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# Membrane Bound NADH Oxidase Complex of Micrococcus Lysodeikticus

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# MEMBRANE BOUND NADH OXIDASE COMPLEX OF MICROCOCCUS LYSODEIKTICUS

by

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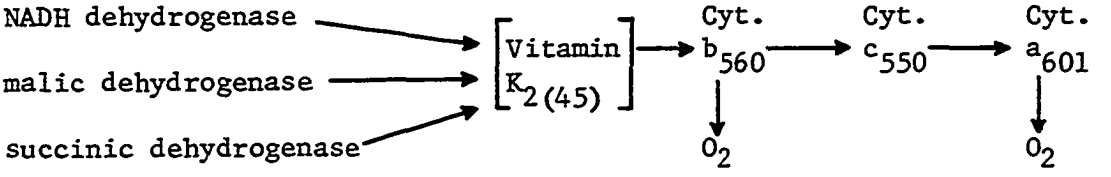
# INTRODUCTION

The mitochondria of plants, animals, and eucaryotic microorganisms have long been known as the site of respiration. Bacteria, which have some structural and functional similarities with mitochondria, also have a respiratory chain, i.e., a multicomponent structure composed of protein and lipid. These components are capable of undergoing reduction and oxidation reactions within a system of spatially organized dehydrogenases and cytochromes which brings about electron transport.

The respiratory chain (electron transfer chain) is known to be associated with the bacterial plasma membrane. A Gram-positive bacterium, Micrococcus lysodeikticus, was adopted in this investigation of the membrane bound electron transfer chain since the membranous complex (mesosomes and cytoplasmic membranes) of Gram-positive bacteria can be isolated in a relatively pure form.

The respiratory chain of Micrococcus lysodeikticus (11, 21) has been described as follows:




Electrons can enter the respiratory chain through these three dehydrogenases. One of these, NADH dehydrogenase, can oxidize NADH and pass electrons via Vitamin $K_{2(45)}$ and cytochromes to molecular oxygen. This particular multi-enzyme complex (NADH

oxidase) is very insoluble and firmly bound to the total membrane structure.

This study of the NADH dehydrogenase and oxidase complex has been attempted by means of sonication, versene, and detergent treatments. These methods provide for solubilization of NADH dehydrogenase away from the NADH oxidase complex. Effects of divalent cations on the NADH dehydrogenase oxidase complex have also been studied in order to effect a reconstitution of this respiratory chain.

## REVIEW OF LITERATURE

It is well known that respiratory particles are localized in the mitochondria of plants, animals, and eucaryotic microorganisms such as yeast (15, 16, 23, 24).

Studies on the membrane associated respiratory chain in bacteria were not achieved until 1953 when Weibull first isolated "membrane ghosts" from Bacillus megaterium (50). These "ghosts" represent the bacterial cytoplasmic membrane obtained by lysozyme treatment (51, 52). Several reports about the isolation of membranous fractions from bacteria have been obtained since Weibull's initial work (14, 37, 49).

Storck and Wachsman, in 1957, found (45) that respiratory enzymes such as succinate oxidase were localized in the ghost fraction of B. megaterium. Weibull (53) also found the bulk of cytochromes, succinic dehydrogenase, and NADH oxidase were localized in the "ghost" of another strain of this organism (B. megaterium M.)

A great deal of work has been done on the localization of respiratory enzymes in Micrococcus lysodeikticus. Lūkoyanova et al. (1961) found that succinate oxidase and succinate dehydrogenase were in the cytoplasmic membrane bounding the protoplast (26). The succinic oxidase activity, however, could not be detected in the lysed ghost (41). Succinic dehydrogenase activity was still detectable (38). Ostrovskii et al. (30) treated the protoplast of M. lysodeikticus with pancreatic lipase and reported that at least

50-70% of the respiratory enzymes were localized in the cytoplasmic membranes. The localization of respiratory enzymes in the intracytoplasmic membrane of other bacteria, such as Azotobacter, has also been studied (31).

It has been found that the bacterial membrane is a "unit" membrane, which consists of bimolecular layers of lipids with two monolayers of proteins attached on both sides (36). In mitochondria, the unit membrane is doubled. The protoplast membrane of Micrococcus lysodeikticus comprises about 10% of the cell dry weight and is a complex of lipid (28%), protein (50%), and carbohydrate (15-20%) (13).

It has been suggested that the physical state of the cytoplasmic membrane in Escherichia coli (19) controls respiratory activity in some way. This is in accordance with Smith's data which showed the effect of swelling and the concomitant deformation of the membrane of Bacillus subtilis on the operation of the respiratory chain (44). Smith found that some weakly bound dehydrogenases detached from the membrane as it swelled; thus, electrons failed to enter the chain and the respiration of endogenous substrates and amino acids was therefore decreased. As the protoplasts further swelled and ruptured, only NADH was oxidized. This suggests that NADH oxidase is firmly bound in the membrane structure. This stability of NADH oxidase can probably be attributed to the special strength of the bond of NADH dehydrogenase with the respiratory chain (11).

Several preparations of NADH dehydrogenase have been isolated from mitochondria, such as Hatefi et al.'s NADH-CoQ reductase (17), Mahler et al.'s dehydrogenase (28), DeBernard's dehydrogenase (6),

Mackler's dehydrogenase (27), King and Howard's dehydrogenase (22), Ringler et al.'s dehydrogenase (35), and Ziegler et al.'s dehydrogenase (54). The properties of NADH dehydrogenase vary with these different preparations (15, 16, 42).

Purification of bacterial NADH dehydrogenases has received little study since these NADH dehydrogenases are firmly bound with the respiratory chain. Also, the composition of phospholipids in bacteria is quite different from that in mitochondria, and binding of lipids with bacterial NADH dehydrogenase protein is stronger than that found in mitochondrial preparations (11). For instance, phosphatidylglycerols (which are common in bacterial membranes) have the ability to form stable complexes with protein by ionic bonds, hydrogen bonds, and by Van der Waals forces (46). Thus, the methods which are easily used in isolating NADH dehydrogenase from mitochondria, such as extracting lipid with cold solvent, are more difficult when applied to the bacterial membranes.

The NADH oxidase and dehydrogenase activities of Bacillus megaterium KM are masked in the membrane (7). Both physical (such as osmotic and sonic shock) and chemical (such as versene, detergent and lipolytic enzyme treatments) methods have been employed to fragment membranes and unmask enzymes. Salton and Netschey (38) reported that treatment with ultrasonic vibration disaggregated over 95% of M. lysodeikticus and Sarcina lutea

bacterial membranes. This treatment gave "subunit"[1] products which were lipid-protein complexes and behaved in a rather uniform manner when examined in the analytical ultracentrifuge (4, 39). The effect of sonic oscillation on Bacillus megaterium membrane bound NADH oxidase and dehydrogenase activities has also been reported (7).

Pangborn's work with membrane envelopes of Azotobacter agilis (31) also showed that treatment in a Mickle disintegrator resulted in the release of NADH oxidase activity.

Another method used to unmask NADH oxidase and dehydrogenase in bacteria is to prepare the ghost membrane in the presence of magnesium ion and then wash these membranes in the absence of this cation (7). This effect suggests an important role for magnesium ions with the membrane bound respiratory enzymes.

As described by Gel'man et al. (11), the bacterial membrane, which contains many acid phospholipids and is poor in basic proteins, requires stabilization to balance the excess negative charges. Stabilization of the bacterial membranes involves divalent cations, including magnesium and calcium, which impart strength to bacterial membranes. According to Gel'man et al. (11), the cations are probably the bridges which neutralize an excess of negative charges, and bind acid phospholipids and acid proteins together

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[1] "subunits" are mushroom-shaped and consist of a head 80-100 Å in diameter and a cylindrical stalk of 50 Å long and 40 Å broad embedded in the membranes. They are found only on the cristae and on the inner side of the mitochondrial membrane, i.e., on the membranes carrying the respiratory chain. The subunits of bacteria are similar to those of mitochondria, the relative fine membrane carrying the subunits lay inside the cytoplasmic membrane (11).

(phospholipid-R-COO-Mg-OOC-R-protein).

Data on the role of cations in membranes have also been obtained for Gram-negative marine bacteria (3). Thus, Brown has suggested that removal of cations results in conformational changes in membrane proteins and the appearance of protease activity (3). This process probably leads to the loss of the cationic binding bridges and to an increase in hydrophilia due to the liberated carboxyl groups. This process results in destruction of the membrane structure.

J. Brown (4) attempted to investigate the mechanism(s) of bacterial membrane stabilization by magnesium. He reported that removal of magnesium from Sarcina membranes or their fragments has an inhibitory effect on the respiratory chain. Brown further showed that sonication of these membranes in a solution containing magnesium gave both 70-S and 5-S particles. Subsequent removal of magnesium by dialysis resulted in the 70-S particles breaking up into 5-S particles. Brown therefore suggested that these membranes consisted of relatively small lipoprotein aggregates (5-S particles) which were bound together by magnesium.

Another method of causing deformation of bacterial membranes is to use a chelating agent which binds ions of divalent metals, e.g., $Mg^{2+}$, thus breaking bonds with the structural membrane components. A chelating agent such as ethylenediamine tetraacetic acid (EDTA) can presumably affect the activity of the respiratory chain by its ability to destroy a portion of the membrane structure.

Inhibition of the respiratory chain of Micrococcus lysodeikticus by EDTA and the concomitant destruction of membranes has been reported (26). In this case, an enzyme-lipid complex containing NADH dehydrogenase, malic dehydrogenase and lipid passed into solution (10, 29).

The ability of EDTA to destroy membranes and release enzyme-lipid complexes or even individual enzymes has been used in mitochondria. Person and Zipper (32) reported that cytochrome oxidase can be extracted from beef heart or yeast mitochondria with 0.05M $Na_4EDTA$ and 0.05M Tris at pH 9.0. Other authors' opinions are that the relative importance of this kind of interaction with cation in mitochondrial membrane is not great since $10^{-3}$M versene (the concentration used to destroy bacterial membrane) does not act on the mitochondrial membranes. In view of their composition, proteins and phospholipids can interact directly with one another and cationic bridges are not necessary. Versene is sometimes used even as a stabilizer of mitochondrial membranes (43).

In addition to divalent cations, hydrophobic interactions also play an important role in the stabilization of mitochondrial and bacterial membranes. The hydrophobic connections in both of these membranes would be ruptured by detergents, whether they are protein-protein or lipid-protein bonds.

Anionic and cationic detergents consist of molecules which have a hydrophobic hydrocarbon moiety and a hydrophilic solubilizing or polar group with strong valence forces. The mechanism of action of these substances consists in the conformation of a monolayer at the

phase boundary owing to the ability of the molecules to adopt an oriented position; this reduces the membrane's surface tension (11).

Investigation of the effect of detergents on the membranous structure of bacteria showed that detergents disaggregated lipoprotein membranes (33) and gave smaller "subunits" which were composed of lipid and protein. These subunits behaved in a uniform manner when examined in the analytical ultracentrifuge (34). Cholate and deoxycholate bile salts resemble detergents in their action. Treatment of Escherichia coli membranes with deoxycholate resulted in dissociation of NADH dehydrogenase and $CoQ_8$ from cytochromes of the electron transfer chain with the inactivation of NADH oxidase (2). Cytochromes a, b, and c, and succinic dehydrogenase activity were found in an insoluble lipid-depleted fraction in the form of membranous sheets when M. lysodeikticus membranes were extracted with deoxycholate (40).

More reports about the action of detergent on the membranes of M. lysodeikticus have been obtained (26, 27). A recent paper by Gel'man et al. (1970), showed that the respiratory chain of M. lysodeikticus membranes can be disrupted by detergents into two blocks, one of which contains malate and NADH dehydrogenases with cytochrome $b_{556}$ and another block containing cytochromes $b_{560}$, $c_{550}$ and $a_{601}$. These blocks appeared to maintain their functional activity and exhibited various degrees of bonding strength with the membranes. Substrate portions of the respiratory chain were more easily solubilized than the terminal portion (12).

The mechanism of the solubilizing effect of detergent (e.g., deoxycholate) on the lipoprotein complexes of membrane has also

been studied in mitochondria (1, 5).

Removal of detergent from bacterial membrane-detergent mixtures led to the formation of lipoprotein material but large uniform lipoprotein aggregates were only obtained in the presence of magnesium (34, 47). In this connection, Eisenberg et al. (8) recently demonstrated that the NADH oxidase chain of Bacillus megaterium KM, which was inactivated by deoxycholate treatment, can be reactivated by divalent cations with the formation of insoluble active NADH oxidase. A partial reassociation of sonicated particles (5-S) into larger aggregated particles (70-S) by the presence of magnesium has been shown in Sarcina lutea (4). The restoration of NADH oxidase activity in sonically fragmented membranes of M. lysodeikticus has not been demonstrated (21).

Reaggregation of the mitochondrial system is different than the bacterial system. The reconstitution of electron transfer chains in beef heart mitochondria was first achieved by Hatefi et al. (18). These investigators successfully reconstituted the electron transfer system either totally or in any desired sequential segment by appropriate combinations of one or more of the four primary complexes (complex I. NADH-CoQ reductase; II. succinic-CoQ reductase; III. $QH_2$-Cyt.c reductase; IV. Cyt.c reductase). These complexes were previously isolated in a highly purified form (17). Hatefi et al. found that the general oxidation-reduction properties of the reconstituted systems are essentially the same as those found in both intact mitochondria and in the integrated particles derived therefrom. The reformation of membranes accompanied functional reassembly.

Reformation was achieved by mixing either complex I + complex III or complex II + complex III with complex IV in concentrated detergent solution. These concentrated individual complexes in detergent were then diluted resulting in reaggregation of the repeating units of the complexes into vesicular membranes (48).

The functional and structural reassembly of Micrococcus lysodeikticus membranes has not been reported. In view of the work described above, it was of interest to investigate some relationships between M. lysodeikticus membranes and its bound respiratory chains. We have thus studied physical and chemical fragmentation procedures which lead to the solubilization of NADH dehydrogenase and inhibition of NADH oxidase. We have also attempted to study reactivation of disrupted NADH oxidase activity in an attempt to establish some basis for understanding how these membranes are organized in vivo.

## MATERIALS AND METHODS

### Source of Organisms

Micrococcus lysodeikticus (strain ISU Ad Pos) was obtained from the culture collection of Dr. W. E. Kloos, Department of Genetics, North Carolina State University, Raleigh, North Carolina.

### Growth Of Organism And Preparation Of Protoplast Ghost Membranes

Micrococcus lysodeikticus was grown on a broth medium (pH 6.9) containing 1.5% trypticase; 0.1% yeast extract; 0.5% sodium chloride; 0.082% $KH_2PO_4$; 0.417% $K_2HPO_4.3H_2O$; and 0.9% glucose which was autoclaved separately. Cells were routinely grown aerobically in batch culture (9 liters) at 32°C in a New Brunswick Microferm fermenter; air flow was 10 liters per minute and foaming was controlled with seven drops of antifoam reagent (Union Carbide SAG-471). Bacteria were harvested by centrifugation at the end of exponential growth and washed twice with a total volume of 2.4 liters (per 9 liter culture) of 0.01M Tris-Cl, pH 7.2.

For the preparation of "ghost" membranes, cells were resuspended to one liter with 0.01M TKM buffer (pH 7.2), which consisted of 0.01M Tris-Cl, 0.01M KCl and 0.01M $MgCl_2$. To the cell suspensions, 100-120 mg crystalline lysozyme (1.0 mg lysozyme per gram wet weight cells) was added with stirring, and followed immediately by adding a small quantity of crystalline DNase. The mixture was incubated at 23°C for 1 hr, with continuous stirring. After cells

were lysed, the suspensions were centrifuged at 27,000xg for 30 minutes and residues (membranes) were washed three times with a total volume of 720 ml 0.01M TKM buffer. Washed membranes were resuspended in 150 ml of double strength TKM buffer containing 30 g glycerol and $2x10^{-4}$M β-mercaptoethanol and then diluted to 300 ml with distilled water.

Aliquots of these membranes were frozen immediately and retained at -60°C until used.

These frozen membrane preparations contained 7-10 mg protein per ml. (Slight variation between cell batches).

## Enzyme Assays

The amount of enzyme activity for both NADH dehydrogenase and NADH oxidase was proportional to the enzyme concentration.

NADH dehydrogenase activity was estimated with 2,6-dichlorophenolindophenol (DCIP) as electron acceptor (NADH:DCIP oxidoreductase). The extinction coefficient employed for DCIP was 15.2 $mM^{-1}cm^{-1}$ at pH 6.1, which was derived from 21 $mM^{-1}cm^{-1}$ at pH 7.0 (Fig. 1) at 600 nm. The assay reaction mixture contained 0.15 μmoles of DCIP, 0.48 μmoles of NADH, and 150 μmoles of phosphate buffer, pH 6.1 [the optimal pH of NADH dehydrogenase (see Fig. 3)] in a total volume of 3.0 ml. NADH dehydrogenase activity was estimated from the rate of DCIP reduction occurring in the first 10 to 15 seconds after addition of enzyme; rate measurements were performed with a Gilford (model 2000) spectrophotometer. A unit of NADH dehydrogenase is the amount of enzyme catalyzing the reduction of 1 μmole

of DCIP in one minute.

NADH oxidase assay mixtures contained 0.48 μmoles of NADH and 150 μmoles of phosphate buffer at pH 7.5, which is the optimum pH of NADH oxidase (see Fig. 4 & 5), in a final volume of 3 ml. The rate of NADH oxidation was measured spectrophotometrically at 340 nm. An extinction coefficient of 6.21 $mM^{-1}cm^{-1}$ at pH 7.5 was used for NADH (20). A unit of NADH oxidase is the amount of enzyme catalyzing the oxidation of 1 μmole of NADH in one minute.

## Sonic Oscillation

Protoplast ghosts were sonically treated in 25-ml stainless-steel centrifuge tubes immersed in ice. A Branson sonifier (model W185) was used at an output of 5.0 amp unless stated otherwise.

## Protein Determination

Membrane protein was estimated by heating membranes in 0.5 N NaOH for 15 minutes at 100°C and assaying the supernatant solution by the Lowry method (25). Bovine serum albumin (Fraction V powder, Sigma Chemical Company), treated in the same way as the membranes, was used as a protein standard.

## Chemicals

The substrate of NADH oxidase and dehydrogenase, NADH (β-Diphosphopyridine Nucleotide, Reduced form), and the electron acceptor of NADH dehydrogenase, DCIP (2,6-dichlorophenolindophenol, sodium salt) were purchased from Sigma Chemical Company. A chelating

agent used to dissociate bacterial membrane, versene (EDTA or Ethylenediaminetetracetic acid), was purchased from Mallinckrodt Chemical Works. Another reagent used to fragment bacterial membrane in this experiment, deoxycholate, sodium salt (Na-DOC), was also obtained from Sigma Chemical Company.

# RESULTS

## Optimum pH Of NADH Dehydrogenase And Oxidase Activities

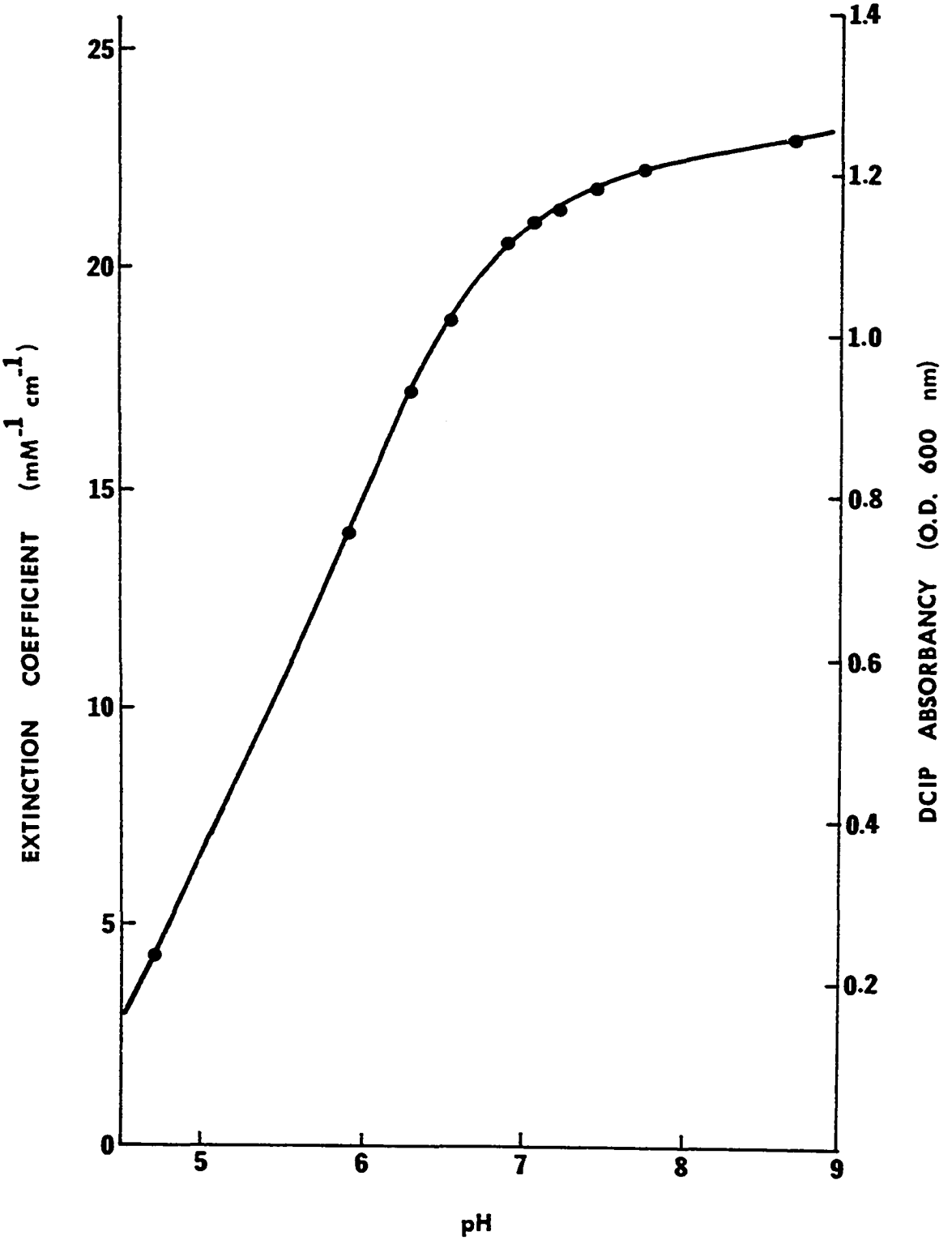
The pH optimum of different enzymes varies. The multi-enzyme NADH oxidase of Micrococcus lysodeikticus was found in this study to have an optimum pH of about 7.5, whereas one of its component enzymes, NADH dehydrogenase, had an optimum pH of 6.1. The pH optimum of M. lysodeikticus membrane bound NADH dehydrogenase (NADH: 2,6-dichlorophenolindophenol oxidoreductase) was established by three steps.

The first step involved establishing the extinction coefficient of 2,6-dichlorophenolindophenol (DCIP) at various pH values. Since DCIP absorbancy varies with pH (Fig. 1), the extinction coefficient of DCIP should also vary at each pH according to the relationship Abs = E·c·l where "Abs" is the absorbancy of DCIP, "c" is the concentration of DCIP [which is a constant in this experiment (0.05 mM)] and "l" is the length of light path. Because c and l are constants, and the extinction coefficient (E) of DCIP is 21 $mM^{-1}cm^{-1}$ at pH 7.0 (Fig. 1), the E values of DCIP at each pH indicated can be easily calculated.

The second step involved establishing the rate of absorbancy change (Δ Abs/min) of DCIP at different pH values in enzyme and non-enzyme preparations. DCIP is a dye which is easily reduced in water from deep blue to a colorless hydroxy compound without enzymatic

Figure 1

DCIP Absorbancy And Extinction Coefficient At Different pH Values. DCIP (0.1 ml of 1.5mM) was added to 2.9 ml of 0.05M phosphate buffers of different pH values, and the absorbancy at each pH was measured at 600 nm in a Gilford spectrophotometer, which was then converted to extinction coefficient by the formula Abs = E•c•1. Concentration of DCIP at pH 7.0 was estimated using an extinction coefficient (E) of $21 mM^{-1} cm^{-1}$.



action. The DCIP reduction is also pH-dependent (Fig. 2). The rate of NADH dehydrogenase-dependent DCIP reduction increased when enzyme was present. The rate difference between the enzyme dependent DCIP reduction and the non-enzymatic dependent DCIP reduction was taken as the true reduction rate of DCIP by NADH dehydrogenase (see Fig. 2).

The last step for establishing the pH optimum of NADH dehydrogenase activity was by correcting for changes in absorbancy and extinction coefficient of DCIP with change in pH. Since Abs = E·c·1, the absorbancy change during a definite time is proportional to the concentration change of the same time period, which is Δ Abs/min = E·Δc/min·1. By rewriting this equation, we can obtain

$$\Delta c/\text{min} = \frac{\Delta \text{ Abs/min}}{E \cdot 1} \quad \text{..... Eq. (1)}$$

where Δc/min is the rate, in μmoles, of NADH oxidized per minute, and is also the rate, in μmoles, of DCIP reduced per minute. A change in concentration of 1 μmole per minute is defined as one unit of activity. Since "1" is still a constant, the different extinction coefficients (Fig. 1) were substituted for "E" in Eq. (1) and the true DCIP reduction rate by NADH dehydrogenase (Fig. 2) was substituted for Δ Abs/min at each pH measured. The NADH dehydrogenase activity units at each pH can be calculated from Eq. (1). Such a curve is presented in Fig. 3. From this curve, the optimum pH of the NADH dehydrogenase was determined to be 6.10.

The optimum pH for the NADH oxidase was found to be about 7.5, which is more neutral than the pH optimum for NADH dehydrogenase. The optimum pH of NADH oxidase was established in both 0.05M phosphate

Figure 2

Rate Of Absorbancy Change Of DCIP By NADH Dehydrogenase And Non-enzymatic DCIP Reduction At Different pH Values. (1) DCIP (0.15mM, 0.1 ml) was added to 2.8 ml of 0.05M phosphate buffers of differing pH values, followed by the addition of 0.1 ml of 4.8 mM NADH at zero time, and reduction was measured at 600 nm at each pH. (2) Frozen TKM ghost membranes were thawed, washed once with 0.01M TKM buffer (pH 7.2), and sonicated for 30 seconds (10 seconds intervals) at a setting of 5 amp. These sonicated membranes (0.02 ml) were added to the reaction mixture (2.88 ml) containing 0.05M phosphate of different pH and 0.05mM DCIP. The rate of enzyme induced DCIP reduction (by enzyme and DCIP) was measured by adding 4.8mM NADH (0.1 ml) at zero time.

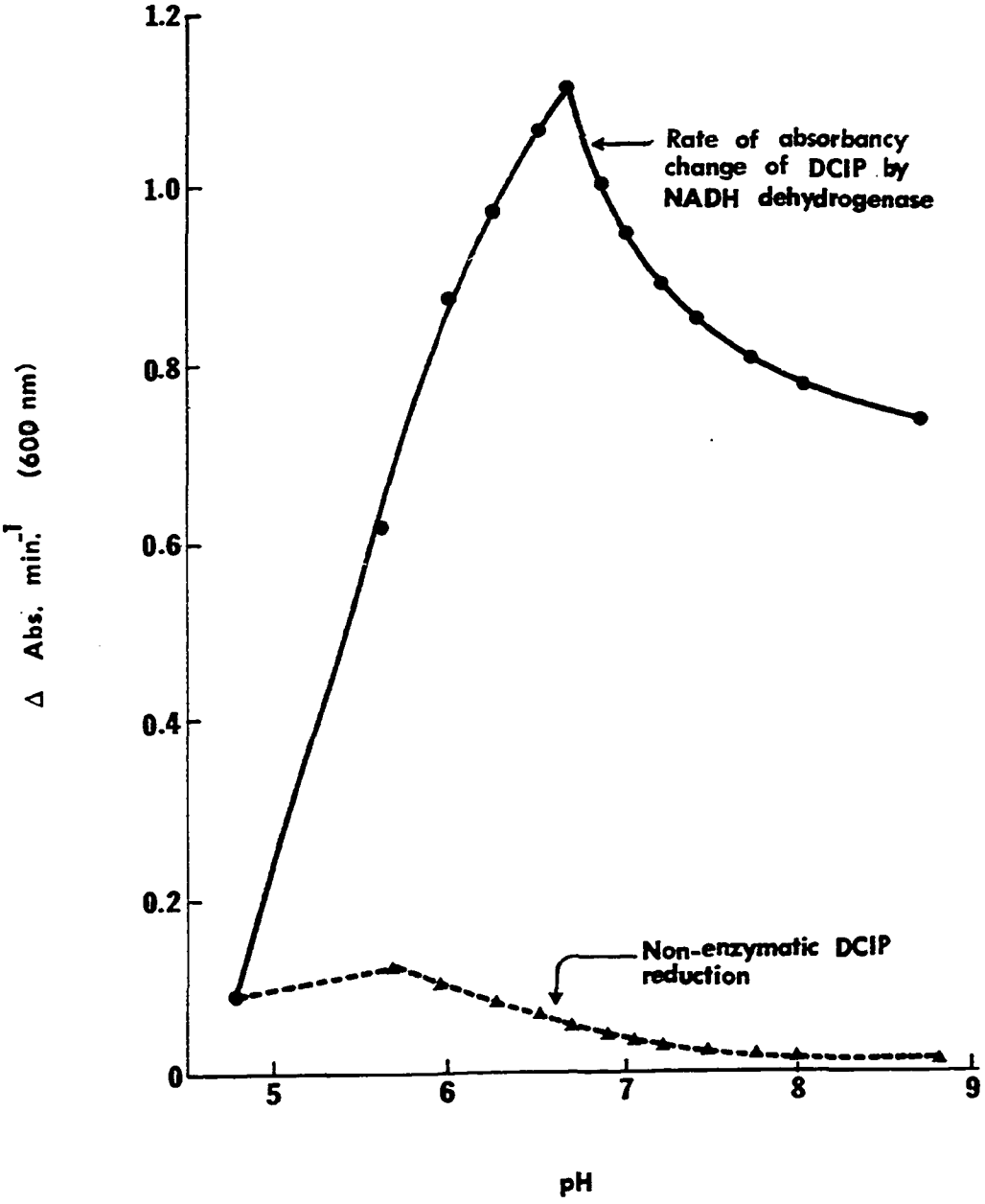


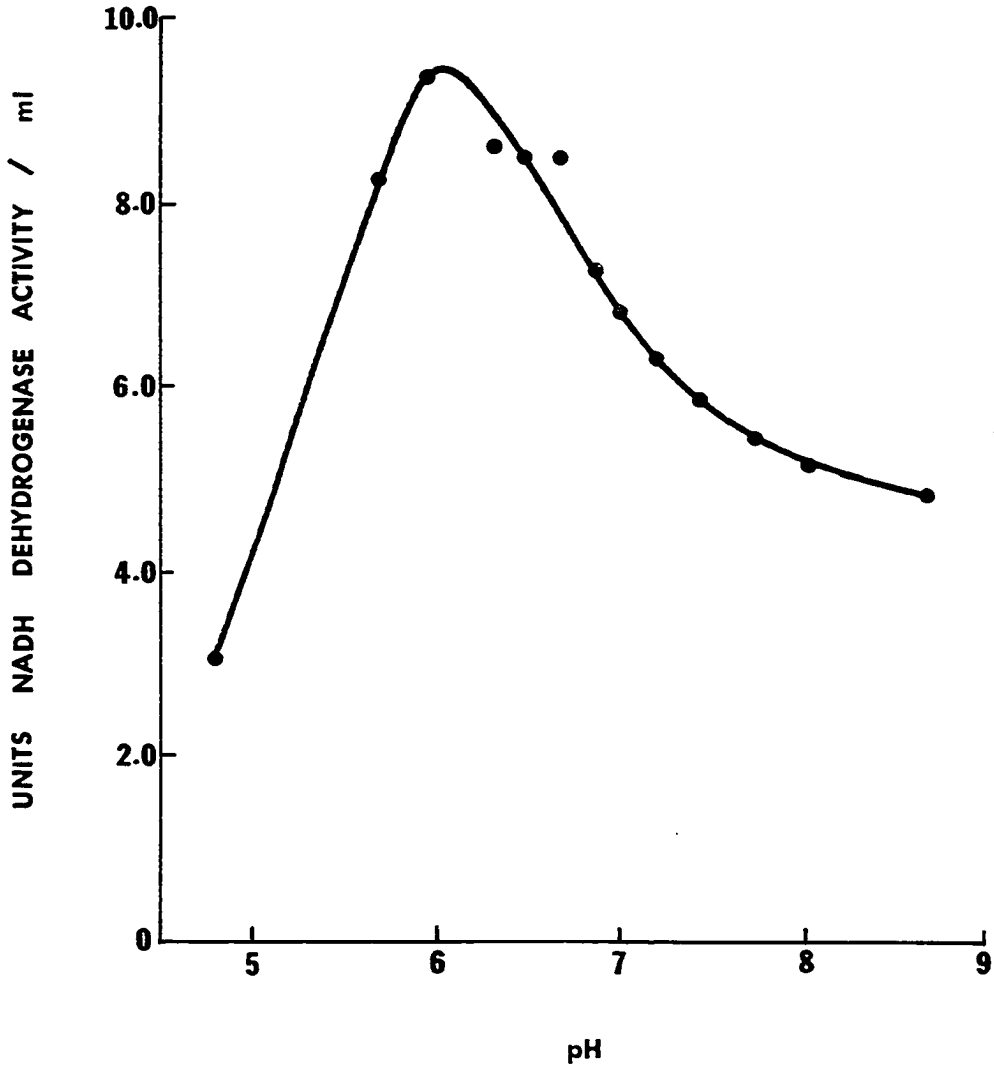
Figure 3

pH Optimum Of NADH Dehydrogenase. pH optimum curve of NADH dehydrogenase was obtained by the formula

$$\text{Units/ml} = \frac{\Delta \text{ Abs/min}}{E \cdot 1 \cdot \text{ml of enzyme}}$$

where ΔAbs/min was obtained from Fig. 2, and E was from Fig. 1.

One unit of dehydrogenase activity is equal to one μmole of DCIP reduced per minute.



and Tris-Tes buffers (Fig. 4 and Fig. 5). It was found that optimum NADH oxidase activity was higher in Tris-Tes buffer than phosphate buffer. This is consistent with the data of Table 1, which indicated that 0.05M Tris-Cl is more favorable to NADH oxidase, than phosphate buffer of the same concentration, at a pH of 7.5. Phosphate buffer was used, however, in almost all the enzyme assays since the pH optimum range of oxidase activity was more broad in phosphate buffer and experimental error due to small pH changes was minimized.

## Effect Of Sonication On Membrane NADH Dehydrogenase And Oxidase Activities

Sonic oscillation was used to fragment B. megaterium membranes and unmask NADH dehydrogenase and oxidase (7). The effect of sonication time on M. lysodeikticus ghost membranes is shown in Fig. 6 and indicates that prolonged sonication results in less turbidity and oxidase activity but higher NADH dehydrogenase activity. This dehydrogenase activity unmasked by sonication was mostly in a solubilized form whereas most of the oxidase activity was still insoluble (Table 2). In most cases, a sonication time of 30 seconds (5 second sonication intervals) at an output of 5 amp was used in investigating the properties of membrane bound enzymes since most of the NADH dehydrogenase was unmasked and solubilized within this time period (Fig. 6). Also, about 35% of NADH oxidase activity was retained after 30 seconds sonication (Fig. 6).

Figure 4

Optimum pH Of NADH Oxidase In Phosphate Buffer. Frozen TKM ghost membranes were thawed, washed once with 0.01M TKM buffer (pH 7.2), and sonicated for 30 seconds (10 seconds intervals) at a setting of 5 amp. Portions of these sonicated membranes (0.1 ml) were added to 2.8 ml of 0.05M phosphate buffer of different pH values. NADH oxidase activity was then measured at 340 nm by the addition of 0.1 ml of 4.8 mM NADH at zero time.

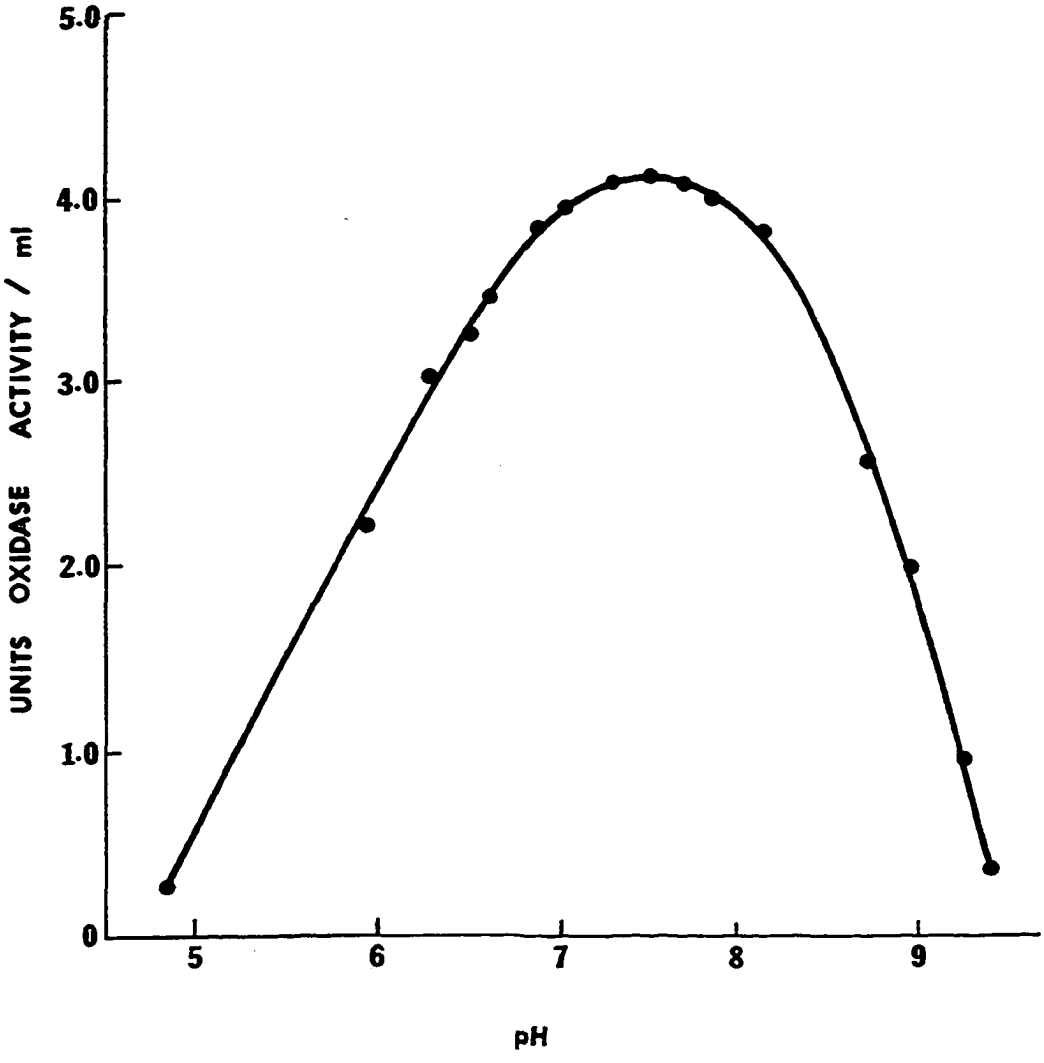


Figure 5

Optimum pH Of NADH Oxidase In Tris-Tes Buffer. The enzyme preparation is the same as described in the legend for Fig. 4. NADH oxidase activity was assayed in 0.05M Tris-Tes buffer at different pH values.

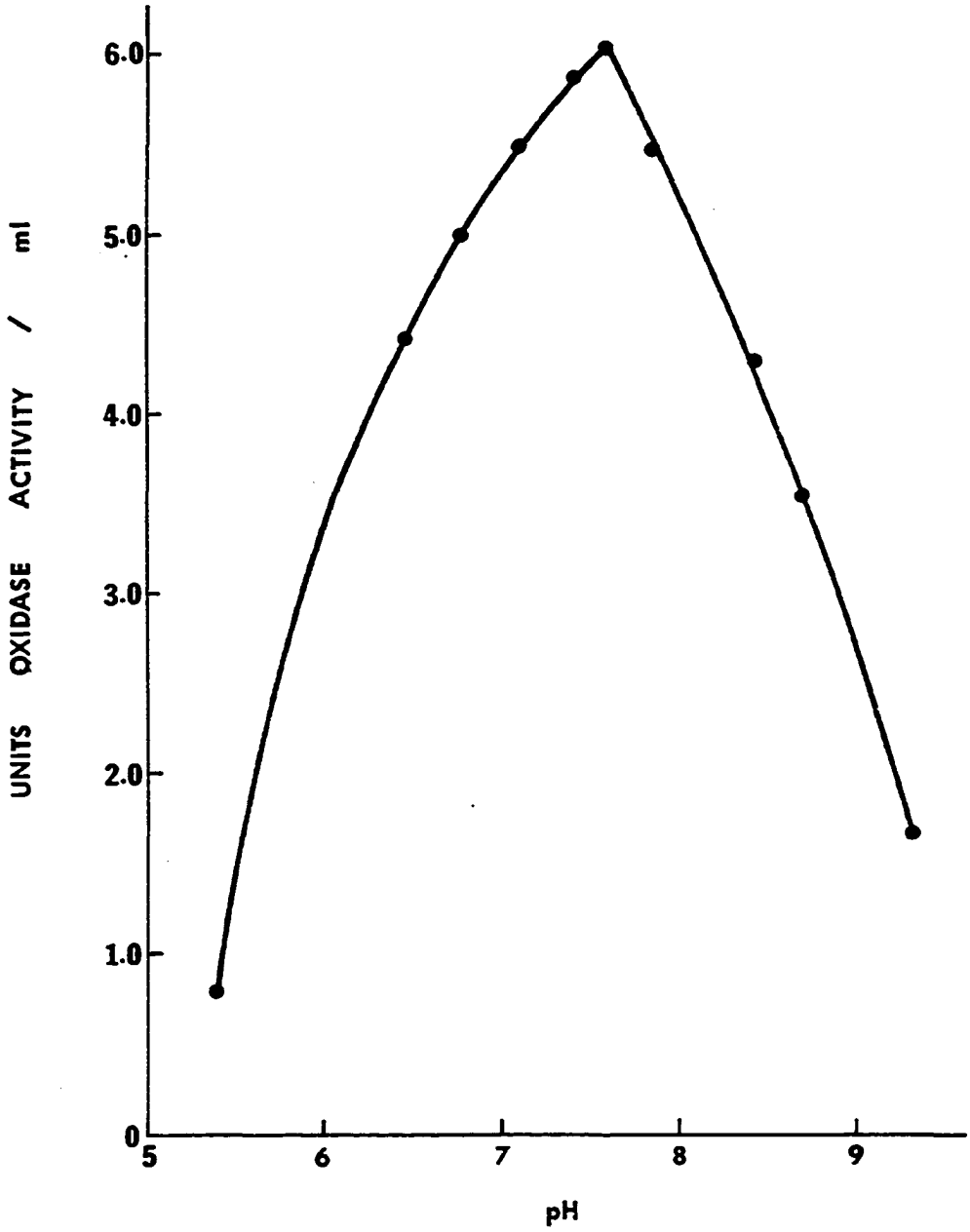


Table 1

Effect Of Various Cations, Sonication, And Different Buffer Systems On *M. lysodeikticus* Ghost Membranes

| Fractions (Salt concentration :0.05M) | Units of NADH oxidase activity/ml | | | |
|---|---|---|---|---|
| | 0.05M phosphate[a] | | 0.05M Tris-Cl[b] | |
| | sonicated | non-sonicated | sonicated | non-sonicated |
| Control | 5.37 | 13.77 | 7.61 | 18.11 |
| NaCl | 6.99 | 13.52 | 8.11 | 17.87 |
| KCl | 6.57 | 12.75 | 7.07 | 14.97 |
| $MgCl_2$ | 9.31 | 2.84 | 13.80 | 3.27 |
| $MgSO_4$ | 10.74 | 2.35 | 13.10 | 3.25 |
| $CaCl_2$ | ----- | ----- | 3.21 | 0.77 |
| $BaCl_2$ | ----- | ----- | 10.67 | 2.59 |
| $MnCl_2$ | ----- | ----- | 2.15 | 0.51 |
| $FeCl_3$ | ----- | ----- | 0.20 | 0.20 |

[a]Frozen TKM ghosts were thawed and washed once with 0.03M Tris-Cl (pH 7.5). The washed ghosts were divided into two parts, one part was sonicated at an output of 5 amp for 30 seconds (5 seconds intervals); the other was left unsonicated. These two different membrane preparations were incubated separately with 0.05M phosphate buffer (pH 7.5) containing 0.05M of various salts at 32°C for 30 minutes. NADH oxidase activities were then assayed by adding 0.1 ml of 4.8mM NADH and following decrease of absorbance at 340 nm.

[b]Similar to "a", except 0.05M Tris-Cl (pH 7.5) was used in assaying oxidase activities instead of 0.05M phosphate buffer.

Figure 6

Effect Of Sonication Time On Micrococcus lysodeikticus Protoplast Membranes. Frozen TKM ghosts were thawed at room temperature and washed twice with 0.05M Tris (pH 7.2) containing $2x10^{-4}$M β-mercaptoethanol at 27,000xg for 30 minutes. Sonic oscillation was carried out with a Branson sonifier at an output of 5 amp in 25-ml stainless-steel centrifuge tubes immersed in ice. Portions (0.05 ml) were removed at the indicated time intervals and turbidity, NADH oxidase, and NADH dehydrogenase activities were assayed. Turbidity was measured at 340 nm by adding 0.1 ml of sonicated membranes to 2.8 ml of 0.05M phosphate buffer and the oxidase activity was assayed by adding 0.1 ml of 4.8mM NADH. NADH dehydrogenase activity was measured at 600 nm as described in "Materials and Methods".

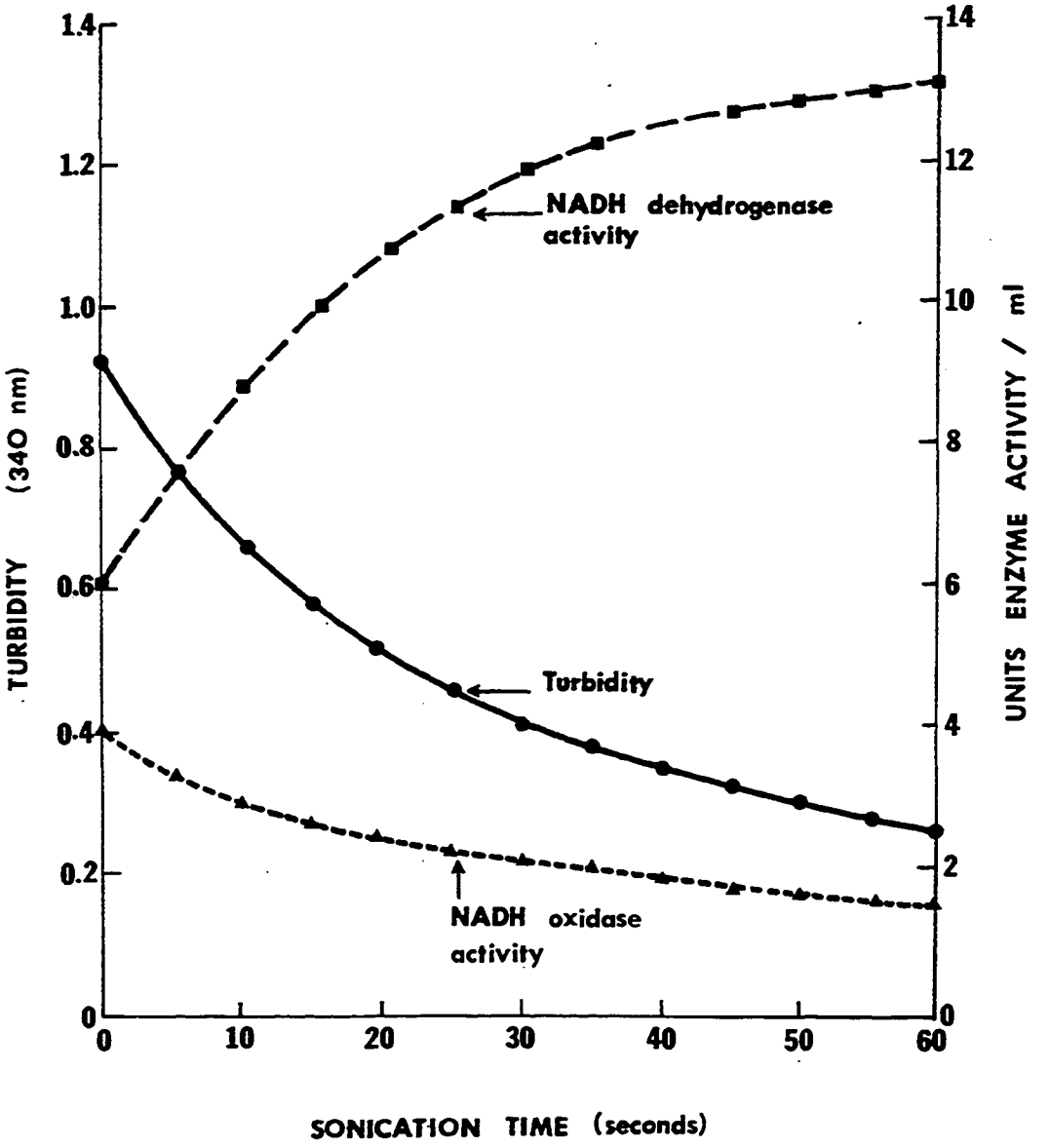


Table 2

Effect Of Washing, Sonication And Magnesium On M. lysodeikticus Membrane Bound NADH Oxidase--Dehydrogenase Activities

| Membrane Preparations | NADH oxidase[d] | | NADH dehydrogenase[d] | |
|---|---|---|---|---|
| | No $Mg^{2+}$[c] | w/ $Mg^{2+}$[c] | No $Mg^{2+}$[c] | w/ $Mg^{2+}$[c] |
| Protoplast ghosts | 4.73 | 2.15 | 11.36 | 2.76 |
| Washed ghosts[a] | 3.56 | 1.66 | 7.98 | 2.25 |
| Sonicated ghosts[b] | 2.17 | 3.13 | 13.40 | 9.96 |
| Supernatant of sonicated ghosts[b] | .39 | .75 | 6.16 | 6.06 |
| Pellet of sonicated ghosts[b] | 1.87 | 2.03 | 4.25 | 4.16 |

[a]Frozen ghost membranes prepared in TKM plus glycerol, were thawed, and washed once with 0.03M Tris-Cl (pH 7.5).

[b]The washed ghosts were sonicated at an output of 5.0 amp for 30 seconds (5 seconds intervals). These sonicated ghosts were then centrifuged at 27,000xg for 30 minutes and the pellet was resuspended in the same buffer (0.03M Tris-Cl, pH 7.5).

[c]Membrane preparations were incubated with 0.05M phosphate buffer containing or not containing 0.03M $MgSO_4$ at 32°C for 30 minutes. The oxidase and dehydrogenase activities were assayed by employing the methods described in "Materials And Methods".

[d]Expressed as units per milliliter.

## Influence Of Magnesium And Other Cations On Membrane NADH Dehydrogenase And Oxidase Activities

Various ions had different effects on NADH oxidase activities (Table 1). In general, divalent cations such as magnesium had a stimulatory effect on the oxidase of sonicated ghost membranes. Oxidase of the non-sonicated ghost membranes, however, was greatly inhibited by $Mg^{2+}$ (Tables 1 and 2). This was true for both buffer systems tested (Table 1). Since sonication fragmented part of the electron transfer chain and solubilized NADH dehydrogenase from the fragmented membrane (Table 2), it is possible to recover the insoluble, active NADH dehydrogenase--oxidase complex by the addition of $Mg^{2+}$ (Table 3), as with Brown's experiments (4).

As shown in Tables 1 and 3, monovalent cation such as potassium also had a stimulatory effect on the reformation of an active and insoluble enzyme complex. Thus, the additive effect of TKM buffer, which consists of Tris-Cl, $Mg^{2+}$ and $K^{1+}$, would give the sonicated membrane particles the highest oxidase restoration, as compared to Tris-$Mg^{2+}$ and Tris buffers (Table 3).

Calcium ions, at 0.05M, showed an inhibition of both the non-sonicated and the sonicated ghost (Table 1). More detail about calcium inhibition effect on the sonicated membrane is presented in Fig. 7. Calcium at all concentrations tested inhibited NADH oxidase and this inhibition was related to the calcium concentration.

In summary (Table 1), oxidation of NADH in non-sonicated ghosts was inhibited by all the ions tested although monovalent cations showed the least inhibition.

Table 3

The Distribution Of Membrane NADH Oxidase And Dehydrogenase In TKM, In Tris-Cl, And In Tris-Cl + Mg

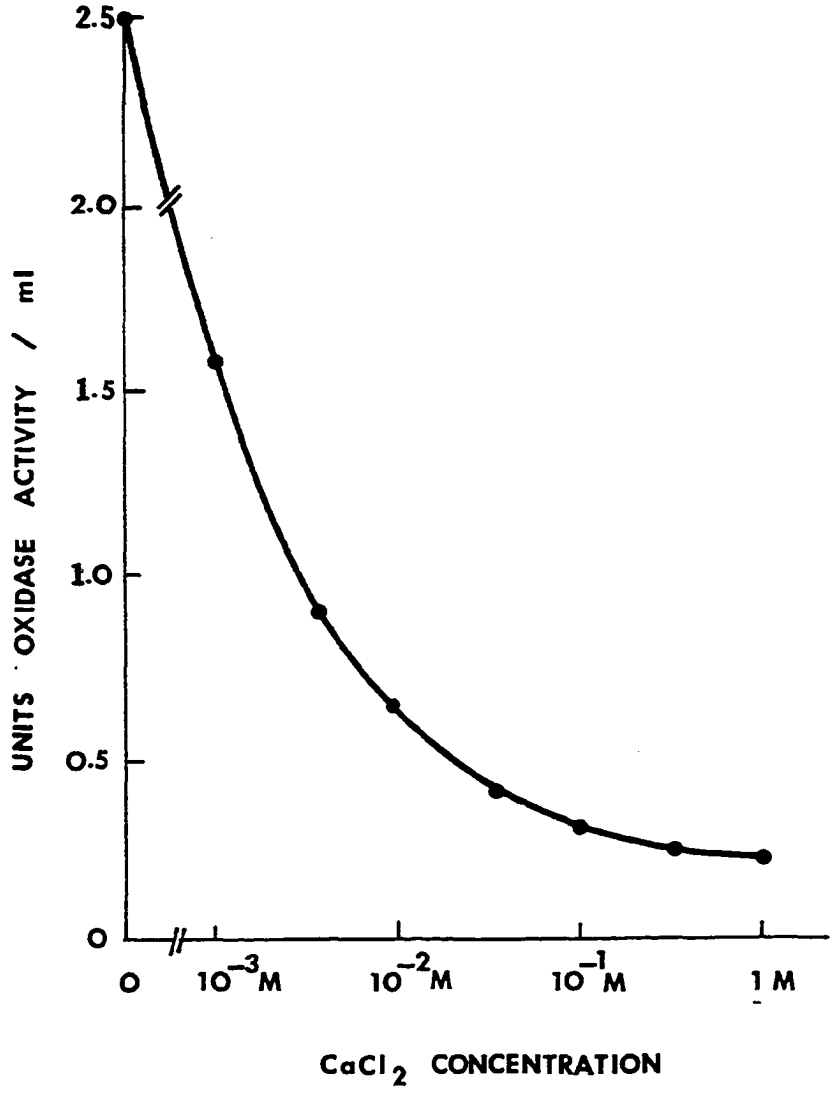
| Fractions[a] | NADH dehydrogenase[b] | | NADH oxidase[b] | |
|---|---|---|---|---|
| | Supernatant | Pellet | Supernatant | Pellet |
| Tris-Cl | 4.20 | 11.25 | .18 | 2.19 |
| Tris-Cl + $Mg^{2+}$ | 2.31 | 13.59 | .08 | 3.64 |
| TKM | 1.86 | 11.72 | .08 | 4.06 |

[a]Frozen TKM ghosts were thawed, centrifuged at 27,000xg for 30 minutes, the pellets were resuspended separately in 0.01M Tris-Cl (pH 7.2), and 0.01M TKM (pH 7.12). These Tris- and TKM-membranes were sonicated at an output of 5 amp for 20 seconds (10 second intervals) and 0.05M $MgCl_2$ was added to half of the sonicated Tris-membranes. These three sonified preparations (TKM, Tris-Cl, Tris-Cl + 0.05M $MgCl_2$) were then centrifuged at 130,000xg for 1 hr. Supernatants and pellets were assayed for NADH oxidase and dehydrogenase activities.

[b]Expressed as units per milliliter.

Figure 7

Effect Of $CaCl_2$ On NADH Oxidase Activities. $CaCl_2$ solutions of various concentration (1.0 ml each) were added to 1.0 ml of 0.15M Tris-Tes buffer (pH 7.6). The volume in the cuvette was brought to 2.9 ml with distilled water. After adding 0.1 ml of sonicated membrane (see legend to Fig. 6) to these mixtures, the NADH oxidase activity was assayed at 340 nm by adding 0.1 ml of 4.8 mM NADH.



## Effect Of EDTA On Membrane Oxidase And Dehydrogenase Activities

As described earlier in the "Review of Literature" section, magnesium ions function as a stabilizing agent of bacterial membranes. Versene (EDTA, or Ethylenediaminetetracetic acid) is able to chelate magnesium and results in the destruction of these membrane structures. Table 4 shows EDTA reduced M. lysodeikticus membrane oxidase activity by about 33% although NADH dehydrogenase activity increased. The dehydrogenase "activated" by EDTA was mostly solubilized[1] (about 82%) in the absence of magnesium (Table 4).

Versene had little effect on the sonicated oxidase, as shown in Table 5. There was, however, a stimulation of NADH dehydrogenase, as in non-sonicated ghosts, after treatment with EDTA. The enzyme distribution of EDTA-treated membranes is shown in Tables4 and 5.

## Effect Of Na-deoxycholate On Membrane Oxidase And Dehydrogenase Activities

The effect of deoxycholate (DOC) on bacterial membranes has been shown for E. coli and B. megaterium (2, 7, 8), as described earlier. The data on the M. lysodeikticus membranes showed (Table 6) that deoxycholate solubilized all the NADH dehydrogenase concomitant

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[1]The enzyme activity in the supernatant obtained from centrifuging membranes at 27,000xg for 30 minutes may not mean "solubilized". Data not presented have shown that only 20-50% of the EDTA-treated oxidase and 33-54% of the DOC-treated oxidase in this supernatant were actually solubilized by re-centrifuging this supernatant at 130,000xg for 1 hr. Only small amounts of EDTA-treated dehydrogenase (10-25%) and DOC-treated dehydrogenase (0-14%) were found to be "insoluble" after a second centrifugation.

Table 4

Effect Of EDTA On NADH Oxidase
And Dehydrogenase Activities
Of Non-sonicated Membranes

| Membrane preparations | NADH oxidase[f] | | NADH dehydrogenase[f] | |
|---|---|---|---|---|
| | No $Mg^{2+}$ | w/ $Mg^{2+}$[e] | No $Mg^{2+}$ | w/ $Mg^{2+}$[e] |
| Control, washed membranes[a] | 4.00 | 2.61 | 9.77 | 2.98 |
| EDTA treated membranes[b] | 2.69 | 6.03 | 17.57 | 10.73 |
| Supernatant of EDTA treated membranes[c] | .11 | .22 | 7.21 | 5.03 |
| Pellet of EDTA treated membranes[c] | 1.93 | 3.97 | 6.17 | 2.47 |
| EDTA-supernatant + EDTA-pellet[d] | 2.04 | 4.11 | 16.99 | 10.39 |

[a]Frozen protoplast membranes were thawed, centrifuged (27,000xg, 30 minutes), and washed once with 0.03M Tris-Cl (pH 7.5). The pellets were resuspended in 0.03M Tris-Cl (pH 7.5).

[b]Similar to a, except washed pellet was resuspended in 5mM EDTA (in 0.03M Tris-Cl) (pH 7.5). The resuspensions were incubated overnight at 0-4°C.

[c]The incubated EDTA-suspensions were centrifuged at 27,000xg for 30 minutes; the pellet was resuspended in 5mM EDTA (pH 7.5) (in 0.03M Tris-Cl).

[d]EDTA-treated supernatant and pellet (0.1 ml each) were added to the reaction mixture, and oxidase activity was assayed.

[e]$Mg^{2+}$ incubation was performed as described in the legend for Table 2[b].

[f]Expressed as units per milliliter.

Table 5

Effect Of EDTA On NADH Oxidase --Dehydrogenase Activities Of Sonicated Membrane Fragments

| Membrane preparations | NADH oxidase[f] | | NADH dehydrogenase[f] | |
|---|---|---|---|---|
| | No $Mg^{2+}$ | w/ $Mg^{2+}$[e] | No $Mg^{2+}$ | w/ $Mg^{2+}$[e] |
| Control, sonicated particles[a] | 1.87 | 2.03 | 4.25 | 4.16 |
| EDTA treated particles[b] | 1.79 | 3.46 | 6.87 | 8.47 |
| Supernatant of EDTA particles[c] | .16 | .28 | 2.39 | 2.24 |
| Pellet of EDTA particles[c] | 1.53 | 2.72 | 3.08 | 2.01 |
| EDTA-supernatant + EDTA-pellet[d] | 1.81 | 3.20 | 6.11 | 4.86 |

[a]The preparation of sonicated membrane suspensions is the same as described in legend for Table 2[a]. These suspensions were centrifuged at 27,000xg for 30 minutes. The pellet, which was resuspended in 0.03M Tris-Cl (pH 7.5), is called "sonicated particles".

[b]Similar to above except the sonicated pellet was resuspended in 5mM EDTA (in 0.03M Tris-Cl, pH 7.5), and incubated overnight in an ice bucket at 0°C.

[c]The incubated EDTA treated membrane particles were centrifuged at 27,000xg for 30 minutes. The pellet was resuspended in 5mM EDTA (in 0.03M Tris-Cl) (pH 7.5).

[d]EDTA-treated supernatant and pellet (0.1 ml each) were added to the reaction mixture and oxidase activity was assayed.

[e]$Mg^{2+}$ incubation was performed as described in the legend for Table 2[b].

[f]Expressed as units per milliliter.

Table 6

Effect Of Deoxycholate On NADH Oxidase --Dehydrogenase Activities Of Non-sonicated Membranes

| Membrane preparations | NADH oxidase[f] | | NADH dehydrogenase[f] | |
|---|---|---|---|---|
| | No $Mg^{2+}$ | w/ $Mg^{2+}$[e] | No $Mg^{2+}$ | w/ $Mg^{2+}$[e] |
| Control, washed membrane[a] | 4.00 | 2.61 | 9.77 | 2.98 |
| DOC treated membrane[b] | .53 | 1.58 | 15.13 | 11.78 |
| Supernatant of DOC-membrane[c] | .23 | .73 | 12.92 | 9.22 |
| Pellet of DOC-membrane[c] | .05 | .12 | 0 | 0 |
| DOC-supernatant + DOC-pellet[d] | .36 | 1.64 | 13.15 | 11.43 |

[a]The washed membrane was prepared as in legend to Table 4[a].

[b]Similar to above except the washed pellet was resuspended in 0.2% Na-deoxycholate (in 0.03M Tris-Cl, pH 7.5) and incubated overnight in 0°C.

[c]The incubated DOC-suspensions were centrifuged at 27,000xg for 30 minutes and the pellet was resuspended in 0.2% Na-DOC (in 0.03M Tris-Cl, pH 7.5).

[d]DOC-treated supernatant and pellet (0.1 ml each) were added to the reaction mixture and oxidase activity was assayed.

[e]$Mg^{2+}$ incubation was performed as described in legend for Table 2[b].

[f]Expressed as units per milliliter.

with a significant inhibition of membrane oxidase. Only 8% of the original oxidase activities was retained after deoxycholate treatment, and most (80%) was also solubilized.

Even when the membrane was fragmented by sonication, deoxycholate still showed the ability of solubilizing most of the NADH oxidase and dehydrogenase, as shown in Table 7. The great solubilization of enzymes by deoxycholate, according to Gel'man *et al.* (11), might be due to breaking of membrane hydrophobic bonds, whether the membrane was intact or fragmented.

## Studies On The Restoration Of Disrupted NADH Oxidase Activities By Divalent Cations

The restoration of sonicated oxidase activities by divalent cations has been briefly described earlier in Tables 1, 2, and 3. The function of magnesium on the chemically disrupted membranes (by versene or detergent) is similar to that of the physically disrupted membranes (such as by sonication). They all show certain degrees of oxidase restoration. The EDTA-disrupted oxidase activity can be fully restored by the addition of magnesium; this re-activated oxidase even showed a higher activity than the control (Table 4). Deoxycholate treated membranes could not be fully restored although there was still about 40% of oxidase re-activated by magnesium (Table 6). Oxidase reactivation is usually accompanied by a loss of soluble dehydrogenase activities (Tables 4, 6 and 7).

Since most dehydrogenase (54%) was solubilized by EDTA, and most oxidase (about 95%) was still retained in the insoluble

Table 7

Effect Of Deoxycholate On NADH Oxidase --Dehydrogenase Activities Of Sonicated Membrane Fragments

| Membrane preparations | NADH oxidase[f] | | NADH dehydrogenase[f] | |
|---|---|---|---|---|
| | No $Mg^{2+}$ | w/ $Mg^{2+}$[e] | No $Mg^{2+}$ | w/ $Mg^{2+}$[e] |
| Control, sonicated particles[a] | 1.87 | 2.03 | 4.25 | 4.16 |
| DOC treated particles[b] | .37 | .97 | 7.04 | 5.97 |
| Supernatant of DOC-particles[c] | .19 | .36 | 4.92 | 5.61 |
| Pellet of DOC-particles[c] | .06 | .12 | .32 | 1.45 |
| DOC-supernatant + DOC-pellet[d] | .33 | .82 | 6.69 | 4.10 |

[a]Sonicated particles were prepared as in legend to Table 5[a].

[b]Sonicated particles were treated with EDTA as described in legend to Table 6[b].

[c,d]Supernatant and pellet of deoxycholate treated particles were obtained as seen in legend to Table 6[c]. The combination mixture of these two fractions was assayed for NADH oxidase activity.

[e]Magnesium incubation was performed as described in legend to Table 2[b].

[f]Expressed as units per milliliter.

fraction, it is possible to get full restoration by mixing these two fractions with the addition of magnesium which is thought to remove the action of EDTA (Tables4 and 8). In addition, when the DOC-treated supernatant, which contains most of the oxidase (82%) and all the dehydrogenase, was mixed with the EDTA-treated pellet, a very high percentage of oxidase reactivation was also observed upon the addition of magnesium (Table 8).

When membranes were first fragmented by sonication and then chemically disrupted, restoration of oxidase was also achieved by the addition of magnesium (Tables 5, 7 and 9).

Table 8

Restoration Of EDTA And Deoxycholate Disrupted Membrane (Non-sonicated) Oxidase Activities By Magnesium

| Membrane preparations[a] | Percent Oxidase Activity[b] |
|---|---|
| Control membrane | 100.0 |
| EDTA-supernatant + EDTA-pellet + $Mg^{2+}$ | 128.0 |
| EDTA-supernatant + DOC-pellet + $Mg^{2+}$ | 7.4 |
| DOC-supernatant + EDTA-pellet + $Mg^{2+}$ | 116.4 |
| DOC-supernatant + DOC-pellet + $Mg^{2+}$ | 23.9 |

[a]Frozen membranes were thawed, centrifuged at 73,500xg for 30 minutes and washed once with 0.03M Tris-Cl (pH 7.5). The pellets were resuspended separately in 0.03M Tris-Cl (pH 7.5) (as control), in 5mM of EDTA (in 0.03M Tris-Cl, pH 7.5), and in 0.2% of Na-deoxycholate (0.03M Tris-Cl, pH 7.5). These preparations were retained overnight at 0°C. The EDTA and deoxycholate treated membranes were then centrifuged at 73,500xg for 30 minutes and pellets were resuspended in 0.03M Tris-Cl, pH 7.5. The membrane preparations (0.1 ml of supernatant and 0.1 ml of pellet) were incubated with 0.05M phosphate containing 0.03M $MgSO_4$ at 32°C for 30 minutes. The NADH oxidase activity was then assayed by adding 0.1 ml of 4.8mM NADH at zero time.

[b]Calculated as compared to the NADH oxidase activity of control membranes (no $Mg^{2+}$) which were established as 4.07 units/ml.

Table 9

Restoration Of EDTA And Deoxycholate Disrupted Membrane (Sonicated) Oxidase Activities By Magnesium

| Membrane preparations[a] | Percent Oxidase Activity[b] |
|---|---|
| Control sonicated particles | 100.0 |
| EDTA-supernatant + EDTA-pellet + $Mg^{2+}$ | 179.7 |
| EDTA-supernatant + DOC-pellet + $Mg^{2+}$ | 25.3 |
| DOC-supernatant + EDTA-pellet + $Mg^{2+}$ | 215.1 |
| DOC-supernatant + DOC-pellet + $Mg^{2+}$ | 60.1 |

[a]Frozen membranes were thawed, centrifuged at 73,500xg for 30 minutes and washed once with 0.03M Tris-Cl, (pH 7.5). The washed membranes were sonicated at a setting of 5 amp for 30 seconds (5 seconds intervals) and centrifuged again at 73,500xg for 30 minutes. The pellets were resuspended separately in 0.03M Tris-Cl (pH 7.5), in 5mM EDTA (in 0.03M Tris-Cl, pH 7.5), and in 0.2% of Na-deoxycholate (0.03M Tris-Cl, pH 7.5). These suspensions were incubated overnight at zero degrees. The EDTA and deoxycholate treated particles were then centrifuged at 73,500xg for 30 minutes and the pellets were resuspended in 0.03M Tris-Cl, pH 7.5. The membrane preparations (0.1 ml of supernatent and 0.1 ml of pellet) were incubated with 0.05M phosphate containing 0.03M $MgSO_4$ at 32°C for 30 minutes. The NADH oxidase activity was then assayed by adding 0.1 ml of 4.8mM NADH at zero time.

[b]Calculated as compared to the NADH oxidase activity of control sonicated pellets (no $Mg^{2+}$) which was established as 1.72 units/ml.

## DISCUSSION

This study has demonstrated that NADH oxidase and dehydrogenase activities are masked in Micrococcus lysodeikticus membranes, as in Bacillus megaterium (7), and this may involve factors of steric hindrance, or of impermeability. Effects of sonic oscillation, deoxycholate, and versene may break impermeability barriers and unmask NADH dehydrogenases which are localized on the inner side of cytoplasmic membranes. These unmasking treatments usually caused inactivation of NADH oxidase and solubilization of NADH dehydrogenase (Tables 2, 4, 5, 6,and 7). The inactivated oxidase could be reactivated upon the addition of magnesium. This suggests an important role for magnesium ion in the stabilization of the membrane bound NADH dehydrogenase-oxidase complex.

Magnesium ion has a different effect on different types of bacteria. For example, respiration of Bacillus megaterium protoplasts and ghosts is inhibited by $Mg^{2+}$, whereas magnesium ions increase the respiration of B. subtilis spheroplasts slightly and do not affect the respiration of the ghosts (44, 50). The general effects of magnesium on Micrococcus lysodeikticus ghost membranes are summarized in Table 10. The inhibition of NADH oxidase and dehydrogenase activities of intact ghost membranes by magnesium suggests that magnesium might enhance the degree of impermeability or steric hindrance of enzyme active sites. Once the ghost membranes were disrupted by sonic oscillation, this impermeability (or hindrance) was destroyed and NADH oxidase and dehydrogenase activities were

Table 10

Summarized Effect Of Magnesium On *Micrococcus lysodeikticus* Membrane NADH Oxidase And Dehydrogenase Activities

| Membrane preparations | Enzyme activities | Control | EDTA | DOC |
|---|---|---|---|---|
| Non-sonicated washed membranes | Oxidase | inhibit[a] | stimulate[b] | stimulate |
| | Dehydrogenase | inhibit | inhibit | inhibit |
| Sonicated particles | Oxidase | stimulate | stimulate | stimulate |
| | Dehydrogenase | inhibit | stimulate | inhibit |

[a]Enzyme activities were inhibited by magnesium.

[b]Enzyme activities were stimulated by magnesium.

then unmasked. Most of the unmasked NADH dehydrogenases were solubilized along with the partial inactivation of NADH oxidase (Table 2). Fig. 6 shows that prolonged sonication of M. lysodeikticus membranes unmasked the NADH dehydrogenase simultaneously with the inactivation of the NADH oxidase; this is different from the data on B. megaterium which showed that NADH oxidase activity was increased above five-fold with 20 seconds of sonication and longer sonic oscillation periods led to a decline in oxidase activity (7). Addition of magnesium ion to the sonicated particles reaggregated most of the fragmented membranes and restored the insoluble, active NADH oxidase complex (Tables 2 and 10). This is in accord with J. Brown's work on Sarcina lutea which showed that larger aggregate particles (70-S) were obtained by dialyzing 5-S particles in the presence of magnesium (4).

Not all the divalent cations tested could restore fragmented membrane oxidase activity. For example, calcium showed a great inhibition of membrane NADH oxidase activity independent of whether or not the membrane was sonicated (Table 1). The reason for this inhibition is not clear, some secondary effects may be involved.

The oxidase reconstitution of deoxycholate treated membrane has been explained by Eisenberg et al. (8) wherein magnesium might serve a role of neutralizing negative charge repulsion, or supplying divalent cations to react with the negatively charged groups. Thus, the effect of magnesium would be primarily important on the EDTA treated membranes where membrane bound divalent cations had been chelated by EDTA and resulted in solubilization of NADH dehydrogenases and inactivation of oxidases (Table 4). The EDTA-activated dehydro-

genases were bound back to the inactivated oxidase by magnesium ion and reform the active oxidase complex which mostly are insoluble (Table 4). The oxidase re-activated by magnesium was shown to have an activity 50% more than the control (Table 4). This suggests that the organization of the recovered membrane, in which the enzymes had been unmasked and stimulated, must be changed in some way that could not be equal to the original ghost membranes.

The effect of magnesium on the EDTA treated sonicated particles is more interesting; it seems to enhance an activation of both the EDTA-activated dehydrogenases and the EDTA-inactivated oxidases (Table 5). This might suggest that magnesium also plays a role in NADH dehydrogenase activity. When this activity was unmasked by sonication, some of the unmasked dehydrogenase became inactive because its component magnesium was chelated by EDTA. Upon the addition of magnesium this inactivated dehydrogenase was then reactivated.

Hydrophobic interaction in membranes or membrane fragments has been shown to be very important due to the action of detergent. Gel'man *et al.* found recently that the respiratory chain of *Micrococcus lysodeikticus* membranes could be disrupted by detergents into two blocks; one contained malate and NADH dehydrogenases with cytochrome $b_{556}$; the other cytochromes $b_{560}$, $c_{550}$ and $a_{601}$ (12). Eisenberg *et al.* reported that deoxycholate can solubilize the total respiratory chain from *B. megaterium* KM ghosts (7, 8). The data presented here (Tables 6 and 7) also suggested that deoxycholate was able to solubilize all of the NADH dehydrogenases from *M. lysodeikticus* ghost membranes. Reasons for small amounts of oxidase found

in the particular fraction are still unknown.

Inhibition of M. lysodeikticus NADH oxidase by detergent could only be partially removed by the addition of magnesium (Tables 6 and 7); this is not like deoxycholate treated B. megaterium KM membranes which could be stimulated for NADH oxidase activity even higher than in the original ghosts (8). The poor restoration of M. lysodeikticus NADH oxidase by magnesium was also shown in the recombination mixture of deoxycholate disrupted soluble and particulate fractions (Tables 6, 7, 8, and 9, also reference 9).

Since there is more dehydrogenase activity solubilized by deoxycholate than by EDTA (Tables 4, 5, 6, and 7), it is possible to get a very high percentage of oxidase re-activation by mixing the deoxycholate treated supernatant and EDTA treated pellet with the addition of magnesium (Tables 8 and 9) which acts mostly on the EDTA-treated pellet. On the contrary, there was only little oxidase reactivated by magnesium in the recombination mixture of EDTA-treated soluble and DOC-treated particulate fractions. The actual mechanisms of oxidase reconstitution are still not clearly understood and are expected to be investigated further.

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