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Separation of the Membrane Associated L-Malate and Reduced Nicotinamide Adenine Dinucleotide Dehydrogenases of Micrococcus Lysodeikticus

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SEPARATION OF THE MEMBRANE ASSOCIATED L-MALATE AND REDUCED NICOTINAMIDE ADENINE DINUCLEOTIDE DEHYDROGENASES OF *MICROCOCCUS LYSODEIKTICUS*

by

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REVIEW OF LITERATURE

Respiration

Production of energy is a vital function for all living systems. Respiration is one of the major metabolic pathways involved in energy production in aerobic organisms (Lehninger, A.L., 1970). Respiration involves the biological oxidation of reduced substrates at the expense of molecular oxygen as a terminal electron acceptor. The energy released by these oxidations is conserved via coupling with ATP synthesis. This coupling system provides a useable form of energy to the living system (Klingenberg, M., 1968).

The specific mechanism of biological respiration involves the transfer of electrons from reduced compounds, through a system of electron carriers, to molecular oxygen. This chain of electron carriers is generally referred to as an electron transport system. More important than the reduction of molecular oxygen is the coupling of oxidative phosphorylation to the transfer of electrons. These two functions, electron transport and oxidative phosphorylation, collectively define respiration in aerobic organisms (Lehninger, A.L., 1965).

The site of respiration in eucaryotic organisms resides in the mitochondria (Green, D.E., 1966; Hatefi, Y., 1966). Mitochondrial electron transport systems are localized within the mitochondrial membranes (Ball, E. and C. Joel, 1962; Criddle, R.

et al, 1962; and Racker, E., 1968). Coupling of electron transport to oxidative phosphorylation is strictly dependent on the integrity of the mitochondrial membranes. Oxidative phosphorylation has never been detected in preparations that do not contain topologically closed vesicles (Packer, L., 1972). According to Mitchell, the driving force for oxidative phosphorylation is a pH gradient by electron flow through the mitochondrial membranes (Mitchell, P., 1969). Vectorial movement is made possible because of the impermeability by simple diffusion of the mitochondrial membranes to hydrogen ions. This allows the accumulation of both an electrochemical and pH gradient between the outside and the inside of the mitochondrial membrane. Hydrogen ions are allowed to re-enter the mitochondria only at certain points along the membrane. As these ions enter, they energize the oxidative phosphorylation function and effect the production of ATP (Mitchell, P., 1969; Wang, J.H., 1970).

Bacteria do not have intracellular organelles such as mitochondria for localization of the respiratory function. It has been assumed, however, that bacterial respiration would be associated with membranous structures since the function of respiration in mitochondria is inherently dependent on membrane phenomenon (Harold, F.M., 1972). Claus Weibull, working with Bacillus megaterium, discovered the association of bacterial electron transport with the plasma membrane (Weibull, C., 1953, a and b). Weibull's success was achieved mainly because of his

ability to form protoplasts of B. megaterium by the action of lysozyme. Lysozyme, in effect, removes the bacterial cell walls by hydrolysis of wall polymers, leaving behind membranes and membrane associated constituents (Shah, S.B. and H.E. King, 1966).

Controversy quickly developed over Weibull's findings because of Chapman and Hillier's discovery of "peripheral bodies" in Bacillus cereus (Chapman, G.B. and J. Hillier, 1953). The term "mesosome" was later substituted for "peripheral bodies" (Fitz-James, P.C., 1966). Mesosomes, membranous structures located on the inner side of the plasma membrane, intrigued many investigators with the idea that these structures might be analogous to eucaryotic mitochondria (Salton, M.R.J., 1967). Since the discovery of mesosomes, much work has been done on the isolation and characterization of these mesosomal structures (Kaback, H.R., 1971; Patch, C.T. and O.E. Landman, 1971; Robertson, J.D., 1959; and Rothfield, L. and A. Finkelstein, 1968). The distribution of electron transport components varies between mesosomal and plasma membranes with the species of bacteria studied (Coleman, R., 1973; Reusch, V.M. and M.M. Burger, 1973). Salton and Ellar have worked directly with Micrococcus lysodeikticus which is the organism used in the current study (Salton, M.R.J., and J.A. Chapman, 1969; Ellar, D.J., et al, 1971). Eller's work, in particular, showed that most of the electron transport components of M. lysodeikticus are not associated with mesosomal structures.

Since Weibull's initial work, many reports have shown that respiration is localized within the bacterial plasma membrane and

that this respiratory activity is related to membrane phenomenon (Mudd, S., et al, 1961; Pangborn, J., et al, 1962). As with mitochondrial systems, much effort has been expended in proving that the vesicles with oxidative phosphorylation activity are topologically closed and have the same outside-inside orientation as the parent cells (Harold, F.M., 1972). The association of respiratory function with membrane phenomenon in bacteria is clearly analogous with the knowledge obtained about mitochondrial respiration.

Respiratory systems are best understood in eucaryotic beef heart mitochondria (Blair, P.U., et al, 1964; Fernandez-Moran, H. et al, 1964; Green, D.E. and D.C. Wharton, 1963). This system offers a number of particular advantages in that there is a very low content of proteolytic enzymes in the preparations and the electron transport system is relatively stable (Keilen, D., 1966). The most important reason for the extensive knowledge of beef heart mitochondria is that this is the system from which the first electron transport particle was isolated (Keilen, D. and E.F. Hartree, 1940).

Experiments Used in Studying Respiratory Mechanisms

The work with beef heart mitochondria paved the way for studying the electron transport systems of other types of cells including bacteria, and led to the development of the types of experiments used in obtaining information on the structure and

function of electron transport systems (Mahler and Cordes, 1966). These types of experiments involve a variety of concepts. Thus, a method must be available for identification of electron transport components. Spectrophotometric techniques have been developed for the identification and quantitation of electron transport carriers. Britton Chance contributed significantly in the development of these techniques (Chance, B., 1951 a and b). D. C. White and P. R. Sinclair have reviewed these techniques, the instrumentation involved, and most importantly, the interpretation of the data (White, D.C. and P.R. Sinclair, 1971). After identification of electron transport carriers, communition of the mitochondrial electron transport chain was achieved along with characterization of the unit building blocks (Hatefi, Y. et al, 1962; Slater, E.C., 1958; Tzagaloff, A. et al, 1967; Ziegler, D.M. et al, 1959). After isolation of the electron transport system components, attempts were made to reconstitute the particulate system both morphologically and functionally. These experiments afforded the advantage of being able to study both the components of the system and their relationship to membrane structure (Racker, E., 1970, a and b; Skulacheu, V.P., 1971; Van Dam, K. and A.J. Meyer, 1971). Respiratory inhibitors divide the electron transport chain, before and after the inhibition site, into segments. In this manner, the inhibitors provide a method for elucidating the sequence of electron carriers (Slater, E.C., 1958). One important criterion in this final type of experiment is the assumption that the

inhibitors act only at specific points in the chain. This must be continually tested in each system in which inhibitors are used (Klingenberg, M., 1968).

The types of experiments described above are currently being applied to the study of bacterial electron transport systems. The variation and number of electron carriers in bacterial systems is quite large. This necessitated the development of more efficient and varied spectrophotometric techniques for studying bacterial electron transport. These techniques for bacterial studies have been reviewed by L. Smith (1968). The most common method for isolating bacterial membranes from Gram negative organisms has been with the use of lysozyme and ethylenediamine-tetracetic acid (EDTA). Gram positive bacteria require only lysozyme for membrane preparation (Salton, M.R.J., 1967; Weibull, C., 1956). Lysozyme treatment, however, has been found to affect the activity of some electron transport systems; this possibility must be kept in mind when studying lysozyme prepared membranes (Shah, S.B. and H.K. King, 1966). The use of detergents has been employed extensively in the disruption of prepared membranes. The most commonly used detergents are the anionic detergents such as sodium dodecyl sulfate (SDS) and sodium deoxycholate (DOC). These anionic detergents disrupt hydrophobic bonds. It is very difficult, however, to measure the completeness of membrane disruption and the amount of enzyme denaturation through the use of these detergents (Smith, L., 1968). Nevertheless,

disruption of the electron transport system of lysozyme membrane preparations with anionic detergents has greatly increased the efficiency with which respiratory chain components can be identified and characterized (Bragg, P.D. and C. Hou, 1967, a and b; Gelman, N.S. et al, 1967; Kamen, M.D. and T. Horio, 1970). With the isolated components of the respiratory chain, experiments again turned to, as with mitochondria, the selective reassembly of the system. The reassembly of the native structure is exceedingly difficult to monitor, especially when the native structure is not known. Instead, the method of monitoring reassembly has been used to monitor the reconstitution of function. This type of monitoring system must then include the assumption that reconstitution of function is a good measure of the reassembly of the structure. This assumption may not always be true (Razin, S., 1972).

The reassembly of membranes accompanied by functional reconstitution was first reported in a mitochondrial system (Tzagaloff, A. et al, 1967). Reassembly of membrane structures following sodium dodecyl sulfate disruption was first reported in a microbial system by work with Mycoplasma laidlawii (Razin, S. et al, 1965 and Terry, T. et al, 1967). Disruption of an NADH oxidase electron transport system by deoxycholate followed by restoration of activity was first reported in work with Bacillus megaterium, strain KM (Eisenberg, R.C. et al, 1970, a and b). The electron transport chain of B. megaterium was disrupted by 0.4%

DOC and restoration of NADH oxidase activity was accomplished by diluting the detergent membrane suspension in the presence of magnesium ions. Since the time of these original experiments, much work has been done with the solubilization and reconstitution of bacterial electron transport components (Kagawa, Y., 1972; Kiszkiss, D.F. and R.J. Downey, 1972, a and b).

Respiratory inhibitors, as described with the mitochondrial system, have been applied to the study of electron transport in bacterial systems. It has been possible to elucidate the sequence of electron carriers in many bacterial systems through the use of respiratory inhibitors (Harold, F.M., 1970; Horio, T. and M.D. Kamen, 1970).

Use of Bacterial Systems in Studying Respiratory Mechanisms

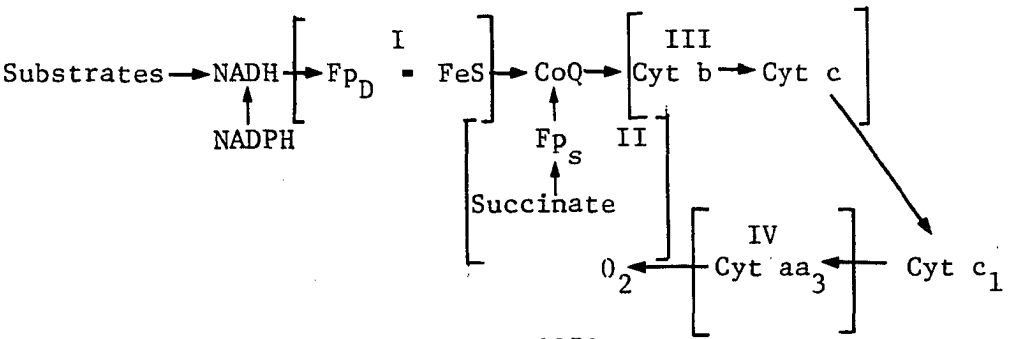
Bacteria are excellent tools for studying respiratory mechanisms. Some of the reasons for their applicability are apparent from the development of the studies referred to above; however, there are some other considerations for their use. A primary consideration is the simple economy with which bacteria can be obtained and their short generation times. Large quantities of bacteria can easily be grown in batch cultures. This affords a consistency in sample material not available in most eucaryotic systems (Salton, M.R.J., 1967). Another aspect of bacterial systems making them advantageous for study is because genetic analysis of specific mutant and wild type strains provides

information not readily available in eucaryotic systems (Manns, B. and H. Gest, 1973 a and b).

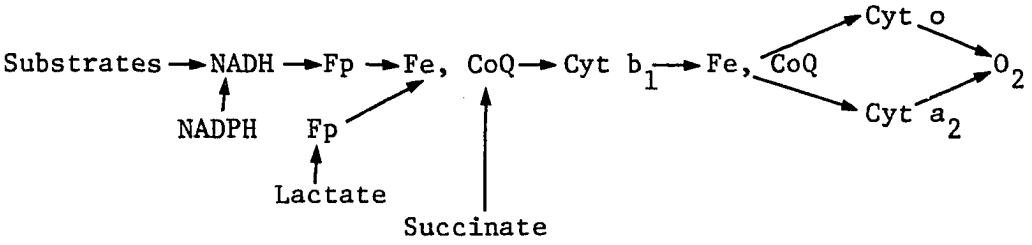
Nutritional requirements and environmental influences, such as carbon sources and oxygen requirements, also affect the pathways of electron transport and oxidative phosphorylation; all of these factors can be controlled very easily in the growth of bacteria (Smith, L., 1968). Finally, as noted earlier, bacterial systems have been quite easily adapted to the types of studies first developed with mitochondria.

Description of Bacterial Electron Transport Systems

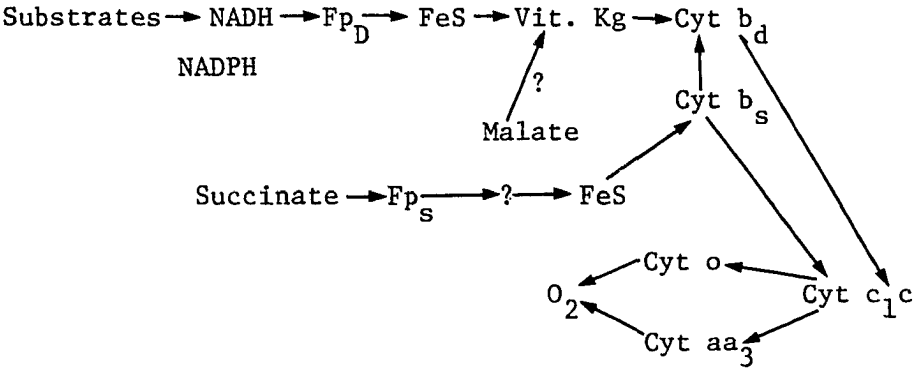
The main features of the architecture of the respiratory chain and its overall electron flow are, within certain limits, similar in both mitochondria and bacteria. To illustrate this, the mitochondrial electron transport system and several bacterial electron transport systems are shown below:



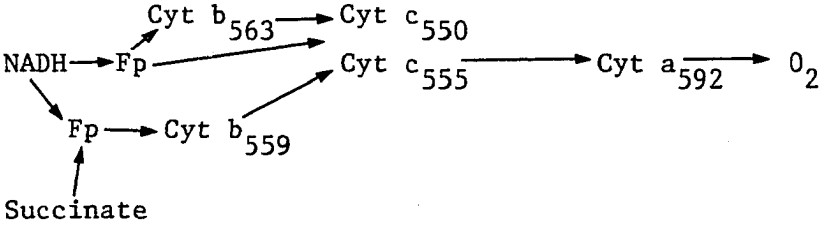
Mitochondria, from Harold, F.M., 1972.



Escherichia coli, from Cox, G.B., et al. 1970.



Mycobacterium phlei, from Brodie, A.F. and D.L. Gutnick, 1972; and Asano, A. and A.F. Brodie, 1964.



Halobacterium cutirubrum, from Lanyi, J.K. 1969.

Abbreviations: Cyt, cytochromes; FeS, nonhaem iron protein; Fp, flavoprotein; and Vit., vitamin.

Electrons enter the chain through the primary dehydrogenases in all of the systems represented above. These dehydrogenases usually require nicotinamide adenine dinucleotide (NAD) as a

coenzyme and are commonly referred to as pyridinoproteins. After reduction of NADH this coenzyme has the ability to move to a second site where a second substrate is reduced and the electrons pass to the next carrier. This second carrier is usually a flavoprotein. Flavoproteins catalyze the oxidation of substrates of intermediate electron potential; they may, however, pass their electrons directly to oxygen. Reduced flavoproteins can pass their electrons to ubiquinones and/or nonhaem iron proteins. These two carriers then pass electrons through a system of cytochromes terminating with cuproproteins. Cuproproteins are terminal dehydrogenases using oxygen as the final electron acceptor and have high redox potentials; they usually serve as the terminal members of respiratory chains (Lehninger, A.L., 1966 and 1970; Mahler, H.R. and E.H. Cordes, 1966; Massey, U. and C. Veger, 1963).

The scheme of electron transport in mitochondrial systems is followed fairly rigidly. This has made possible the isolation and characterization of specific complexes from the solubilized mitochondrial electron transport system (Hatefi, Y., 1966; Tzagaloff, A. et al., 1967). These specific complexes are indicated by brackets in the scheme on page 9 and serve as a general reference for solubilization and reconstitution of bacterial electron transfer systems (Harold, F.M., 1972). The schemes of electron transfer in bacteria are not nearly so uniform as in mitochondria. Some of the differences are illustrated in the schemes on the preceding pages. There are

primary dehydrogenases in bacterial systems which do not require NADH (Deley, J. and J. Schel, 1959, Cohn, D., 1956). In such systems, it is suggested that these primary dehydrogenases are closely and obligately bound to the second electron carrier in order for transfer of electrons to occur (Gel'man, N.S. et al., 1959). Ubiquinones are sometimes replaced by napthoguinones in bacterial systems and as shown in the scheme for E. coli., there may be more than one quinone in the transfer chain (Brodie, A.F. and D.L. Gutnick, 1972, Cox, G.B. et al., 1970). Many times there is more than one terminal oxidase leading to molecular oxygen. Branched chain electron transport systems, therefore, do exist in bacteria in which electrons once entering via dehydrogenases, have a number of pathways available to reach molecular oxygen (Marrs, B. and H. Gest, 1972b). Such a system is illustrated by the scheme of Halobacterium cutirubrum (Lanyi, J.K., 1969). It should be kept in mind that the function of producing energy via oxidative phosphorylation is the same independent of the sequence of electron carriers. There is no reason to expect the coupling of electron transport to oxidative phosphorylation in bacteria to be mechanistically different than in mitochondria (Brodie, A.F. and D.L. Gutnick, 1972).

Choice of Organism for Study

Many types of bacteria have been employed in the study of respiration (White, D.C. and P.R. Sinclair, 1971). Some of these

have proven to be more suitable than others. Gram negative bacteria have a trilaminar cell envelope structure consisting of an outer lipolysaccharide layer, a middle peptidoglycan layer, and the inner plasma membrane. In order to obtain the membrane preparations needed for study, combined treatment with lysozyme, EDTA, and either osmotic lysis, sonication, or chemical disruption of the cells is needed. These preparations are invariably heterogeneous and contain fine structured layers (lipopolysaccharides) and varying amounts of the underlying plasma membranes (Salton, M.R.J., 1964). Data on the chemical structure of the membrane may be misleading until it is possible to distinguish between the components of the organized lipopolysaccharide layers and the plasma membrane components (Salton, M.R.J., 1967).

Gram positive bacteria present simpler systems in that the cell walls consist of a single peptidoglycan layer surrounding the plasma membrane. Weibull has shown that the plasma membrane can be separated from the peptidoglycan layer by the use of lysozyme (Weibull, C., 1956). Furthermore, following treatment with deoxyribonuclease, the membrane preparations can be separated from the cell sap by washing with suitable buffers. By utilizing carefully controlled methods, removal of conspicuous membrane markers such as carotenoids, quinones, and cytochromes is prevented (Salton, M.R.J., 1967).

The simplest bacterial membrane systems appear to be those in the genus Mycoplasma. Mycoplasma are naturally occurring

organisms bounded by a single membrane and there is little evidence of the internal membranes seen in those bacteria possessing an outer rigid cell wall (Salton, M.R.J., 1967). Because of their membrane simplicity, Mycoplasma have been used extensively in the study of membrane structure and function. Razin, Morowitz, and Terry (1965) demonstrated the complete disruption of the plasma membrane of Mycoplasma laidlawii by the action of sodium dodecyl sulfate. Reaggregation of the disrupted membranes was accomplished by exposing them to magnesium ions. Electron micrographs have shown these reaggregations to be similar to the native membranes (Razin, S. et al., 1967). More recent studies have led to the characterization of the disrupted membrane fragments solubilized by sodium dodecyl sulfate (Engleman, P.M. and J. Morowitz, 1968 a and b; Engelman, D.M. et al., 1967). Work in Razin's laboratory has shown the similarity between the native membranes and the disrupted membrane fragments with the use of immunological analysis (Argaman, M. and S. Razin, 1969; Kahane, I. and S. Razin, 1969). Most of the work with Mycoplasma has been oriented around reformation of membrane structure and has not included reconstitution of normal enzymatic activities (Razin, S., 1972).

Asporogenic Gram positive bacteria of the genus Bacillus have been used extensively in the study of membrane structure and function (Razin, S., 1972). Anionic detergents have also been used to solubilize the membrane components of these bacteria (Eisenberg, R.C. et al., 1970a). Protoplast membranes were

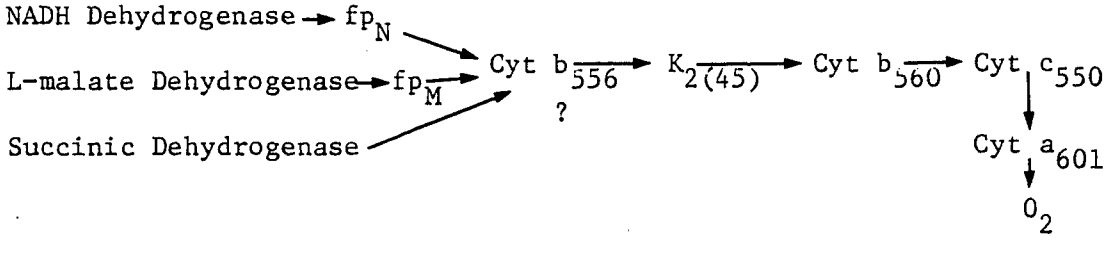
prepared by using the methods of Weibull (1956) and sodium deoxycholate was then used to disrupt the membrane components. This formed a condition in the solubilized preparations which provided for divalent cation dependent reaggregation and restoration of a functional electron transport complex (Eisenberg, R.C. et al., 1970b). The studies of Bacillus megaterium KM membrane associated electron transport have illustrated the importance of divalent cations, especially magnesium, in the reconstitution of electron transport activity (Yu, L. and M.J. Wolin, 1970). The effect of divalent cations may be a catalytic one or they may act to change the physical structure of the protein (Ne'eman, Z. et al., 1972). Yu and Wolin (1970) have given evidence that the effect of Mg^{2+} in the reconstitution process of electron transport in B. megaterium is probably a complex one. This process could involve the reassociation of the solubilized proteins due to the presence of Mg^{2+} via Mg^{2+} acting as a precipitant in allowing protein fragments to come together. Activation of the system also occurs spontaneously in the absence of Mg^{2+} at pH 5.0. Spontaneous reactivation was not found to occur at pH 7.0 in the absence of Mg^{2+}. This seems to suggest a role for Mg^{2+} other than one of physical reassociation (Yu, L. and M.J. Wolin, 1970). Some of the solubilized components of the electron transport system of B. megaterium treated with DOC have been isolated and characterized (Yu, L. and M.J. Wolin, 1972b). Furthermore, it has been shown that the reactivation of electron

transport in B. megaterium causes the formation of membranous structures similar to the native membranes (Yu, L. and M.J. Wolin, 1972a). This agrees with the data of Engelman and Morowitz (1968), and reaffirms the hypothesis of a correlation between membrane structure and function.

Micrococcus lysodeikticus is a Gram positive cocci that has been the subject of much research in the area of bacterial respiration (Gel'man, N.S. et al., 1967). This organism serves as an index for lysozyme activity (Salton, M.R.J., 1952) and thus is quite suitable for this type of research since membranes can be prepared with considerable ease (Mitchel, P. and J. Moyle, 1956).

Electron Transport in Micrococcus lysodeikticus

A proposed scheme of electron transport (Eisenberg, R.C., 1972; and Gel'man, N.S. et al., 1970) in Micrococcus lysodeikticus is given below:



Abbreviations: fp, flavoprotein; cyt, cytochrome.

Electrons enter the system through either of the three dehydrogenases (Oparin, A.I. et al., 1958; Ishakawa, S. and

A.L. Lehninger, 1962). From two of the enzymes, reduced nicotinamide adenine dinucleotide (NADH) and L-malate dehydrogenases, the electrons flow through cytochrome $\underline{b}_{556}$ to vitamin $K_{2(45)}$ (Gel'man, N.S. et al., 1970). The fate of electrons entering via succinic dehydrogenase is not known. Cytochrome b has always been detected by dithionite reduction in the purified preparations of succinic dehydrogenase although a physiological association of the enzyme with a b bype cytochrome has not been made (Pollock, J.J. et al., 1971). Vitamin $K_{2(45)}$ was detected and isolated by M. Fujita et al. (1966). Later work placed the napthoquinone in its natural position with the reconstituted electron transport chain (Eisenberg, R.C., 1972). Cytochromes of a, b, and c types were first detected in M. lysodeikticus by F.L. Jackson and V.D. Lawton (1959). The presence of two cytochromes of the b type was first suggested by D.N. Ostrovskii et al. (1968). The characterization of both cytochrome b types was later made with high resolution liquid nitrogen difference spectra (Gel'man, N.S. et al., 1970; Shipp, W.S., 1972).

Use of pancreatic lipase showed that most of the respiratory enzymes of M. lysodeikticus were bound to the plasma membrane (Ostrovskii, D.N. et al., 1961). However, upon treatment with lysozyme, succinic oxidase was destroyed (Shah, S.B. and H.K. King, 1966). Further studies with lysozyme prepared membranes have shown the functional presence of the rest of the electron transport components as shown in the proposed scheme. It has also been

shown that this bacterium contains two separate L-malate dehydrogenases (Cohn, D., 1956 and 1968). Cohn showed that whole cells contained both NADH requiring and NADH independent L-malate dehydrogenases (Cohn, D., 1958; Gel'man, N.S. et al., 1960). The NADH independent L-malate dehydrogenase was found to be a membrane associated enzyme while the NADH requiring enzyme was cytoplasmic and lost during membrane preparation.

Membrane solubilization studies have been used extensively in determining the functional aspects and sequence of electron transport in M. lysodeikticus. The two most commonly used extraction agents have been EDTA and anionic detergents (Gel'man, N.S. et al., 1970, Nachbar, M.S. and M.R.J. Salton, 1970). It has also been shown that the membrane bound cytochromes of the electron transport system are bound exclusively by hydrophobic interactions (Simakova, I.M. et al., 1969, Oparin, A.I. et al., 1971). These findings agree with T. F. Butler et al. (1967) who found the major types of membrane protein bonds to be hydrophobic. Hydrophobic binding of the electron transport system would make the use of anionic detergents very helpful in determining electron transport component sequence. Sodium dodecyl sulfate solubilization of M. lysodeikticus membranes has been used to determine some of the physical characteristics and to determine, with disc gel electrophosesis, the number of protein species present in these membranes (Estrugo, S.F. et al., 1972). Functional studies of the electron transport system have not been made after

solubilization with sodium dodecyl sulfate, probably because of the drastic denaturing action of this detergent. The action of sodium dodecyl sulfate has been found to disrupt cytochromes and therefore is not suitable for these experiments (Tikhonova, G.V. et al., 1970). Salton has shown that cytochromes a, b, and c and succinic dehydrogenase are not solubilized by the action of 0.2% deoxycholate (Salton, M.R.J. et al., 1968). Work by G. V. Tikhonova et al. (1970) using the same concentration of deoxycholate, reports the solubilization of the entire electron transport system. In both cases it has been found that L-malate and Nicotinomide Adenine Dinucleotide dehydrogenases are solubilized by even low concentrations of deoxycholate. These findings have been confirmed by R. C. Eisenberg (1971 and 1972). Furthermore, attempts to separate L-malate and NADH dehydrogenase activities have not been successful (Ostrovskii, D.N. et al., 1968, Tsfasman, I.M. et al., 1970 a and b). This inability to separate these activities led N. S. Gel'man et al. (1970) to propose that L-malate and NADH dehydrogenases are solubilized as a unit block and this association helps to maintain functional activity of the enzymes. It is the purpose of this study to re-evaluate the possibility of separating L-malate and NADH dehydrogenases solubilized by deoxycholate from M. lysodeikticus membranes.

MATERIALS AND METHODS

Source of Organism

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 Membranes

Cells were grown in 1.5% (w/v) Trypticase Soy Broth without glucose (Bioquest, Cockeysville, Md.) as the basal medium. Glucose (autoclaved separately) was added to 0.04 M; final pH 6.9. The cells were grown in nine liter batch cultures at 32 C in a New Brunswick Microferm fermenter, air flow 10 liters per minute with seven drops of antifoam agent (Union Carbide SAG-471) added to control foaming. Bacteria were harvested at the end of logarithmic growth by centrifugation with the use of a "Szent-Gyorgyi Blum" Continuous Flow System (Ivan Sorvall Inc., Norwalk, Conn.). The cells were then washed with a 2.4 liter total volume 0.01 M tris(hydroxymethyl) aminomethane,(Tris)-hydrochloride, pH 7.2.

Membrane preparation consisted of suspending the cells to 1 liter of TKM buffer (0.01 M each Tris-hydrochloride, KCl, $MgCl_2$, pH 7.2) and then adding 100 to 120 mg of crystalline lysozyme [1 mg lysozyme per g of cells (wet weight)] with stirring for 1 hour at 23 C. A small amount of deoxyribonuclease was added

immediately after addition of lysozyme. The lysed cell suspensions were collected by centrifugation at 27,000 x g for 30 min at 5 C and the pellet material (membranes) were washed 3 times with 720 ml (total volume) TKM buffer. The washed membranes were suspended in double strength TKM buffer (total volume 150 ml) containing 30 g glycerol and 2 x 10^{-4} M β-mercaptoethanol. The suspension was then diluted to 300 ml with distilled water. Seven ml aliquots were then collected in test tubes and frozen at -60 C until further use. The frozen membrane preparations contained 7 to 10 mg protein per ml; this value varied slightly between membrane batches.

Preparation of Disrupted Membranes

To prepare the disrupted membranes, a frozen preparation was first thawed and washed twice with 0.05 M Tris-hydrochloride, pH 7.2, and resuspended to a volume containing 8 mg protein per ml. An equal volume of buffer solution containing 6 mg sodium deoxycholate (DOC) per ml was added to the protein suspension (final concentrations, 4 mg protein per ml and 3 mg DOC per ml). The membranes were incubated in the detergent solution for 15 min at 23 C with stirring. The DOC was immediately removed from the disrupted membrane preparation by desalting through a Sephadex G-25 column (the disrupted membranes eluting in the void volume with the DOC being retained). The void volume, collected from the column, routinely contained approximately 1 mg protein per ml in the Tris-hydrochloride buffer and protein recovery was quantitative.

Preparation of Supernatant and Particulate Disrupted Membranes

The particulate fraction of the disrupted membranes was collected by centrigugation for 1 hour at 50,000 revolutions per minute (rpm) in a Beckman L3-50 Preparative Ultracentriguge. The supernatant was poured off and used to obtain the dehydrogenase enzymes. The pellet formed the particulate fraction of the disrupted membranes.

Further purification of the dehydrogenase enzymes was attempted by salting out the proteins in the disrupted membrane supernatant with 45% (277 g per liter) ammonium sulfate saturation. Ammonium sulfate was added to the supernatant and allowed to incubate with stirring for 30 min at 23 C. After incubation, the precipitate containing the dehydrogenase enzymes was spun down at 27,000 x g for 60 min at 5 C. The precipitate was resuspended in 0.05 M Tris-hydrochloride, pH 7.2, and dialyzed overnight against Tris-hydrochloride buffer to remove the ammonium sulfate. Further purification and concentration of the dehydrogenase enzymes was attempted with a 27.5% (160 g per liter) ammonium sulfate saturation of the dialyzed sample. The precipitate and the supernatant fractions of the 27.5% ammonium sulfate saturation were obtained in the same manner as described for the 45% ammonium sulfate saturation. The resuspended and dialyzed precipitate of the 27.5% ammonium sulfate saturation served as

the purified enzymes and was used as a source for the dehydrogenase enzymes in the electrophoretic studies that followed.

Enzyme Assays

The activity of both L-malate and NADH dehydrogenases was estimated by using dichlorophenolindophenol, sodium salt (DCIP) (Sigma Chemical Co., St. Louis, Mo.) as an artificial electron acceptor. Reduction of DCIP was measured at 600 nm with a Gilford 2000 Recording Spectrophotometer. A Lauda Temperature Control Unit was used to maintain the temperature at 32 C. NADH dehydrogenase was assayed in a 3 ml reaction mixture containing 0.14 µmoles DCIP, 1.0 µmoles NADH, and 145 µmoles Tris-hydrochloride at pH 7.2. The rate of DCIP reduction was measured from the first 5 seconds following substrate (NADH) addition and corrected for low nonenzymatic reduction of DCIP by NADH. The amount of enzyme activity was proportional to enzyme concentration. Assuming a millimolar absorbancy of DCIP to be 21 per cm, one unit of dehydrogenase activity was taken as 1 µmole of DCIP reduced per minute. The L-malate dehydrogenase activity assay was identical except that 10 µmoles of L-malate, the substrate, was added to start the reaction. Nonenzymatic reduction of DCIP by L-malate was not detected. L-malate dehydrogenase activity was proportional to enzyme concentration.

Protein Estimation

Membrane protein concentrations were estimated by cold trichloroacetic acid (5% w/v) precipitation followed by hot alkali extraction (1.0 M NaOH, for 15 min at 100 C) and assaying the solubilized material by the method of Lowry et al (1951). Bovine serum albumin (fraction V powder, Sigma Chemical, St. Louis, Mo.) served as the standard and was treated in the same manner as described for the membrane proteins.

Polyacrylamide Disc Gel Electrophoresis

Methods for disc gel electrophoresis were as described by K. Weber and M. Osborn (1969).

Materials used specifically for this procedure included the following: Buchler Polyanalyst Disc Gel Electrophoresis Apparatus, Buchler Corp., Fort Lee, N.J.; Heathkit Regulatory H.V. Power Supply, Heathkit, Benton Harbor, Mi.; Acrylamide, N,N'-Methylenebis-acrylamide, and Coomassie blue, Bio-Rad, Richmond, Calif.; N,N,N',N'-Tetramethyl-ethylenediamine (Temed), Matheson, Coleman, Bell, Cincinnati, Ohio.

The separating gel stock solution contained 22.2 g acrylamide and 0.6 g N,N'-methylenebis-acrylamide made to 100 ml with distilled water. The mixed solution was filtered through Whatman #1 paper and stored in a light proof bottle at 4 C until use.

The polymerizing catalyst was made fresh for each experiment and contained 1.0 g anhydrous ammonium sulfate per 100 ml distilled water.

A number of different buffer systems were employed to determine the most suitable for enzyme separation. The gel buffers were diluted 1:1 with distilled water to form the chamber buffers in all cases. A list of the gel buffers is given below:

1) Tris-boric acid, 0.36 M, running pH 8.28.

2) Tris-hydrochloride, 0.1 M with varying concentrations of DOC (0.05 to 8 mg DOC per ml) or varying concentrations of sodium dodecyl sulfate (0.01 to 10 mg sodium dodecyl sulfate per ml), running pH from 6.8 to 8.6 (varied in different experiments).

3) Tris-hydrochloride, 0.1 M with 0.5% (w/v) Triton X-100, running pH 8.6.

4) Potassium phosphate, 0.15 M, with varying concentrations of DOC (0.05 mg DOC per ml, running pH from 4.4 to 7.0 (varied in different experiments).

5) Potassium hydroxide, 1 N, 90% (v/v) formic acid with 4.8 g urea per liter, running pH 3.5.

The 10% separating gels were prepared by combining the following ingredients in order: 10 ml deaereated gel buffer, 9 ml acrylamide stock solution, 25 liters Temed, and 1.0 ml catalyst (enough for 10 to 12 tubes). After mixing the gel solution thoroughly, each electrophoresis tube was filled with 1.2 ml of the gel solution. Three ml of water was gently layered on top of the gel solution to form a flat top on the gel. Polymerization occurred 15 to 20 minutes after adding the catalyst.

The gel tubes were placed in the electrophoresis apparatus after polymerization of the gels. Purified enzyme samples were

mixed with the following ingredients and layered on top of the gels: 100 µliters purified enzyme solution, 5 µliters β-mercaptoethanol, 10 µliters 0.05% (w/v) bromophenol (tracking dye), and 2 drops glycerol. The chamber buffers were added to the system and the samples were electrophoresed at 2 to 3 ma per gel for 20 minutes. This allowed the tracking dye to enter the gel and completed the cross linking of the gel system. The amperage was then adjusted to 8 ma per gel and continued until the tracking eye neared the bottom of the gel tubes. The gels were removed from their tubes at the completion of the electrophoresis and stained.

Two methods were employed in staining the gels. The first was a general protein stain, 0.25% Coomassie brilliant blue in 5:1:5 methanol, acetic acid water. After 2 hours of staining, the gels were destained for 1 week in a solution containing 3:2:35 acetic acid, methanol, water. The gels were stored in 7.5% (v/v) acetic acid at 4 C following destaining. An artificial electron acceptor, 3-(4,5-Dimethyl Thiazolyl-2)-2,5-diphenyl Tetrazolium bromide (MTT Tetrazolium) (Sigma Chemical, St. Louis, Mo.) was used to selectively stain the dehydrogenase enzymes by adding the dye with the proper substrate. Staining solutions for each enzyme contained the following ingredients: 1) NADH dehydrogenase; 1 ml 0.01 M NADH in 0.05 M Tris-hydrochloride, and 4 ml Tris-hydrochloride, pH 7.2, 8 to 12 drops saturated aqueous MTT tetrazolium and 2) L-malate dehydrogenase; 1 ml 0.05 M L-malate in Tris-hydrochloride, 4 ml Tris-hydrochloride, pH 7.2,

.1 ml 0.1 mg Phenazine methosulfate (PMS) (Sigma Chemical, St. Louis, Mo.) in Tris-hydrochloride, and 8 to 12 drops aqueous MTT tetrazolium.

The purple color of the reduced MTT tetrazolium developed in 5 to 10 minutes after addition of the substrate and was stable for 24 hours. No color developed without addition of the substrate.

DEAE Sephadex Ion Exchange

Diethylaminoethyl- (DEAE) Sephadex A-50 (Pharmacia Fine Chemicals, Piscataway, N.J.) was used as an anion exchanger in attempts to separate and purify the two dehydrogenase enzymes. Both batch and column methods were used in these experiments.

DEAE Sephadex was prepared by allowing the beads to swell in distilled water for 2 hours and then repeatedly washed in a Buchner funnel with 0.05 N NaOH until the wash was free of chloride ions. Excess NaOH was rinsed out with distilled water. This was followed by several rinses with the counter-ion solution, Tris-acetate (0.05 M, pH 7.2). Rinses were continued until the proper pH (7.2) was obtained.

For batch operation, a small amount (5 to 10 g) of the prepared DEAE Sephadex was placed in a centrifuge tube and a known amount of the resuspended and dialyzed 45% saturated ammonium sulfate precipitate was added. Following thorough mixing, the sephadex with the dehydrogenases attached was centrifuged at 27,000 x g for 15 minutes. The free fluid was

then removed. Elution of the dehydrogenases from the DEAE Sephadex was attempted with washes containing progressively higher concentrations of NaCl and DOC. Elution volumes were collected in the same manner as the first wash. The elution volumes were assayed for dehydrogenase activity and protein concentration.

For column operation, the prepared DEAE Sephadex was placed in a 10 g capacity column of 2.5 mm diameter. The dehydrogenase enzymes were attached by applying the resuspended and dialyzed 45% saturated ammonium sulfate precipitate to the top of the column and washing with 100 ml 0.05 M Tris-hydrochloride, pH 7.2. Elution of the dehydrogenases was attempted with pH, NaCl, and detergent gradients. Elution volumes were collected as 10 ml fractions and assayed for dehydrogenase activity and protein concentration.

Elutants for both operations were dissolved in 0.05 M Tris-hydrochloride.

Microzone Electrophoresis

The materials used for the microzone electrophoresis included a Beckman Model R-101 Microzone Electrophoresis Cell, a Beckman Model R2-2 Duostat Regulated Power Supply, Beckman Acetate Electrophoresis Membranes, and Beckman Barbitol B-2 buffer (Beckman Instruments, Inc., Fullerton, Calif.). The Tris-barbitol buffer system used in all of the electrophoretic studies

0.1 M Tris-hycrochloride, pH 8.6, mixed 1:1 with the contents of 1 Beckman Barbitol B-2 buffer packet dissolved in 1 liter (total volume) distilled water, pH 8.6.

Purified enzyme samples served as the source of the dehydrogenase enzymes in these experiments. Enzyme samples were placed on the acetate membranes with a Beckman .25 µliter sample applicator. Three microliter samples were routinely used. The electrophoresis was run at 150 volts constant voltage for 90 minutes at 4 C. After completion of the electrophoresis, the dehydrogenase enzymes were selectively stained with substrate and MTT-tetrazolium dye. An acetate membrane with three equally spaced enzyme samples was electrophoresed in order to demonstrate separation of these enzymes. After completion of electrophoresis, the membrane was cut into three strips so that each strip contained the migration of one of the enzyme samples. One membrane strip was stained for NADH dehydrogenase in a solution containing 24 ml Tris-barbitol, 24 drops saturated aqueous MTT tetrazolium, and 1 ml 8 mg NADH per ml in Tris-barbitol. Another membrane strip was stained for L-malate dehydrogenase in a solution containing 24 ml Tris-barbitol, 24 drops saturated aqueous MTT tetrazolium, 1 ml 0.1 M L-malate in distilled water, and 0.1 ml 0.1% (w/v) PMS in Tris-barbitol. The third membrane strip was stained for both enzymes by placing it in the NADH dehydrogenase system and allowing the color to develop, gently washing in Tris-barbitol, and placing the strip in the L-malate dehydrogenase

system. Stains on membrane strips were allowed to develop for 5 minutes and then fixed in 5% (v/v) acetic acid for 15 minutes. Photographs were taken of the membrane strips to record the results. Densitometric tracings of photographic transparencies were also made to show separation of the dehydrogenase enzymes.

Isoelectric Focusing

Isoelectric focusing procedures followed O. Vesterburg (1971) and the LKB 8100 Ampholine Electrofofocusing Instruction Manual. The following materials were obtained from LKB Produkter, Stockholm, Sweeden; Ampholine Column LKB 8101 (110 ml), gradient mixer, LKB 8121 (110 ml), LKB 10200 Perpex Pump (peristaltic pump), and Ampholine Carrier Ampholytes. Other materials included a LDC-UV monitor, Laboroatory Data Control, Rivera Beach, Fl.; a Toyo-Pulsa-HP-10 power supply, Toyo Kagaku Sang Yo Co. Ltd., distributed by Rainin Instrument Co. Inc., Boston, Mass.; and a Kontron recorder 1100, W-W Scientific Instruments Inc., Basel, Switzerland.

Two pH ranges, 3 to 10 and 4 to 6, were used in separating and determining isoelectric points of the two dehydrogenase enzymes. The broad pH range ampholyte mixture was used initially to find approximate isoelectric values. The narrow pH range ampholyte mixture was employed to effect better separation of the dehydrogenases. A linear sucrose gradient in the ampholyte mixture was used to help prevent diffusion of the protein bands.

The solutions used in the gradient mixer to prepare the column contained 1.9 ml carrier ampholytes, 37 ml distilled water, and 26 g sucrose in the dense solution mixing chamber and 0.6 ml carrier ampholytes and 51.5 ml distilled water with purified enzyme solution in the less dense solution mixing chamber. The electrodes were placed with the anode in the central tube (down) and the cathode at the top of the column. The anodic electrolyte solution contained 0.05 ml concentrated phosphoric acid in 15 ml distilled water with 11 g sucrose. Diethylamine (2 g per 100 ml distilled water) was used as the cathodic electrolyte solution. A focusing experiment lasted for 36 hours at 600 volts and 5 C. Fractions of 1 ml were collected from the column and pH measured at 5 C. The dehydrogenase enzyme activities were estimated at pH 7.2 with the DCIP reduction assay and total proteins estimated with a UV monitor and recorder at 280 nm as they eluted from the column.

Starch Gel Electrophoresis

The method of O. Smithies (1955) with modifications as suggested by A. H. Gordon (1969) was used in the horizontal starch gel electrophoresis experiments. Hydrolyzed starch was obtained from Electrostarch Co., Madison, Wis.

Preparation of the starch gel consisted of mixing 400 ml of the Tris-barbitol buffer with 40 g of the hydrolyzed starch. Mixing was continued until a lump free suspension was formed.

The suspension was then heated over a bunsen burner in a vacuum flask with continuous swirling. Heating was continued until the suspension became viscous and then changed to a less viscous state. The less viscous starch suspension was then degassed with a partial vacuum for a period of 60 to 90 seconds. The hot degassed starch suspension was then evenly poured into a plexiglass mold (19.5 mm x 17.5 mm x mm deep). After 45 minutes cooling at room termperature, the gels were covered with plastic wrap and allowed to set at least 2 hours before use. Gels were always used within 12 hours after preparation.

Sections of filter paper (Whatman #1) saturated with the purified enzyme sample were inserted into a narrow slit cut the width and depth of the gel. The sections of filter paper were placed so that they did not extend outside the dimensions of the gel slit. Enzyme samples were electrophoresed for 24 hours with 50 ma at 4 C. A Heathkit Regulated H.V. power supply served as the power source. The buffer that consistently gave the best separation of the dehydrogenase enzymes was Tris-barbitol (Materials and Methods, Microzone electrophoresis, page 28) with 0.025% (w/v) Triton X-100 at a running pH of 8.6. Horizontal gel slices were stained with MTT tetrazolium as given in the methods for Microzone electrophoresis (page 29).

Chemicals

Lysozyme (3 x crystallized) and deoxyribonuclease (1 x crystallized) were purchased from Sigma Chemical, St. Louis, Mo. All other chemicals, unless specifically referred to, were of reagent grade quality.

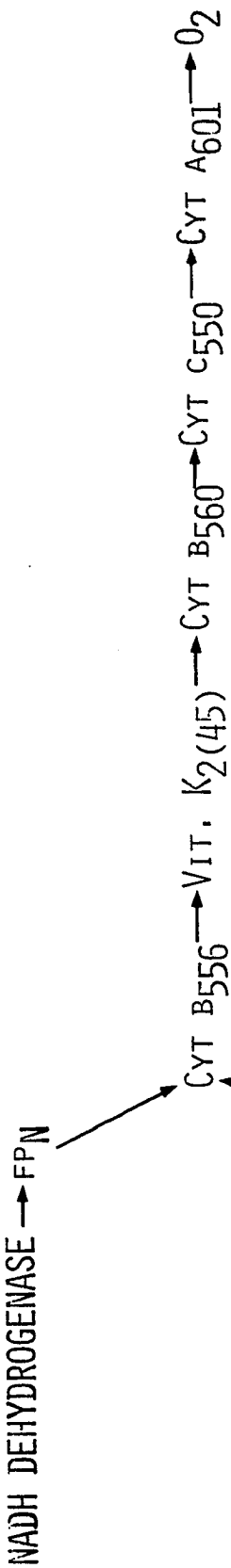
RESULTS

Proposed Electron Transport Scheme of L-malate and NADH Oxidases in Micrococcus lysodeikticus

A proposed shceme of electron transport for L-malate and reduced nicotinamide adenine dinucleotide oxidases is depicted in figure 1. Electrons enter the cytochrome system through either of the dehydrogenase enzymes. Current evidence suggests that these enzymes are flavoproteins and have been labeled fp_M and fp_N for L-malate and NADH dehydrogenases respectively (Eisenberg, R.C., 1972). The electrons flow from the primary dehydrogenase enzymes through a b type (probably b_{556}) cytochrome. Gel'man et al. has suggested that both of the dehydrogenase enzymes pass their electrons to a common cytochrome b indicated in figure 1 as Cyt b_{556} (Gel'man, N.S., et al., 1970). However, there is now current data which make this interpretation somewhat questionable (Eisenberg, R.C. and B.B. Smith, unpublished data) since these dehydrogenase enzymes may have b type cytochrome(s) as a functional prosthetic group. High resolution liquid nitrogen difference spectra of purified dehydrogenases with both physiological and dithionite reduced minus minus oxidized samples are needed before the correct sequence can be established. After reduction of cytochrome b, electrons from both of the dehydrogenase enzymes flow through vitamin $K_{2(45)}$. M. Fujita et al. (1966) characterized vitamin $K_{2(45)}$ in M. lysodeikticus. R. C. Eisenberg (1972)

Figure 1

Proposed Electron Transport Scheme of L-malate and NADH Oxidases in Micrococcus lysodeikticus. Abbreviations: fp_N, NADH dehydrogenase flavoprotein; fp_M, L-malate dehydrogenase flavoprotein; Cyt, cytochrome.



confirmed that napthoquinone is an obligatory component of the electron transport system; this work has also shown the correct position of the napthoquinone in the electron transport chain through black light inactivation of the napthoquinone and reduced minus oxidized difference spectra. Thus, electrons flow from vitamin $K_{2(45)}$ through cytochromes b, c, and a to molecular oxygen (Eisenberg, R.C., 1971 and 1972; Gel'man, N.S. et al., 1970). Cytochrome a is apparently the only terminal oxidase in this system since cytochrome o has not been detected in M. lysodeikticus (Smith, L., 1954).

Distribution of Deoxycholate Extracted and Subsequent Ammonium Sulfate Precipitated L-malate and NADH Dehydrogenases from Micrococcus lysodeikticus Membranes

Deoxycholate extraction of M. lysodeikticus membranes solubilized both L-malate and NADH dehydrogenases along with a cytochrome b (Eisenberg, R.C., 1972). Centrifugation of the disrupted membranes at 100,000 x g for 60 minutes left most of the dehydrogenase activities in the disrupted membrane supernatant as indicated by results shown in figure 2 and table 1. Further procedures were performed to purify the dehydrogenase enzymes and to separate them. These procedures are shown in the flow diagram presented in figure 2 and the distribution of the dehydrogenases in the fractionation procedure is given in table 1.

There was essentially no loss of protein in the purification procedure shown in figure 2. The total protein per fraction in

Figure 2

Flow diagram of the Deoxycholate Extractions and Subsequent Ammonium Sulfate Precipitations of Micrococcus lysodeikticus Membranes. Disrupted membranes, supernatant and particulate fractions, and ammonium sulfate precipitations were prepared as indicated; further experimental details are given in the Materials and Methods, pages 21 and 22. The enzyme activity and amount of purification at each numbered step in the flow diagram is given in table 1. Abbreviations: DOC, sodium deoxycholate; Tris-hydrochloride, 0.05 M Tris-hydrochloride.

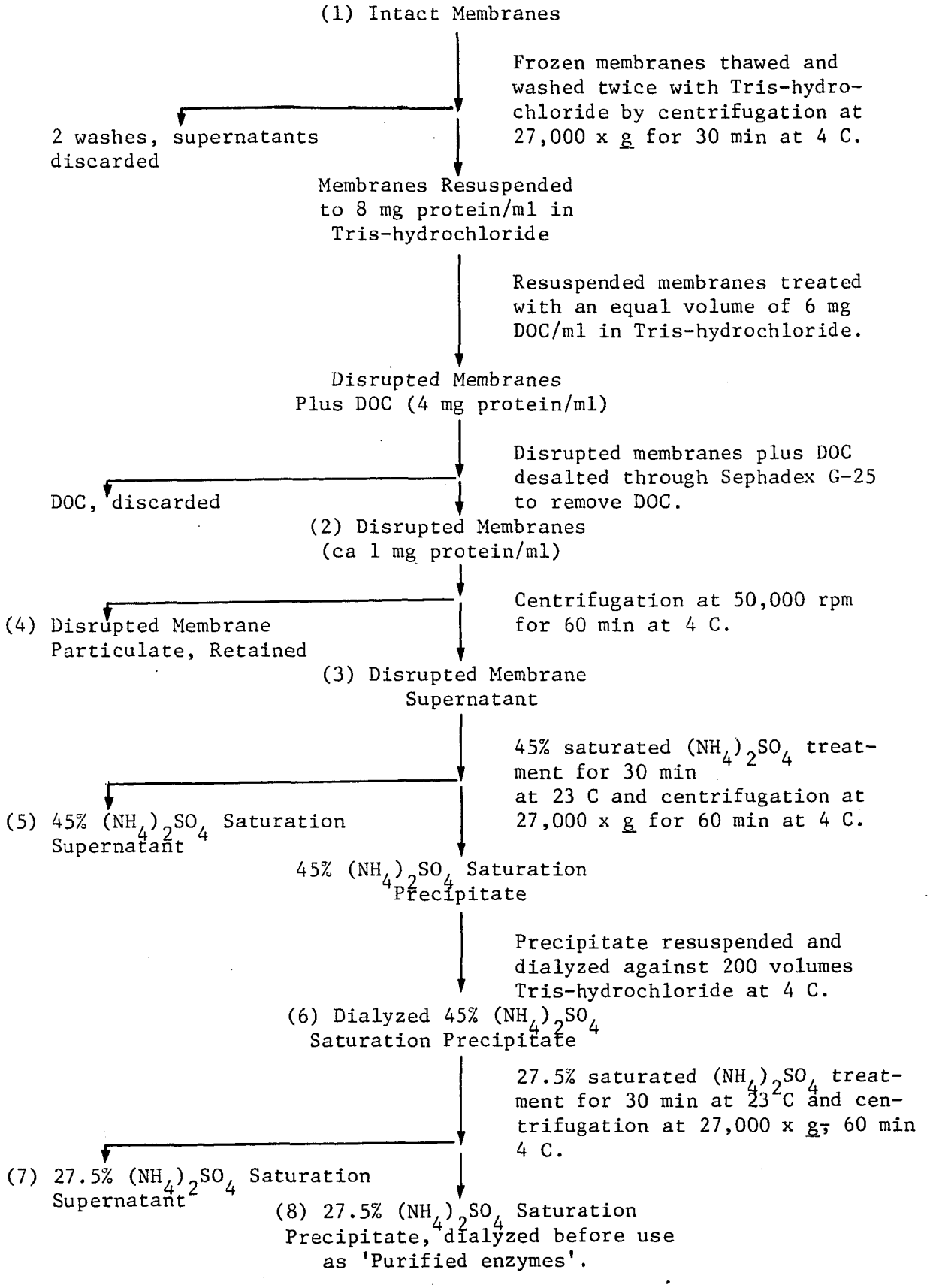


Table 1

Distribution of Deoxycholate Extracted and Subsequent Ammonium Sulfate Precipitated L-malate and NADH Dehydrogenases from Micrococcus lysodeikticus Membranes. Disrupted membranes, supernatant and particulate fractions, and ammonium sulfate fractions were prepared as indicated in figure 2. Particulate material was diluted to the original volume of the disrupted membranes with Tris-hydrochloride before assaying for enzyme activity. Intact membranes were sonicated 4 times for 10 seconds each before assaying for enzyme activity. Specific activities are units dehydrogenase per mg protein. Abbreviations: LM, L-malate dehydrogenase, NADH, NADH dehydrogenase.

Fraction	Fraction Volume, ml	Total Units Enzyme Activity Per Fraction		Total Protein Per Fraction, mg	Specific Activity		Ratio
		LM	NADH		LM	NADH	NADH/LM
1. Intact Membranes	10.0	31.60	95.47	93.60	0.338	1.020	3.02
2. Disrupted Membranes	80.0	14.72	76.16	95.20	0.155	0.800	5.17
3. Disrupted Membrane Supernatant	78.67	12.27	59.40	62.94	0.195	0.944	4.84
4. Disrupted Membrane Particulate	80.0	2.56	8.40	28.80	0.089	0.292	3.28
5. 45% $(NH_4)_2SO_4$ Saturation Supernatant	90.0	0.21	4.22	18.90	0.011	0.223	1.97
6. 45% $(NH_4)_2SO_4$ Saturation Precipitate	80.0	16.80	46.56	47.04	0.357	0.990	2.77
7. 27.5%$(NH_4)_2SO_4$ Saturation Supernatant	86.0	15.27	36.46	29.93	0.510	1.218	2.39
8. 27.5%$(NH_4)_2SO_4$ Saturation Precipitate	80.0	8.16	28.40	19.20	0.425	1.480	3.48

steps 4, 5, 7, and 8, in table 1, (96.83 mg) compared fairly well with the total protein in the intact membranes, i.e., fraction 1 (93.60 mg). There was a slight difference between the two figures but it was believed to be within the limits of accuracy of the methods for protein determination.

L-malate dehydrogenase was greatly inhibited by the action of DOC. Approximately 50% of the initial total L-malate dehydrogenase activity was lost between steps 1 and 2. All of the L-malate dehydrogenase activity was conserved in the disrupted membrane supernatant and particulate fractions 3 and 4. Ammonium sulfate treatment of the disrupted membrane supernatant appeared to stimulate L-malate dehydrogenase with an increase of 14%. Even after allowing a 5% error (as was allowed for the interpretation of all enzyme activity measurements) there was still a significant total activity increase from step 3 to steps 5 and 6 in table 1. A second ammonium sulfate treatment caused further stimulation of L-malate dehydrogenase. There was a 1.4 fold increase in total activity between steps 6 and 7 plus 8 in table 1. The mechanism of stimulation of L-malate dehydrogenase activity by ammonium sulfate was not elucidated. It may be a catalytic function or one of physical rearrangement of protein conformation. There was an optimal concentration of ammonium sulfate stimulation of L-malate dehydrogenase activity between the two concentrations of ammonium sulfate used. The 27.5% ammonium sulfate saturation treatment caused the greatest stimulation of activity.

NADH dehydrogenase total activity was also inhibited by DOC treatment in steps 1 and 2 as seen in table 1. This inhibition was not as great as for L-malate dehydrogenase between steps 1 and 2. There was a loss of total NADH dehydrogenase activity between the disrupted membranes, step 2, and the disrupted membrane supernatant and precipitate, steps 3 and 4. This effect has been noticed previously (Eisenberg, R.C., 1972). The 45% ammonium sulfate saturation treatment inhibited total NADH dehydrogenase activity. This was in contrast to the stimulatory effect on L-malate dehydrogenase. Approximately 9% of the total NADH dehydrogenase was lost between steps 3 and 5 plus 6. The 27.5% ammonium sulfate saturation treatment had a contrasting effect on total NADH dehydrogenase activity as compared to the first ammonium sulfate treatment. There was a 1.4 fold stimulation of total NADH dehydrogenase activity between steps 6 and 7 plus 8 in table 1. The mechanism of inhibition and stimulation of NADH dehydrogenase activity by the two concentrations has not been determined. The mechanism may be one of differential conformational changes with the lower concentration of ammonium sulfate forming a more active conformation of the enzyme.

Some of the changes in dehydrogenase activities were also reflected in the ratios of total NADH dehydrogenase activity to total L-malate dehydrogenase activity. This ratio increases from 3.02 to 5.17 from the intact membranes to the disrupted membranes, steps 1 and 2 respectively in table 1. Again, this reflected the

greater lability of L-malate dehydrogenase activity to the effect of DOC. The ratios for the disrupted membrane supernatant and particulate showed the loss of NADH dehydrogenase activity from the disrupted membranes at step 1. A slightly greater proportion of NADH dehydrogenase activity remained in the disrupted membrane supernatant after the centrifugation at step 2. This caused a higher ratio at step 3 as compared to step 4. This may mean that the amount of solubilization of the two enzymes may not have been the same. The 45% ammonium sulfate saturation treatment inhibited NADH dehydrogenase activity and stimulated L-malate dehydrogenase activity. This was again reflected in the ratios for steps 5 and 6. The 27.5% ammonium sulfate saturation treatment stimulated both L-malate and NADH dehydrogenase activity but the ratio values at steps 7 and 8 were found to be relatively close to the ratio for the intact membranes at step 1 in table 1. This indicated little separation of the dehydrogenase enzymes.

Specific activity calculations (units enzyme activity per mg protein) were made to determine purification of the dehydrogenase enzymes. No significant purification of the dehydrogenases enzymes was achieved as found by comparing the specific activities at steps 1, 7, and 8; however, a significant portion of the total protein was removed before the final ammonium sulfate treatment. A significant percentage of the dehydrogenase activities was destroyed by the action of DOC. This accounted for the drop in specific activities between the intact and disrupted membranes.

Ammonium sulfate stimulated L-malate dehydrogenase activity but this resulted in specific activities at steps 7 and 8 that were only slightly higher than in the intact membranes. NADH dehydrogenase was both inhibited and stimulated by ammonium sulfate treatment. The combined effect of the stimulation and inhibition of NADH dehydrogenase activity increased the specific activity only slightly in the purified samples at steps 7 and 8. However, the greatest loss of both enzyme activities occurred with DOC treatment. This appeared to be the main reason for the lack of purification of the dehydrogenase enzymes.

Fraction 8 was found to contain less than 1% nucleic acid as determined by a 280/260 ratio. This was considerably lower than nucleic acid concentrations in the other fractions and was therefore used as the 'purified enzymes' in the electrophoretic studies that followed.

Use of Sepharose, Sephadex G-200, and Sucrose Gradients in the Separation of L-malate and NADH Dehydrogenases

A number of attempts have been made using Sepharose and Sephadex G-200 column chromatography as well as sucrose gradient centrifugation to separate L-malate and NADH dehydrogenase activities. I. M. Tsfasman et al. (1971) attempted separation with negative results of these enzymes with Sepharose 4B chromatography after solubilization of the membrane proteins with versene and Tween 80. Similar negative results were

obtained by I. M. Tsfasman et al. (1971) with Sephadex G-200 and sucrose gradient fractionations. D. N. Ostrovskii et al. (1968) has also performed the same types of experiments using sodium cholate solubilized membrane proteins and found no separation of these dehydrogenase activities. R. C. Eisenberg and B. B. Smith (unpublished data) obtained the same negative results with DOC extracted dehydrogenases. L-malate and NADH dehydrogenase activities were found to elute in the void volume of Sephadex G-200 columns indicating molecular weights of over 1 million. In sucrose gradient studies, both in the presence and absence of magnesium ions, both dehydrogenases occurred in the same fractions (Eisenberg, R.C., 1972).

Polyacrylamide Disc Gel Electrophoresis of L-malate and NADH Dehydrogenases

Both L-malate and NADH dehydrogenases have large and very similar molecular weights. Other methods based on different criteria for separation were employed since separation based on differences in molecular size did not seem feasible.

Disc gel electrophoresis is a standard means of protein separation based on differences in electrophoretic mobility of proteins within a cross linked molecular sieve of polyacrylamide. This technique has been used in the separation of many types of proteins (Gordon, A.H., 1969). Since both L-malate and NADH dehydrogenases can be stained selectively via MTT tetrazolium dye reduction it was believed that this method would be ideal for

separation. The results obtained from such experiments were inconclusive. Both enzymes failed to migrate in a number of different buffer systems containing either detergent or urea. All enzyme activity was located at the top of the gels with the MTT tetrazolium staining procedure. Use of sodium dodecyl sulfate in the disc gel electrophoresis buffer system eliminated all L-malate dehydrogenase activity; it was not known therefore if the L-malate dehydrogenase did migrate. NADH dehydrogenase activity was not eliminated by sodium dodecyl sulfate but the enzyme did not migrate in the presence of this detergent. An interesting observation was that dehydrogenase activity always occurred in the top millimeter of the gel in the systems where the dehydrogenase activity was detected. The enzymes entered the gel but migration abruptly ceased. It was not known whether these enzymes have a particular affinity for the polyacrylamide gel or if the large molecular size of the enzymes precluded migration through these gels.

It became apparent from the above results that future success in separating these dehydrogenase enzymes via other methods might depend on the presence of detergent throughout the separating procedure. This possibility necessitated a study of the effect of the detergents on enzyme activity before the dehydrogenases could be retained in the presence of detergent for extended periods of time. Two detergents were tested, a slightly anionic detergent, sodium deoxycholate (DOC) and a nonionic detergent,

Triton X-100. Sodium dodecyl sulfate was not tested since it was already known to inactivate L-malate dehydrogenase (as described above).

Effect of Deoxycholate and Triton X-100 on L-malate and NADH Dehydrogenase Activities

The 'purified enzyme sample' (fraction 8, table 1), as used in the electrophoretic studies, was used in these experiments. This material was used to facilitate a direct comparison between immediate and long term effects of the detergents on dehydrogenase activity. By using this material, results were not complicated by unmasking effects found in untreated membranes (Eisenberg, R.C., 1970a).

Figure 3 shows the effect that varying concentrations of DOC had on L-malate and NADH dehydrogenase activities. The effect of the lower concentrations of DOC (up to 3 mg DOC per ml) on NADH dehydrogenase activity was minimal although the enzyme was significantly inhibited by higher concentrations of DOC. The inhibition of L-malate dehydrogenase activity by DOC was more drastic since at low detergent concentration (2 mg per ml) 50% of the initial L-malate dehydrogenase activity was lost. Further inhibition was not observed at detergent concentrations greater than 6 mg per ml.

The effect of DOC concentration on dehydrogenase activity versus time is presented in figure 4. A concentration of 10 mg DOC per ml was used. Both L-malate and NADH dehydrogenase

Figure 3

Effect of Varying Concentrations of Deoxycholate on L-malate and NADH Dehydrogenase Activities. 'Purifies enzymes', fraction 8 in figure 2, served as the enzyme samples. DOC solutions of various concentrations (1.4 ml each in 0.05 M Tris-hydrochloride) were added to 1.4 ml enzyme samples (in Tris-hydrochloride). DOC concentrations indicated are those obtained after dilution and are final detergent concentrations. Enzyme-detergent mixtures were incubated for 5 min at 32 C before enzyme assay. DCIP [0.1 ml, (0.14 µmoles)] and the substrate, NADH [0.1 ml (1.0 µmole)] or L-malate [0.1 ml, (10 µmoles)] were added at the end of 5 min. Enzyme activity was assayed immediately following addition of the substrate. Enzyme activity in the absence of DOC was made with enzyme samples treated with 1.4 ml buffer in the same manner as the detergent treated samples. Symbols: △ , NADH dehydrogenase; ○ , L-malate dehydrogenase.

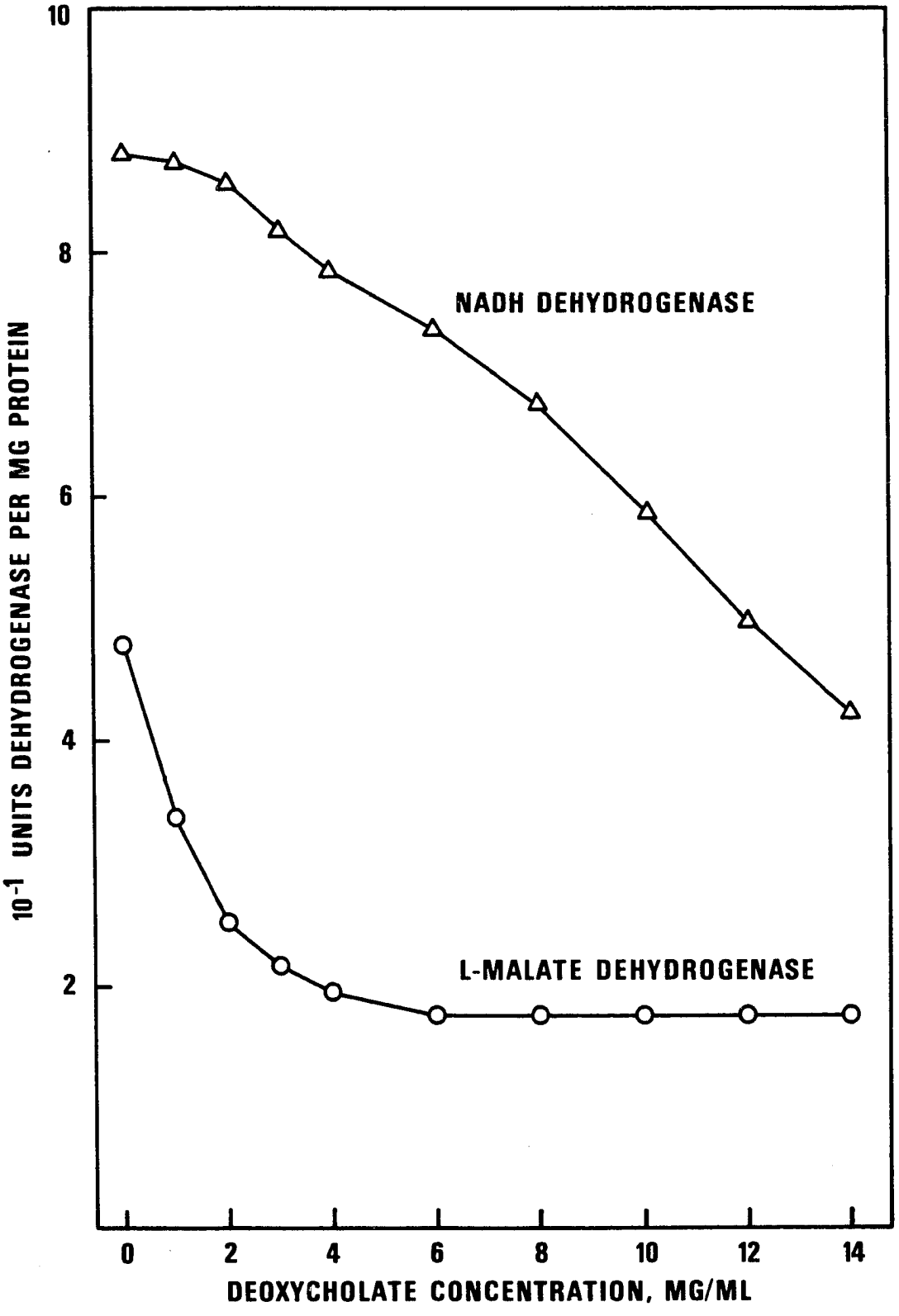
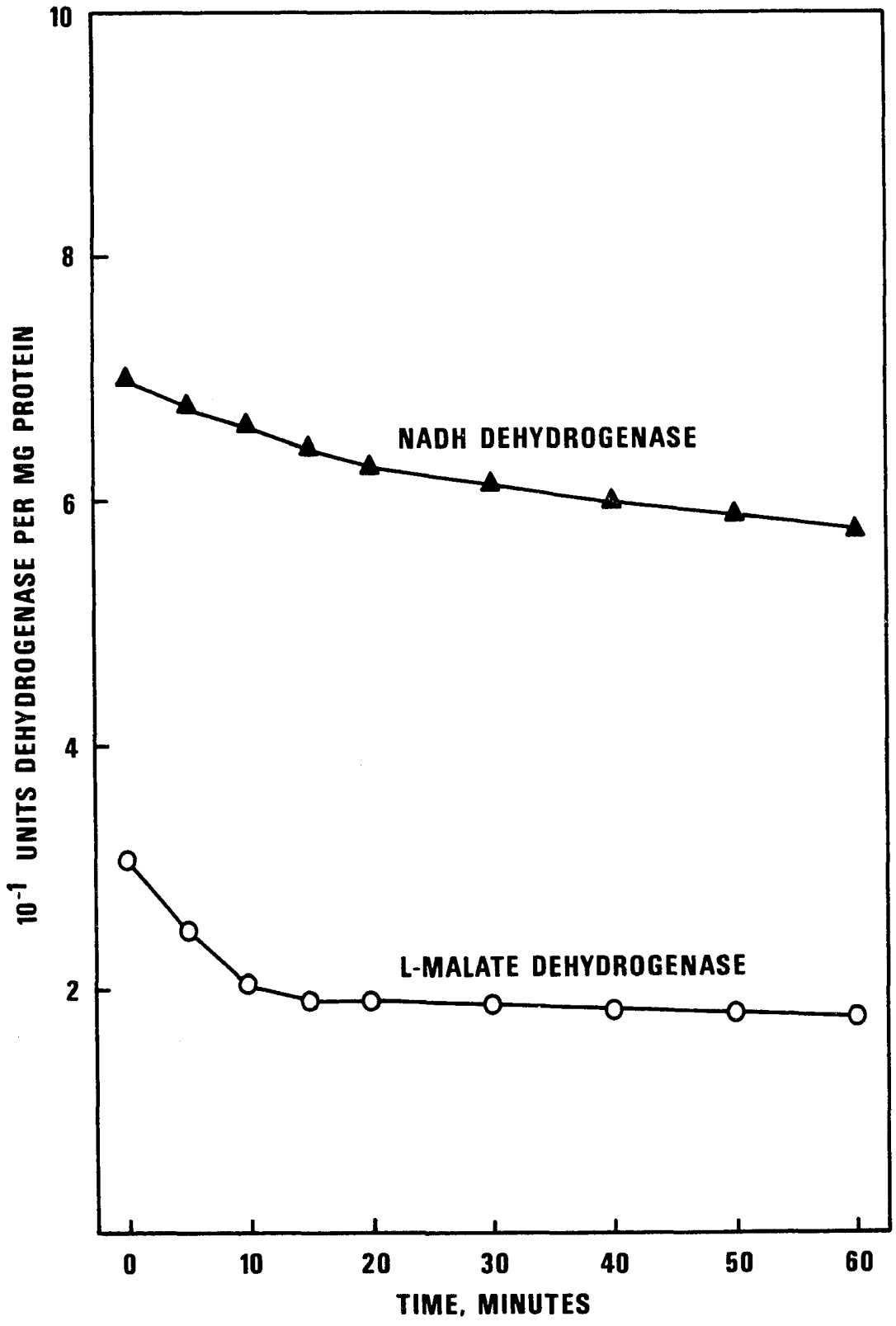


Figure 4

Effect of Deoxycholate on L-malate and NADH Dehydrogenase Activities Versus Time. 'Purified enzymes' in step 8 figure 2 served as the enzyme sample. The enzyme sample was treated with 10 mg DOC per ml with stirring at 32 C. At the time intervals indicated, 2.8 ml of the enzyme detergent mixture was assayed for enzyme activity by adding 0.1 ml (0.14 µmoles) DCIP and the substrate, NADH [0.1 ml (1.0 µmole)] or L-malate [0.1 ml (10 µmoles)]. Enzyme activity was assayed immediately after addition of substrate. Controls: Initial enzyme activities before addition of DOC were 0.86 and 0.49 units per mg protein for NADH dehydrogenase and L-malate dehydrogenase respectively. Symbols: ▲ , NADH dehydrogenase; ○ , L-malate dehydrogenase.



activities were rapidly inhibited by DOC since control experiments, no DOC present, showed more activity present than was found after addition of detergent. Thus, addition of DOC and rapid initial dehydrogenase enzyme assays, indicated as zero time in figure 4, showed an initial loss of 19% NADH dehydrogenase and 39% L-malate dehydrogenase when compared to the enzyme activities present before addition of DOC (legend to figure 4). After the initial mixing time inhibition indicated above, both dehydrogenase activities were slowly lost as a function of time as indicated in figure 4.

Further investigations have shown that loss of initial L-malate dehydrogenase activity via deoxycholate inhibition can be partially restored by removal of deoxycholate. This partial reversal of DOC inhibition of L-malate dehydrogenase activity is shown in table 2. Incubation of enzyme with detergent reduced the L-malate dehydrogenase activity to ca 28% of the initial activity whereas removal of detergent after DOC treatment gave ca 50% of the initial activity. These results indicate that although DOC inhibits L-malate dehydrogenase a partial restoration can be achieved by removing detergent and thus separation procedures using DOC may be tenable if the detergent is removed before assaying for L-malate dehydrogenase.

The effect of Triton X-100 on these dehydrogenase activities was also studied. These experiments were performed similar to the DOC experiments described above. Figure 5 shows the effect of varying concentrations of Triton X-100 on the dehydrogenase

Table 2

Partial Restoration of DOC Treated L-malate Dehydrogenase Activity by Removal of Detergent

Enzyme Sample	Units of Dehydrogenase Activity per mg Protein	
	L-malate dehydrogenase	NADH dehydrogenase
Initial Activity [a]	0.707	1.905
DOC Treated Enzymes [b]	0.198	1.715
Desalted Enzymes [c]	0.350	1.719

[a]Enzymes samples before treatment with DOC: prepared from the 'Purified enzymes'. All samples assayed by the DCIP reduction assay.

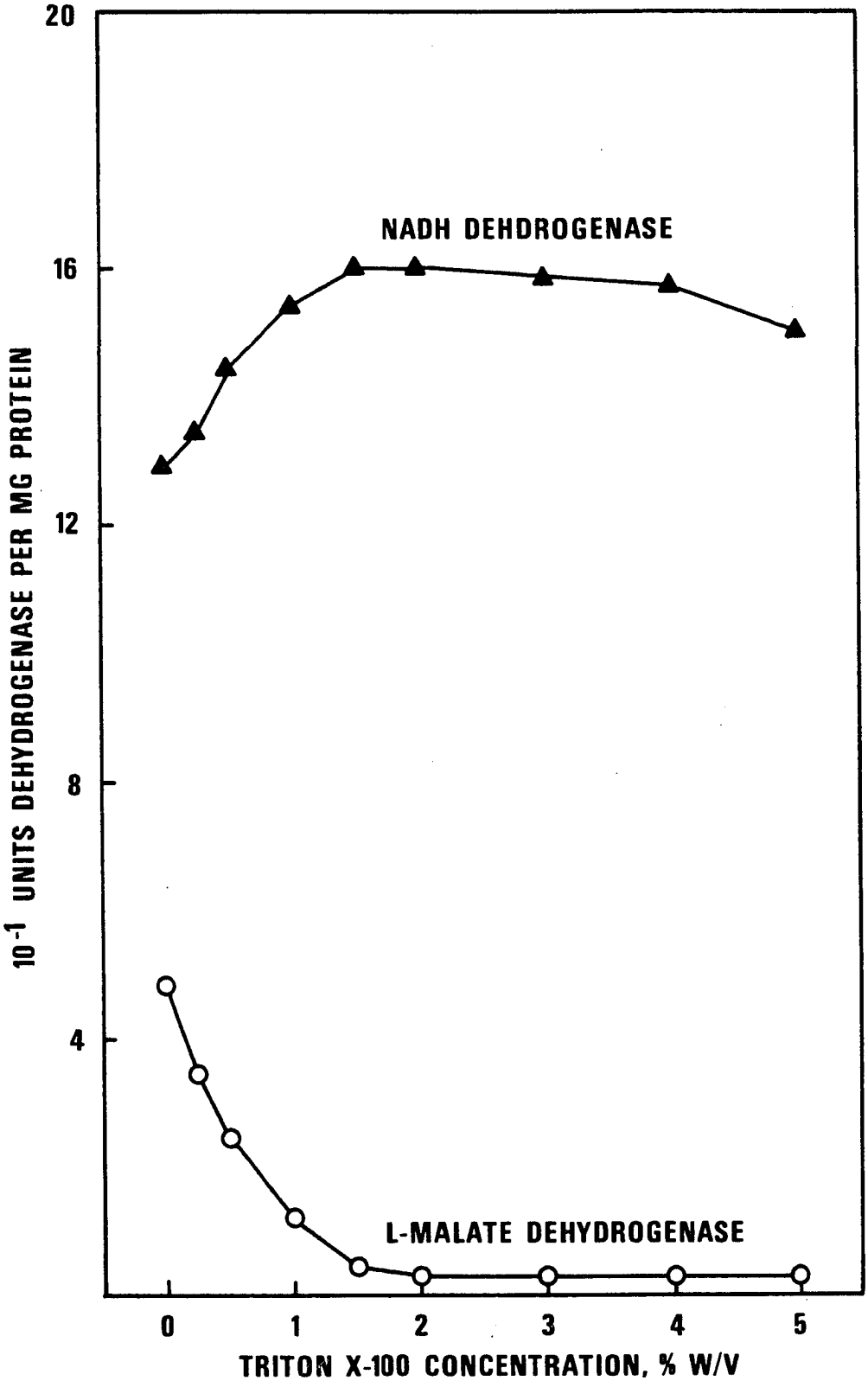
[b]Enzyme samples treated with 6 mg DOC per ml for 15 min at 32 C. DOC concentration was not altered before enzyme assays.

[c]DOC treated enzymes desalted by passing through a Sephadex G-25 column with the proteins coming out in the void volume.

Figure 5

Effect of Varying Concentrations of Triton X-100 on L-malate and NADH Dehydrogenase Activities. 'Purified enzymes', fraction 8 in figure 2 served as the enzyme samples. Triton X-100 solutions of various concentrations (1.4 ml each in 0.05 M Tris-hydrochloride) were added to 1.4 ml enzyme samples (in Tris-hydrochloride). Triton X-100 concentrations indicated are those obtained after dilution and are final detergent concentrations. Enzyme-detergent mixtures were incubated for 5 min at 32 C before enzyme assay. DCIP [0.1 ml, (0.14 µmoles)] and the substrate, NADH [0.1 ml (1.0 µmole)] of L-malate [0.1 ml, (10 µmoles)] were added at the end of 5 min. Enzyme activity was assayed immediately following addition of the substrate. Enzyme activity in the absence of DOC was made with enzyme samples treated with 1.4 ml buffer in the same manner as the detergent treated samples.

Symbols: ▲ , NADH dehydrogenase; ○ , L-malate dehydrogenase.



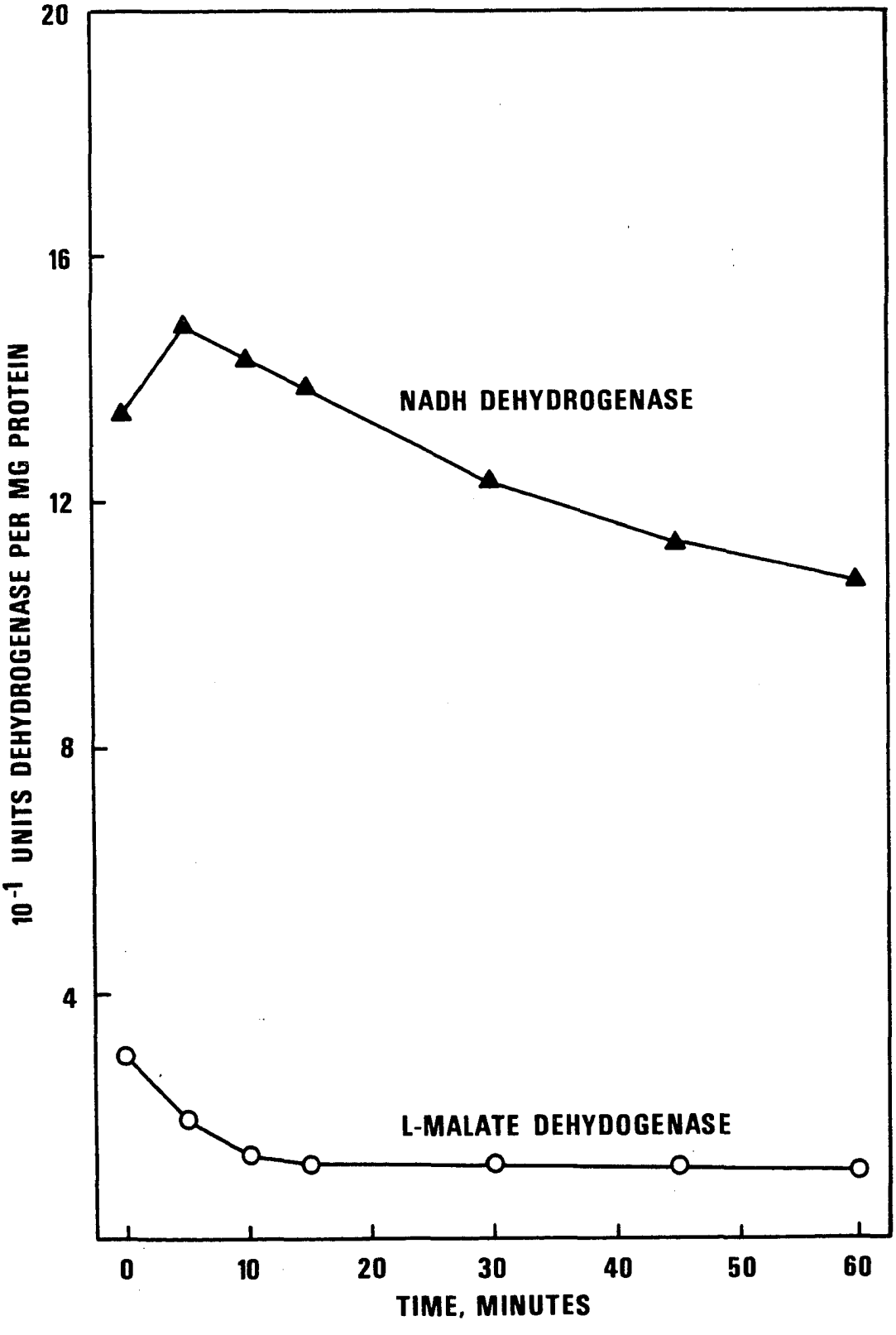
activities. NADH dehydrogenase was stimulated by low concentrations of Triton X-100. At higher concentrations this activity was slightly inhibited. Triton X-100 affected L-malate dehydrogenase activity in the same manner as did DOC although there were two important differences which should be noted. L-malate dehydrogenase inhibition was not nearly so great at the lower concentrations of Triton X-100 as with the lower concentrations of DOC. However, at the higher concentrations of Triton X-100 almost total inhibition of L-malate dehydrogenase activity was found.

Figure 6 shows the effect of a constant concentration of Triton X-100 on dehydrogenase activity versus time. A concentration of 0.5% (w/v) Triton X-100 was used in such a manner that detergent concentration was not altered before assaying for enzyme activity. NADH dehydrogenase activity was stimulated by low Triton X-100 concentration. This stimulation was temporary, however, since the activity began to decrease slowly after 5 minutes. At the end of 60 minutes, ca 89% of the initial NADH dehydrogenase activity (see legend to figure 6) still remained. L-malate dehydrogenase activity was inhibited with drastic inhibition occurring immediately upon addition of the detergent. Approximately 50% of the initial activity was lost within 5 minutes. The L-malate dehydrogenase activity was reduced ca 70% after 15 minutes and no further loss was observed up to 1 hour.

Figure 6

Effect of Triton X-100 on L-malate and NADH Dehydrogenase Activities Versus Time. 'Purified enzymes' in step 8 figure 2 served as the enzyme sample. The enzyme sample was treated with 0.5% (w/v) Triton X-100 with stirring at 32 C. At the time intervals indicated, 2.8 ml of the enzyme detergent mixture was assayed for enzyme activity by adding 0.1 ml (0.14 µmoles) DCIP and the substrate, NADH [0.1 ml (1.0 µmoles)] or L-malate [0.1 ml (10 µmoles)]. Enzyme activity was assayed immediately after addition of substrate. Controls: Initial enzyme activities before addition of Triton X-100 were 1.20 and 0.40 units per mg protein for NADH dehydrogenase and L-malate dehydrogenase respectively.

Symbols: ▲ , NADH dehydrogenase; ○ , L-malate dehydrogenase.



DEAE Sephadex Ion Exchange of L-malate and NADH Dehydrogenases

Past attempts at separating L-malate and NADH dehydrogenase activities with DEAE Sephadex ion exchange have met with little success (Ostrovskii, D.N. et al., 1968; Eisenberg, R.C. and B.B. Smith, unpublished data). However, this method was re-evaluated since success should allow a relatively simple preparative separation of the enzymes. Initial experiments involved a batch method of elution. Elution was attempted with progressively higher concentrations of NaCl and DOC. Some dehydrogenase activities were removed from the DEAE Sephadex but most of the initial activities remained either tightly bound to the column or was destroyed by the action of the detergent. The results are shown in figure 7. It can be seen in figure 7 that most of the total protein (see legend) was removed from the DEAE Sephadex by the elutants. This would seem to confirm the possibility that a great deal of the dehydrogenase activity was being inhibitied by the detergent. It should be noted that a relatively high concentration of detergent was required to elute the dehydrogenase enzymes. No separation of the dehydrogenase activities was found with the batch method.

A column of DEAE Sephadex was prepared to further investigate the possibilities of using ion exchange as a separation procedure. It was hoped that this method would provide a more efficient elution and recovery of enzyme activity.

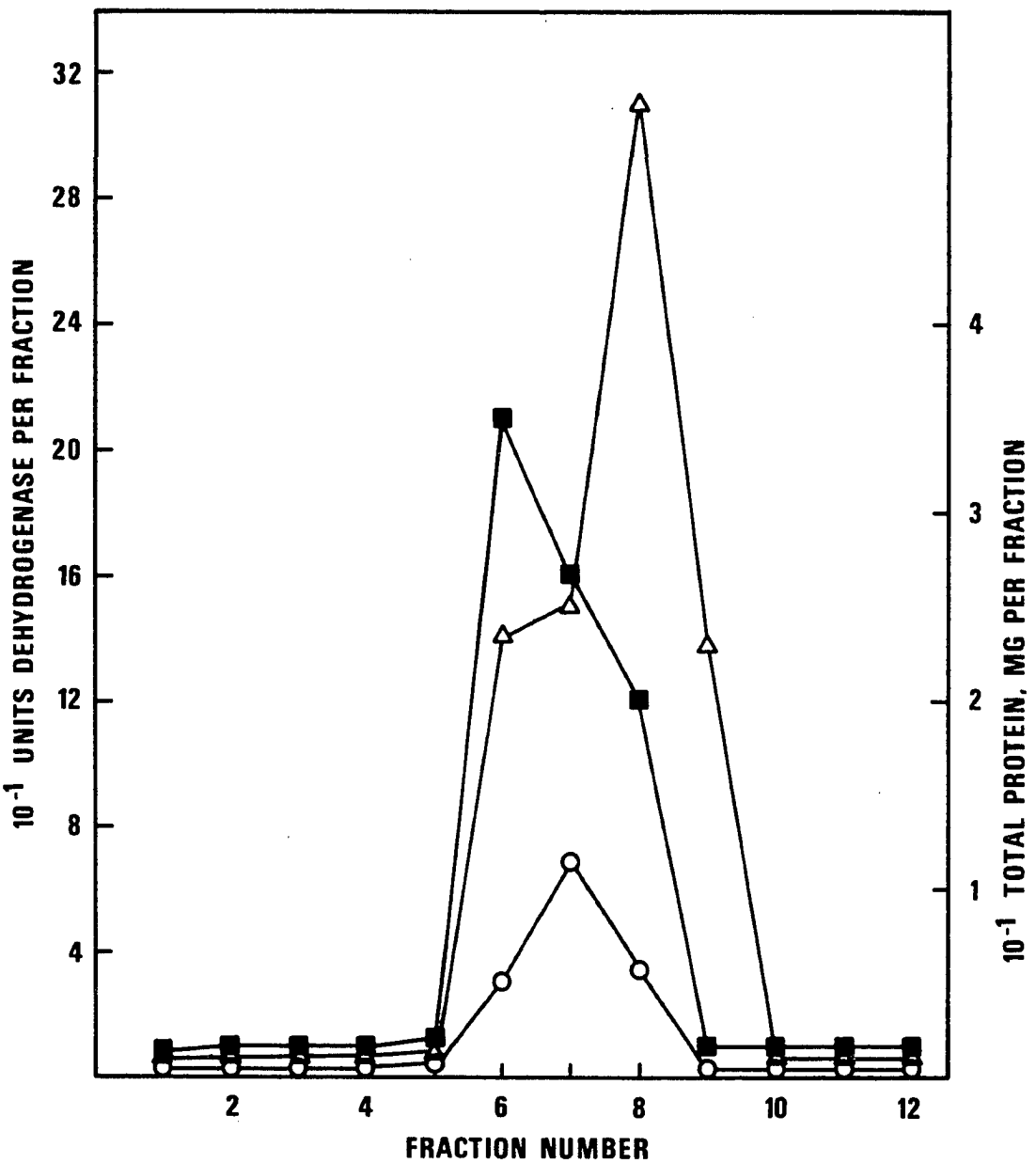
Figure 7

Elution of L-malate and NADH Dehydrogenases from DEAE Sephadex by a Batch Method. Enzyme solutions of known enzyme activity were added to the prepared DEAE Sephadex in a centrifuge tube. After mixing the enzymes with Sephadex, the mixture was centrifuged at 27,000 x g for 15 min and the supernatant decanted. All elutants were dissolved in 0.5 M Tris-hydrochloride, pH 7.2. Elutants were added to the protein-Sephadex precipitate, mixed, and the suspensions centrifuged with the elution volumes decanted as described above. All centrifugations were at 4 C. The elutant fractions were assayed for dehydrogenase activity and protein concentrations were estimated. Controls: Total enzyme activity units placed in the centrifuge tube were 30.73 and 10.15 for NADH and L-malate dehydrogenases respectively. Total protein placed in the centrifuge tube was 2.05 mg.

Symbols: △ , NADH dehydrogenase; ○ , L-malate dehydrogenase; ■ , protein.

Elutants for each fraction are listed below:

Fraction #	Elutant	Fraction #	Elutant
1.	Tris-hydrochloride	7.	2 M NaCl
2.	"	8.	0.5 mg DOC/ml
3.	"	9.	1 mg DOC/ml
4.	"	10.	2 mg DOC/ml
5.	0.5 M NaCl	11.	2 mg DOC/ml
6.	1 M NaCl	12.	2 mg DOC/ml



Three different elution systems were used in attempts to elute the dehydrogenase enzymes from the column in three separate experiments.

The first system employed 0.2 M acetate and 0.05 M Tris-hydrochloride buffers in a pH range from 4.0 to 9.5 as the elutants. Enzyme removal by these elutants was not detected (results not shown).

The second system consisted of progressively higher concentrations of NaCl followed by progressively higher concentrations of DOC. Both elutants, NaCl and DOC, were dissolved in 0.05 M Tris-hydrochloride and the pH was held constant at 7.2 at 23 C. No selective removal of either dehydrogenase enzyme was obtained by NaCl or DOC elution. Fraction 2, the second buffer wash, did contain NADH dehydrogenase in relatively high concentration while there was very little L-malate dehydrogenase activity in this fraction. It is possible that the column was saturated with NADH dehydrogenase and that the activity in fraction 2 was the excess NADH dehydrogenase. Further experiments would be needed to test this hypothesis; however, the amount of activity collected in fraction 2 was only a small fraction of the initial NADH dehydrogenase activity (see figure 8 and the legend to figure 8). Approximately 20% of the initial total protein was removed from the column by the NaCl and DOC elutants. Only a small fraction of the initial total dehydrogenase activities was detected in the elution volumes. Either the dehydrogenase

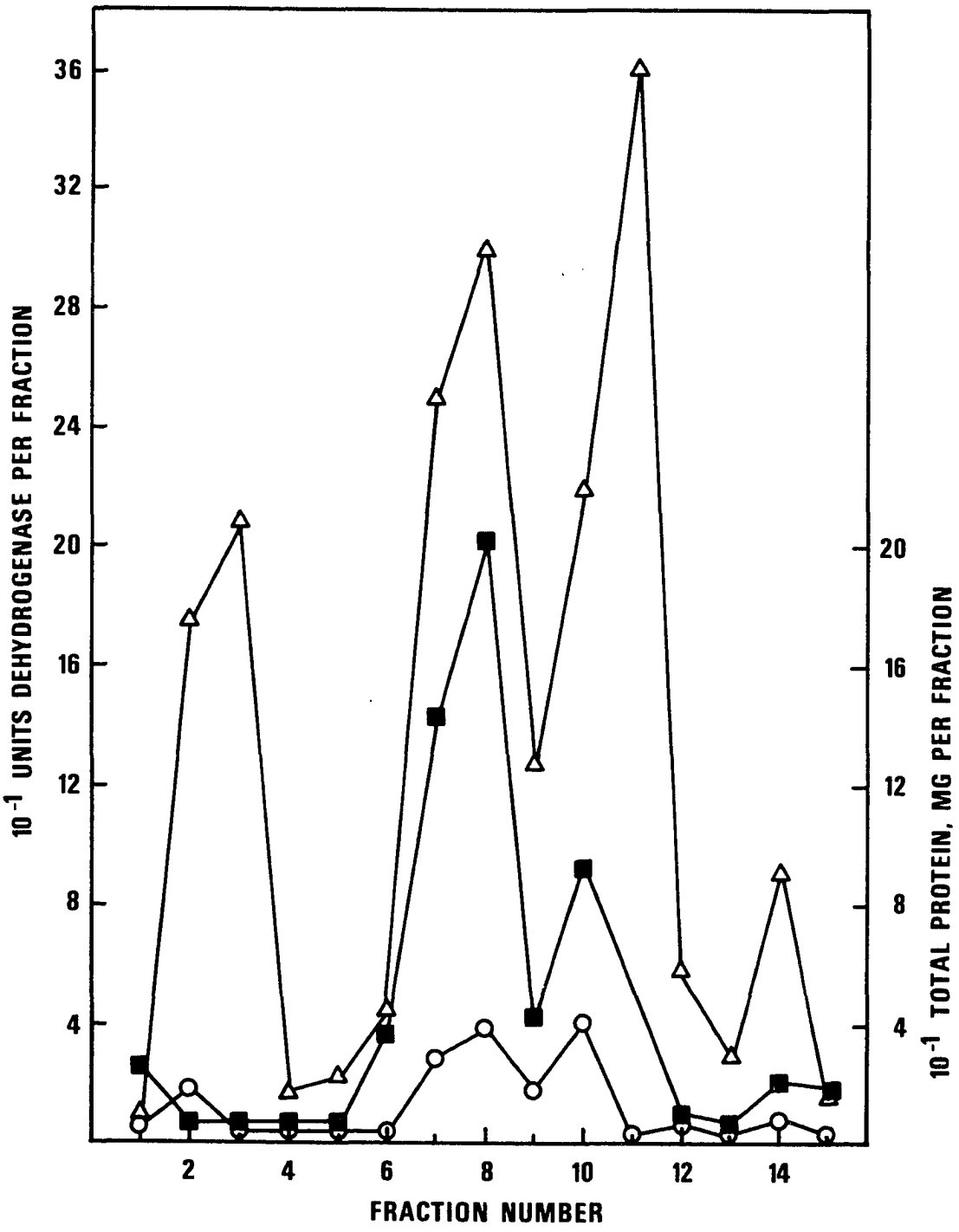
Figure 8

Elution of L-malate and NADH Dehydrogenases from DEAE Sephadex by a Column Method. The enzyme solution was added to the top of the column and washed with 50 ml 0.05 M Tris-hydrochloride (pH 7.2 at 23 C). Various elutants were passed through the column in attempts to elute the dehydrogenase activities. All elutants were dissolved in the Tris-hydrochloride buffer. Ten ml fractions were collected from buffer washes and other elutants. The eluted fractions were assayed for dehydrogenase activities and protein concentration was estimated. Controls: Total enzyme activity units placed on the column were 41.15 and 13.04 for NADH and L-malate dehydrogenases respectively. Total protein placed on the column was 2.23 mg.

Symbols: △ , NADH dehydrogenase; ○ , L-malate dehydrogenase; ■ , protein.

Elutants for each fraction are listed below:

Fraction #	Elutant	Fraction #	Elutant
1.	Tris-hydrochloride	9.	0.5 M NaCl
2.	"	10.	1 M NaCl
3.	"	11.	"
4.	"	12.	2 M NaCl
5.	"	13.	"
6.	0.5 M NaCl	14.	1 mg DOC/ml
7.	"	15.	2 mg DOC/ml
8.	"		



activities were retained on the column or they were retained on the column. In either case, as can be seen in figure 8 from the enzyme activity that was eluted, there was no complete separation of the dehydrogenase activities.

The final elutant system consisted of 0.5% (w/v) Triton X-100 in 0.05 M Tris-hydrochloride (pH 7.2 at 23 C). Essentially all of the protein placed on the column was eluted with Triton X-100. This did not serve the desired goal since both of the dehydrogenase enzymes were found in the elution volume (results not shown).

Microzone Electrophoresis of L-malate and NADH Dehydrogenases

No separation of the dehydrogenase enzymes was obtained using polyacrylamide disc gel electrophoresis. Only partial separation was obtained in the buffer wash of the DEAE Sephadex ion exchange. No complete separation of the dehydrogenases had been demonstrated. Gel'man and Ostrovskii's hypothesis that L-malate and NADH dehydrogenase activities were solubilized as a single block did seem to hold (Gel'man, N.S. et al., 1970; Ostrovskii, D.N. et al., 1968). Therefore, a purely analytical method of separation, Microzone electrophoresis, was attempted.

Microzone electrophoretic separation as with polyacrylamide disc gel electrophoresis is based on differences in protein mobility in an electric field. However, Microzone electrophoresis does not have the added parameter of molecular sieving as does polyacrylamide disc gel electrophoresis. The cellulose acetate

membranes employed in Microzone electrophoresis are very nonionic so that electrostatic binding of proteins to the membranes is not likely (Gordon, A.H., 1969).

Initial studies employing Microzone electrophoresis in the separation of L-malate and NADH dehydrogenases involved the use of various buffer systems at pH 7.2. It was known from prior work that both of the dehydrogenases were anionic. Therefore, higher pH buffers would place more charge on the proteins and increase their electrophoretic mobility. The Tris-barbitol buffer system (Materials and Methods, page) at a pH of 8.6 gave the best separation of the dehydrogenase enzymes. Figure 9 is a photograph of acetate membrane strips showing the separation of L-malate and NADH dehydrogenase. Both enzymes migrated towards the anode with NADH dehydrogenase migrating slightly faster. Dehydrogenase enzyme bands were stained selectively by adding MTT tetrazolium with the proper substrate, either L-malate or reduced nicotinamide adenine dinucleotide. In figure 9, strip A was stained for NADH dehydrogenase alone, strip B for both L-malate and NADH dehydrogenase, and strip C for L-malate dehydrogenase alone.

Normal clearing methods and hence normal densitometric tracings could not be made from the acetate membrane strips since the stain was only the deposition of reduced MTT tetrazolium in the band area of the enzymes. The reduced MTT tetrazolium stain was quickly washed away by the clearing procedures. This

Figure 9

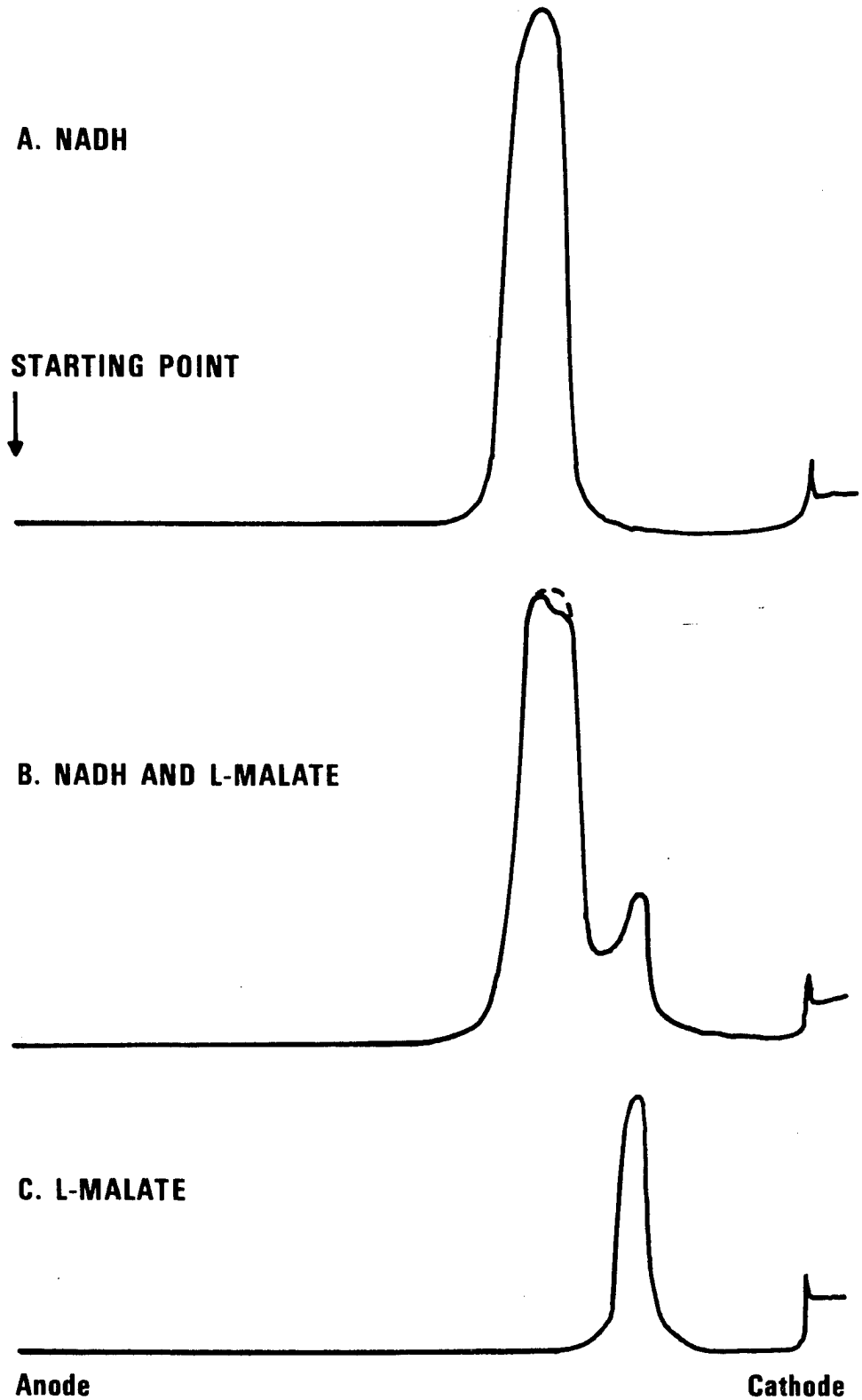
Microzone Electrophoresis of L-malate and NADH Dehydrogenases in the Absence of Mg^{2+}. A photograph of the acetate membranes shows the separation of NADH dehydrogenase from L-malate dehydrogenase. The electrophoresis was run at 150 volts constant voltage at 4 C for 90 minutes. The running pH was 8.6 in the Tris-barbitol buffer. Following electrophoresis, the acetate membrane was cut into three strips so that each strip had the migration pattern of one enzyme sample. With MTT Tetrazolium dye and by adding the proper substrate, each strip was stained for a particular enzyme. Strip A shows NADH dehydrogenase alone; strip B, both L-malate and NADH dehydrogenases; and strip C, L-malate dehydrogenase alone. No color developed without the substrate present.

necessitated finding an alternative method for obtaining densitometric tracings of the membrane strips. This was achieved by making a transparency from the photograph in figure 9. The densitometric tracings were then made from the transpacency. These tracings are presented in figure 10. Part A shows NADH dehydrogenase alone; part B, both L-malate and NADH dehydrogenase; and part C, L-malate dehydrogenase alone. The starting point for the tracings was determined on each strip by taking equidistant points from the origin of the enzyme samples (before electrophoresis). In trace B, NADH dehydrogenase is represented by a shouldered peak. This was probably due to an uneven deposition of the reduced MTT tetrazolium during staining. Also, the staining of this particular strip required an extra wash which may have removed some of the reduced MTT tetrazolium. Due to the nature in which the tracings were made, the areas under the peaks are not proportional to enxyme concentration; they serve only to show the separation of the dehydrogenase enzymes.

It is important to note that the enzyme samples did not come into contact with magnesium ions during the above separation. Magnesium ions have been shown to precipitate the DOC solubilized dehydrogenase enzymes (Eisenberg, R.C., 1972). It was therefore suspected that Mg^{2+} ions would bind the enzymes and prevent their separation. This postulation has been confirmed by the results shown in figure 11. A 'purified enzyme' sample was treated with 0.1 M $MgSO_4$ for 30 min at 32 C. The Mg^{2+} treated samples were

Figure 10

Densitometric Tracings of the L-malate and NADH Dehydrogenase Separations with Microzone Electrophoresis. Tracings were made from transparencies made from the photograph in figure 9, page Trace A shows NADH dehydrogenase alone; trace B, both L-malate and NADH dehydrogenases; and trace C, L-malate dehydrogenase alone. The starting points for the traces were determined by taking equidistant points from the origins of the enzyme samples (before electrophoresis). Further experimental procedures are given in Materials and Methods, Microzone electrophoresis section, pages 28-30.



then electrophoresed in the same manner as before. In figure 11, strip A was stained for NADH dehydrogenase alone, strip B for both NADH and L-malate dehydrogenases, and strip C for L-malate dehydrogenase alone; strip D was stained with a general protein stain. The results presented in figure 11 show no separation of the dehydrogenase enzymes and furthermore that the dehydrogenase enzymes fail to migrate. These effects were probably due to the formation of large protein complexes with Mg^{2+}. In strip D, it is evident that some of the Mg^{2+} treated proteins were able to migrate.

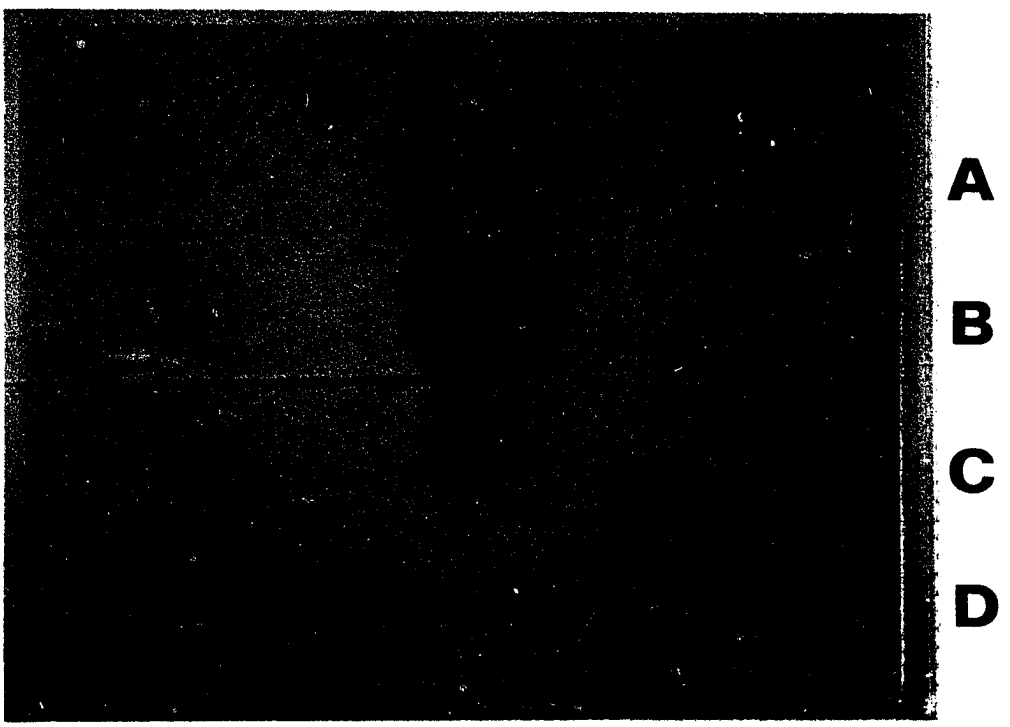
Isoelectric Focusing of L-malate and NADH Dehydrogenases

Isoelectric focusing provides a means of analytical separation of high molecular ampholytes based on their isoelectric points. Focusing of proteins is obtained by imposing a direct current potential on an electrolyte system where the pH steadily increases from the anode to the cathode. Since separation of two proteins requires a difference in isoelectric points of only 0.02 pH units, this method was attempted as a second possible means of showing the separation of L-malate and NADH dehydrogenase activities. This method also provides for the characterization of the enzymes by establishing their isoelectric points and is a possible means of preparative separation.

The first experiments were performed in the expanded pH range of 3 to 10 in order to determine the approximate pH at

Figure 11

Microzone Electrophoresis of L-malate and NADH Dehydrogenases in the Presence of Mg^{2+}. A sample containing both of the dehydrogenase enzymes was treated with .1M $MgSO_4$ for 30 minutes at 32 C. Samples were then electrophoresed at 150 volts constant voltage at 4 C for 90 minutes. The running pH was 8.6 in Tris-barbitol buffer. Following electrophoresis, the acetate membrane was cut into four strips so that each strip had the migration pattern of one enzyme sample. With MTT Tetrazolium dye and by adding the proper substrate, each strip was stained for a particular enzyme. Strip A shows NADH dehydrogenase alone; B, both L-malate and NADH dehydrogenases; C, L-malate dehydrogenase alone; strip D is a general protein stain. No color developed on the MTT stained strips without the substrates present.



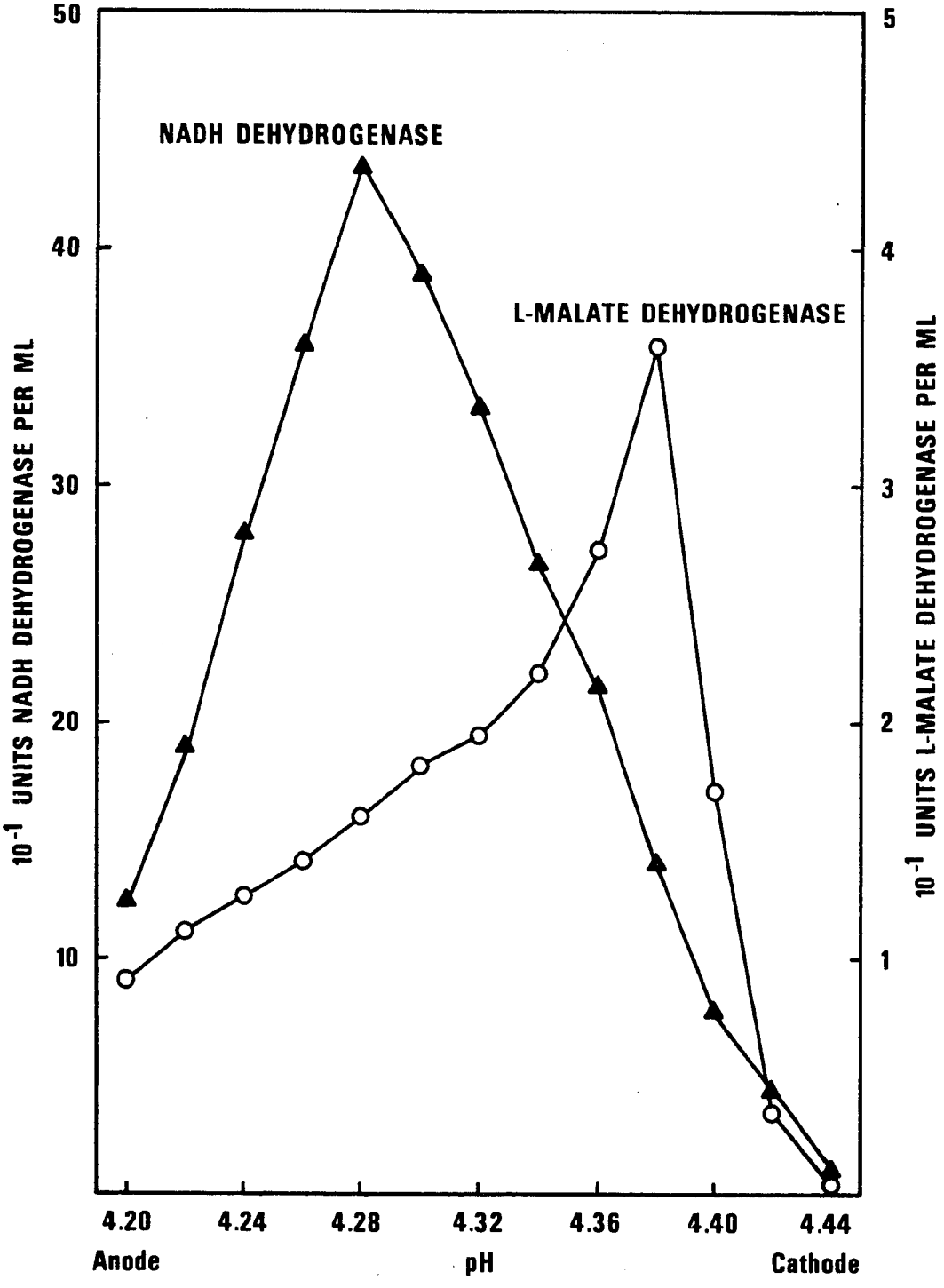
which the enzymes would focus. Both enzymes were found to migrate to a pH of about 4.5. The more narrow pH range, 4 to 6, was chosen for the other experiments in order to test for separation of the dehydrogenase activities. Figure 12 shows the results of the second experiment. Both dehydrogenase enzymes were found to be anionic since they both focused in the acid pH range. The resolved enzyme peaks occurred at a pH of 4.28 and 4.38 for NADH and L-malate dehydrogenases respectively. These points are assumed to most closely approximate the isoelectric points of the enzymes; however, there was overlapping of the dehydrogenase activities. Preparative separation could probably be accomplished by removing the fractions containing the greatest quantities of both enzymes and refocusing these fractions. A complete separation might entail doing this several times. Procedures for the refocusing of samples are given by O. Vesterberg (1971).

All pH readings were made at 5 C, the temperature at which the isoelectric focusing was run. In this manner, a temperature dependent pH correction was not needed. The pH of the one ml fractions was adjusted to pH 7.2 in Tris-HCl before assaying for dehydrogenase activity. This step eliminated correction of enzyme activity due to pH differences.

Additional observations were made with the fractions obtained by isoelectric focusing that did not involve separation of the enzymes. Through the use of difference spectra, both a and b type cytochromes and a soluble flavine were observed in the

Figure 12

Isoelectric focusing of L-malate and NADH Dehydrogenases. Isoelectric focusing in the pH range 4-6 (Ampholine Carrier Ampholytes) was run at 600 volts for a period of 36 hours at 5 C. A sucrose gradient was used to help prevent diffusion. One ml fractions were assayed for enzyme activity via DCIP reduction at pH 7.2. All pH determinations were made at 5 C. Symbols: ▲ , NADH dehydrogenase; ○ , L-malate dehydrogenase. Further experimental details are given in Materials and Methods, Isoelectric focusing section, pages 30-31.



same fractions that contained the dehydrogenase enzymes. Since there was not complete separation of the dehydrogenase enzymes, it cannot be stated if the cytochrome and the flavine were associated specifically with either of the dehydrogenase enzymes.

Starch Gel Electrophoresis of L-malate and NADH Dehydrogenases

Starch gel electrophoresis was employed as a less expensive and more accessible means of separating L-malate and NADH dehydrogenases. Initial experiments were done with the Tris-barbitol buffer system (Materials and Methods, Microzone electrophoresis section, page 28) and a running pH of 8.6 in the absence of any detergent. Separation of the enzymes in the absence of any detergent would have given substantial supporting evidence for the separability of the two dehydrogenase enzymes. The results shown in figure 13 were taken from an experiment performed in the absence of any detergent. There was no banding of the NADH dehydrogenase activity. The L-malate dehydrogenase activity remained very close to the origin.

A significant separation of the dehydrogenase enzymes was obtained by adding 0.25% (w/v) Triton X-100 to the buffer system. This separation is shown in figure 14. NADH dehydrogenase activity (part A) banded in two different areas (marked by the arrows). The major band of NADH dehydrogenase was 15 to 20 mm into the gel and thus separated from any L-malate dehydrogenase activity. L-malate dehydrogenase (part B) moved only 1 to 2 mm

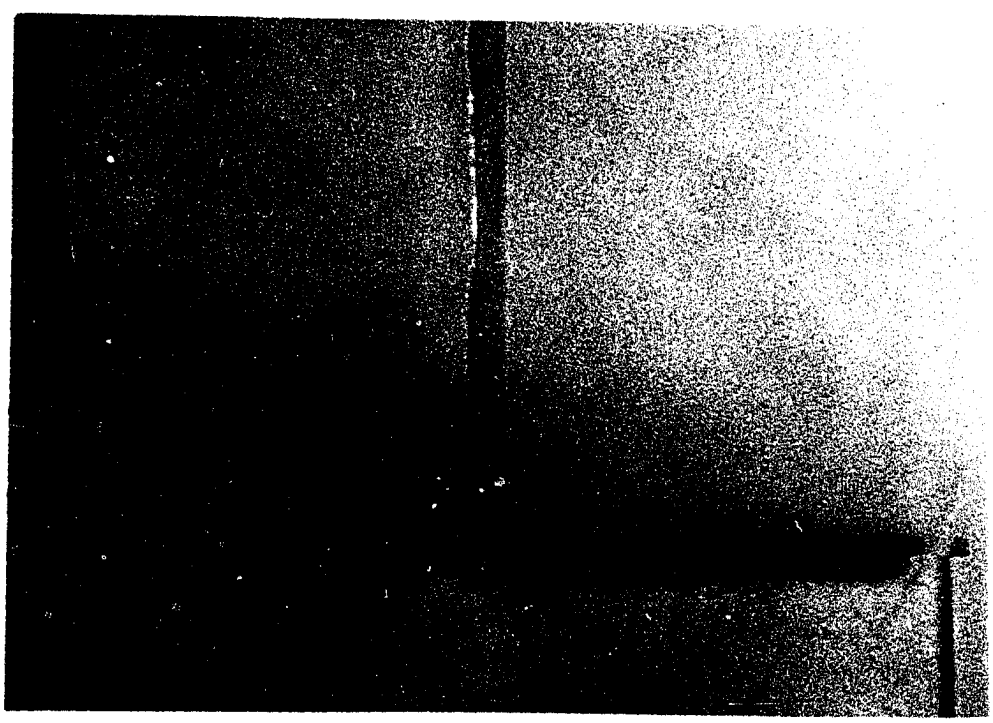
Figure 13

Starch Gel Electrophoresis of L-malate and NADH Dehydrogenases in the Absence of Triton X-100. The photograph shows the migration patterns of NADH dehydrogenase (A) and L-malate dehydrogenase (B). 'Purified enzyme' samples were electrophoresed in a horizontal 10% starch gel for 24 hours at 4 C. Running pH of the system was 8.6 with Tris-barbitol buffer. The enzymes were selectively stained in horizontal starch gel slices by adding MTT tetrazolium with the proper substrate. PMS was added to the L-malate dehydrogenase staining bath to speed up the development of the color. No color developed without the substrate present. Further experimental details are given in Materials and Methods, Starch gel electrophoresis section, pages 31-32.

A B

Figure 14

Starch Gel Electrophoresis of L-malate and NADH Dehydrogenases in the Presence of Triton X-100. The photograph shows the separation of NADH dehydrogenase (A) from L-malate dehydrogenase (B). Enzyme samples were electrophoresed in a horizontal 10% starch gel for 24 hours at 4 C. The Tris-barbitol buffer contained 0.25% (w/v) Triton X-100 at a running pH of 8.6. The enzymes were selectively visualized by staining horizontal gel slices with MTT Tetrazolium and the proper substrate. PMS was added to the L-malate dehydrogenase staining bath to speed up development of the color. No color developed without the substrate present. Further experimental details are given in Materials and Methods, Starch gel electrophoresis section, pages 31-32.



A B

from the origin. Both enzymes moved towards the anode. There was also a small amount of trailing of NADH dehydrogenase activity in the presence of Triton X-100.

Heparin, as suggested by the starch manufacturer, was added to the starch suspension in some of the starch gel electrophoresis experiments. Heparin was not found to promote the banding of either of the dehydrogenase enzymes. Triton X-100 was then added along with heparin to the buffer system. The combined effect of Triton X-100 and heparin completely inhibited L-malate dehydrogenase activity. A similar effect was found by the combined action of EDTA and Triton X-100. NADH dehydrogenase activity was not affected by either of these two conditions. The mechanism of inhibition is not clearly understood but the observations are consistent with the individuality of L-malate and NADH dehydrogenases.

DISCUSSION

Deoxycholate extraction of Micrococcus lysodeikticus membranes has been shown to solubilize both L-malate and NADH dehydrogenases (Eisenberg, R.C., 1972, Gel'man, N.S. et al., 1970). Previous attempts to separate these activities using Sepharose 4B and Sephadex G-200 column chromatography have met with no success (Ostrovskii, D.N. et al., 1968b, Tsfasman, I.M. et al., 1970 a and b). R. C. Eisenberg and B. B. Smith (unpublished data) have obtained similar negative results. These findings led Gel'man to propose that NADH and L-malate dehydrogenases were solubilized by deoxycholate as a complex with cytochrome b_{556}. Furthermore, it was proposed that the functional activity of these two dehydrogenases was related to a complexed structure (Gel'man, N.S. et al., 1970).

D. N. Ostrovskii et al. (1969) showed that L-malate and NADH dehydrogenases were differentially inactivated by high velocity electrons. Radiation inactivation of L-malate and NADH dehydrogenases was used in determining their molecular weights, 73,000 and 70,000 respectively. The determination of these molecular weights assumes a direct relationship between radiation dose and radiation inactivation. This assumption ensues from target theory (Ostrovskii, D.N. et al., 1969). This data, coupled with the differential rates of reconstitution of L-malate and NADH dehydrogenase activities (Eisenberg, R.C., 1972),

suggested that these enzymes were indeed separate. Therefore, methods of protein separation were undertaken to better establish either Gel'man's proposal or to show separation of these enzymes.

Starch gel electrophoresis of the enzyme preparation has shown a differential mobility of L-malate and NADH dehydrogenases (figures 13 and 14). However, complete resolution of NADH dehydrogenase activity from L-malate dehydrogenase activity was accomplished only in the presence of Triton X-100 (figure 14). Separation of the enzyme activities with starch gel electrophoresis was not accomplished in the presence of deoxycholate. The possibility exists, therefore, that Triton X-100 somehow changes the physical structure of the enzymes, especially NADH dehydrogenase, making the electrophoretic mobility greater.

There was also a difference in the effect of deoxycholate and Triton X-100 on NADH dehydrogenase activities. This difference was established in the experiments dealing with the effects of detergent on enzyme activity (figures 3-6). In native membranes, there is often an increase in enzyme activity upon detergent solubilization of the membranes (Razin, S., 1972). Such a case has been found sepcifically in Micrococcus lysodeikticus since solubilization of the membranes with deoxycholate results in a significant increase of NADH dehydrogenase activity (Hsia, K., 1972). This effect is best explained by unmasking of the enzyme so that active sites are more available for catalytic function. This type of action has also been described for NADH oxidase in

Bacillus megaterium KM (Eisenberg, R.C. et al., 1970a). However, the effects of unmasking catalytic sites within a membrane matrix would not be expected to occur in previously solubilized proteins. The effects of deoxycholate on solubilized enzyme activity (figures 3 and 4) indicate, as expected, no stimulation of enzyme activities by deoxycholate. Triton X-100, however, does appear to stimulate NADH dehydrogenase activity (figure 5). One possibility for explaining this slight stimulation of NADH dehydrogenase by Triton X-100 would be satisfying a lipid requirement; this would be analogous to stimulation of purified mitochondrial cytochrome oxidase by Triton X-100 as described by F. F. Sun et al. (1968 and 1969).

An observation made during the starch gel electrophoresis experiments was that the combined effect of Triton X-100 and either Heparin or EDTA completely inhibited L-malate dehydrogenase activity. Both Heparin and EDTA are known to be chelating agents. It may be that Triton X-100 changes the conformation of the L-malate dehydrogenase so that it is made susceptible to the action of chelating agents. Requirement of Triton X-100 for inhibition was established since only slight inhibition of L-malate dehydrogenase by EDTA or Heparin occurred in the absence of detergent. NADH dehydrogenase activity was not inhibited by Triton X-100 and chelators. Although it is not clear what these differences in inhibition and stimulation really involve, the data does suggest individuality of the dehydrogenase enzymes.

Microzone electrophoresis provides the best evidence for separation of individual L-malate and NADH dehydrogenase activities (figure 9) since separation was completed in the absence of added detergent. Therefore, further treatment beyond deoxycholate solubilization is not needed for separation. These results are not in accord with Gel'man's hypothesis that L-malate and NADH dehydrogenase enzymes are solubilized as a unit block (Gel'man, N.S. et al., 1970).

Further studies with Microzone electrophoresis have been made with enzyme samples treated with $MgSO_4$. Magnesium sulfate treated samples precluded separation of the enzymes (figure 10). Magnesium ions have been found to be of significant importance in the reaggregation of bacterial membrane components after detergent solubilization (Razin, S. et al., 1965, Butler, T.F. et al., 1967). If the magnesium ions do cause reaggregation of the solubilized dehydrogenase proteins, lack of separation of these enzymes would be expected. Preliminary electron microscopic investigations have indicated the presence of vesicular-like structures in the Mg^{2+} treated samples (Dills, S.S. and R.C. Eisenberg, unpublished data).

These enzymes moved towards the anode in both microzone electrophoresis and starch gel electrophoresis. This confirms the anionic nature of these membrane proteins as being similar to those proteins that have been described in many other bacterial membranes (Razin, S., 1972). Furthermore, it was also

found that an increase in pH of the buffer system consistently gave better separations. This is probably due to the fact that alkaline conditions enhanced a greater charge on the proteins which resulted in increasing their electrophoretic mobility. Increasing the negative charges on the proteins may facilitate separation of the enzymes due to repulsion. The repulsion forces could possibly overcome electrostatic bonds binding the enzymes together. Electrostatic bonds, possibly involving Mg++, could bind the enzymes at low pH. A system similar to the one described here has been described by J. Kahane et al. (1973) in Mycoplasma membranes. In this system, with EDTA extraction, there was a small but constant amount of Mg++ that was retained within the membrane proteins. Such a Mg++ complex might also exist in the solubilized complexes containing L-malate and NADH dehydrogenases. This possibility might give credence to Gel'man's "unit block" hypothesis and definitely expresses the need for quantitative measurements of Mg++ in the solubilized material (Gel'man, N.S. et al., 1970).

Results from polyacrylamide disc gel electrophoresis experiments were inconclusive. Significant migration of these enzymes did not occur even in the presence of detergent. These results were difficult to reconcile with the results obtained with starch gel electrophoresis (figure 14). Several possibilities might account for the difference between polyacrylamide and starch gel electrophoresis. First, it should be recognized that

the basis of separation in starch and polyacrylamide gel electrophoresis is somewhat different. Both methods do separate proteins on the basis of differences in electrophoretic mobility (charge on the proteins). However, polyacrylamide electrophoresis separation is dependent on both the change and molecular size of proteins (Davis, B.J., 1964, Ornstein, L., 1964). A 10% polyacrylamide gel was used routinely in the present study. This gel has a pore size of approximately 46 A°. D. N. Ostrovskii et al. (1968) have determined that both L-malate and NADH dehydrogenases are eluted in the void volume of a Sepadex G-200 column. This would give the complex a molecular weight of over 1 million. Further work in the same laboratory has shown that these dehydrogenase complexes have a Stokes radius (Rs) of about 100 Å (Ostrovskii, D.N. et al., 1968). Clearly, a 10% gel would offer extreme frictional resistance to complexes with a Stokes radius of 100 Å.

Nachbar and Salton have been able to obtain migration of NADH dehydrogenase with 7% apolyacrylamide gels (Nachbar, M.S. and M.R.J. Salton, 1970). Therefore, the proteins containing NADH dehydrogenase activity would have to have an Rs value of 55 Å or less. One important difference in Nachbar and Salton's system and the preparations used in this study is that Nachbar and Salton employed EDTA in the solubilization of the membranes. The chelating agent, EDTA, may have an action totally different than that of anionic detergents since it dissociates electrostatic bonds (Butler, T.F. et al., 1967). If there are electrostatic

bonds binding dehydrogenase complexes EDTA may serve to break these complexes. This same effect might also be accomplished by a high pH in the buffer system although high pH buffer systems used in disc gel electrophoresis did not promote migration in the present study. Both L-malate and NADH dehydrogenases may be solubilized by DOC in fairly large complexes. These complexes might be broken down further by EDTA.

Isoelectric focusing was employed as a third means for achieving separation of L-malate and NADH dehydrogenases. This separation, unlike the first three electrophoretic methods with separation based on charge differences, is based on protein isoelectric points (lack of charge on the proteins). Thus, an electrophoretic field is only the vehicle of moving the enzymes to their isoelectric points where migration ceases to occur because of a lack of charge (Vesterberg, O., 1971).

Isoelectric focusing, as with microzone electrophoresis, separates enzymes in the absence of added detergent. This data confirms the prior results showing that the solubilized NADH and L-malate dehydrogenases are separable without further treatment. Again, this is in conflict with Gel'man's hypothesis that both enzymes are solubilized as a single block.

Results of isoelectric focusing, figure 12, show considerable overlap of dehydrogenase activities. A Gausian distribution of enzyme concentration is normally expected in tyis type of separation (Rilbe, H., 1973). The distribution of dehydrogenase

activities is to diffuse for the best resolution of the enzymes. The width of enzyme distribution might be decreased by focusing for longer periods of time, higher voltages, or a combination of both time and higher voltages. A more practical approach might rather be refocusing of those samples containing the greatest amount of each of the enzymes. It is believed that the data shows enough concentration of the enzymes that the pH at which maximum activity occurs closely reflects the isoelectric points of the enzymes (Rilbe, H., 1973).

Two observations were made with the fractions obtained from the isoelectric focusing experiments. It was found that both flavine and a cytochrome of the b type occurred in those fractions containing the greatest amount of enzyme activities. These cofactors were detected by dithionite reduced minus oxidized difference spectra. Since there was a considerable overlapping of the enzymes, it was impossible to determine if either cytochrome or flavine was associated preferentially with either of the enzymes. A preparative and complete separation of the enzymes followed with high resolution liquid N_2 difference spectra will be needed to ascertain the distribution of b type cytochromes.

Separation of the enzymes on DEAE Sephadex by elution with NaCl and detergent was not achieved. From results obtained in Microzone and starch gel electrophoresis, it was established that both of the enzymes are anionic at pH 7.2 and above. It is further known from the isoelectric focusing data that the charges

on the enzymes are relatively close. Results obtained with DEAE Sephadex ion exchange experiments are amenable to several explanations. The first possibility is that the enzymes are retained on the column by ionic bonds too strong to be disrupted by high salt concentrations. Hydrophobic bonds would of course be broken by the detergent elutants and the enzymes would pass through the column. This is probably the case since it was found that only detergents would release significant amounts of the enzymes. Unfortunately, with DOC or Triton X-100 as elutants, there is no selective removal of either enzyme. A considerable amount of dehydrogenase inactivation also occurs due to detergent action. Use of DEAE Sephadex was also unsuccessful in more extensive studies by D. N. Ostrovskii et al. (1968b).

The microzone, starch gel, and isoelectric focusing data clearly show definite separation of L-malate dehydrogenase from NADH dehydrogenase. Further work must be done to show the possible association of cytochrome b or flavine with either of the enzymes. The best method for approaching these questions is through the use of preparative separations and high resolution liquid N_2 difference spectra. It is suggested that the most workable preparative separation would involve the methods discussed for isoelectric focusing.

Further quantitative difference spectra should be made to determine if the dehydrogenases both deliver electrons to the same b type cytochrome. Black light inactivation of the

Vit. $K_{2(45)}$ followed by physiological reductions would help to answer these questions. Certainly, high resolution liquid N_2 difference spectra would be needed to accurately identify the cytochromes.

Further aspects must also be considered for evaluating the overall electron transport system of M. lysodeikticus. M. R. J. Salton et al. (1968) has stated that all of the membrane lipid is solubilized by deoxycholate treatment. This would mean that the deoxycholate disrupted desalted membranes could be deficient in some lipids which may be needed for dehydrogenase activity and are somehow bound to the enzyme. Lipid analysis of the enzyme preparations should be made to determine if catalytic activity is enhanced by lipid. This topic leads to another aspect that should be considered. Reconstitution of the electron transport system should consider what components are needed for reconstitution of the dehydrogenase with the cytochrome oxidase activity. It may be necessary to characterize all of the components needed for reconstitution which might include more than just the dehydrogenase enzyme proteins.

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