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Studies on the Membrane Associated L-Malate Dehydrogenase-Oxidase Complex of *Micrococcus Lysodeikticus*

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STUDIES ON THE MEMBRANE ASSOCIATED
L-MALATE DEHYDROGENASE-OXIDASE
COMPLEX OF MICROCOCCUS
LYSODEIKTICUS

by
Jerry James Johnson

A Thesis
Submitted to the
Faculty of The Graduate College
in partial fulfillment
of the
Degree of Master of Arts

Western Michigan University
Kalamazoo, Michigan
April 1974

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Jerry J. Johnson

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INTRODUCTION

L-malate oxidoreductase (E.C.1.1.1.37) was discovered independently by Batelli and Stern in 1910 (3) and by Thunberg in 1911 (29). The enzyme was first purified by Straub in 1942 (26). In 1961, Davies and Kun (6) investigated the specificity of animal L-malate dehydrogenase and showed that it catalyzed the following reactions:

1. L-malate + $\text{NAD}^+ \rightleftharpoons$ oxaloacetate + reduced NAD ($\text{NADH} + \text{H}^+$)
2. D (-) or meso tartrate + $\text{NAD}^+ \rightleftharpoons$ oxaloglycollate + $\text{NADH} + \text{H}^+$
3. Oxaloglycollate + $\text{NAD}^+ \rightleftharpoons$ dioxysuccinate + $\text{NADH} + \text{H}^+$
4. Tartarate + $\text{NAD}^+ \rightleftharpoons$ mesoxalate + $\text{NADH} + \text{H}^+$
5. α -hydroxyglutarate + $\text{NAD}^+ \rightleftharpoons$ α -oxoglutarate + $\text{NADH} + \text{H}^+$

This demonstrated the enzyme was an α -hydroxydicarboxylic acid dehydrogenase. Before the enzyme specificity was defined several investigators found evidence for two forms of L-malate dehydrogenase. Price and Thinmann (25) reported the presence of L-malate dehydrogenase in both mitochondrial and supernatant fractions of pea plant stems. Davies (5), also working with pea plant stems, found approximately 75 percent of the L-malate dehydrogenase activity in the supernatant fraction and the remaining 25 percent in the mitochondrial fraction. In 1959 Delbruck, et al., (7) proposed that animal cells also contained L-malate dehydrogenase in both mitochondrial and supernatant (cytoplasmic) fractions.

While investigating these two cellular forms of L-malate dehydrogenase in rat liver Kaplan (21) found that the addition of oxaloacetic acid (OAA) inhibited the soluble L-malate dehydrogenase 13 percent while the mitochondrial enzyme was inhibited 66 percent. Addition of excess

L-malate reduced the mitochondrial enzyme by 50 percent of its maximum rate and the soluble enzyme to 30 percent. The differential effects of OAA and L-malate on the mitochondrial and cytoplasmic enzymes led the author to propose the mitochondrial enzyme as catalyzing the oxidation of L-malate to OAA whereas the cytoplasmic L-malate dehydrogenase catalyzed reduction of OAA to L-malate.

The occurrence of L-malate dehydrogenase in mitochondrial and cytoplasmic cellular fraction together with the differential substrate effects led to the scheme shown in Figure 1 wherein Kaplan proposed a physiological explanation for these observations. Thus, the participation of both L-malate dehydrogenases were implicated in coupling oxidation of cytoplasmic NADH to ATP synthesis via mitochondrial oxidative phosphorylation. Kaplan's scheme indicated that OAA and L-malate acted as shuttles between cytoplasmic and mitochondrial L-malate dehydrogenases. Accordingly, cytoplasmic NADH was oxidized by OAA to form L-malate which then entered the mitochondria and was oxidized to OAA by the mitochondrial L-malate dehydrogenase and NAD^+ . Oxaloacetate then entered the cytoplasm and was reduced to L-malate via cytoplasmic NADH. The mitochondrial NADH would then be oxidized by the mitochondrial respiratory chain enzymes and be coupled with ATP formation. This scheme was also supported by Grim on the basis of work with beef heart L-malate dehydrogenase (18).

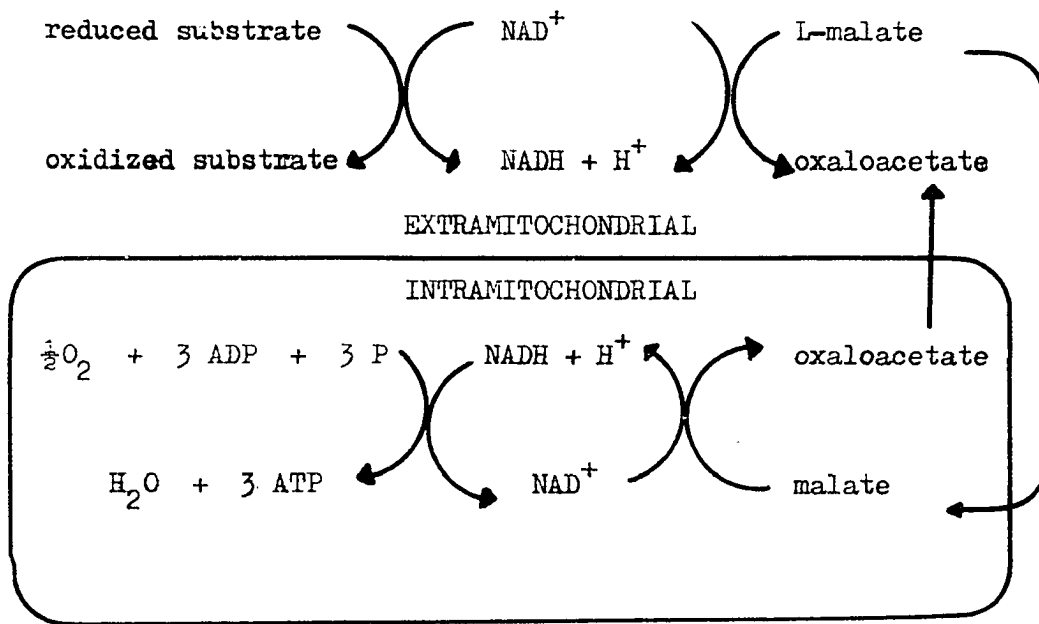
Another distinction between soluble and mitochondrial L-malate dehydrogenase, isolated from rat liver, was reported by Thorne (28) in 1960. Thorne found significant differences in the chromatographic behavior of these two forms of L-malate dehydrogenase. Using ammonium

Figure 1

Kaplan's Scheme for the Role of L-malate Dehydrogenase

Scheme for the role of L-malate dehydrogenase activity as a shuttle mechanism between cytoplasmic and mitochondrial oxidative phosphorylation in eucaryotic cells according to Kaplan (21).

Figure 1



sulphate fractionation, calcium phosphate adsorption and elution, and chromatography on amberlite resin and on DEAE-cellulose, Thorne effected a 100-fold purification of the mitochondrial enzyme and a 250-fold purification of the cytoplasmic enzyme. After isolation of the enzymes it was found that the elution of the mitochondrial enzyme was retarded more than the cytoplasmic L-malate dehydrogenase by the amberlite resin. The reverse was true when DEAE-cellulose chromatography was used. The author found that elution patterns were reproducible and characteristic when the enzymes were chromatographed either separately, as a mixture, or in the form of a crude rat liver homogenate. His investigation also showed that pig heart mitochondrial L-malate dehydrogenase had the same elution pattern as the rat liver mitochondrial enzyme.

L-malate Dehydrogenase in Bacteria

Bacteria exhibit a variety of mechanisms for the oxidation of L-malate to OAA which Francis grouped into four general categories of L-malate oxidation (10). The first, and most common mechanism, involves an initial oxidation of L-malate mediated by a cytoplasmic, or soluble, NAD^+ linked L-malate dehydrogenase. Evidence for this mechanism was presented by Kornberg and Massen (22) who prepared cell free extracts of L-malate dehydrogenase from Escherichia coli and Pseudomonas fluorescens. After disrupting the bacterial cells in a Hughes press and centrifugation at 25,000 x g, the supernatant fraction was shown to contain all the cellular L-malate dehydrogenase activity as assayed spectrophotometrically by following the oxidation of NADH on addition of OAA.

A second mechanism of bacterial oxidation of L-malate involves both a soluble L-malate dehydrogenase and a particulate L-malate oxidizing system. This system has been found in Micrococcus lysodeikticus and in Mycobacterium phlei (1, 10). A NAD^+ linked L-malate dehydrogenase is present in the soluble fraction of M. phlei and the particulate fraction contains a membrane-bound L-malate dehydrogenase which utilizes flavoprotein in lieu of NAD^+ and donates electrons directly to vitamin K (naphthoquinone). These two L-malate dehydrogenase enzymes may be significant physiologically in providing a mechanism for reoxidizing cytoplasmic NADH (1). The OAA formed from the membrane-bound L-malate reductase could, therefore, be reduced to L-malate by cytoplasmic L-malate dehydrogenase and NADH.

A third mechanism was demonstrated in Pseudomonas ovalis and Mycobacterium avium in which the oxidation of L-malate to OAA was catalyzed by a flavine adenine dinucleotide (FAD) linked particulate system (10, 23). The investigators could not detect soluble L-malate dehydrogenase in the extracts of these organisms.

A fourth, less common, L-malate dehydrogenase occurs in organisms such as the obligate anaerobe Chromatium (10). This organism does not possess either a particulate or cytoplasmic L-malate dehydrogenase. However, there is a L-malate enzyme which converts L-malate and NADP^+ to pyruvate, carbon dioxide, and NADPH via oxidative decarboxylation.

L-malate Oxidation in Micrococcus lysodeikticus

The L-malate oxidizing system of M. lysodeikticus fulfills the conditions for Francise's second category of bacterial L-malate oxidation systems since the organism possesses both a soluble NAD^+ linked

L-malate dehydrogenase and a particulate L-malate dehydrogenase which is not dependent on NAD^+ (10, 4).

Cohn (4) purified the two above mentioned L-malate dehydrogenase enzymes by ammonium sulphate fractionation. The non- NAD^+ dependent enzyme precipitated between 0-20 percent (w/v) ammonium sulphate concentration and the NAD^+ dependent L-malate dehydrogenase between 30-40 percent ammonium sulphate fraction. Other investigators have also found two L-malate dehydrogenase enzymes in M. lysodeikticus. Principle among these have been Gelman and Oparin (12, 13, 17, and 25). These investigations have suggested that a flavoprotein is the intermediate electron acceptor for the non- NAD^+ dependent membrane-bound L-malate dehydrogenase (15, 17, and 25). This membrane-bound L-malate dehydrogenase is part of a respiratory multi-enzyme complex which involves flavin, naphthoquinone, and cytochromes of the a, b, and c types as intermediary electron carriers to oxygen (14). Gelman (14) has reported α -band absorbancy maxima for a, b, and c type cytochromes at 590-66, 560, and 550 nm respectively; a β -band absorbance maximum for b and c type cytochromes occurs at 520 nm. Thus, this L-malate dehydrogenase enzyme is only a part of a membrane-bound L-malate oxidase (L-malate: O_2 oxidoreductase, E.C.1.1.1.37) enzyme activity.

M. lysodeikticus L-malate oxidase, on the basis of electron carrier components, would appear to be analogous to a variety of respiratory complexes described for bacteria and mitochondria. However, an important difference between M. lysodeikticus L-malate oxidase and mitochondrial respiratory chains is the insensitivity of M. lysodeikticus oxidase to cyanide. Several investigators have reported that

10^{-3} M KCN has no effect on M. lysodeikticus L-malate oxidase (4, 12, 13, and 16). Cohn (4) reported a two-fold stimulation of M. lysodeikticus L-malate oxidase by 6×10^{-2} M cyanide. This lack of oxidase inhibition by cyanide could suggest the action of a flavoprotein oxidase in the electron transport chain (16). Cohn also suggests that this possibility might be due to an atypical cytochrome oxidase in M. lysodeikticus since this organism possesses a cytochrome a which has a maximum absorbancy at 600 nm instead of the typical cytochrome a₃ absorbance maximum at 605 nm.

The present investigation was undertaken to aid in the elucidation of the characteristics of the membrane-bound L-malate dehydrogenase-oxidase complex of M. lysodeikticus with the realization that any research designed to illuminate the function of membrane subcomponents will ultimately aid in the understanding of membranes and their structure.

MATERIALS AND METHODS

Growth of Bacteria

Micrococcus lysodeikticus ISU Ad+ was obtained from James B. Evans, Department of Microbiology, North Carolina State University, Raleigh. Stock cultures were maintained on slants of trypticase soy broth (Bioquest) plus 1.5 percent (w/v) agar and kept at 4°C; cultures were routinely transferred at two month intervals.

The following procedure was routinely employed in order to obtain a suitable amount of cells in an actively growing state: 40 ml of a 24-48 hour trypticase soy broth culture was used to inoculate 350 ml of TYE basal medium in a one liter shake flask. TYE basal medium contained trypticase, 15 g; yeast extract, 1.0 g; and sodium chloride, 5 g per liter. Sodium phosphate buffer was added to a final concentration of 0.02 M at pH 7.2 and sterile glucose (autoclaved separately) was added to a final concentration of 0.05 M. After incubation for 24-30 hours at 32°C 50 ml of these cells were used to inoculate 500 ml of fresh TYE medium in 1 L shake flasks. After 13 hours incubation at room temperature the cells were in the late logarithmic growth phase. They were then harvested by centrifugation and resuspended to the original volume in fresh TYE medium. After an additional hour of incubation at room temperature the cells were again harvested by centrifugation and used to prepare cell membranes.

Membrane Preparation

Freshly harvested cells were washed twice in 10 ml of 0.01 M Tris (hydroxymethyl) aminomethane (Tris-HCl) buffer, pH 7.2, weighed and resuspended to 280 ml in TKM buffer. TKM buffer contained 0.01 M each of Tris, KCl, and $MgCl_2$, adjusted to a pH of 7.2 with HCl. One mg lysozyme was added for each gram of bacterial wet weight. A knife point of DNase was added and the cells were incubated with gentle stirring until lysis was completed (approximately one hour). Lysis was monitored throughout the incubation period utilizing the phase contrast microscope; complete lysis was estimated by the observation of no refractile bodies remaining in four or five randomly selected microscopic fields.

The resulting lysate was centrifuged 40 minutes at 20,000 x g in a Sorvall SS-34 rotor and washed twice with TKM buffer. The lysed membranes (ghosts) were suspended in 100 ml of storage media consisting of 0.01 M TKM buffer, ten percent glycerol (w/v) and 10^{-4} M β -mercaptoethanol. This membrane preparation was stored in 2 ml portions at -60° C. The frozen TKM ghosts were thawed and washed twice in 10 ml Tris-HCl buffer and resuspended in appropriate buffer to a protein concentration of 1.0-1.5 mg per ml before use.

Enzyme Assays

L-malate: 2,6-dichlorophenolindophenol oxidoreductase (E.C.1.1.1.37) was assayed colorimetrically by following the reduction of 2,6-dichlorophenolindophenol at 600 nm with a Gilford model 2000 recording spectrophotometer. The temperature of the reaction was maintained

at 30° C with a Lauda K-2/R circulating water bath. The assay mixture consisted of: 0.05 M Tris-HCl buffer, pH 7.2, 0.05 mM DCI (adjusted to give the total mixture an absorbance of 1.0-1.5), 3.3 mM L-malate (final concentration) and 0.1 ml enzyme preparation containing 1.0-1.5 mg protein per ml. Distilled water was added to bring the final volume of the assay mixture to 3.0 ml. The reaction was started by the addition of substrate. One unit of dehydrogenase activity is one μ mole DCI reduced per minute. This value was calculated using an extinction coefficient for DCI of $21 \text{ mM}^{-1} \text{ cm}^{-1}$ at 600 nm.

L-malate oxidase activity was estimated with a Gilson model KM Oxygraph equipped with a Clark-type electrode. Temperature of the reaction chamber was maintained at 30° C with a Lauda K-2/R water bath. The 1.5 ml assay mixture contained 0.05 M Tris-HCl buffer, pH 7.2, 0.05 ml enzyme suspension containing approximately 1.5 mg protein per ml, and 3.3 mM L-malate.

Protein Determination

Protein content of membrane preparations was estimated by the procedure of Lowry, et al., (24) using bovine serum albumin as a standard. Each sample was diluted to 5.0 ml with 1.0 N NaOH and heated in a boiling water bath for fifteen minutes. The sample was then cooled, centrifuged ten minutes at 10,000 rpm in a Sorvall SS-34 rotor, and the supernatant fraction employed for protein estimation.

RESULTS

Permeability Barriers in Protoplast Membrane Ghosts

Earlier work with Bacillus megaterium KM has shown that protoplast ghosts may retain permeability barriers to exogenous substrates (8). This suggests a retention of membrane vesicular structure. The permeability barriers of these structures were enhanced by divalent cations, particularly magnesium, and were eliminated by sonication. Therefore, it was of interest to determine whether similar permeability barriers were present in the protoplast membrane ghosts of M. lyso-deikticus since such permeability factors could influence observed rates of enzyme activity.

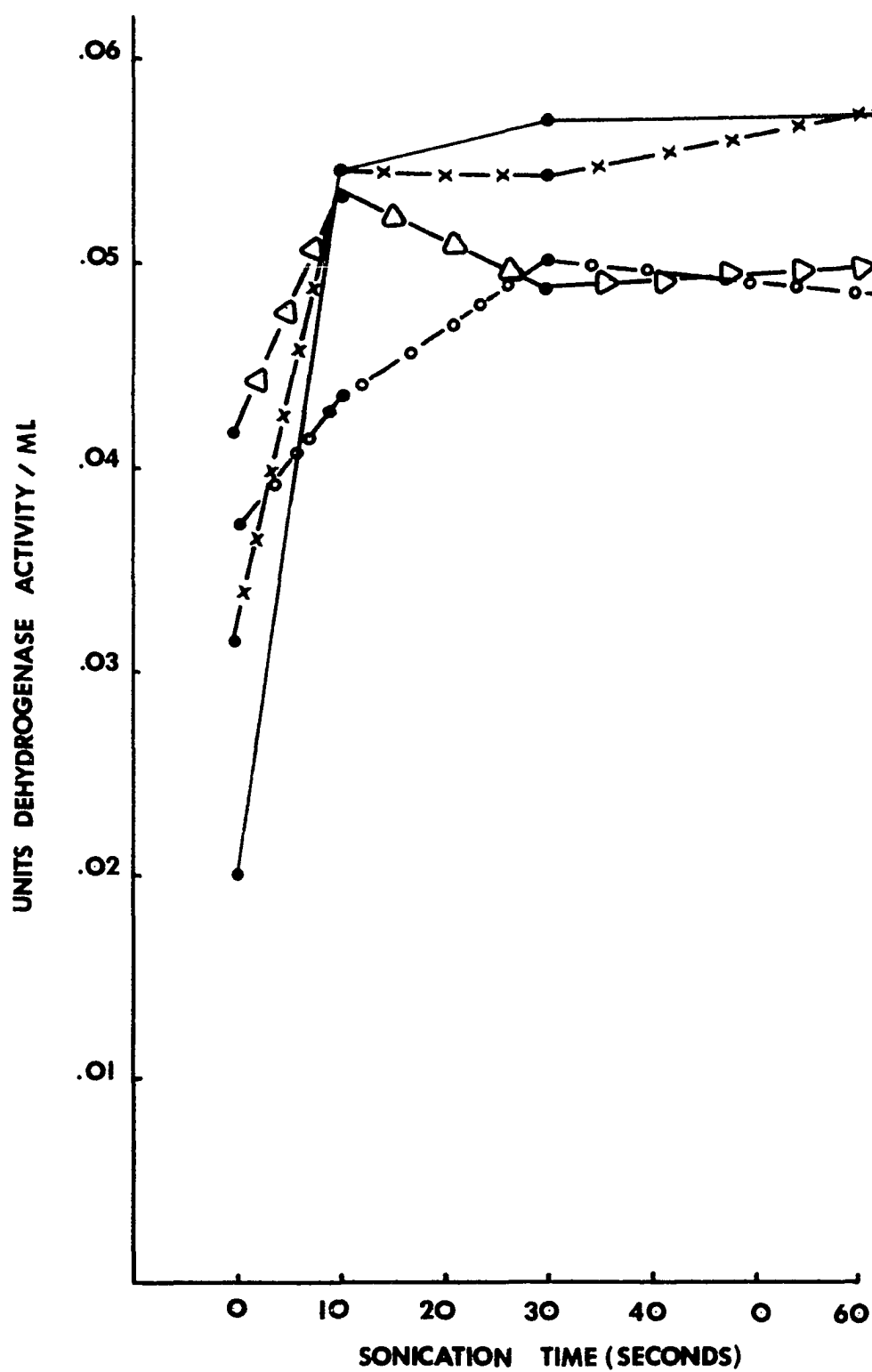
Data presented in Figure 2 show a small increase in dehydrogenase activity when Tris-HCl membranes were sonicated for ten seconds; longer periods of sonication did not significantly change levels of the L-malate dehydrogenase activity. Addition of Ca^{++} to Tris-HCl membranes lowered the level of enzyme activity 0.02 enzyme units (see zero time in Figure 2), and the addition of Mg^{++} lowered the initial activity 0.01 enzyme units. However, 10 to 60 seconds of sonication time increased the activity of the Mg^{++} and Ca^{++} membranes approximately 0.01 enzyme units more than the Tris-HCl membranes or the K^+ treated membranes which were sonicated for the same period of time.

Although not shown in Figure 2, the divalent cations Ba^{++} and Mn^{++} , as well as the monovalent cations Na^+ and NH_4^+ , were also tested. Their effect was much like that exhibited by Mg^{++} , Ca^{++} , and K^+ . The dival-

Figure 2

Effect of Sonication on Micrococcus Lysodeikticus
Membrane-Bound L-malate Dehydrogenase Activity

Initial L-malate dehydrogenase activity (time zero) was assayed before sonication. Sonication was then carried out for the time indicated in Tris-HCl buffer, pH 7.2. Divalent and monovalent cations were added to each assay mixture just prior to assay. Membranes were assayed in Tris-HCl buffer, $\Delta\text{-}\Delta\text{-}$; Tris-HCl buffer plus 1.0 mM MgCl_2 , -x-x-x ; Tris-HCl buffer plus 1.0 mM CaCl_2 , ----- ; and Tris-HCl buffer plus 1.0 mM KCl, -o-o- .



ent cations all inhibited the dehydrogenase activity of the unsonicated membranes 0.01 to 0.02 enzyme units, and they all responded to sonication very closely to Ca^{++} and Mg^{++} . The sonicated and unsonicated activities of the monovalent cations was quite similar to that shown by K^+ in Figure 2.

These data indicate that there is a permeability barrier to exogenous substrate in the protoplast ghosts of M. lysodeikticus. This barrier can be eliminated by sonication in the presence of divalent cations such as Mg^{++} .

Solubilization of Membrane Associated L-malate Dehydrogenase Activity

The solubilization of membrane associated enzymes and restoration of their activity in the solubilized state has been used to gain insight into the composition of the membrane (8). Therefore, it was of interest to examine the behavior of the membrane-bound L-malate dehydrogenase of M. lysodeikticus under these conditions.

Several agents (see Table I) were used to attempt the solubilization of the membrane-bound enzyme from protoplast ghosts. The membranes were suspended in the solubilization agents, centrifuged 30 minutes at 20,000 x g, and the supernatant and pellet assayed for L-malate dehydrogenase activity. Table I shows that deoxycholate produced the best solubilized activity.

Further work was then undertaken to determine the best deoxycholate concentration and ratio to membrane protein. To accomplish this, β -TKM ghosts of M. lysodeikticus were washed in Tris-HCl buffer, resuspended in 6 ml aliquots with Tris-HCl buffer and treated with deoxycholate (see

TABLE I

Solubilization of Membrane-Bound L-malate Dehydrogenase

Fraction	Enzyme Units					
	H ₂ O	Tris-HCl buffer	EDTA	Deoxy- cholate	Triton x 100	TKM buffer
Supernatant #1	.036	.036	.043	.250	.210	.064
Supernatant #2	.029	.022	.036	.043	0	.046
Supernatant #3	.022	.027	.086	0	0	.050
Supernatant #4	.022	.029	.093	0	0	.036
Washed pellet	1.43	1.57	1.57	0	0	1.57

β -TKM ghosts were resuspended to 5 ml in the solubilizing agent and centrifuged 30 minutes at 20,000 x g. The supernatant was retained for assay and the pellet resuspended to 5 ml with fresh solubilizing agent and the process repeated for a total of four washings. Each supernatant fraction was assayed for dehydrogenase activity, and the washed pellet was resuspended to 5 ml in 0.01 M Tris-HCl buffer and assayed for retention of unsolubilized L-malate dehydrogenase activity. The following agents were used as trial solubilizing agents: water, Tris-HCl buffer (0.01 M), EDTA (10^{-4} M), deoxycholate (1 mg/ml), Triton x 100 (1 mg/ml), and β -TKM buffer (0.01 M TKM buffer plus 10^{-4} M β -mercapto ethanol).

Table I). One ml of this suspension was retained for assay of total L-malate dehydrogenase activity, and the remaining 5 ml aliquots centrifuged at 100,000 x g for one hour. The supernatant containing solubilized L-malate dehydrogenase was then decanted and the residual pellet resuspended to 5 ml with Tris-HCl buffer. Assays for L-malate dehydrogenase were then performed on the initial deoxycholate- β -TKM ghost suspension, the solubilized enzyme (supernatant), the resuspended particulate material (pellet), and a 1:1 mixture of the supernatant and resuspended pellet.

Samples two and three (see Table II) revealed that a protein to detergent ratio of 3:1 solubilized more L-malate dehydrogenase than a protein to detergent ratio of 1:5:1. When the deoxycholate concentration was increased from 0.6 mg per ml to 1.0 mg per ml the enzyme activity was inhibited. This is demonstrated by the lower specific activity for the β -TKM ghost and deoxycholate suspension in sample one compared to activity measured for samples two and three. Although the percentage of protein solubilized was higher in samples one and two, the specific activity for both the solubilized and particulate L-malate dehydrogenase is higher for sample three.

These data show that the range of deoxycholate concentration appropriate for deoxycholate use in solubilization is quite narrow. The best solubilization of particulate L-malate dehydrogenase was accomplished with a deoxycholate concentration of 0.6 mg/ml at a protein to detergent ratio of 3:1.

The pH Optima of L-malate Dehydrogenase and Oxidase Activities

The absorbance of 2,6-dichlorophenolindophenol (DCI) at various

TABLE II

Detergent Concentrations Tested for Solubilization
of Membrane-Bound L-malate Dehydrogenase

Enzyme Sample	mg protein per ml	mg DOC per ml	percent protein solubilized	specific activity
Number 1	-	-	66.5	-
Deoxycholate and β -TKM ghosts	1.82	1.0	-	2.12
Solubilized	1.12	-	-	2.12
Particulate	1.00	-	-	1.07
Number 2	-	-	52.0	-
Deoxycholate	0.91	0.6	-	2.21
Solubilized	0.47	-	-	1.40
Particulate	0.73	-	-	2.93
Number 3	-	-	33.0	-
Deoxycholate	1.81	0.6	-	2.59
Solubilized	0.47	-	-	2.03
Particulate	1.48	-	-	2.39

Frozen β -TKM ghosts of *M. lysodeikticus* were thawed, washed twice in 0.01 M Tris-HCl buffer, pH 7.2, and resuspended in the same buffer which contained 1.0 or 0.6 mg per ml deoxycholate (DOC). One ml of each DOC membrane suspension was retained for assay and five ml aliquots of each suspension were then centrifuged at 100,000 x g for one hour. After centrifugation, the supernatant was decanted and the pellet resuspended to 5 ml with the Tris-HCl buffer. Assays for L-malate dehydrogenase were then run on the initial deoxycholate β -TKM ghost suspension, the solubilized enzyme (supernatant), and the resuspended particulate material (pellet).

hydrogen concentrations had to be determined prior to determining the relationship between pH and L-malate dehydrogenase activity. At pH 6.7 or higher, DCI has an extinction coefficient of $21 \text{ mm}^{-1} \text{ cm}^{-1}$ (constant absorbance) and an absorbance of 1.0 at 66 nm. However, as the pH is lowered the absorbance (and the extinction coefficient) decreases proportionately. Figure 3 shows this relationship.

The pH values for this experiment were obtained by combining varying quantities of 0.2 N HCl with 0.05 Tris-HCl buffer for a pH of 7.0 and above, and 0.5 M N-Tris (hydroxymethyl) methyl-2-aminoethane sulfonic acid (TES) buffer plus quantities of 0.05 M Tris for the pH values below 7.0. The Tris buffer was used because it had a high pH which could be adjusted downward with HCl and it had been shown to work as an efficient buffering system in the previous L-malate dehydrogenase assays contained within this study. Tes buffer was used for the pH ranges below 7.0 as it had a low pH which could be adjusted upward with Tris buffer. Trial runs with this Tris-Tes combination showed no L-malate inhibition. Therefore, these two buffers were used because they did not interfere with the enzymatic activity while maintaining the required pH range.

The pH optimum for the solubilized and particulate L-malate dehydrogenase was assayed to determine if there was a variation in the characteristics of the enzyme when removed from the membrane by deoxycholate. The pH optimum for the detergent solubilized enzyme did prove to be different from the membrane-bound enzyme. The solubilized enzyme had an optimum pH of 6.3, while the particulate enzyme had a slightly higher pH optimum of 6.9 (see Figure 4). This could indicate that the

Figure 3

Effect of pH on Absorbance and Extinction Coefficient
of 2,6-dichlorophenolindophenol at 600 nm

Variation of pH was accomplished by adding 0.2 N HCl to 0.05 M Tris-HCl buffer for a pH of 7.0 and above, and adding 0.05 M Tris to 0.05 M TES buffer for lower pH values.

The extinction coefficient for 2,6-dichlorophenolindophenol (DCI) was calculated as follows: $Abs = E \cdot c \cdot l$. Abs is the absorbance of DCI, E is the extinction coefficient of DCI, c is the concentration of DCI (0.05 mM for this experiment), and l cm the length of the light path. Since c and l are constants the μ moles DCI reduced per minute for a 3 ml assay = $3(\Delta Abs \text{ per minute}) E^{-1} l^{-1} \text{ cm}$.

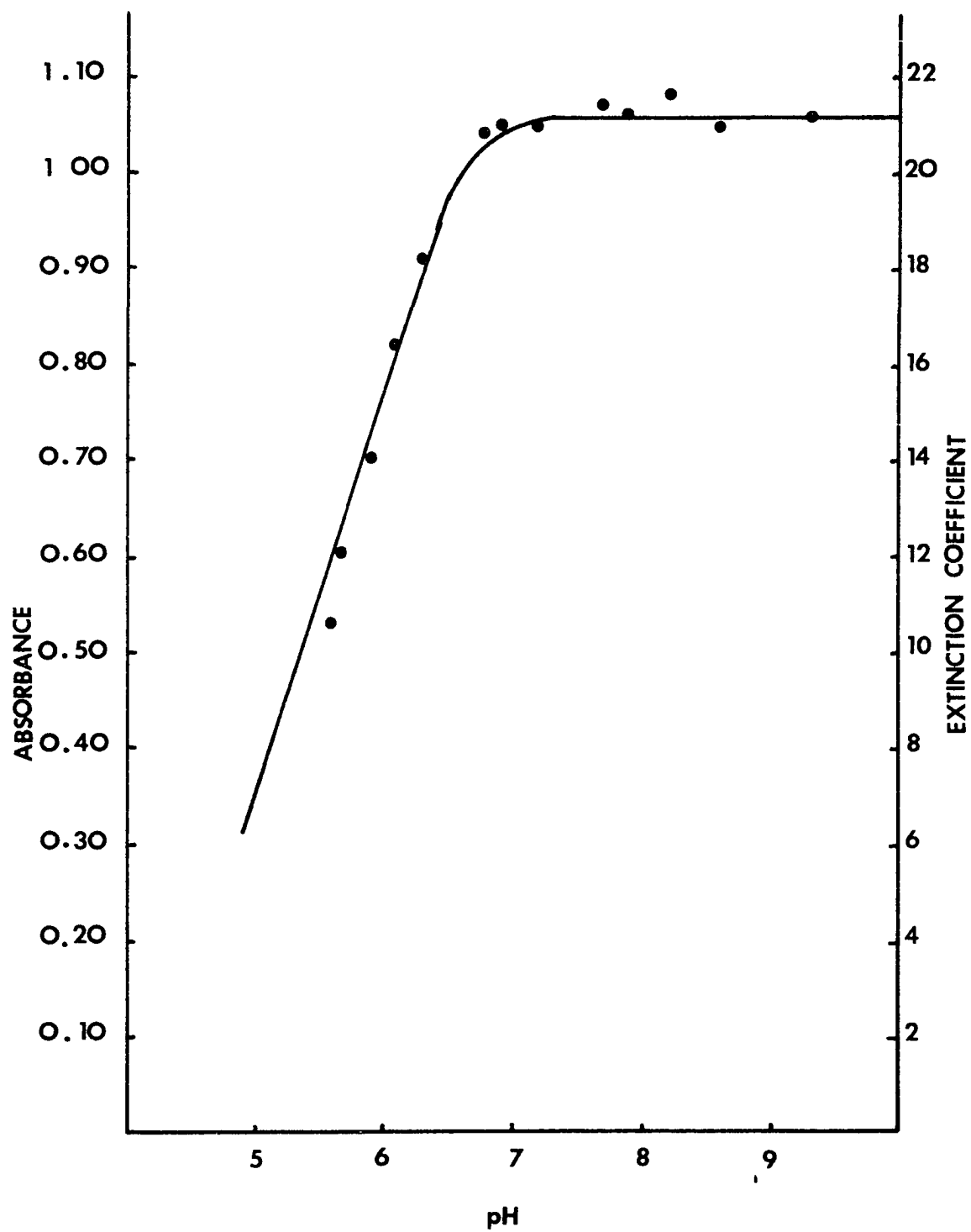
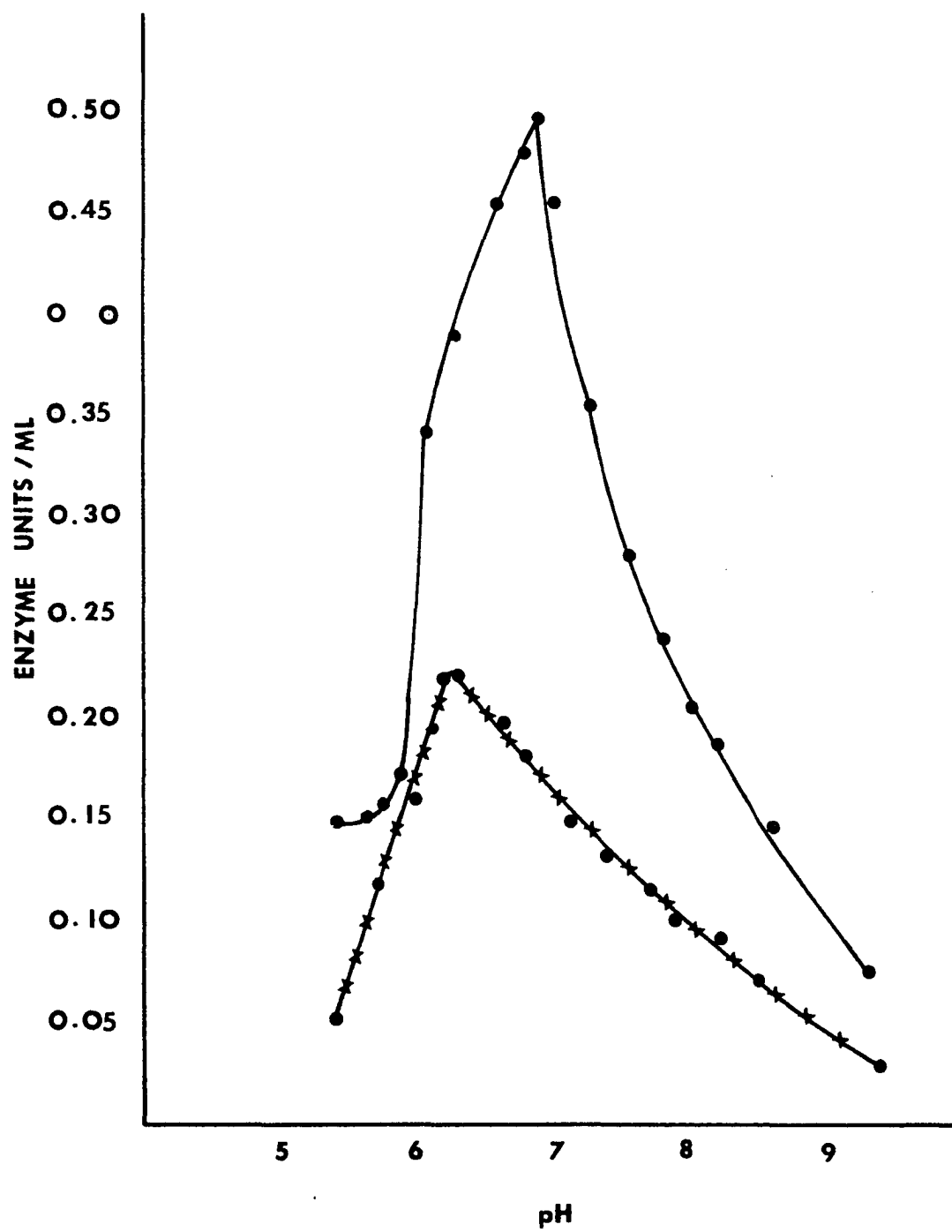


Figure 4

The pH Optimum for Solubilized and Particulate
L-malate Dehydrogenase

Symbols: particulate fraction, ———; detergent solubilized fraction, -x-x-x. See legend to Figure 3 for method of pH adjustment.



enzyme structure was altered by the detergent or that the absence of a surrounding membrane caused a change in the characteristics of the enzyme.

L-malate dehydrogenase is part of a multienzyme system which oxidizes L-malate and transfers the resulting electrons to oxygen. The pH optimum for this L-malate oxidase system was 7.2, higher than the optimum of either the solubilized or particulate L-malate dehydrogenase (see Figure 5). Since the optimum pH for L-malate oxidase is the pH optimum for an entire series of enzymes, the higher value could be caused by some other rate limiting enzyme and not be a direct result of L-malate dehydrogenase activity.

Effect of Cyanide on L-malate Dehydrogenase and L-malate Oxidase

β -TKM ghosts of M. lysodeikticus were treated with cyanide to test for cytochrome oxidase inhibition. This was done to determine if DCI reduction could be improved by inhibiting electron flow through the cytochrome system. This inhibition by cyanide would thus assure that all of the electrons removed from L-malate by L-malate dehydrogenase would be used to reduce DCI, and not be shunted to oxygen via the cytochrome system. To investigate this question, the oxidase and dehydrogenase activity of β -TKM ghosts were assayed in the presence of several concentrations of KCN. Table III shows that there is no inhibition of the L-malate oxidase except at very high cyanide concentrations. The dehydrogenase enzyme demonstrated a related response to cyanide concentration. At high concentrations there was an increase in dehydrogenase activity, but at lower concentrations there was only a slight elevation of activity (see Table III).

Figure 5

Optimum pH for L-malate Oxidase

The pH curve for L-malate oxidase was measured by oxygen uptake with a Gilson Oxygraph. See legend to Figure 3 for pH variation. Enzyme units as μ moles O_2 taken up per minute.

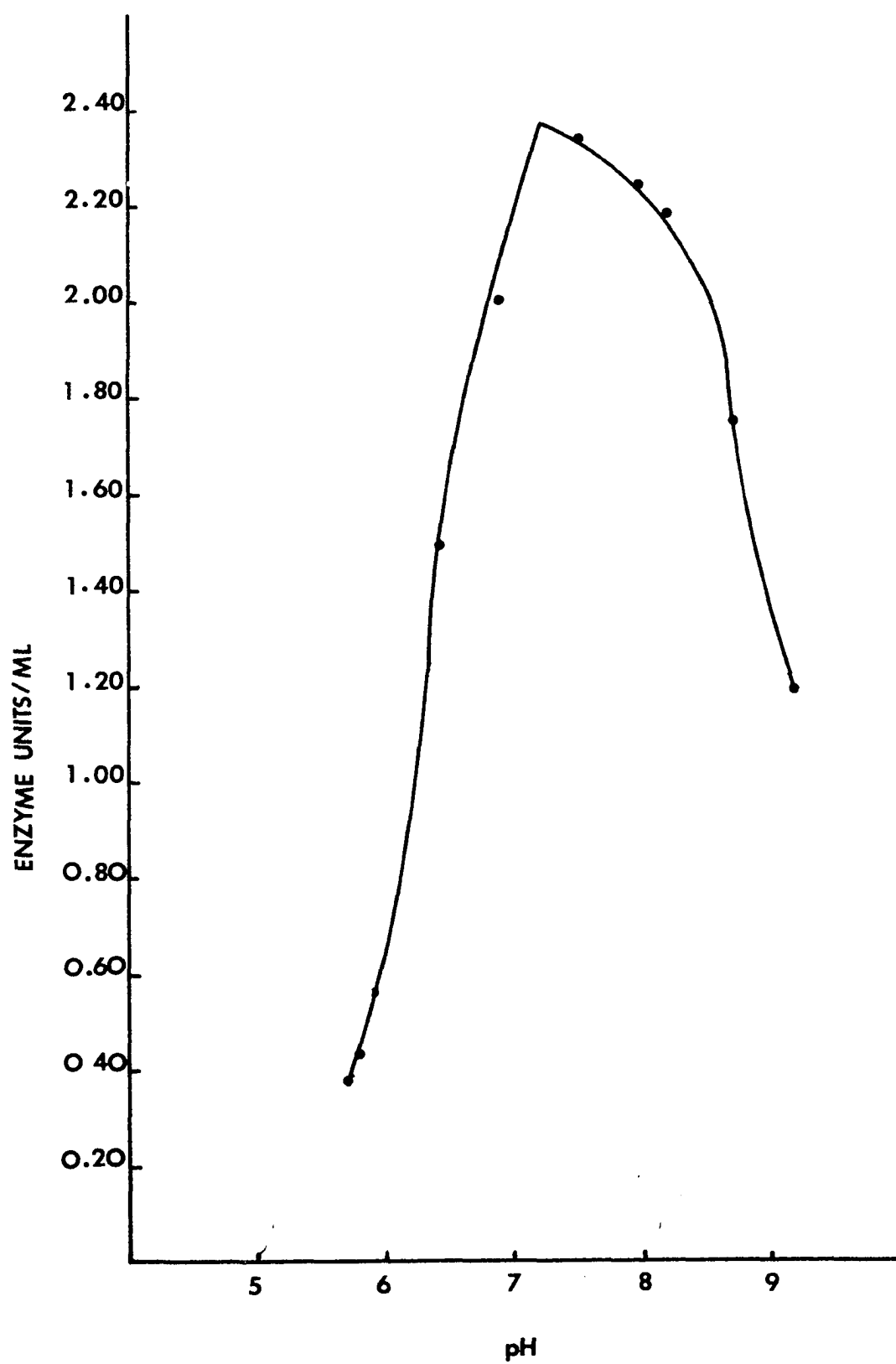


TABLE III

Effect of KCN on the Activity
of L-malate Oxidase and Dehydrogenase

Concentration of KCN	μ moles oxygen consumed per minute	μ moles DCI reduced per minute
control	0.311	4.350
10^{-2} M	0.251	5.920
10^{-3} M	0.300	5.660
10^{-4} M	0.311	5.430
10^{-6} M	-	4.520

Activity of L-malate oxidase assayed by measuring dissolved oxygen uptake with a Gilson Oxygraph. Dehydrogenase activity assayed by measuring the reduction of 0.05 mM DCI with a Gilford spectrophotometer. Only a slight inhibition of cytochrome system or increase of dehydrogenase activity was found at high concentration of KCN.

These data then posed new questions as to why there was no cyanide inhibition of the L-malate oxidase system. Low concentrations of cyanide (10^{-3} M or below) can inhibit cytochrome oxidase, while higher concentrations (10^{-2} M) can inhibit other enzymes in the oxidase system (13). Therefore, the lack of cyanide sensitivity could be due to a direct link between the dehydrogenase and oxygen, or simply a lack of a cyanide sensitive cytochrome a₃. To investigate this possibility, spectra of reduced cytochromes were run on a Coleman 124 double beam spectrophotometer.

β -TKM ghosts of M. lysodeikticus were resuspended in 0.01 M Tris-HCl buffer, pH 7.2, to a protein concentration of 1.5 mg/ml. Membranes were then sonicated twenty seconds and centrifuged ten minutes at 12,000 x g. An initial spectrum was determined on a 3.0 ml sample from 640 to 400 nm. The suspension was then placed in test tubes and aerated vigorously on a vortex mixer for twenty seconds. The enzyme suspension was then returned to the cuvette and the spectrum determined over the same range. These steps were done to ensure that none of the cytochromes remained reduced prior to the addition of substrate. Ten μ moles of L-malate were then added to the cuvette and the same volume of Tris-HCl buffer to the reference cuvette. The spectrum of the reduced minus oxidized cytochromes was then obtained. Successive aerations and spectra recordings were made for one hour.

Figure 6, summarizing these results, shows reduced cytochromes a, b and c as indicated by the appearance of bands at 600, 559 and 550 nm respectively. A β -band for b and c type cytochromes appeared at 520 nm. Repeated aerations of these reduced cytochromes reoxidized the cytochromes within one hour.

Figure 6

Reoxidation of L-malate Reduced Cytochromes
of Micrococcus Lysodeikticus

Sonicated β -TKM ghosts suspended in Tris-HCl buffer, pH 7.2, were scanned from 400 to 640 nm with a Coleman 124 double beam spectrophotometer. Scan A shows the oxidized cytochromes in the sonicated membranes. Scan B shows cytochromes a, b, and c which were reduced by 3.3 μ moles per ml L-malate. Scan C shows the reoxidized membranes 160 seconds after aeration (eight periods of 20 seconds each) over a time span of one hour.

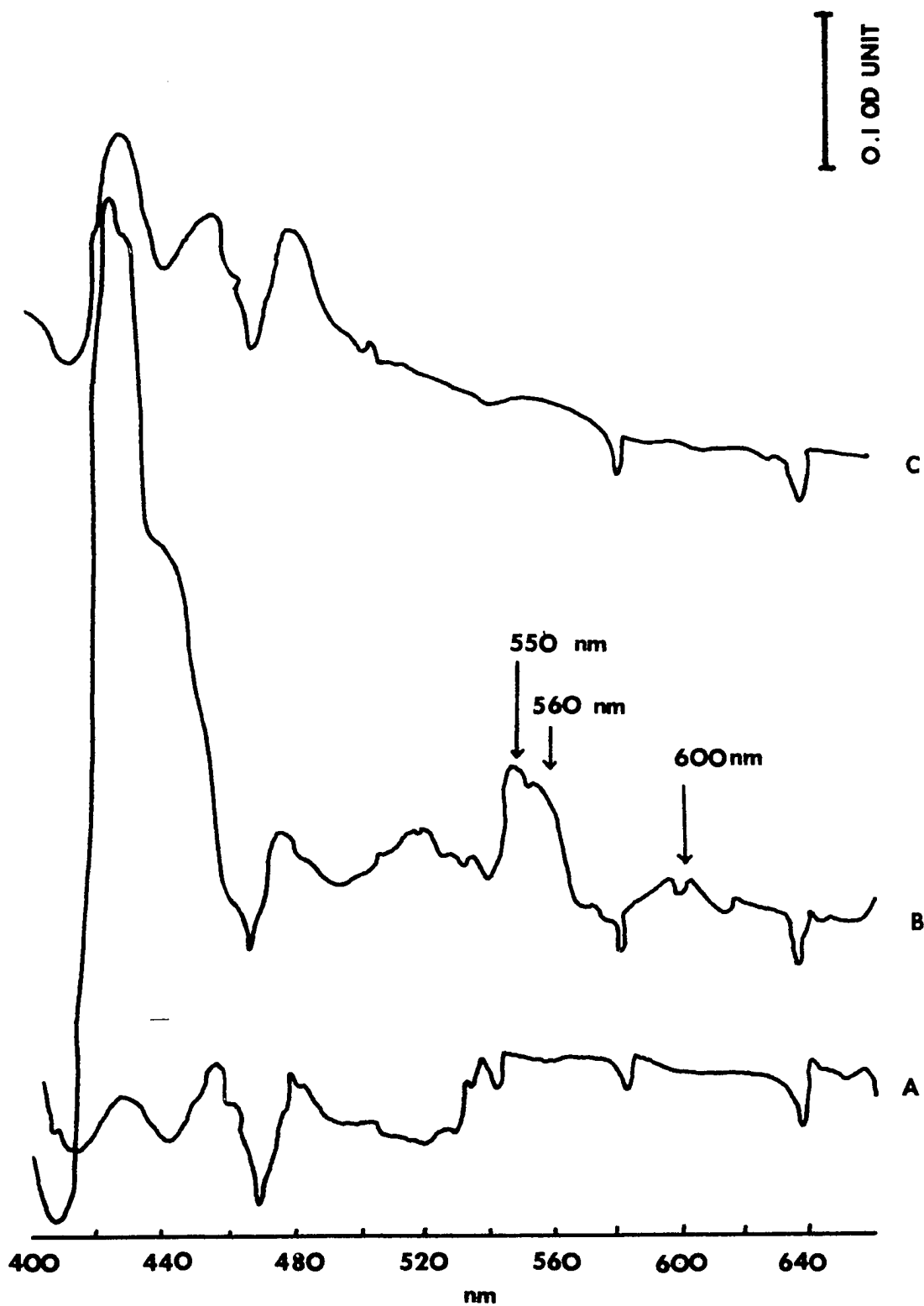


Figure 7

Reoxidation of L-malate Reduced Cytochromes of Micrococcus lysodeikticus
in the Presence of Cyanide

Sonicated β -TKM ghosts were suspended in Tris-HCl buffer, pH 7.2, and scanned from 400 to 640 nm with a Coleman double beam spectrophotometer. Scan A shows the oxidized membranes. Ten μ moles L-malate and 2.5 μ moles KCN were then added to the 3 ml assay mixture and Scan B run. This showed the reduced cytochromes a, b and c. Aeration for 160 seconds (Scan C) reoxidized the cytochromes in the presence of the KCN.

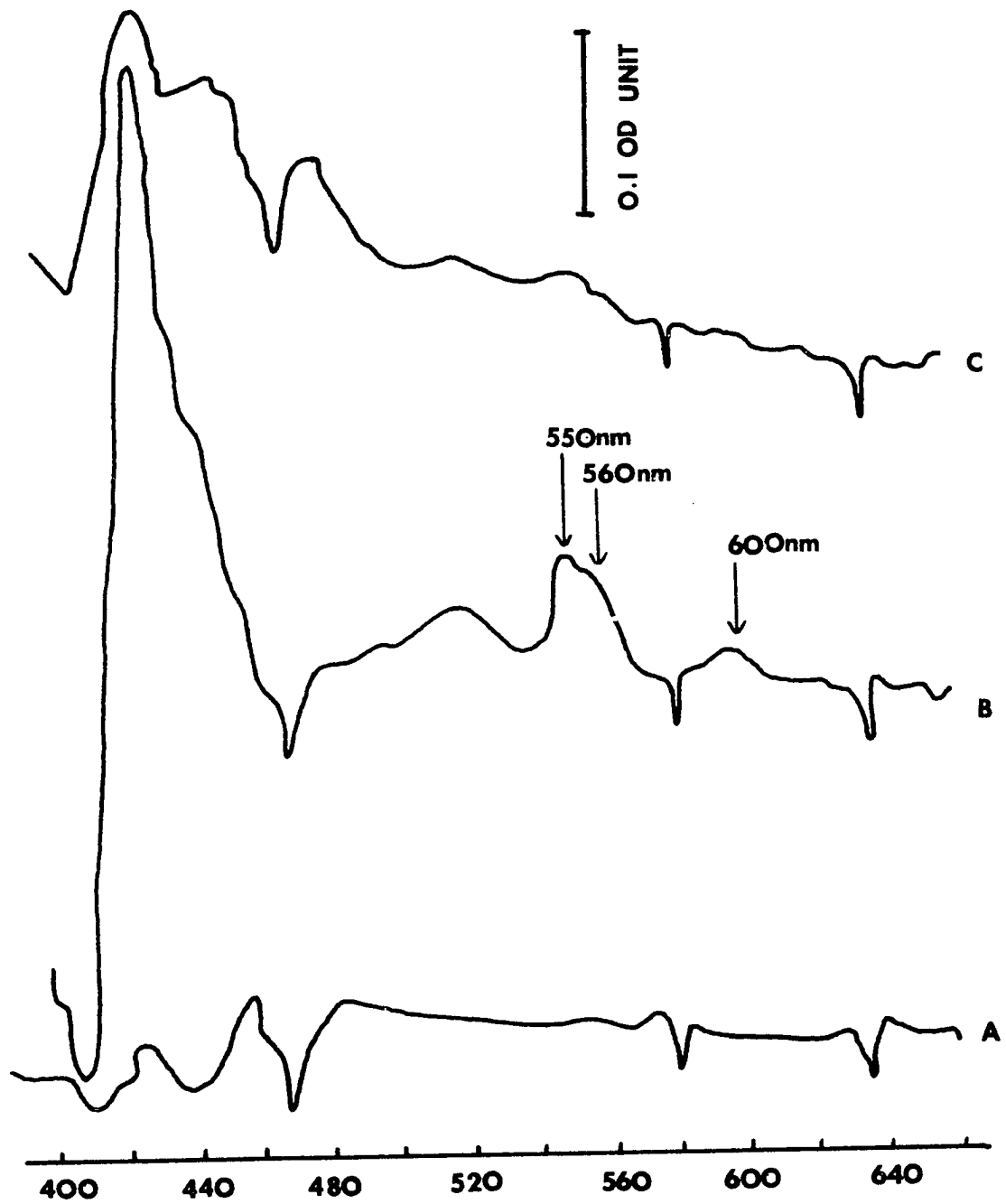


Figure 7 shows that cyanide added prior to L-malate had little or no effect on the reduction of the cytochromes or on their reoxidation.

Artificial Electron Acceptors for Soluble and Particulate L-malate Dehydrogenase

Various artificial electron acceptors have been utilized for the estimation of dehydrogenase activity. This investigator chose to use 2,6-dichlorophenolindophenol since it has a high extinction coefficient, and thereby provides a sensitive means for simple spectrophotometric dehydrogenase assays.

Table IV compares DCI with ferricyanide which has a lower extinction coefficient ($1 \text{ mM}^{-1} \text{ cm}^{-1}$). These data demonstrate that both the particulate and the solubilized L-malate dehydrogenase is more active with DCI as an electron acceptor than with ferricyanide.

A more sensitive L-malate dehydrogenase assay system was tested by adding phenazine methosulphate (PMS) to the colorimetric assay mixture. This PMS-DCI system was expected to be more efficient as PMS accepts electrons from reduced L-malate dehydrogenase more efficiently than DCI. The reduced PMS then releases electrons to DCI, reducing it to a colorless compound. Figures 8 and 9 show the increase in the rate of 0.05 mM DCI reduction by particulate and solubilized L-malate dehydrogenase in combination with increasing concentrations of PMS. One ml of the particulate L-malate dehydrogenase suspension reduced 4.83 μ moles of DCI per minute without PMS. The stepwise increase in PMS concentration from 0.1 to 1.0 mM increased the reduction rate of DCI to 7.76 μ moles per minute. The addition of the same quantity of PMS to the assay of the deoxycholate solubilized L-malate dehy-

Table IV

Comparison of 2,6-dichlorophenolindophenol
and Ferricyanide as Artificial
Electron Acceptors

Enzyme Preparation	moles L-malate oxidized per ml per minute	
	DCI as e^- acceptor	$\text{Fe}(\text{CN})_6^{3-}$ as e^- acceptor
Particulate L-malate dehydrogenase	4.209	0.295
Solubilized L-malate dehydrogenase	0.758	0.099

The activity of particulate L-malate dehydrogenase was determined using sonicated ~~Δ~~-TKM ghosts, and the solubilized enzyme was obtained by treatment with deoxycholate. Micromoles L-malate oxidized was calculated as μ moles DCI reduced per ml per minute, or as two μ moles ferricyanide reduced per ml per minute. A concentration of 0.05 mM DCI or 1.0 mM $\text{K}_2\text{Fe}(\text{CN})_6$ produced an absorbance of 1.0. The DCI assay was at a wavelength of 600 nm and the ferricyanide assay was at 420 nm.

Figure 8

Effect of Phenazine Methosulphate Concentration on the Reduction
of DCI by Particulate L-malate Dehydrogenase

Sonicated β -TKM ghosts of Micrococcus lysodeikticus were suspended in Tris-HCl buffer, pH 7.2, and the L-malate dehydrogenase activity estimated by the reduction of DCI in the presence of 0.0 to 1.0 mM PMS. The initial optical density was adjusted to 1.0 with 0.05 mM DCI.

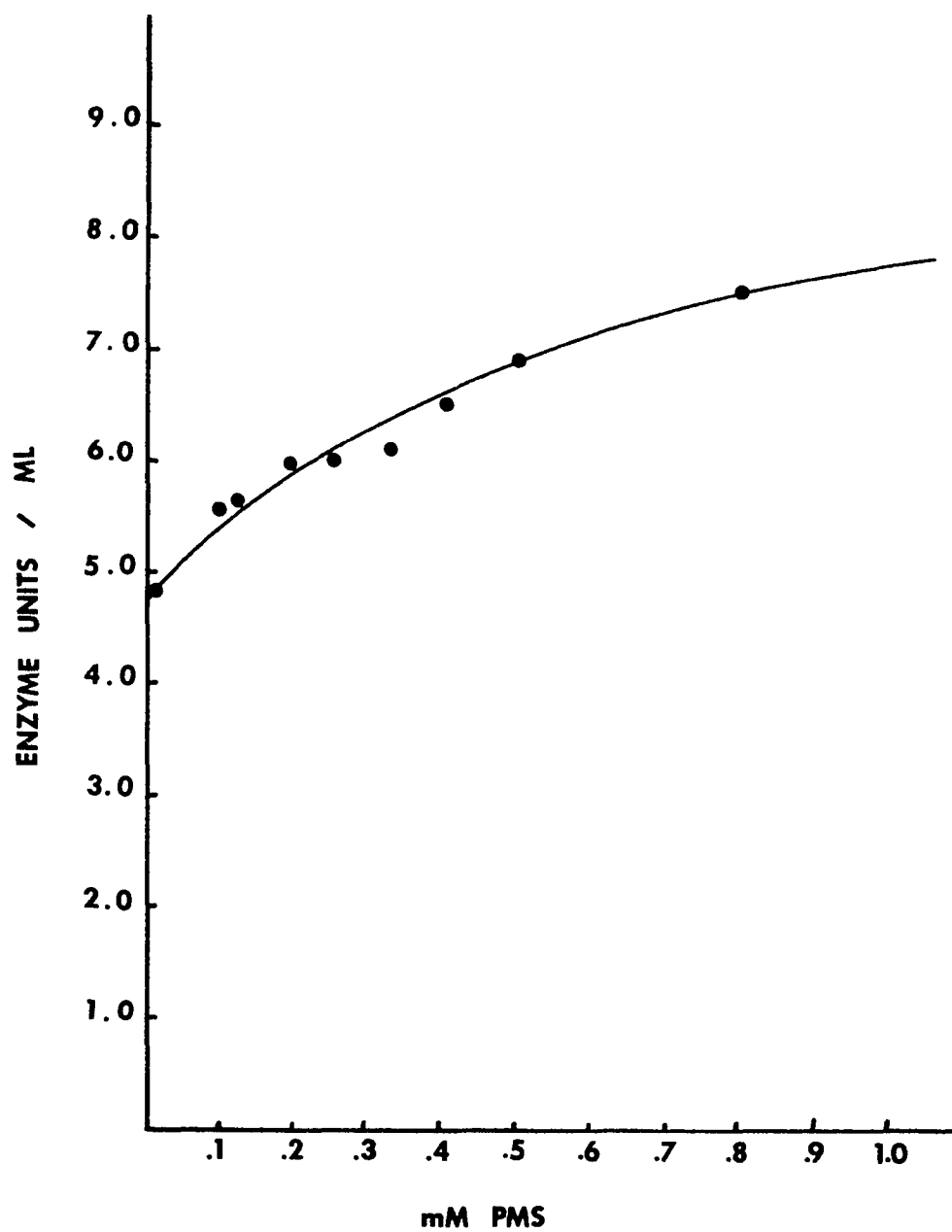
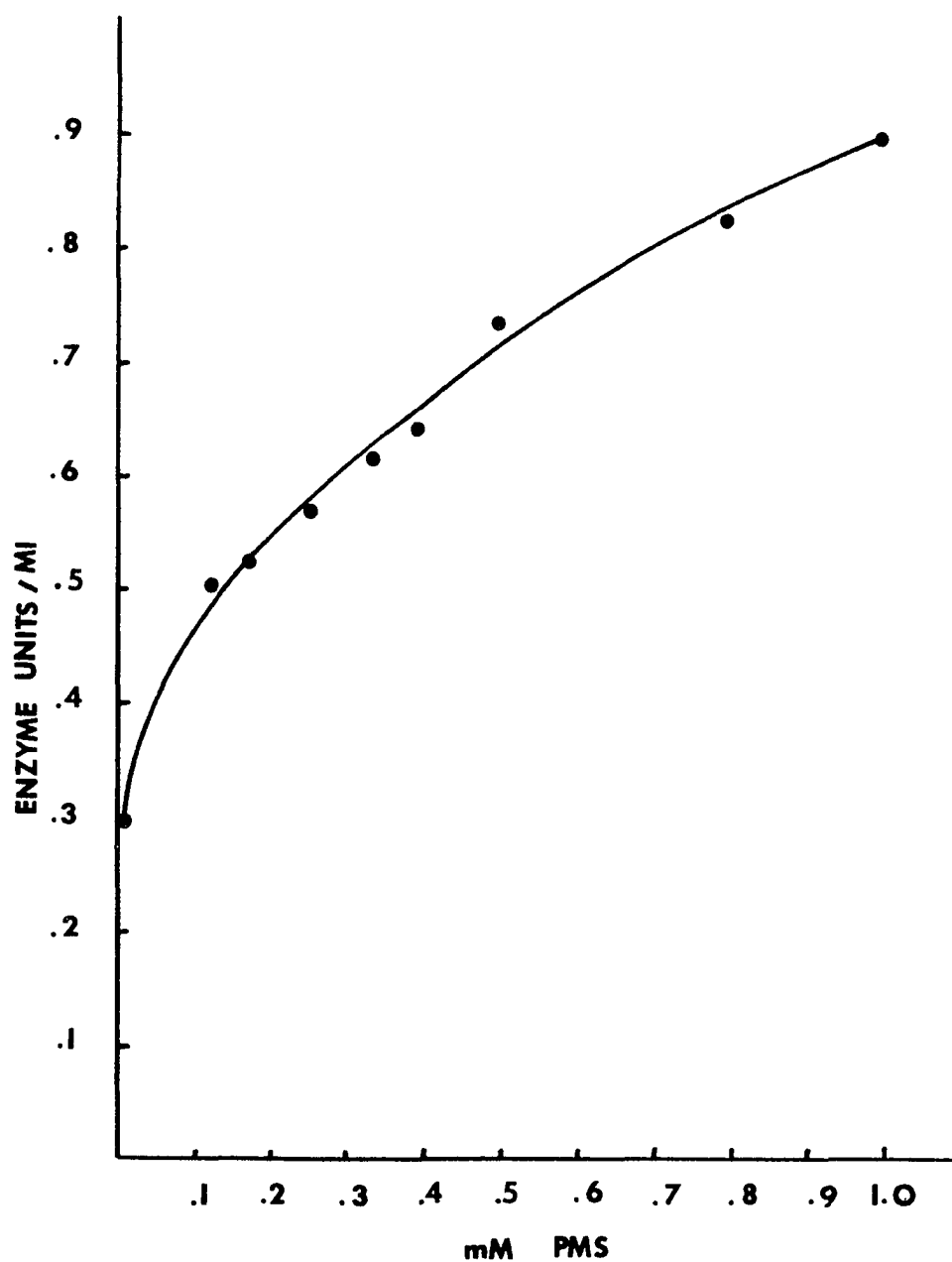


Figure 9

Effect of Phenazine Methosulphate Concentration on the Reduction
of DCI by Solubilized L-malate Dehydrogenase

5 -TKM ghosts of Micrococcus lysodeikticus were removed from storage, thawed, and resuspended in Tris-HCl buffer, pH 7.2. Deoxycholate (1.0 mg per ml) was added to the resuspended membranes and the mixture centrifuged at 100,000 x g for one hour. The supernatant was decanted and retained for assay of solubilized L-malate dehydrogenase. The assay was performed using a constant concentration of DCI (0.05 mM) and PMS concentrations varying from 0.0 to 1.0 mM.



drogenase increased the reduction rate of DCI from 0.300 to 0.889 micromoles per minute.

A Lineweaver-Burk plot of these data (see Figures 10 and 11) demonstrates that the V_{\max} for the particulate L-malate dehydrogenase is 8.84 enzyme units and 3.33 units for the detergent solubilized enzyme.

These data showed that DCI was a more efficient electron acceptor than ferricyanide and that the efficiency of DCI could be further increased by using PNS as a coupling agent between the reduced L-malate dehydrogenase and DCI. However, the DCI-PMS system was not used for this investigation as the direct reduction of DCI by L-malate dehydrogenase provided a sensitive assay in which the units of enzyme activity were proportional to its concentration.

Km of Particulate and Solubilized L-malate Dehydrogenase and L-malate Oxidase

The Km was determined for the particulate and solubilized L-malate dehydrogenase and for the L-malate oxidase system of M. lysodeikticus. The enzyme suspensions were prepared in Tris-HCl buffer as previously described, and the actual Km was calculated from Lineweaver-Burk plots using the least squares method. The Michaelis constant was then determined to see if there was any difference in the kinetics of these enzymes. The Lineweaver-Burk plots in Figures 12, 13, and 14 show that there are different Km values for the particulate and solubilized L-malate dehydrogenase and the L-malate oxidase system. A Km of 3.61×10^{-4} M was demonstrated by the particulate dehydrogenase, 1.98×10^{-4} M for the solubilized enzyme, and 7.81×10^{-4} M for the L-malate oxidase system.

Figure 10

Km for Particulate L-malate Dehydrogenase Using 0.05 mM DCI
With Varying Concentrations of PMS

β -TKM ghosts of Micrococcus lysodeikticus were removed from storage, thawed, and resuspended in Tris-HCl buffer, pH 7.2. This membrane resuspension was then used for the colorimetric assay of particulate L-malate dehydrogenase. The DCI reduction was assayed at an initial optical density of 1.0 in the presence of 0.0 to 1.0 mM PMS. The V_{\max} was determined to find the rate of DCI reduction at infinite PMS concentration.

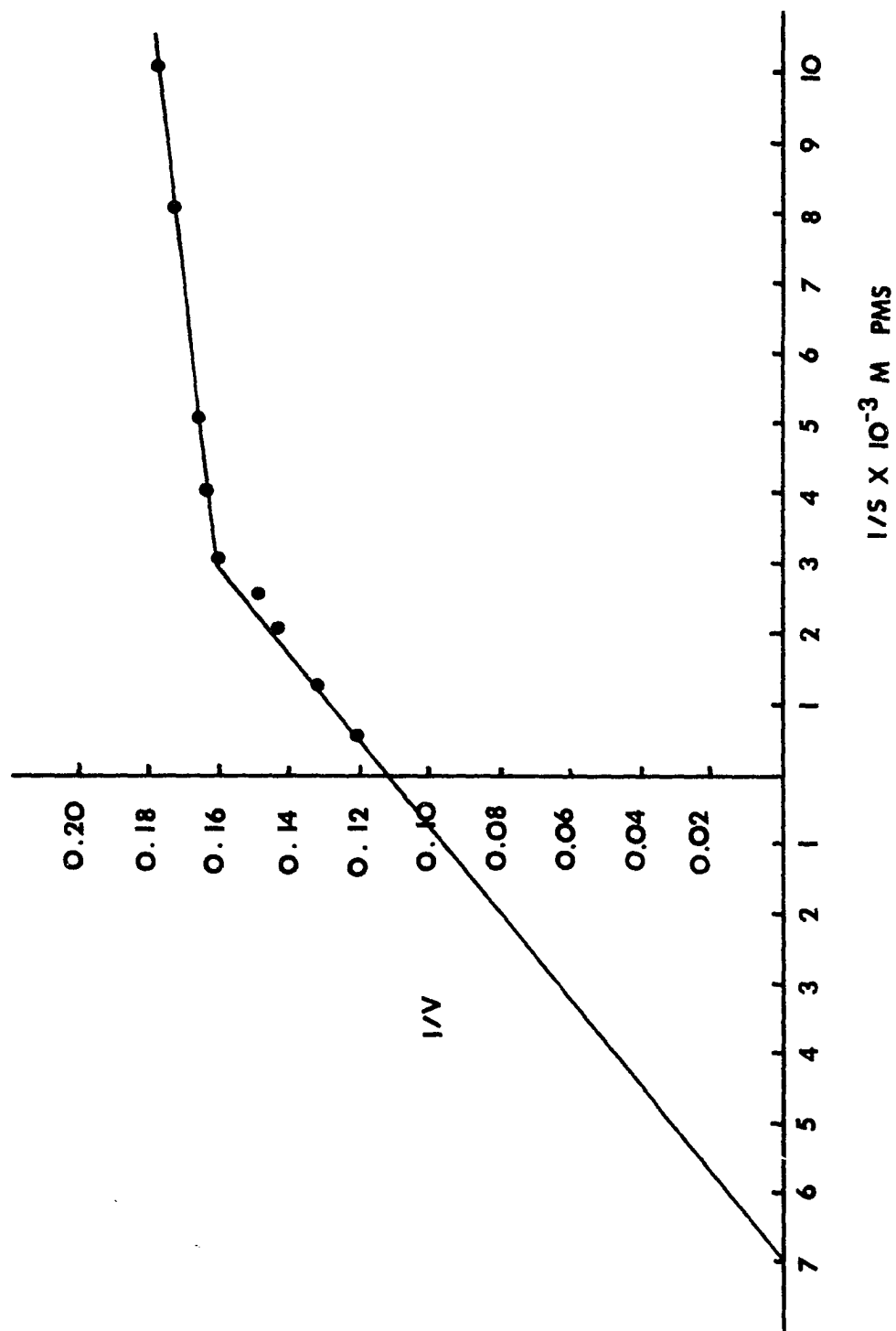


Figure 11

K_m for Solubilized L-malate Dehydrogenase Using 0.05 mM DCI
With Varying Concentrations of PMS

β -TKM ghosts of Micrococcus lysodeikticus were resuspended in Tris-HCl buffer, pH 7.2, and deoxycholate (1.0 mg per ml). The solubilized enzyme was prepared and assayed with PMS as described in the legend for Figure 9. The V_{\max} was determined to find the rate of DCI reduction at infinite PMS concentration.

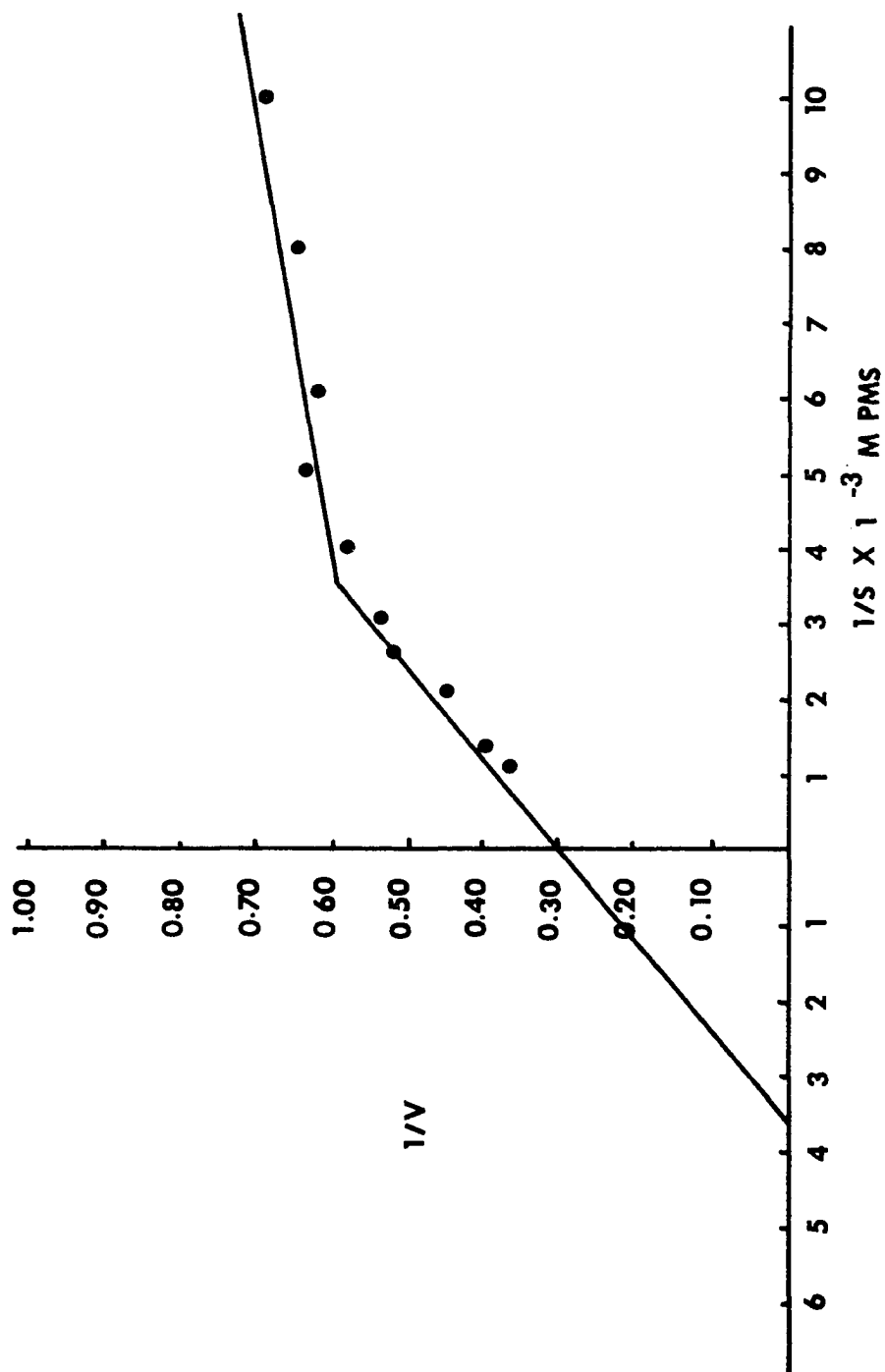


Figure 12

Km of Particulate L-malate Dehydrogenase

S-TKM ghosts of Micrococcus lysodeikticus were removed from storage, thawed and resuspended in Tris-HCl buffer, pH 7.2. This membrane resuspension was then used for the assays which were run with varying concentrations of L-malate. V = enzyme units, and S = molar concentration of substrate (L-malate).

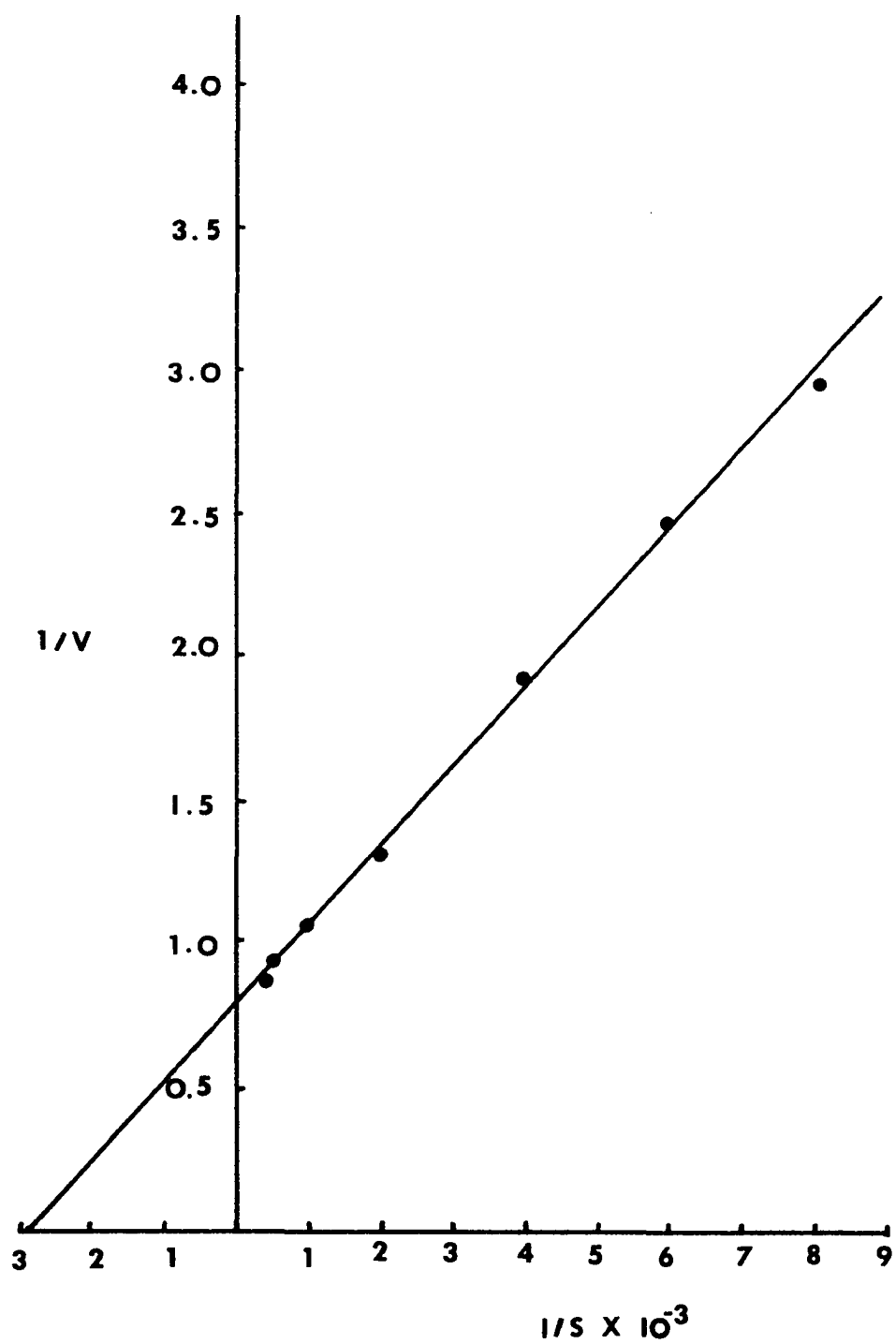


Figure 13

Km of Solubilized L-malate Dehydrogenase

β -TKM ghosts of Micrococcus lysodeikticus were removed from storage, thawed, and resuspended in Tris-HCl buffer, pH 7.2. The solubilized enzyme was prepared as described in the legend for Figure 9. Km was developed using a constant concentration of DCI (0.05 mM) and varying concentrations of L-malate. V = enzyme units, and S = molar concentration of L-malate.

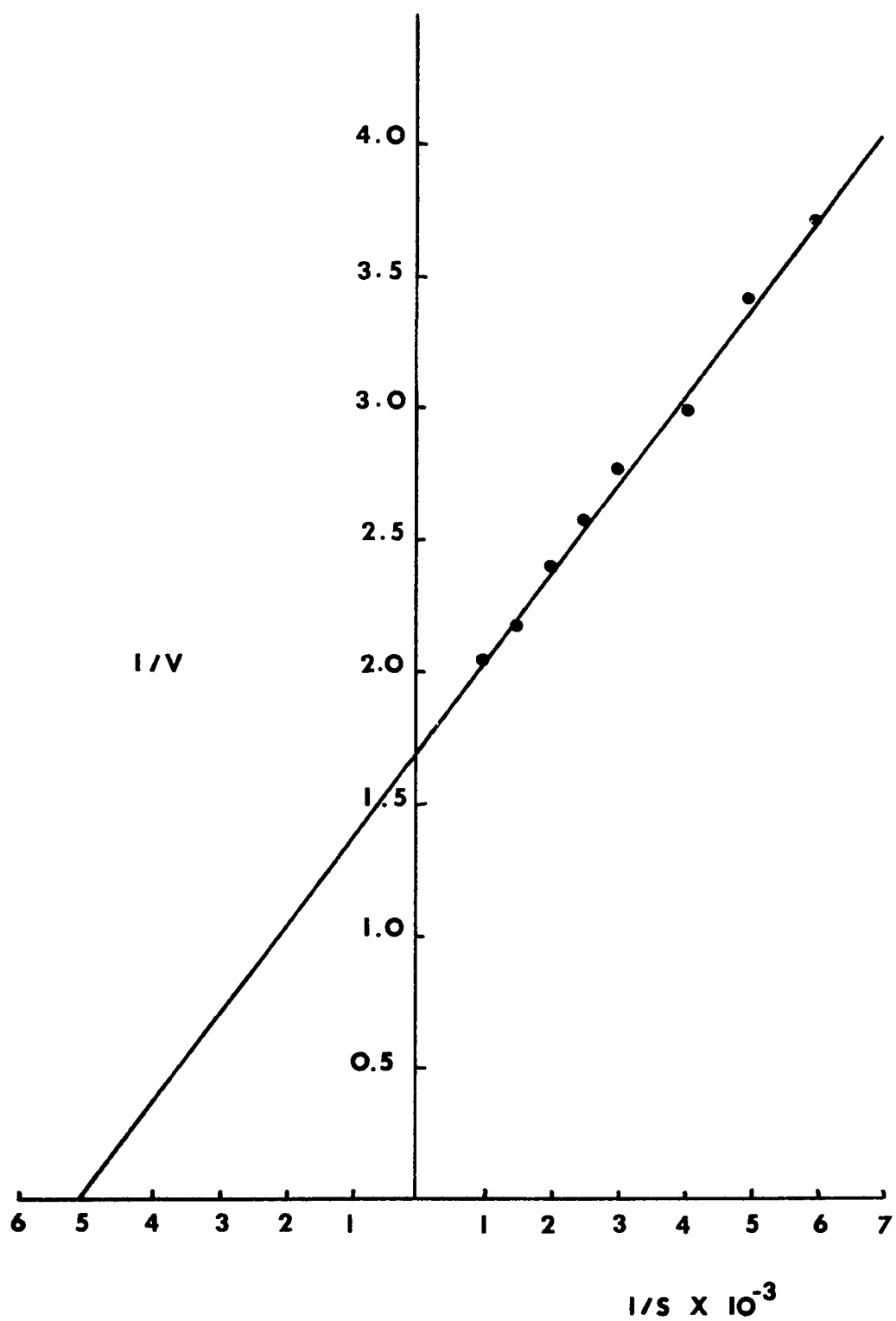
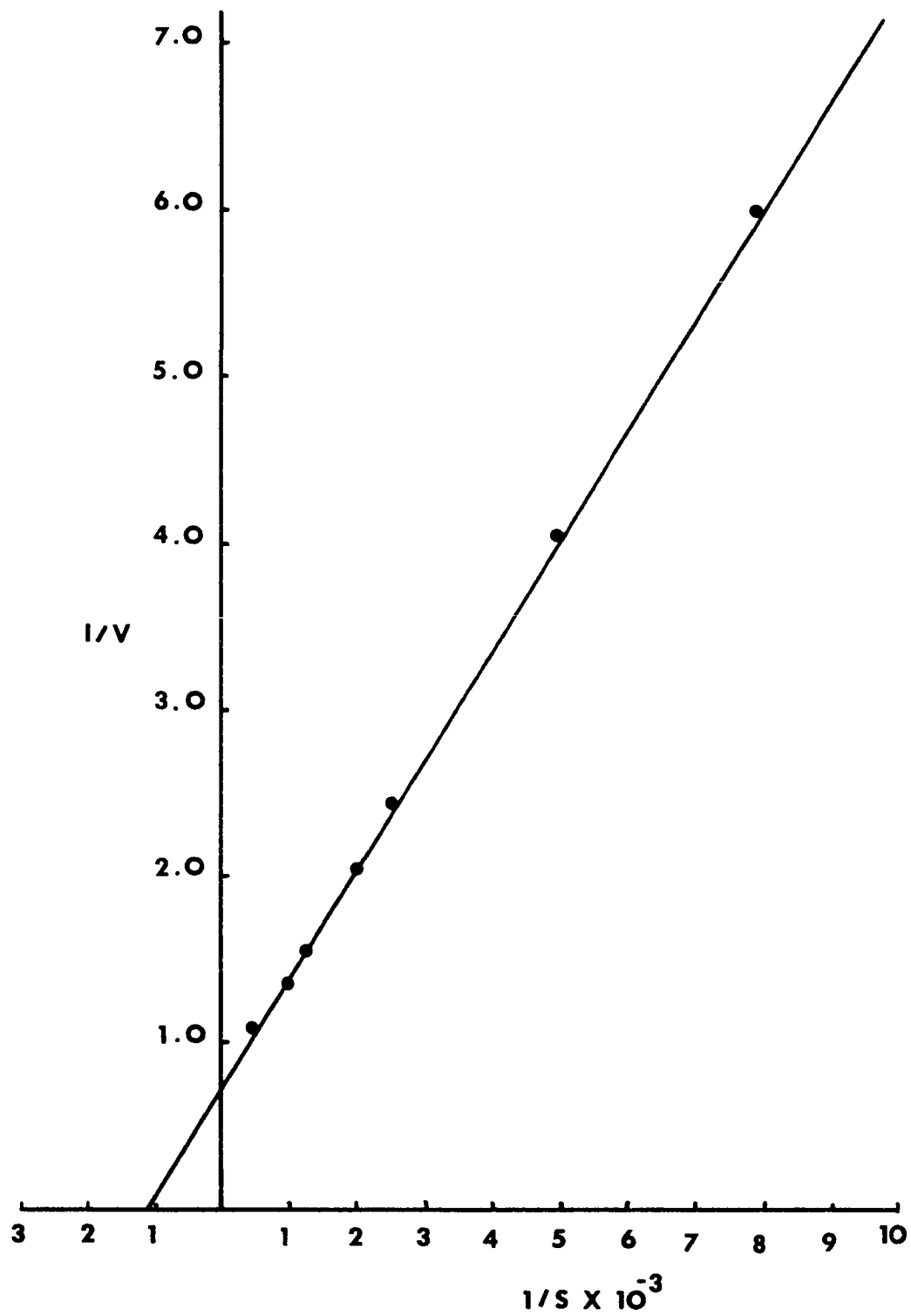


Figure 14

Km of L-malate Oxidase

β -TKM ghosts of Micrococcus lysodeikticus were removed from storage, thawed, and resuspended in Tris-HCl buffer, pH 7.2. Oxidase activity was measured by uptake of dissolved oxygen with a Gilson Oxygraph. $V = \mu\text{moles O}_2$ taken up per minute, and S = molar concentration of substrate.



The above data indicate that there may be a slight change in the enzyme-substrate affinity when the dehydrogenase enzyme is solubilized with deoxycholate. The slightly lower K_m for the solubilized dehydrogenase may indicate a small change in the state of the enzyme. The small difference in the K_m 's, however, do not indicate that there was a major change in the physical state of the enzyme when it was solubilized. The higher K_m for the oxidase system was expected since the oxidase is a multienzyme system, or complex, instead of a single enzyme such as the dehydrogenase.

DISCUSSION

This study was undertaken to survey the basic characteristics of the membrane-bound L-malate dehydrogenase and oxidase system of Micrococcus lysodeikticus. These enzymes are part of the protein components of the plasma membrane of this bacteria. Any insight gained through the study of this enzyme system will help in elucidating the structure and function of bacterial membranes.

Preliminary studies in our laboratory had determined that the L-malate dehydrogenase and oxidase complex of M. lysodeikticus ISU Ad + were proteins that were bound to the plasma membrane. The L-malate dehydrogenase proved to be an active enzyme that could be assayed via a simple colorimetric assay. The multienzyme oxidase system could be assayed by measuring consumption of dissolved oxygen with an oxygraph. Since this was a membrane associated enzyme system that was both active and convenient to assay, it was chosen for an examination of the L-malate dehydrogenase and oxidase complex as one means of investigating membrane phenomena.

Protoplast ghosts of M. lysodeikticus were used routinely for the enzyme studies as they contained all of the membrane-bound L-malate dehydrogenase and oxidase activities and could be stored at -60° C for four months without significant loss of activity.

Earlier work with protoplast ghost preparations similar to those utilized in this study had demonstrated substrate permeability barriers which were overcome by sonication (8). Figure 2 shows the M. lysodeikticus β -TKM ghosts also exhibited this trait as the L-malate dehydrogen-

ase activity was increased to a maximum by ten seconds sonication. It has been indicated (8) that sonication physically disrupts the protoplast membranes permitting the substrate more direct access to the dehydrogenase enzyme. Figure 2 also demonstrates that divalent cations decrease the dehydrogenase activity of the unsonicated membranes. However, after ten seconds sonication the dehydrogenase activity increased, possibly by some removal of permeability barriers through the disruption of vesicles. In some cases a slightly higher activity was observed with sonication in the presence of divalent cations.

These data indicate that divalent cations enhance permeability barriers in the intact membranes. However, once this barrier is removed by sonication, the activity of the dehydrogenase enzyme is increased by the presence of divalent cations, possibly due to reaggregation of the sonicated protein. A similar example of this has been demonstrated with detergent solubilized NADH oxidase of Bacillus megaterium KM (9). In view of these findings, the protoplast ghosts used in this study were sonicated routinely for ten seconds in the presence of 0.01 M MgCl_2 .

The L-malate dehydrogenase used in this study was active without the addition of NAD^+ . This finding indicates that the enzyme is a non- NAD^+ dependent L-malate dehydrogenase such as Cohn (4) found in his 0.0 to 20 percent (w/v) ammonium sulphate fraction. No attempt was made to isolate the L-malate dehydrogenase and determine whether or not bound NAD^+ was present or absent. However, subsequent work has shown that there was NADH dehydrogenase and oxidase present in the protoplast membranes of this organism (19).

One approach which has been used to study the molecular composition of a membrane is the solubilization and separation of the components followed by the reestablishment of the components function (8). Therefore, it was necessary to examine solubilization procedures and the characteristics of the solubilized and reconstituted enzyme. Table I shows that deoxycholate proved to be an effective agent for solubilizing the membrane-bound L-malate dehydrogenase. Table II indicates that 0.6 mg per ml deoxycholate at a protein to detergent ratio of 3:1 effectively solubilized the enzyme without activity destruction. If the concentration of the deoxycholate was increased, the enzyme was inhibited, a possible result of detergent caused denaturation.

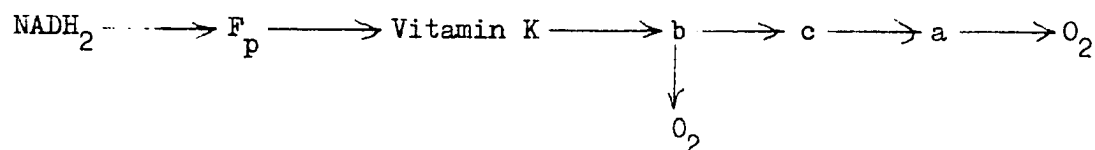
Once the permeability and solubilization characteristics of the L-malate dehydrogenase was determined, the pH characteristics of the enzyme system was examined. Prior to determining the pH dependences of L-malate dehydrogenase it was necessary to measure the effect of pH change on DCI. As shown in Figure 3, the absorbance of DCI is constant at a pH of 6.7 and above. However, below 6.7 there is a linear decrease in DCI absorbancy which correlates directly with decreasing pH. This produces a corresponding drop in the extinction coefficient of DCI which necessitated its recalculation for all pH values below 7.6

Using the corrected values found above it was determined that the optimum pH for each physical state of the L-malate dehydrogenase was in fact different. The pH values for solubilized, membrane-bound, and L-malate oxidase were 6.3, 6.9, and 7.2 respectively. These differences might be explained by the differences in the physical state of the en-

zymes, e.g., the particulate enzyme could be buried in a lipid matrix on the membrane while the detergent solubilized enzyme could be free of any such shield resulting in a variance of pH characteristics.

On the other hand, L-malate oxidase is a multienzyme system which removes electrons from L-malate and transports them through the cytochrome system to molecular oxygen as a final electron acceptor. The pH optimum for each enzyme in the system will influence the pH optimum for the total complex shown to be 7.2, a value which might be explained as the result of a rate limiting step within the system. A pH optimum was not determined for solubilized L-malate oxidase because the L-malate dehydrogenase component cannot transfer its electrons to the cytochrome system unless it is physically associated with a particulate system (23). Thus, a pH determination on solubilized L-malate oxidase would be meaningless or not feasible experimentally.

In an attempt to improve the efficiency of the colorimetric dehydrogenase assay, cyanide was tested as an agent to inhibit cytochrome oxidase. This would assure that all electrons were utilized to reduce DCI and not be shunted to oxygen via the cytochrome oxidase. However, Table III demonstrates that cyanide has no effect on the cytochrome system of M. lysodeikticus. This lack of cyanide inhibition is unusual in bacteria, but it has been shown by other investigators. For example, Fujita, et al., (11) reported cyanide insensitivity in M. lysodeikticus in 1966. Based on spectral data, he proposed the following scheme:



in which he thought that there was a break in electron transfer between cytochrome b and c, and that cytochrome b was acting as a cytochrome oxidase. If this cytochrome b was not sensitive to cyanide this might possibly explain the lack of cyanide inhibition in the oxidase system.

Further evidence for this conclusion was presented by Gelman (12) who found an atypical cytochrome a which absorbed at 600 nm instead of 605 nm which is usual for a₃ cytochrome oxidase. He proposed that this atypical enzyme was cyanide insensitive which gave M. lysodeikticus its insensitivity to KCN.

Gelman's findings of cytochrome a, b and c are in complete agreement with the results of this investigation. Figures 6 and 7 show that all three of these cytochromes are present, reduced by L-malate and re-oxidized by aeration either in the presence or absence of cyanide. If Fujiata's proposed system were correct it would be probable that cytochromes c and a would remain reduced when in the presence of KCN. However, were an insensitive cytochrome a present, the flow of electrons from cytochrome b to c to a and on to oxygen would remain uninterrupted, and the spectra would have demonstrated a permanently reduced cytochrome oxidase. Another possibility which could account for the cyanide insensitivity data is reversed electron flow. This possibility cannot be evaluated, however, from this data.

An efficient colorimetric dehydrogenase assay depends upon an artificial electron acceptor which readily accepts electrons from the reduced enzyme. This electron acceptor must then be reduced to a colorless state, or at least change its optical density a measurable amount. Several colorimetric assay systems were evaluated in this study in order to

find an efficient means of measuring the activity of L-malate dehydrogenase.

DCI was chosen as the electron acceptor for this work as it has a high extinction coefficient and thereby provides a sensitive means for a simple spectrophotometric assay. Ferricyanide has a lower extinction coefficient and, therefore, provides a less sensitive assay. Both solubilized and particulate L-malate dehydrogenases were assayed using these two electron acceptors. Table IV shows that the ratio of solubilized to particulate enzyme is 1:3 using ferricyanide as an electron acceptor and 1:5.6 using DCI. On the other hand, the ratio of ferricyanide to DCI as an electron acceptor is 1:7.7 for the solubilized enzyme and 1:14 for the particulate L-malate dehydrogenase.

Phenazine methosulphate is also an efficient electron acceptor and in addition it is autooxidizable. This means that it accepts electrons from a reduced dehydrogenase more efficiently than DCI and then is reoxidized by passing the electrons to O_2 or an artificial acceptor such as DCI. It provides a very efficient coupling between a dehydrogenase and an electron acceptor. Figures 8 and 9 show the increase in the rate of DCI reduction when the concentration of PMS is increased from zero to 1.0 mM in the assay mixture for the particulate and solubilized enzyme. The Lineweaver-Burk plots in Figures 10 and 11 show that an infinite concentration of PMS would increase the rate of DCI reduction by the particulate enzyme 83 percent, and the solubilized enzyme by 338 percent. However, PMS was not routinely used in this investigation since the direct reduction of DCI by L-malate dehydrogenase provided an efficient assay for measuring dehydrogenase activity.

In order to further explore the characteristics of these enzymes, a Michaelis-Menton constant was determined for the L-malate oxidase system,

as well as for the solubilized and particulate dehydrogenases. Figures 12 and 13 demonstrate a K_m of 3.61×10^{-4} M for the particulate and 1.98×10^{-4} for the solubilized enzyme. The slightly higher K_m for the particulate L-malate dehydrogenase might be due to permeability barriers created by the membrane. However, the K_m 's for these two enzymes are similar enough to indicate that there is not a major change in the physical state of the enzyme when it is detergent solubilized. The K_m of 7.81×10^{-4} M for the L-malate oxidase system is higher than either the solubilized or particulate dehydrogenase. This could be explained by the fact that the oxidase is a multienzyme complex instead of a single enzyme such as the dehydrogenase.

Table V summarizes the characteristics of the particulate and deoxycholate solubilized L-malate dehydrogenase. These data show that the enzyme is slightly modified by treatment with deoxycholate, but its physical state is not significantly altered.

This preliminary characterization of the membrane-bound L-malate dehydrogenase-oxidase complexes of M. lysodeikticus has indicated that this is a readily prepared and active system for study of membrane-bound enzymes. By developing the characteristics of this system as a functional component of the plasma membrane, insight can be gained into the molecular structure of the membrane. At this level of biological complexity, structure and function are inseparable and additional insight into one contributes into knowledge of the other.

Table V

Characteristics of Particulate and Solubilized
L-malate Dehydrogenase Enzyme

Characteristic	Particulate	Solubilized
pH optimum	6.9	6.3
Enzyme activity (ferricyanide as e^- acceptor)	0.30	0.10
Enzyme activity (DCI as e^- acceptor)	4.21	0.76
Percent increase using PMS with DCI	83	338
$K_m \times 10^{-4} \text{ M}$	3.61	1.98

Summary of the characteristics of the particulate L-malate dehydrogenase and the deoxycholate solubilized dehydrogenase enzyme.

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