water, divided into three-milliliter portions, and frozen. No solution was used after sitting six hours.

The 1,4-naphthoquinone and menadione were dissolved in one milliliter of ethanol and rapidly diluted to the desired volume with water.

The p-hydroxymercuribenzoate was dissolved in the minimum amount of 0.1 M NaoH and then diluted up to the desired volume.

NADH and NADPH were dissolved in 0.002 M potassium phosphate buffer at pH 7.5.

Chemical Determinations

Protein concentration was determined by the method of Lowry et al. (50) and/or by 280-260 mμ ratios according to Warburg and Christian (51).

For quantitative estimation of the following compounds by optical absorbance, the values shown were used for the molar extinction coefficients: FAD, 11.2×10^6 cm^2/mole at 450 mμ (52); FMN and riboflavin, 12.2×10^6 cm^2/mole at 450 mμ (52); cytochrome c, oxidized, 0.96×10^7 cm^2/mole; reduced, 2.81×10^7 cm^2/mole at 550 mμ (53); NADH, 6.22×10^6 cm^2/mole at 340 mμ (49); $Fe(CN)_6$, 1×10^6 cm^2/mole at 420 mμ (54); 2,6-dichlorophenolindophenol, 2.1×10^7 cm^2/mole at 600 mμ (55).

Cell Growth

The medium used for the growth of Lactobacillus casei ATCC 7469 contained the following components per liter:

Tryptone (Difco)	10.0 grams
Yeast extract (Difco)	10.0 grams
Dextrose	5.0 grams
K_2HPO_4	2.6 grams

The pH was approximately 7, and no adjustment was required.

The procedure for the growth of this organism was as follows: All of the ingredients for 18 liters of medium with the exception of the dextrose, was placed in a five

gallon pyrex carboy and 18 liters of water was added. Enough dextrose for 18 liters of medium was put in a 500 ml Erlenmeyer flask and made up to 360 milliliters with water. The dextrose was autoclaved separately, since dipotassium hydrogen phosphate is a catalyst for its caramelization. It can also be heated in a more precise manner in a small volume.

One and one-half liters of the dextrose-free medium in the carboy was put into a two liter Erlenmeyer flask and thirty milliliters of dextrose solution was added. Then150 milliliters and 15 milliliters of dextrose-containing medium was put in a 250 milliliter Erlenmeyer flask and a test tube, respectively. All containers were plugged with cotton, surrounded with gauze, then covered with brown paper and tied with string. The carboys were autoclaved for 1 hour after they had reached 121°C. The rest of the flasks were autoclaved for 15 minutes at 121°C.

In order to build up a vigorous carboy inoculum, the test tube was first inoculated from a stab culture and, after the appearance of visible turbidity, its contents were poured aseptically into the 250 milliliter flask. This serial transfer of the inoculum was repeated through the two liter flask and finally into the carboy. The remaining dextrose solution was added to the carboy along

with the inoculum. The turbidity of the culture in the carboy was checked periodically by sight and when no further increase occurred, the contents were harvested in a Sharples continuous centrifuge. The growth period in the carboy was about 16-20 hours depending upon the number of organisms in the inoculum.

Isolation Procedures

Cell Rupture

Several procedures were attempted for cell rupture. Breakage was attempted first in a model 45 Virtis Homogenizer. It was found that suitable breakage could be obtained only in the presence of glass beads. The major problem encountered with this procedure, however, was keeping the Virtis flask cooled. The flask was packed in ice and the breakage carried out in the cold room. Still, the heat generated in the presence of the glass beads was not conducted away fast enough, so that the solution warmed to forty or fifty degrees Centigrade. As a result the procedure was abandoned in favor of sonic oscillation.

Variations in temperature, time, glass bead concentration, etc. were tried with the sonic oscillation process. The following procedure appeared to give the best results: After harvesting, the whole cells were suspended in phosphate

buffer pH 7.0, 0.02 M to give a thick slurry. This slurry was subjected to sonic oscillation in a Raytheon 10 kc sonic oscillator for 55 minutes. The sonicate was removed and diluted with the above buffer. This was followed by repeated centrifugation at 28,000 RCF in an International HR-1 centrifuge until the supernatant solution was almost clear.

The debris was made into a slurry again with phosphate buffer and subjected to a further period of sonic disruption. The supernatant solution was cleared by repeated centrifugation at 28,000 RCF.

An example of a typical run would be as follows: 154.2 grams of wet L. casei (corresponding to 49.3 grams, dry weight) were mixed with forty milliliters of phosphate buffer. The resulting slurry was disrupted by sonic oscillation as outlined above. The final volume of the supernatant solution was 360 milliliters, which contained approximately 18 milligrams of protein per milliliter of solution.

Nucleic Acid Removal

The sonic supernate contained a high nucleic acid content. Using 230 mμ to 260 mμ ratios according to Warburg and Christian (51), the nucleic acid content was calculated

to be in the range of 50 to 60%.

The nucleic acid was enzymatically hydrolyzed by incubation with deoxyribonuclease and ribonuclease. Two to five milligrams each of ribonuclease and deoxyribonuclease were added to the sonic supernatant, the exact amount depending upon the volume of the solution. This solution was then incubated in a low-actinic flask at 37°C for six to eight hours. Similar results could be obtained by allowing the flask to sit overnight at room temperature.

Upon incubation a precipitate usually formed, which was removed by centrifugation. The resulting supernate was a clear yellow solution.

The nucleotides which resulted from hydrolysis were removed during the ammonium sulfate fractionation and Sephadex columns stages of purification.

Ammonium Sulfate Fractionations

All ammonium sulfate fractionation procedures were carried out at 0°C. The percent-saturation values were calculated from the data in Table 1. The calculated number of grams of finely-ground solid ammonium sulfate were then added to the solution with gentle continuous stirring. After stirring for forty-five minutes, the precipitates were spun down in a refrigerated centrifuge by spinning for 15 minutes at 18,000 RCF.

Table 1. Calculation of ammonium sulfate saturation.[1]

Temp.	0°	10°	20°	30°
Molarity of sat. soln.	3.9	4.0	4.1	4.2

Salt Content in Wt% (G) of $(NH_4)_2SO_4$ Soln's of Various Molarities (m) at 20°

m	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
G	1.3	2.6	3.8	5.1	6.4	7.6	8.8	10.0	11.1	12.3
m	1.1	1.2	1.3	1.4	1.5	1.6	1.7	1.8	1.9	2.0
G	13.4	14.6	15.7	16.8	17.9	19.0	20.1	21.2	22.2	23.3
m	2.1	2.2	2.3	2.4	2.5	2.6	2.7	2.8	2.9	3.0
G	24.3	25.3	26.4	27.4	28.4	29.4	30.3	31.3	32.2	33.2
m	3.1	3.2	3.3	3.4	3.5	3.6	3.7	3.8	3.9	
G	34.2	35.1	36.1	37.0	38.0	38.9	39.8	40.7	41.6	

Formula: $$X = V\left[\frac{G_2(100-G_1)}{G_1(100-G_2)} - 1\right]\frac{m}{7.6}$$

but when $G_1 = 0$ one uses

$$X = \left[\frac{G_2}{100-G_2}\right] V$$

[1] G. Beisenherz, et al., Z. Naturfarsch 86, 555 (1953).

Various fractionations were attempted. The three enzymes under study were found to precipitate in the 40-75% precipitate fraction. Therefore, the best fractionation seems to be 0-40, 40-75, and 75-100%. In some of the runs 0-50, 50-80 and 80-100% fractionations were used. In these cases parts of each of the enzyme activities found were in the 0-50 fraction; the bulk of the activity, however, was found in the 50-80 fraction.

Sephadex Columns

Preparation of Sephadex Columns: The dry Sephadex was thoroughly suspended in a dilute sodium chloride solution. The gel was then washed repeatedly by decantation with distilled water in order to remove the finer gel particles and most of the sodium chloride.

The proper size chromatographic column was filled with distilled water and mounted in a vertical position. The gel suspension was added to the column through a funnel which had been mounted in the top of the column. When several centimeters of gel had settled, the outlet of the column was opened. After the gel had completely settled, a piece of circular filter paper was placed on top of the column to prevent disturbance of the gel surface.

The packed column was then equilibrated with 0.002 M potassium phosphate buffer, pH 7.33. The buffer contained eight drops of octanoic acid and eight drops of zephiran chloride solution in each four liters to discourage bacterial and mold growth.

The "void volumes," the volume of solution outside of the gel, of the columns were determined by placing a narrow zone of India ink on the top of each of the columns and eluting with buffer. This elution volume is equal to the void volume.

The internal volume was determined by adding a small volume of sodium chloride solution to the top of the column and eluting with the usual buffer. This elution volume is equal to the void volume plus the internal volume. Also, in some cases the internal volume was calculated from the following equation:

$$V_i = a \times W_r$$

V_i = internal volume

a = dry weight of gel used

W_r = water regain[1]

---

[1] Average values for water regain, in grams of water per gram of dry gel, are given by the supplier.

Sephadex Separation Procedures: In general, the protein solutions were placed on top of the Sephadex column in a narrow zone. The column was then eluted with 0.002 M phosphate buffer, pH 7.33. Unless stated otherwise, the elutions were carried out at room temperature. Constant volume fractions were obtained by using a fraction collector with drop counter.

A typical example of a G-100 run would be as follows: One tube of the 50-80% ammonium sulfate precipitate was dissolved in phosphate buffer 0.002 M, pH 7.33, to give a final volume of 4.6 milliliters with a protein concentration of 66.6 milligrams of protein per milliliter. 4.4 milliliters of this solution was placed on a Sephadex G-100 column, 4.5 x 48 centimeters, and the column eluted with the same buffer.

The best separation was obtained when the flow rate was approximately 0.5 milliliters per minute. When the flow rate was faster and/or the protein volume larger, poorer separations were obtained. It was found that the results are highly reproducible; a typical pattern is shown in the section on Results as Figure 4.

The general procedure for G-200 columns is the same as for G-100. A typical run was carried out as follows: Two milliliters of the protein preparation (which contained

33.5 milligrams of protein per milliliter), was placed on a Sephadex G-200 column 30 x 2.2 centimeters. The components were eluted from the column using 0.002 M potassium phosphate buffer at pH 7.3. Three-milliliter fractions were collected. The flow rate was adjusted to approximately 0.5 milliliters per minute. The results are shown in Figure 6 of the Results section.

DEAE-Cellulose Columns

Preparation of Columns: A weighed amount or dry DEAE-cellulose was dispersed in a Waring Blendor for approximately one minute. A slurry was prepared by adding potassium phosphate buffer, 0.05 M, pH 6.8 to the DEAE-cellulose. This slurry was then poured into a suitable column and allowed to settle. The column was then brought to equilibrium by washing with the same buffer. After each use, the columns were regenerated by washing with this same buffer overnight.

Some of the small columns were packed with the dry DEAE-cellulose directly by repeated gentle tamping of small amounts until the desired height was obtained.

Separation Procedures: The protein solution was usually placed on top of a DEAE-cellulose column which had been pre-washed with 0.05 M phosphate buffer, pH 6.8. Various

elution procedures were used in the attempt to obtain maximal separation of the proteins. Again, unless stated otherwise these columns were eluted at room temperature.

A gradient elution system, Figure 2, was set up which consisted of three chambers. The first two were mixing chambers while the third was a reservoir.

The best elution tried was the following:

Chamber I --- 0.05 M phosphate buffer, pH 6.8

Chamber II --- 0.1 M phosphate buffer, pH 7.0

Chamber III --- 0.2 M phosphate buffer, pH 7.2

Equal volumes of each buffer were used in a given run. A volume of 250 milliliters of each buffer gave satisfactory results with a column 32 x 1.2 centimeters.

A typical run would be as follows: 35 milliliters of the combined fractions from a G-100 column were placed on a DEAE cellulose column, 33 x 1.2 centimeters, which had been pre-washed with 0.05 M phosphate buffer pH 6.8. The column was then eluted by gradient elution. (See Figure 2 for elution apparatus) in the following manner:

Chamber I --- 250 milliliters 0.05 M buffer pH 6.8

Chamber II --- 250 milliliters 0.10 M buffer pH 7.0

Chamber III--- 250 milliliters 0.20 M buffer pH 7.2

Five-milliliter fractions were collected. The results are shown in Figure 7 of the Results section.

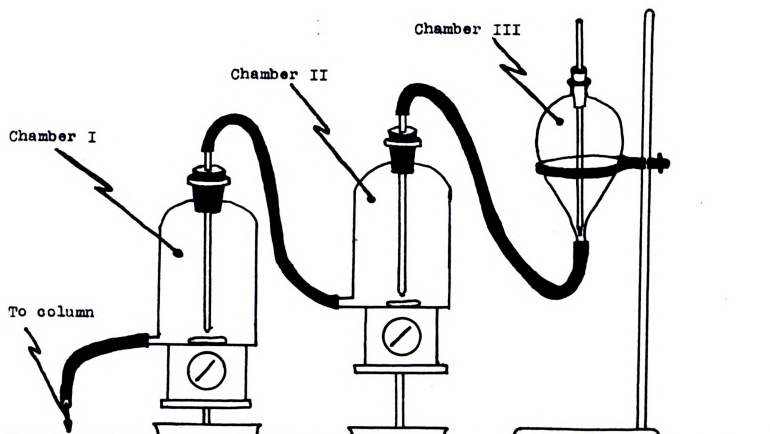


Figure 2 Gradient Elution Apparatus

Calcium Phosphate Gel Separations

The calcium phosphate gel was prepared according to the method of Keilin and Hartree (56).

Several different procedures were tried. First, attempts were made to selectively adsorb one of the enzymes to the gel by addition of varying amounts of gel. The general procedure employed was as follows: The combined fractions (280 milliliters) from a G-100 column separation were precipitated by adding 117 grams of solid ammonium sulfate. The precipitate was stirred for one hour, collected by centrifugation, then dissolved in water and dialyzed overnight. The dialyzed solution was adjusted with sodium acetate buffer to give a pH of 5.0 in a final volume of 135 milliliters, which contained 1.7 milligrams of protein per milliliter. Seventy milligrams of gel were added with stirring. After stirring for twenty minutes the gel was collected by centrifugation and designated "Gel I." To the supernatant solution, 165 milligrams of gel was added, the above steps repeated, and this gel designated "Gel II." This procedure was repeated twice more to give "Gell III" and "Gel IV." Each gel was then eluted in succession with 0.1 M potassium phosphate buffers at pH 5.4, 6.0, and 6.8. The procedure for elution was as follows: The gel was suspended in 25 milliliters of buffer (pH 5.4) for five

minutes. The gel was then collected by centrifugation and the supernatant assayed. The gel was then eluted with buffer at pH 6.0 and then pH 6.8. This procedure was repeated for gels II, III, and IV.

It was found that the peroxidase, oxidase and ferricyanide activities were scattered throughout the elution steps.

Secondly, attempts were made to selectively elute the enzymes from the gel by either changing pH or ionic strength.

The general procedure employed was as follows: The dialyzed G-100 fractions were adjusted with 1.0 M potassium phosphate buffer pH 6.75 to give fifty milliliters of protein solution 0.001 M in phosphate at pH 6.75. To forty-five milliliters of such a solution, 700 milligrams of gel were added and the gel was collected by centrifugation. The resulting supernatant solution contained no peroxidase, oxidase or ferricyanide activities. The gel was successively eluted with potassium phosphate buffers at pH 6.75 of the following molarities: 0.005 M, 0.01 M, 0.02 M, 0.04 M and 1.0 M. The elution procedure is the same as that stated above. (In another run the gel was eluted with 0.1 M potassium phosphate of varying pH: 6.8, 7.0, 7.2 and 7.5. In this case the protein had been absorbed

at pH 6.0.)

It was found that the results again were varied and, most important, they could not be successfully reproduced.

Heat Denaturation

Attempts were made to selectively heat denature one or more of the enzymes. The protein solution was heated in a water bath at the following temperatures:

1. 40°C for 10 minutes
2. 50°C for 5 minutes
3. 60°C for 5 minutes
4. 70°C for 5 minutes

After heating the solution at each of the above temperatures, the preparation was immediately chilled and any coagulated material removed by centrifugation. An aliquot was removed for assaying, and the remaining solution was heat treated at the next temperature.

A Typical Purification Procedure

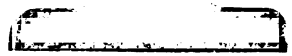
After harvesting the L. casei cells, forty milliliters of 0.02 M potassium phosphate buffer at pH 7.0 were added to 154 grams wet weight of L. casei which corresponded to 49.3 grams dry weight. The resulting slurry was subjected to sonic oscillation for fifty-five minutes. The debris was spun down as stated in Procedures. The final volume of the supernatant solution was 365 milliliters.

To 360 milliliters of the sonic supernatant 4 milligrams each of ribonuclease and deoxyribonuclease were added and the solution was left to incubate at room temperature overnight, approximately 15 hours. The precipitate which was found after incubating was removed by centrifugation. The final volume was 345 milliliters.

Ninety-nine grams of solid ammonium sulfate was added to 340 milliliters of the incubated supernatant. The precipitate was collected by centrifugation after stirring for 45 minutes. The precipitate was stored at 4°C until further used.

Eighty-nine grams of solid ammonium sulfate was added to the 0-50 supernatant with stirring. After stirring for 45 minutes, the precipitate was collected by centrifugation at 18,000 RCF. Again, the precipitate was stored at 4°C until further used.

Approximately one-tenth of the 50-80% ammonium sulfate precipitate was dissolved in 0.002 M potassium phosphate buffer at pH 7.3 to give a final volume of 4.6 milliliters which contained 66.6 milligrams of protein per milliliter. 4.4 milliliters were placed in a narrow band on a Sephadex G-100 column, 4.5 x 48 centimeters. The column was eluted at room temperature with the same buffer at a flow rate of 0.5 milliliters per minute. Five milliliter



fractions were collected. The peroxidase and oxidase were located primarily in fractions 45 to 60, while the diaphorase activity was found in the 64 to 72 fractions. These fractions were combined.

The final volume of the combined 45-60 fractions was 59 milliliters. Fifty-four milliliters of this solution was placed on a DEAE-cellulose column, 33 x 1.2 centimeters and eluted with the following gradient: 250 milliliters of 0.05 M phosphate buffer at pH 6.8; 250 milliliters of 0.1 M phosphate buffer at pH 7.0; and 250 milliliters of 0.2 M phosphate buffer at pH 7.2. The gradient set-up is depicted in Figure 2. Five-milliliter fractions were collected. The peroxidase and oxidase were found in fractions 36 to 50.

The final volume of the combined 64 to 72 fractions was 42 milliliters. Thirty-eight milliliters were placed on a DEAE-cellulose column, 33 x 1.2 centimeters and eluted with the above gradient. The ferricyanide activity was found in fractions 40 to 60. This was used as the "purified diaphorase" for property studies.

The purification procedure is outlined in Figure 3.

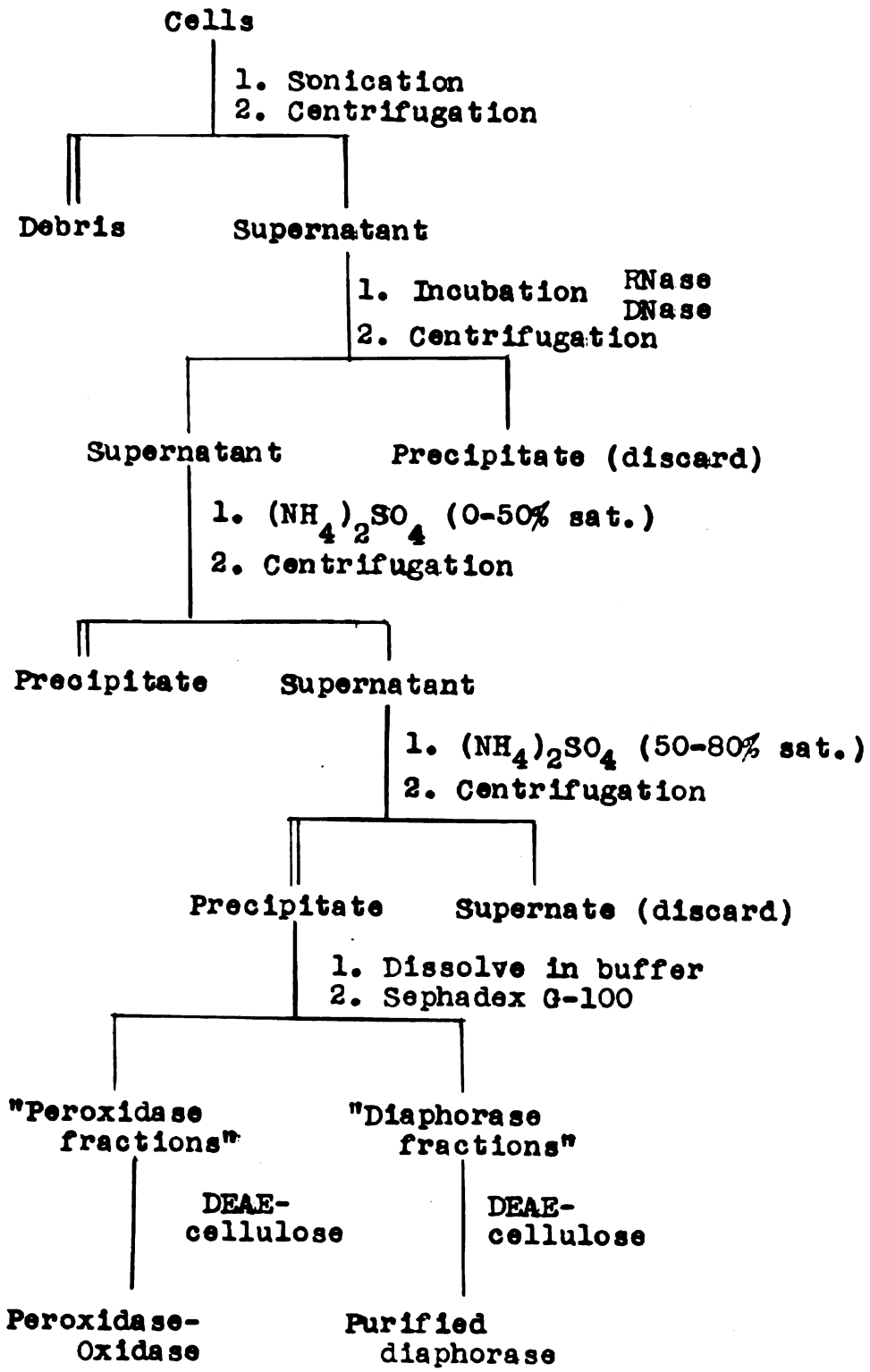


Figure 3 Schematic Diagram of Enzyme Isolation Procedures

RESULTS AND DISCUSSION

Isolation and Purification of the Enzymes

The main objective of this phase of the project was the separation of the three NADH-oxidizing activities of *L. casei*, and the purification of each of these enzymes.

A major problem encountered in working with the cell-free sonic supernatant was the high nucleic acid content. On a weight basis, the nucleic acid content was 1.5 times that of protein (as determined from 280 mμ - 260 mμ ratios (51)). After incubation with ribonuclease and deoxyribonuclease, followed by ammonium sulfate fractionation and Sephadex filtration, the nucleic acid concentration was reduced to only one percent of that of the protein.

Diaphorase

The diaphorase or ferricyanide activity has been successfully separated from the NADH peroxidase and NADH oxidase activities. The best separation was obtained with Sephadex G-100 and G-200 columns. Using the conditions listed under Sephadex Column Procedures in the Experimental section, complete separation was obtained, as shown in Figures 4 and 5. When the conditions were varied, such

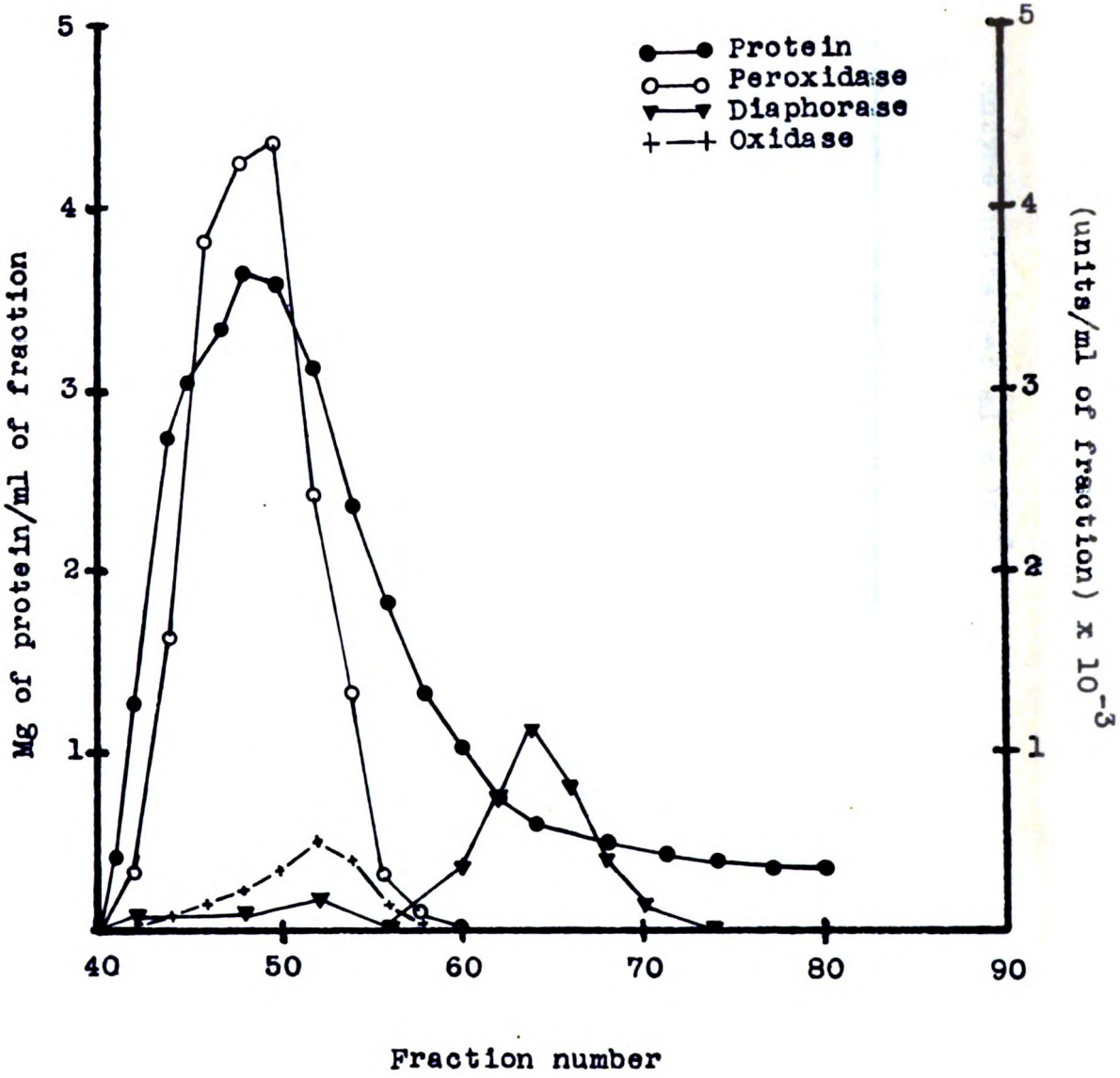


Figure 4 Typical Separation Obtained on Sephadex G-100 Columns under Optimal Conditions

A total of 302 mg of protein in 4.4 ml was added to the column; eluted with potassium phosphate buffer, 0.002 M, pH 7.3. Five ml fractions were collected.

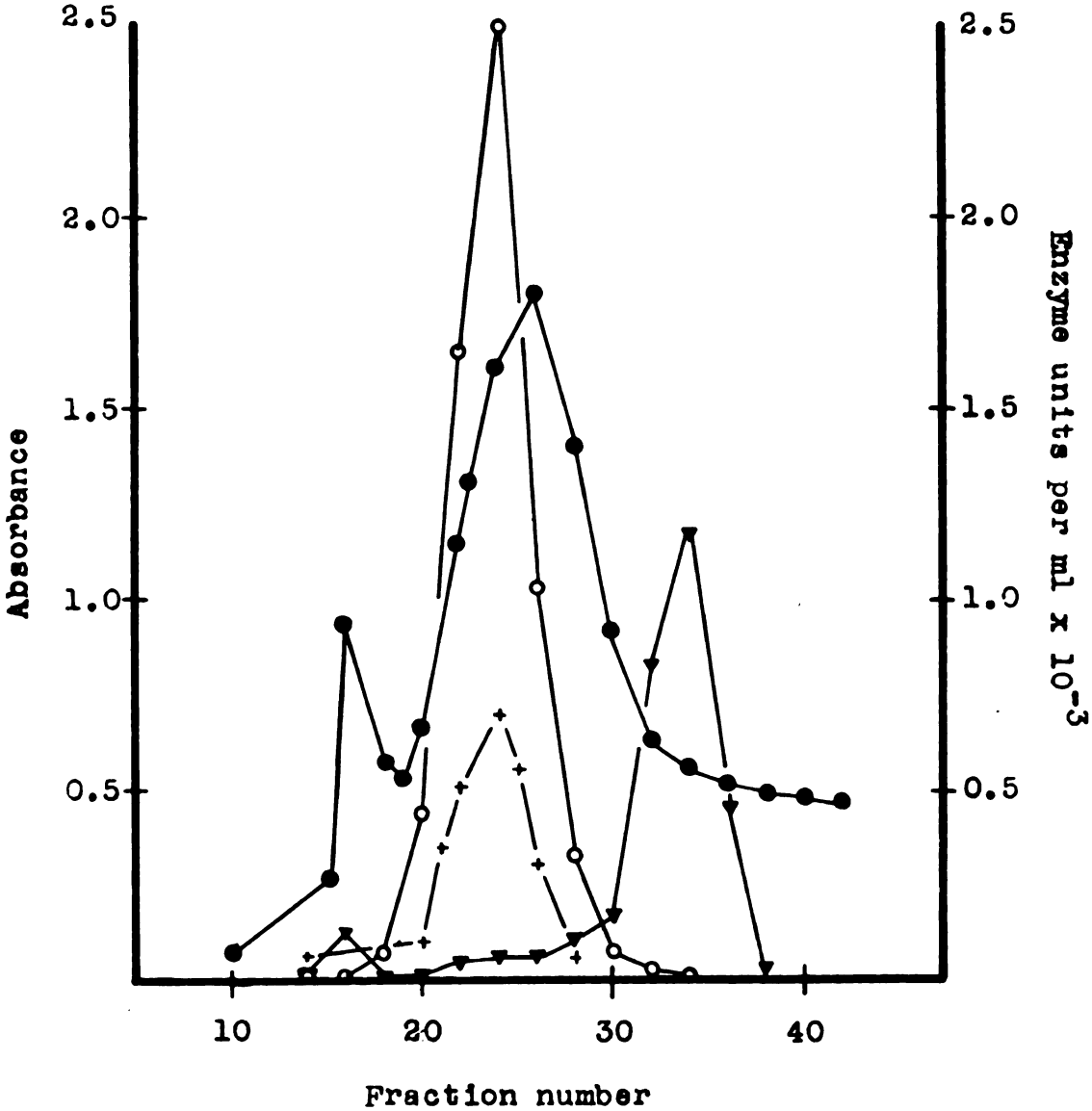


Figure 5 Typical Separation Obtained on Sephadex G-200 Columns

The following symbols are used: absorbance at 280 mμ, ●—●; absorbance at 450 mμ, +—+; peroxidase, o—o; diaphorase, ▼—▼.

as a larger volume of column feed and/or a faster flow rate, incomplete separations were obtained; a typical result is shown in Figure 6.

It was clear from data such as those shown in Figure 7 that the DEAE-cellulose columns also separated the ferricyanide activity from the peroxidase and oxidase. Of more importance, however, was the finding that passage through the DEAE-cellulose procedure also resulted in a considerable increase in specific activity. Thus the figures in Table 2 indicate approximately a nine-fold increase in specific activity for this step alone.

With the Sephadex column separation, there is also a ferricyanide peak which corresponds to the peroxidase-oxidase peak. Since this persists in preparations from which the other ferricyanide peak has been removed completely, it is probable that this peak represents a "secondary activity" of either the peroxidase or oxidase, and does not represent incomplete separation.

The summary for purification of the ferricyanide (diaphorase) activity is found in Table 2. A 94-fold purification has been obtained with approximately 2.4% recovery of the total activity.

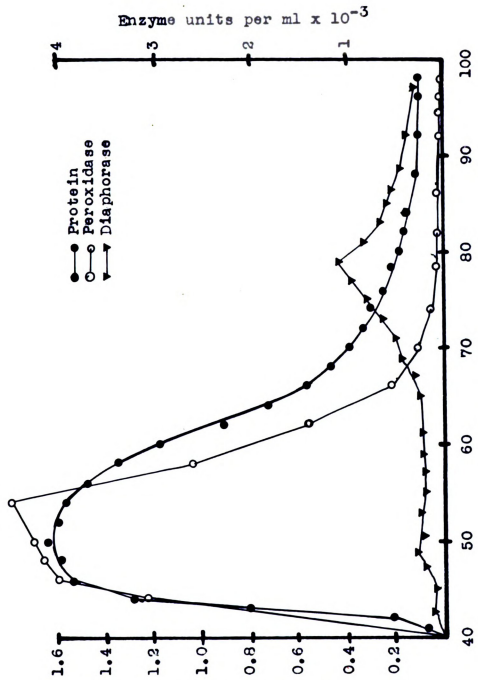


Figure 6 Typical Separation Obtained on Sephadex G-100 Columns under Sub-optimal Conditions

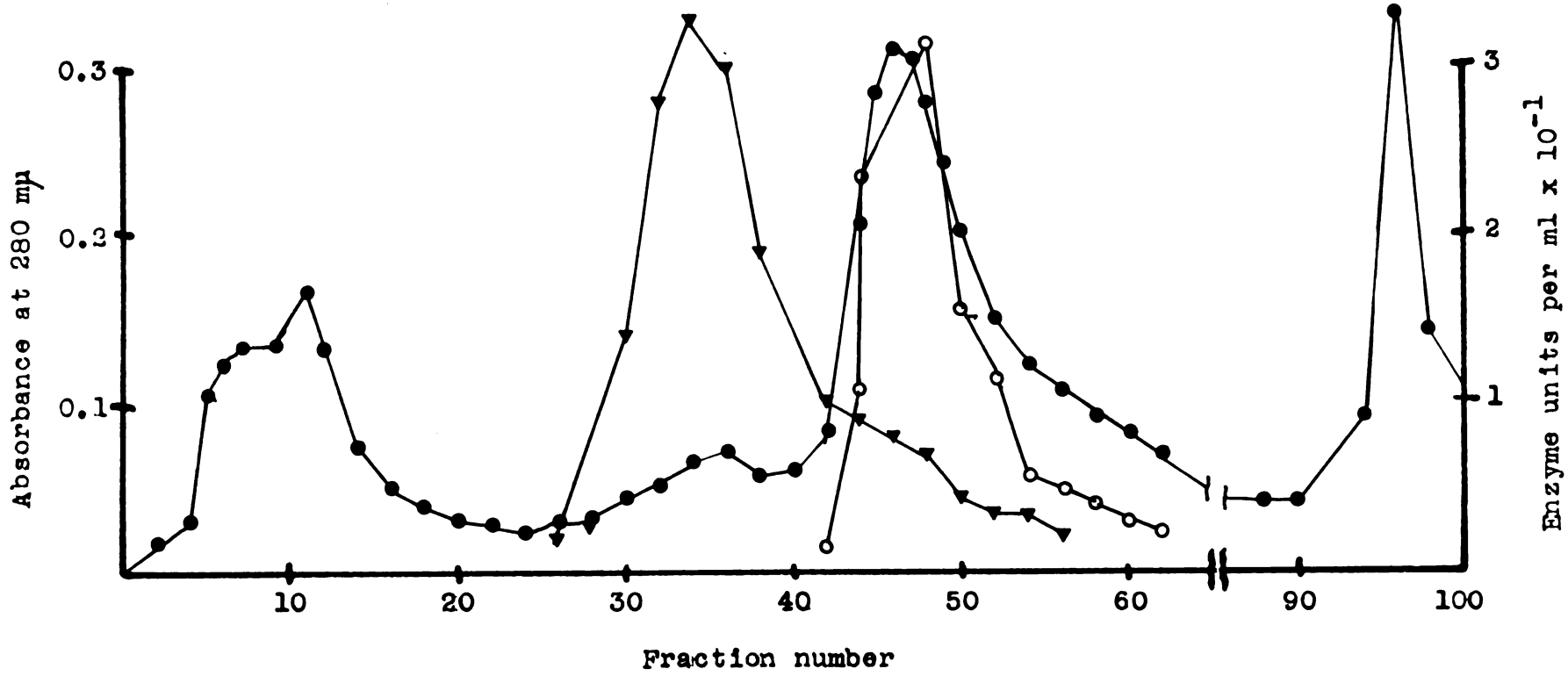


Figure 7 Typical Separation Obtained on DEAE-cellulose Columns

The following symbols are used: protein, ●—●; peroxidase, o—o; diaphorase, ▼—▼. The peroxidase activities have been reduced by one-half; oxidase activity coincided with the peroxidase peak. Enzyme activities where not shown were approximately zero.

Table 2. Purification of diaphorase.

Fractions	Protein conc. mg/ml	Specific Activity	Total Activity
Original	17.5	85	550,000
Incubated	20.0	80	550,000
50-80% sat. $(NH_4)_2SO_4$	68.7	300	93,000
Combined fractions Sephadex G-100	0.48	920	34,300*
DEAE-cellulose Frac. 45	0.036	8000	1,440

*Only one-tenth of the 50-80% saturation ammonium sulfate precipitate was carried through the column steps.

Peroxidase and Oxidase

To the present time it has not been possible to separate the peroxidase and oxidase activities. Ammonium sulfate fractionation, Sephadex G-100, heat denaturation, calcium phosphate gel adsorption, and DEAE-cellulose chromatography have proven unsatisfactory for separation.

Dolin (22) has reported that the NADH oxidase of S. faecalis is precipitated in the 0-50% ammonium sulfate fraction. Mackler, et al. (47) has taken the 0-50% ammonium sulfate precipitate from S. faecalis and purified the oxidase 50-fold. They reported that the purified oxidase

catalyzed the reduction of oxygen to water, which is different from the results of Dolin (22), without the obligatory participation of a NADH peroxidase.

Dolin also reported (23) the purification of the NADH peroxidase from S. faecalis. It is of interest to note that the oxidase was not assayed according to the procedure of Mackler et al. (47) for reconstitution.

Lightbown and Kogut reported (26) that the peroxidase and oxidase activities of B. subtilis could not be separated by any of the procedures which they tried. They were able to separate the diaphorase from the peroxidase and oxidase by DEAE-cellulose anion exchange chromatography.

Properties of the Diaphorase

Demonstration of NADH Oxidation

When the reaction mixture contained NADH, buffer and enzyme, the disappearance of NADH, as measured by optical density at 340 mμ, was very slow. Upon the addition of potassium ferricyanide an appreciable rate was observed, (Point I of Figure 8--the increase in absorbancy is due to the ferricyanide.) After the reaction had proceeded to completion, the pH was adjusted to pH 8.5 $\pm$ 0.5 with NaOH, and 0.1 milliliter of 95% ethanol was added (Point II of Figure 8). Upon addition of crystalline alcohol dehydrogenase,

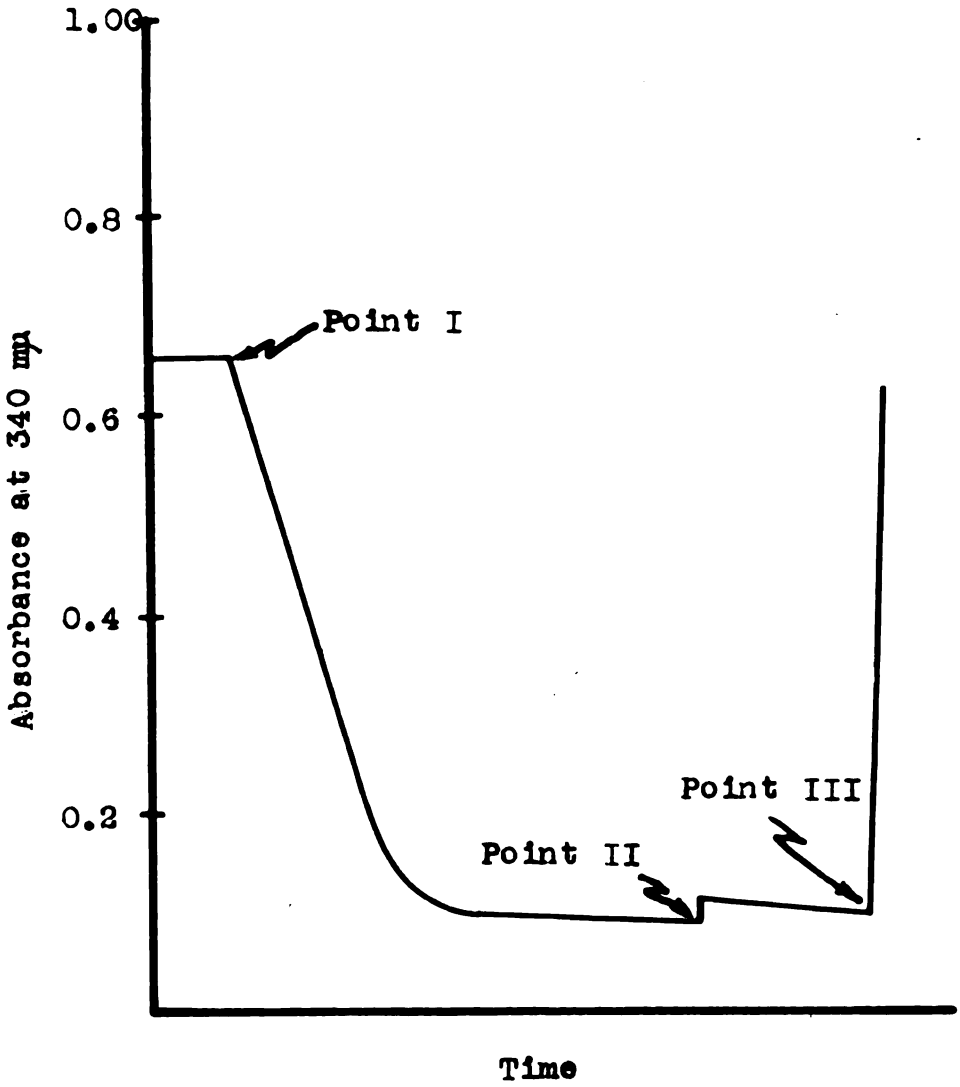


Figure 8 Illustration of the Stoichiometry of the Diaphorase Reaction.

the absorbancy returns immediately to a value corresponding to 90% or the decrease (Point III of Figure 8). (Dolin (22) has shown that 90% of the decrease in absorbance at 340 mμ in the diaphorase assay is due to NADH oxidation, and the other 10% to ferricyanide reduction.) This demonstrates that the disappearance is due to the oxidization of the NADH to NAD^+ and is not due to destruction of the NADH or conversion to a modified NADH product (57, 58).

The effect of variation in enzyme concentration is shown in Figure 9.

The enzyme appears to be specific for NADH. The rate with NADPH as the substrate under identical conditions is only 7% of that with NADH.

Cofactors

When FMN is added to a reaction mixture containing NADH, buffer, enzyme and ferricyanide, the decrease in absorbance at 340 mμ is approximately tripled. However, there is no appreciable change in rate when FMN is added to a mixture containing only NADH, buffer and enzyme. The nonenzymatic rate is similarly not affected by the presence of FMN.

Addition of riboflavin had no effect upon the rate. The action of added FAD was found to vary with the age of the

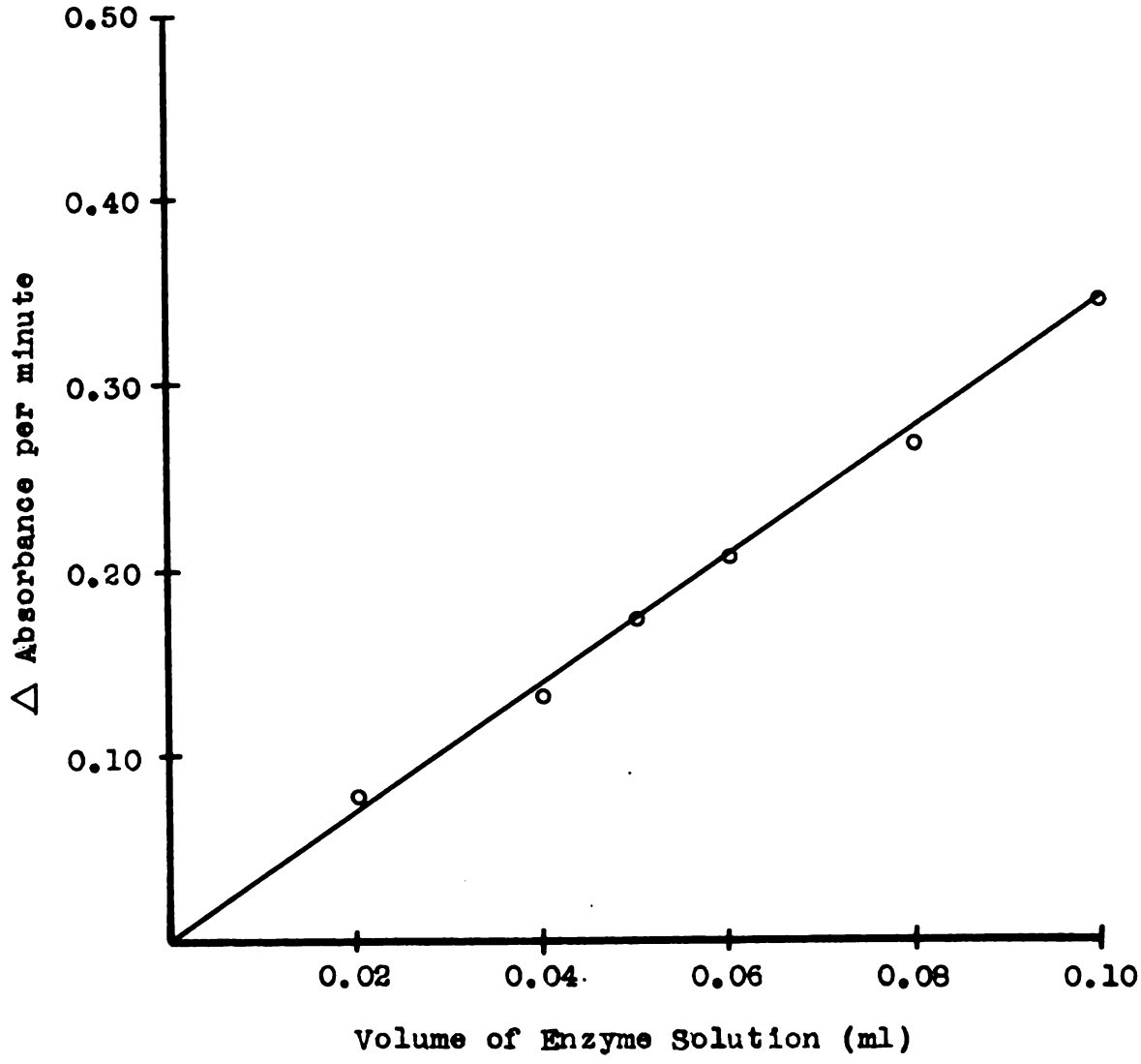


Figure 9 Relationship Between Enzyme Concentration and Rate for the Diaphorase.

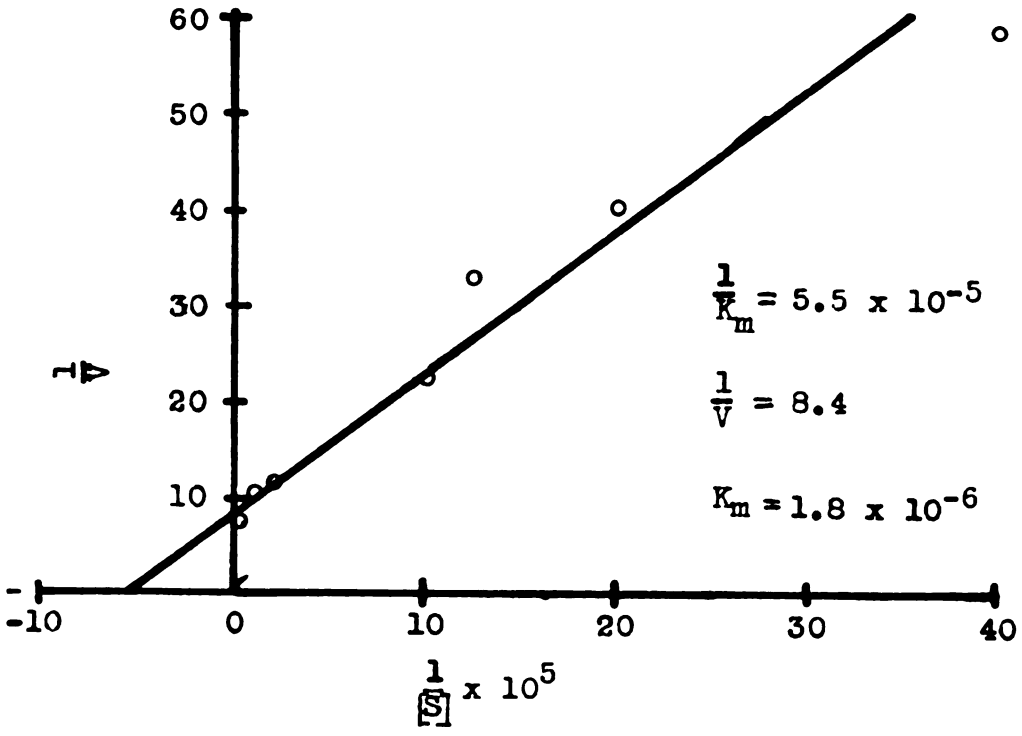
Protein concentration in the enzyme solution was sixty micrograms per milliliter.

solution. A fresh solution, showing no significant amounts of FMN on chromatography in 5% Na_2HPO_4 solution, gave about a 20% stimulation of rate. Older preparations gave up to 50% stimulation, but were found to contain traces of FMN on chromatographic analysis. This is a particularly troublesome problem because of the relatively low apparent K_m for FMN.

An attempt was made to calculate a K_m for the FMN. In order to make this calculation it was assumed that FMN had been completely split from a portion of the enzyme and that the added FMN was reactivating this portion of the enzyme. Therefore, the "inherent" rate was subtracted from the rates found with varying concentrations of added FMN. Using this approach, Figure 10 gives the 1/v versus 1/[S] and [S]/v versus [S] plots. From these plots the "apparent" K_m was determined as 1.8×10^{-6}M.

Acceptors

Various electron acceptors were tested. It was found that riboflavin, excess FMN, and FAD are inactive. The purified preparation did not act as an NADH peroxidase nor as an NADH oxidase, even when the preparation was incubated with FAD and cysteine. It is of interest that methylene blue, which is regarded as a typical flavoprotein



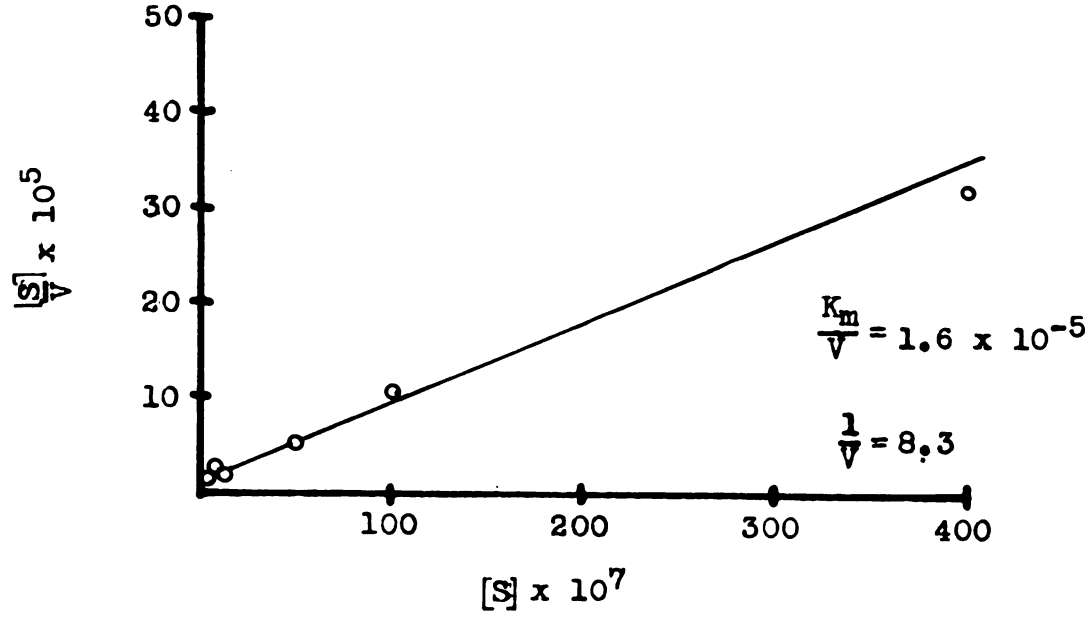


Figure 10 Reciprocal Plots for Determination of Michaelis Constant for FMN with Purified Diaphorase

oxidant, is not an acceptor.

The best acceptors of those tried were p-benzoquinone and indophenol. At equal concentrations, the rate with p-benzoquinone was seven times that with ferricyanide. The rate with indophenol was three times that with ferricyanide even though the indophenol concentration was one-tenth that of the ferricyanide. Dolin (24) has similarly found that benzoquinone and toluquinone are the best oxidants for the diaphorase from S. faecalis.

Inhibitors

Table 3 shows the effect of several inhibitors upon the ferricyanide activity. For this study the buffer, NADH, FMN enzyme and inhibitor were added to the cuvette, mixed, and allowed to incubate for ten minutes at room temperature. The reaction was then started by addition of ferricyanide.

When p-hydroxymercuribenzoate was used, an anomalously high non-enzymatic rate was obtained with ferricyanide. Because of this anomalous rate, it was impossible to interpret the p-hydroxymercuribenzoate effects on the enzymatic reaction. As indicated from Table 3, no major degree of specific inhibition is obtained. These results are in full agreement with those of Dolin (24).

Table 3. Diaphorase inhibition studies.

Inhibitor	Concentration (Molarity)	% Inhibition
Amytal	5×10^{-4}	0
	1×10^{-3}	0
N-ethyl maleimide	5×10^{-4}	11
	1×10^{-3}	16
EDTA	5×10^{-4}	0
	1×10^{-3}	0
Cyanide	5×10^{-4}	6
	1×10^{-3}	17
Hydrogen peroxide	1×10^{-3}	11

Standard Diaphorase Assay - (NADH conc. = 1.3×10^{-4}M)

Molecular Weight

Because of the limited amount of purified enzyme available, no detailed studies of molecular weight could be carried out by physical methods, such as ultracentrifugation and/or electrophosis. However, using Sephadex results, it is possible to make an estimate of the molecular weight.

The minimum weight has to be greater than 25,000 since the protein is not held up on G-25. The maximum weight

is less than 75,000 since diaphorase is held up somewhat on G-75. This would place it in the range of 30,000 to 70,000. It is interesting to note that Dolin has reported (26) a molecular weight of 50,000 for the diaphorase from S. faecalis, based on bound flavin determinations.

Stability

The enzyme is stable at 4°C for at least a month.

The effects of heating the enzyme at different temperatures are shown in Table 4.

Table 4. Heat stability of diaphorase.

Treatment	% of Original Activity
40°C for 10 minutes	100%
50°C for 5 minutes	100%
60°C for 5 minutes	70%
70°C for 5 minutes	6%

Properties of the Peroxidase

Although it has not proved possible to separate the oxidase and peroxidase activities, it has been possible to study the properties of each in the partially purified system. The oxidase is assayed at pH 6.8, where peroxidase activity is negligible; the peroxidase activity at pH 5.4, where the oxidase rate is only 5 per cent of the peroxidase value.

In fact, the slight oxidase activity at pH 5.4 was a problem only in attempts to study alternate electron acceptors for the peroxidase. It did not interfere in the other studies.

Demonstration of NADH Oxidation

When the reaction mixture contained NADH, buffer and enzyme, the disappearance of NADH, as measured by optical density at 340 mμ, was very slow. Upon the addition of hydrogen peroxidas an appreciable rate was observed, (Point 1 of Figure 11). After the reaction had proceeded to completion, the pH was adjusted to pH 8.5 ± 0.5 with sodium hydroxide, and 0.1 milliliter of 95% ethanol was added (Point 2 of Figure 11). Upon addition of crystalline alcohol dehydrogenase, the absorbancy returns immediately to a value corresponding to the original (Point 3 of Figure 11). This demonstrates that the disappearance is due to the oxidation of NADH to NAD^+, as stated earlier.

The enzyme appears to be specific for NADH, since the rate with NADPH as the substrate under identical conditions is only 8% of that with NADH.

Stoichiometry of the Reaction

The balance study for the peroxidase reaction is shown in Table 5.

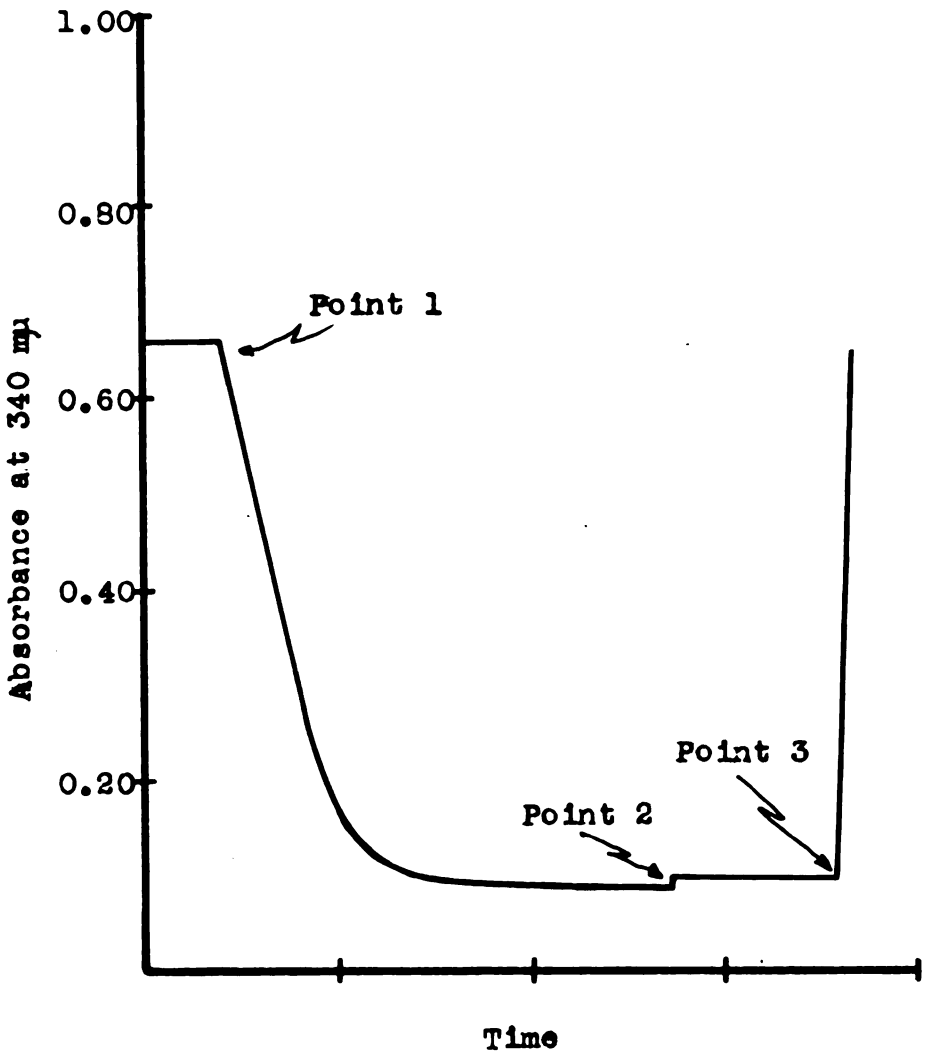


Figure 11 Illustration of the Stoichiometry of the Peroxidase Reaction

Table 5. Stoichiometry for the peroxidase reaction.

H_2O_2 moles	NADH Oxidized moles	Ratio H_2O_2/NADH
4.5×10^{-8}	5.1×10^{-8}	0.9/1
2.7×10^{-8}	2.9×10^{-8}	0.93/1

As stated above it was shown that NADH was converted to NAD^+. Therefore, these results demonstrate that the peroxidase reaction may be formulated as shown in Equation (1).

$$NADH + H^+ + H_2O_2 \longrightarrow NAD^+ + 2H_2O \qquad (1)$$

Spectrum of Enzyme

The visible portion of the spectrum of peroxidase is shown in Figure 12. The enzyme gives a spectrum similar to that of FAD in regard to the maximum at 450 mμ. There is no 370 mμ peak, only a shoulder. Also of interest is the broad absorption band from 525 mμ to 600 mμ. Dolin reported (23) the appearance of a band from 520 to 600 mμ when the peroxidase from S. faecalis is reduced by either substrate or hydrosulfite. There is no explanation at present concerning the shoulders on both sizes of the 450 mμ peak. There are no apparent heme absorption bands.

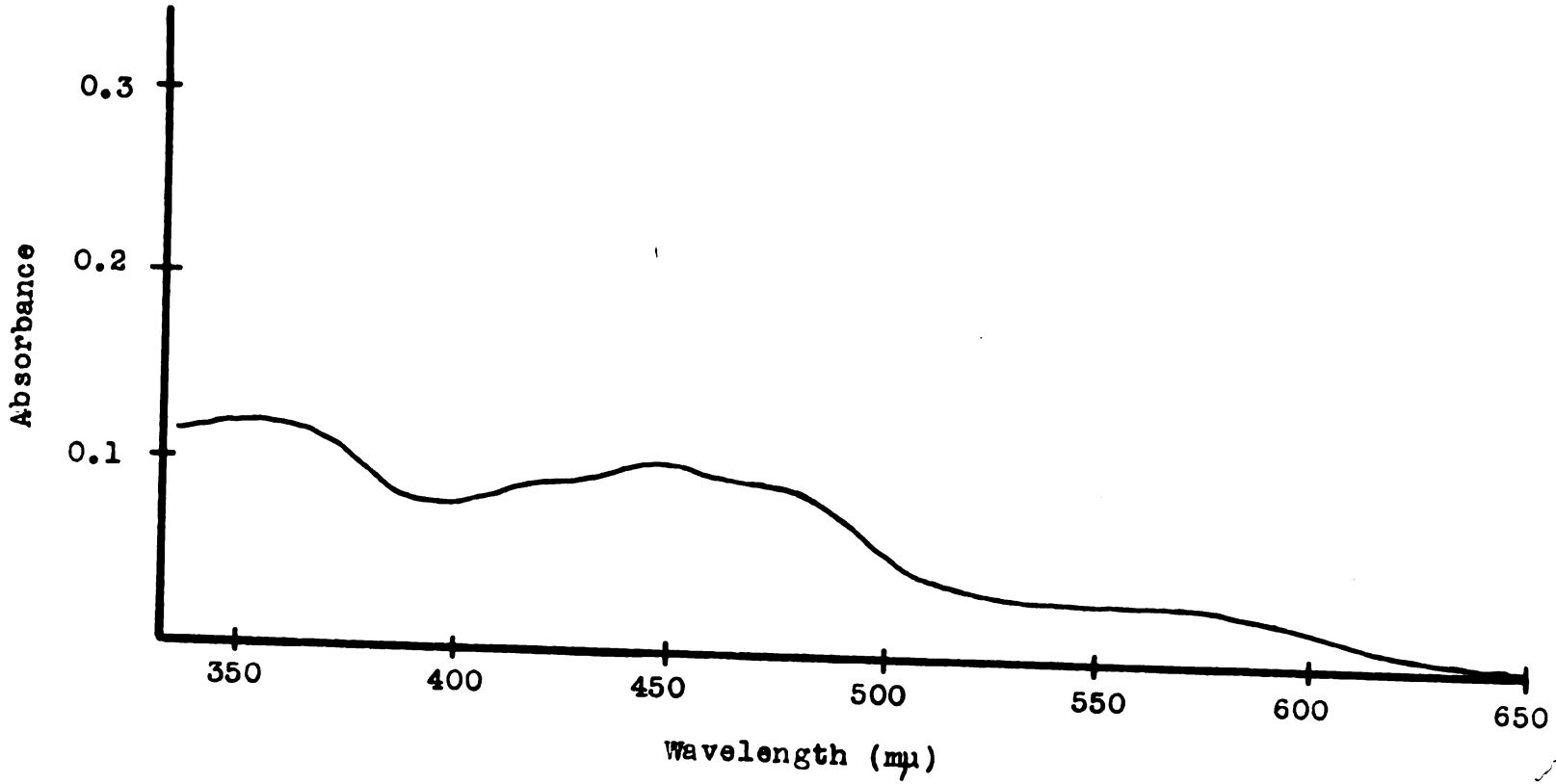


Figure 12 Spectrum of the Peroxidase

Cofactors

Heat denaturation and acid precipitation does not appear to release the flavin from the protein. It was also found that the presence of FAD, FMN or riboflavin in cofactor amounts has no appreciable effect upon the rate of the peroxidase.

It is of interest that the flavin from the S. faecalis peroxidase (23) can be released quantitatively by heat denaturation at 100° for ten minutes or by acid precipitation of the protein. It was identified as FAD.

Also, Lightbown and Kogut reported (26) that the oxidase from B. subtilis required FAD.

Acceptors

Various electron acceptors have been tried, as shown in Table 6. Methylene blue, ferricyanide and substrate-level concentrations of FAD were found to approximately double the rate obtained without any acceptors present; but the rates with these acceptors are only one-seventh of that obtained with hydrogen peroxide. Benzoquinone and indophenol were found to be completely inactive.

It would seem quite likely that the stimulation of the rate caused by the various acceptors in Table 6 is due to stimulation of the oxidase. The stimulation in rate

Table 6. Electron acceptors for peroxidase.

Acceptor	Concentration (Molarity)	Rate units/0.05 ml enzyme
None	----	0.9
H_2O_2	4.5×10^{-4}	18
FAD	1×10^{-4}	3
Ferricyanide	5×10^{-4}	2.1
Methylene Blue	1×10^{-4}	2.5
Benzoquinone	5×10^{-4}	0.3
Indophenol	1×10^{-4}	0.5

Standard Peroxidase Assay - NADH concentration = 1.3×10^{-4}M. Enzyme was the peak fractions from DEAE-cellulose, diluted 1 to 10. Protein concentration in diluted preparation: 0.094 mg/ml.

obtained in the presence of these acceptors is by no means comparable with the rate of the peroxidase, and can readily be explained by the data to be presented on effects of alternate acceptors on oxidase activity.

Dolin reported(23) that FMN, FAD, methylene blue and indophenol did not function as oxidants for the S. faecalis peroxidase. He found menadione and 1,4-naphthoquinone functioned as oxidants. The solubility was a big problem in our studies. Menadione was found to have a very slow rate at the highest levels we could achieve (on the order of 10^{-5}M).

Inhibitors

For the inhibition studies the standard peroxidase assay was used, except that the buffer, NADH, enzyme and inhibitor (1×10^{-3}M) were preincubated for ten minutes at room temperature. The reaction was started by addition of hydrogen peroxide.

It was found that amytal, EDTA and cyanide were ineffective, while p-hydroxymercuribenzoate inhibited 100% and N-ethylmaleimide approximately 50%.

The peroxidase from S. faecalis is inhibited 20% by p-hydroxymercuribenzoate and is completely inhibited by heavy metal ions. It is difficult to conclude if sulfhydryl inhibition is the mechanism of the heavy metal ions. Our studies would seem to implicate -SH groups.

Stability

The effects of heating the enzyme at different temperatures are shown in Table 7.

Table 7. Heat stability of the peroxidase.

Treatment	% of Original Activity
40°C for 10 minutes	100
50°C for 5 minutes	100
60°C for 5 minutes	80
70°C for 5 minutes	0

Properties of the Oxidase

Reconstitution of Activity

It was found that upon storage the oxidase lost activity. Mackler et al. reported (47) that the oxidase from S. faecalis lost enzymatic activity upon storage. They found that activity could be restored by addition of a variety of thiols together with FAD. Table 8 demonstrates that similar results are found when this oxidase is pre-incubated with cysteine and FAD for ten minutes at 37°C.

Table 8. Reconstitution studies.

Reconstitution Mixture	Rate units/0.1 ml reconst. oxidase
Enzyme	1.5
Enzyme, cysteine and FAD	9.0
Enzyme, and cysteine	2.5
Enzyme and FAD	3.7
Enzyme, cysteine and FMN	3.5

The reconstitution is relatively specific for FAD, since reconstitution with FMN gives only a fraction of the rate obtained with FAD. The oxidase from B. subtilis requires FMN (26); that from S. faecalis requires FAD (47).

Acceptors

The acceptors were tried anaerobically with reconstituted enzyme. (It should be noted that the excess flavin and cysteine were not removed. The results were corrected for non-enzymatic rate in the presence of FAD and cysteine in corresponding amounts.) It was found that FMN at 1×10^{-4}M and ferricyanide at 5×10^{-4}M were ineffective as electron acceptors.

In addition to molecular oxygen, the NADH oxidase catalyzed the reduction of excess FAD or methylene blue. Methylene blue appears to be an excellent acceptor even before reconstitution.

The NADH oxidase from S. faecalis has similar acceptor properties. Methylene blue was not tried in this case.

Huennekens et al. reported (12) that the NADH oxidase isolated from pig heart could use methylene blue in cofactor amounts, but could not utilize FAD. (It should be noted that no reconstitution experiments were carried out by this group.)

Inhibitors

The inhibition studies were carried out on the enzyme before reconstitution using 5×10^{-5}M methylene

blue as the electron acceptor. The preincubation of the enzyme, inhibitor (1×10^{-3}M in each case), NADH and buffer was carried out for five minutes at room temperature.

Cyanide, N-ethylmaleimide, p-hydroxymercuribenzoate and EDTA had no significant effect upon the rate. Mackler et al. (47) found NADH oxidase from S. faecalis was insensitive to iodoacetate, EDTA and cyanide, but was inhibited approximately 60% by p-hydroxymercuribenzoate.

Muennekens et al. (12) found 100% inhibition with p-hydroxymercuribenzoate, 22% with iodosobenzoate and 19% with cyanide (1×10^{-4}M).

Thus there appear to be some differences among the enzymes to the typical thiol inhibitors.

Stability

The effects of heating the enzyme at different temperatures are shown in Table 9.

Table 9. Heat stability of the peroxidase.

Treatment	% of Original Activity
40°C for 10 minutes	100
50°C for 5 minutes	100
60°C for 5 minutes	45
70°C for 5 minutes	2

SUMMARY

The diaphorase from Lactobillus casei has been isolated and purified approximately ninety-fold. It has been shown to be free from NADH oxidase and NADH peroxidase activity.

The diaphorase appears to be specific for NADH. It requires FMN for maximal activity; with an apparent K_m for FMN of 1.8×10^{-6}M.

Of the various electron acceptors tested, p-benzoquinone and indophenol gave the highest rates, followed by ferricyanide.

The diaphorase activity is not appreciably affected by hydrogen peroxide, cyanide, EDTA or N-ethylmaleimide.

The molecular weight appears to be in the range of 30,000 to 70,000.

The properties of this diaphorase are in good agreement with those of the diaphorase isolated from S. faecalis by Dolin (24). There are, however, several major differences between the properties of this diaphorase and those of the classical diaphorase of Straub (18): first, Straub's enzyme required FAD as cofactor and second,

methylene blue was an excellent acceptor in his system. Neither is true of the L. casei diaphorase.

It was found that the peroxidase and oxidase activities could not be separated by ammonium sulfate fractionation, Sephadex G-100 and G-200 filtration, DEAE-cellulose chromatography, heat denaturation or calcium phosphate gel adsorption.

Even though separation of these activities could not be accomplished, their properties could be studied independently, due to differences in their pH optima.

With a number of acceptors tried the stimulation was double that without acceptor but it was found to be only twenty percent of that with hydrogen peroxide. Since these rates are not comparable with that of peroxide it is probable that they are stimulating the slight oxidase activity present at this pH.

The peroxidase was completely inhibited by p-hydroxymercuribenzoate and only 50% with N-ethyl maleimide. EDTA, cyanide and amytal were found to be ineffective.

It was found that the oxidase lost activity during the isolation steps. The majority of the activity could be restored by preincubation with cysteine and FAD. (Mackler reported (47) the same phenomenon with the oxidase

from S. faecalis.) Preincubation with FMN and cysteine under the same conditions produced approximately 15% of the activity obtained when preincubated with FAD and cysteine.

The oxidase appears to be able to use excess FAD and methylene blue as electron acceptors as well as molecular oxygen.

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