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Studies of the effect of lipid micelles on mitochondrial malate dehydrogenase.

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STUDIES OF THE EFFECT OF LIPID MICELLES ON MITOCHONDRIAL MALATE DEHYDROGENASE

BY

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ABSTRACT

M-MDH is known to be very unstable in aqueous solution. This instability may be ascribed to removal of the enzyme by severe extraction procedures, from its bound state in the mitochondrion.

Whole heart lipid micelles, mitochondrial lipids and fatty acids increase the stability of the enzyme. The degree of lipid ability to increase activity of M-MDH is dependent on the ionic character of the environment. Thus the enzyme exhibits higher activity in a lipid-phosphate system than in lipid-Tris-acetate or lipid-KCl. Likewise, the enzymatic activity is higher in lipid than in non-lipid media.

The ability of lipid to stimulate M-MDH activity is independent of pH, ionic strength and temperature (between 6° and 37° C).

Preliminary evidence suggests that lipid acts by altering the conformation of the tertiary structure of the enzyme.

The results, in general, suggest that hydrophobic group interactions increase enzyme stability. These interactions may, in part, simulate the actual type of binding which stabilizes the enzyme activity in vivo.

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Acknowledgements must also be made to the National Research Council of Canada for financial assistance.

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1.	Alb.	=	Bovine serum albumin
2.	DCA	=	Deoxycholic acid
3.	LM	=	Lipid micelles
4.	M-MDH	=	Mitochondrial malate dehydrogenase
5.	NAD^+	=	Nicotinamide adenine dinucleotide (Oxidized form)
6.	NADH	=	Nicotinamide adenine dinucleotide (Reduced form)
7.	OAA	=	Oxalacetate
8.	P	=	Phosphate (as in Lipid-P)
9.	S-MDH	=	Cytoplasmic malate dehydrogenase

CHAPTER 1

INTRODUCTION

Malate dehydrogenase (E.C.1.1.1.37) catalyses the reaction:

L-malate + NAD^+ + MDH $\rightleftharpoons$ OAA + NADH + MDH (1)

MDH exists in mammalian tissues in two chemically and physically distinct species — a mitochondrial (M) and a cytoplasmic (S) form (1,2). The enzyme has been isolated by many workers (3-12). Kun (13) has written a review of the early history of MDH.

M-MDH requires relatively drastic procedures for isolation including an acetone-dried powder preparation and an ethanol fractionation (1,2,9). The difficulty of M-MDH isolation suggests that the enzyme is firmly bound in the intact mitochondrion. Griffiths and Robertson (14) have isolated from pig heart mitochondria a particulate fraction capable of electron transfer activity which has MDH activity firmly bound to it. This MDH activity could not be washed out of the particulate fractions by repeated aqueous extraction, nor was there considerable loss of the particulate activity by this treatment. The enzyme is presumably firmly bound to the mitochondrial framework, which consists of a network of lipid and protein interactions (15,16). Green *et al.* have isolated a water

insoluble protein of 20,000-30,000 M.W. which has a high hydrophobic character (17,18,19). Amino acid analyses of this "structural" protein (S.P.) indicated that 51% of the molecule has a non-polar character. Further studies showed that the S.P. from beef heart mitochondria can form stable complexes with cytochromes a and b. It was thus concluded that hydrophobic protein-protein interactions conferred a high degree of stabilization on mitochondrial structure.

The other mitochondrial component capable of hydrophobic interactions is phospholipid. Phospholipid constitutes 20% of the total weight of the mitochondrion (15,20) and has been shown to be present in particulate fractions isolated from pig heart mitochondria (21). The role of phospholipid in the mitochondrion is not structural (22) but catalytic (23-27). Extraction of sub-mitochondrial particles with an acetone-water mixture (90:10, v/v) removes approximately 80% of all phospholipids present and results in loss of electron transfer activity. However, readdition of phospholipids as optically clear micelles, supplemented with coenzyme Q, restored electron transfer activity (28,29). Since MDH has been found bound to such particulate fractions, it is reasonable to assume that MDH resides in a phospholipid-protein hydrophobic framework.

Succinic dehydrogenase (SDH) has been shown to be firmly bound to sub-mitochondrial particles (21,22,26-30).

Extraction of these particles with organic solvents ie. butanol, or bile salts can solubilize SDH. However, SDH in its water soluble form is unstable as shown by a number of criteria. SDH showed a loss of its Electron Spin Resonance signal on "aging" and this result lead King (31,32) to postulate a labile site of the enzyme which may be involved in reconstitution of the tertiary structure but not participant in electron transfer. Kimura and Hauber (33) postulated at least three different forms of SDH to account for changes of the enzyme on aging in the phenazine methosulfate assay, the succinoxidase assay and ability to bind in an alkali-treated assay (Keilin-Hartree preparations). The authors suggest that enzyme aging is due to the changes in tertiary structure resulting from the drastic extraction procedures. Cerletti et al. (34,35) support the above findings but have found that incubation of "soluble" SDH in the presence of lipid micelles prepared according to Fleischer and Klouwen (28) compared to a non-lipid control, produced a twofold increase in the stability of the enzyme. Cerletti et al. (34,35) suggest that the instability is in part due to the removal of some essential component from the surface of the enzyme during the extraction procedure which was present in the particulate fraction. The increased stability of the enzyme in lipid was thought to be the result of lipid either producing a favourable medium for the enzyme or by modifying the protein (35).

Similarly, MDH is isolated by a procedure known to remove phospholipids and is very unstable in its water soluble form. An amino acid analysis of pig heart M-MDH reveals that there is a high degree of non-polar side chains thus making it possible that hydrophobic interaction stabilizes MDH in the mitochondrion (19).

Indirect evidence for a role for phospholipids in MDH has been presented by Fujimoto and Harary (36). They found that isolated rat heart cells when cultured in a medium of lipid-deficient serum albumin, lost their ability to "beat" and also MDH and Ca-ATPase activities, while Glucose-6-phosphate dehydrogenase activity increased. Addition of a 1:1 mixture of human serum and foetal pig lipids restored "beating" and also restored the enzyme activity levels to normal. The effect observed by these workers probably involved mitochondria which are known to lose catalytic activity in lipid deficient media.

Gamble et al. (37) demonstrated that isolated heart mitochondria have the capacity to accumulate citrate and malate and to retain them through washing procedures with isotonic sucrose. Accumulation of these acids resulted in a stoichiometric loss of inorganic phosphate. These carboxylic acids appear to have been incorporated into a portion of the mitochondrial substance which might involve a "steric fit" in a phospholipid or lipoprotein matrix (37).

The circumstantial evidence, especially the instability of the enzyme in aqueous solution after drastic

extraction procedures, as well as the amino acid analysis, suggests that phospholipids or other agents capable of hydrophobic interactions, should have a role in the stability of M-MDH. A possible mechanism for MDH stabilization has been investigated and the results are reported.

CHAPTER II

EFFECT OF LIPID MICELLES ON MITOCHONDRIAL MALATE DEHYDROGENASE

Methods

Mitochondrial malate dehydrogenase was isolated from acetone-dried powder of whole pig heart according to the method of Wolfe and Neilands (9) with slight modifications as reported by Thorne (19) and Kitto (38) (See APPENDIX I). The yield of malate dehydrogenase obtained during the isolation procedure was expressed in units which corresponded to the number of μmoles of NADH oxidized per minute at 340 mμ using the molar absorbancy of 6.22×10^6 per M. per cm. Specific activity was expressed in units (μmoles / min.) per milligram of protein. Protein was determined spectrophotometrically by measuring light absorption at 280 mμ and 260 mμ with a correction for the nucleic acid content from the data given by Warburg and Christian (39). The protein concentration of the solution in the cuvette was determined from the nomograph by E. Adams as distributed by the California Corporation for Biochemical Research.

Spectrophotometric measurements were made with a Beckman model DU spectrophotometer and with a Gilford model 2000 absorbance recorder (Beckman monochromator). The temperature of all assay systems was maintained at

25.0 ± 0.5°C by circulating water from a thermostat through thermospacers at the end of the cell compartments. All pH determinations were made with a Beckman Model G pH meter standardized to pH 7.0 with Beckman standard buffer.

Enzyme activity measurements were made by determining the rate of oxidation of NADH at 340 mμ with a constant concentration of coenzyme and substrate. Initial reaction rates were measured by observing the rate of change of the optical density at 340 mμ. Unless otherwise specified all initial rates were measured at concentration ranges in which oxalacetate inhibition occurred but at which no NADH inhibition was present.

NADH was obtained from Boehringer and Son (Mannheim). Oxalacetate and bovine serum albumin were purchased from California Corporation for Biochemical Research. Tris-(hydroxymethyl)aminomethane was primary standard from the Fisher Scientific Co. The fatty acids [stearic, palmitic, oleic, linoleic and linolenic] were obtained from Sigma Chemical Co. and were stored in a desiccator at -10°C until needed. Egg yolk lecithins, as a chloroform extract, (95% pure), was a gift from Dr. W. Lands of the University of Michigan.

The oxalacetate solution was prepared fresh daily and neutralized to pH 7.0-7.2 with $NaHCO_3$. The spontaneous decarboxylation of OAA was negligible over the experimental interval at this pH (40). The NADH solution was prepared immediately before use (41). Both OAA and NADH stock

solutions were kept at 0°C during the experimental interval.

In the standard assay system #1, each spectrophotometric quartz cuvette (1.0 ml volume and 1.0 cm light path) contained per ml : 100 μmoles of phosphate or Tris-acetate buffer, pH 7.4, 0.76 μmoles of OAA (pH 7.0), and 0.38 μmoles NADH. The spectrophotometer was balanced at 340 mμ and the reaction initiated by the addition of enzyme suitably diluted to a measureable level as dictated by the limits of the recorder.

For the lipid preparations, the amount of lipid present was determined using the Fiske and SubbaRow test for phosphate (42) and expressed as the number of μg of lipid-P per milliliter with KH_2PO_4 as the standard reference compound. For the extraction of pig brain and whole heart lipids, the procedure of Entenman (52) was used (see APPENDICES 2,4). Mitochondria and mitochondrial lipids were isolated according to the procedure of Marinetti *et al.* (20) (See APPENDIX 3). The whole heart lipids, preparations #1 and #2, were dispersed according to the procedure of Fleischer and Klouwen (28). Preparation #1 has 800 μg lipid-P per ml and preparation #2 has 613 μg lipid-P per ml.

Fatty acid micelles were formed by sonication, in a basic medium containing 0.0025 M NaOH per ml, with a Biosonik Ultrasonic at 90 kilocycles for 2-3 minutes. The sonicates in all cases were homogeneous and opalescent.

Oleic acid was dissolved into 95% alcohol and injected into water in a fine stream to form a stable dispersion (43). All such solutions when suitably diluted were homogeneous and of optical clarity.

Results

The initial phases of the study concerned the stability of M and S-MDH in various media. Figure 1 presents a time-activity study of M and S forms of MDH in the presence of serum albumin. While both enzyme forms are more stable in serum albumin, the M form is much less stable. In the presence of buffer alone, the M form decays at a faster rate than does the S form. In all cases, a plateau of activity is attained after an exponential decay. Figures 2 and 3 present time-activity plots for the enzyme in the presence of mitochondrial phospholipid (0.25 μmoles lipid-P). This lipid preparation was useful to stabilize the enzyme (as well as serum albumin) even in the absence of buffer (Figure 3). Incubation of the M form in Trisacetate buffer, pH 7.4, and subsequent addition of mitochondrial lipid increased the activity twofold above the level of activity prior to lipid addition. This stimulation persisted at this higher level for at least 30 minutes. The data in Table 1 substantiate this effect. A sample of pig brain lipid stabilized the enzyme best of all conditions examined at 25°C.

Since the enzyme is constantly losing activity it was allowed to reach a plateau level; at this level the effect of increasing amounts of solubilized whole pig heart lipid

was examined and compared to Tris-acetate and serum albumin controls (Table 2). While the ratio $\frac{\text{lipid rate}}{\text{Tris-acetate rate}}$ was expected to change as the lipid concentration changed, all other ratios were expected to be constant. However, the ratio of the control systems changed also, but the ratio of $\frac{\text{lipid rate}}{\text{albumin rate}}$ approached constancy at higher concentrations of lipid. This observation suggests that above a certain lipid level (15.5 µg lipid-P), the effects of lipid and serum albumin on the enzyme are similar and hence the reaction rate is a property of the state of the enzyme.

In Figure 2, the addition of mitochondrial lipid to the enzyme incubation system resulted in an increase in activity suggesting that the lipid may activate the enzyme. To investigate this further, the effect of lipid on the enzyme was studied using catalytic amounts of OAA. Figure 4 illustrates this effect. In the presence of moderate amounts of lipid (21-28 µg lipid-P), the rate increases above the control at each specific OAA concentration. From a Lineweaver-Burk plot (not shown) the K_m values were calculated: for the non-lipid system, the K_m value was 8.4×10^{-5}M while in the presence of lipid (21.7 µg lipid-P) the value decreased to 6.94×10^{-5}M per mole of enzyme.

Previously (44) it was shown that the initial rate of oxidation of NADH was a function of the order of

addition of assay components. The order of addition of lipid micelles to the assay system was therefore investigated (Table 3). The addition of lipid to the assay increased the initial rate of reaction regardless of when added to the assay system, if the essential components, OAA and NADH, were added in the usual order. If the NADH was added last to a non-lipid assay system, the initial rate decreased to about 90% of the control rate whereas in the presence of, under otherwise similar conditions, no deviation from the control was observed. On the other hand, if OAA was added last to the assay, the initial rate decreased to 94% of the control regardless of the presence or absence of lipid. The results thus indicate that the apparent decrease in the K_m values in a lipid system can not be attributed to an artifact in the assay. The ability of lipid to restore the rate to the control level even when NADH was added last, suggests a possible interaction of lipid with the active site region of M-MDH to enhance NADH binding to the active site -SH groups.

The possibility that the lipid affected the thiol groups in the active site as suggested by the order-of-addition experiment was investigated (Table 4). The rate of appearance of thiol groups was obtained in a non-lipid medium, using the denaturation effect of urea (5.6 or 4.0M). Addition of lipid to the urea assay resulted in an increased rate of -SH appearance. When urea was ommitted from the assay, addition of enzyme to a lipid system resulted in an

immediate increase in absorption at 412 mμ and also an increase in the rate of appearance of thiol groups over the rate observed in a non-lipid assay. The immediate increase in absorption at 412 mμ in the absence of urea, but with lipid included, is reproducible and is equivalent to 2-3 sulfhydryl groups assuming a molar absorbancy for DTNB of 13.6×10^3/M/cm.

Previous data have indicated that M-MDH loses activity on dilution into water. It was shown earlier in this laboratory (44) that M-MDH could be activated by phosphate and by serum albumin. Figure 5 presents the effect of dilution of M-MDH by various media. Dilution with water inactivated the enzyme, while dilution with phosphate, Tris-acetate, and KCl protect against enzyme activity losses in descending order of effectiveness. Dilution of M-MDH with a medium of lipid previously incubated in the presence of 100 μmoles KCl, protects best against the loss activity. The lipid effect follows a time dependency not unlike that seen for phosphate and Tris-acetate, but the initial activity in the lipid-KCl medium is 60% higher than in the KCl medium alone. In every case examined, after the initial stimulation on exposure to the medium, the activity decays to a plateau level. The rate of loss of activity depends on the type of buffer in the enzyme environment.

Lipid can stimulate MDH after the enzyme has been

exposed to an ionic environment. At an appropriate time a known amount of lipid was added to the solution of the enzyme previously exposed to 100 μmoles of KCl. Figure 6 presents the results. Lipid stimulation of the activity occurs only after a 2-3 minute lag period of incubation and the activation lasts 4-5 minutes. Similar effects can be found in Tris-acetate and phosphate environments. The increase in activity appears independent of lipid preparation but dependent on the protein concentration.

The effect of lipid on the reconstitution of M-MDH was investigated (Figure 7). The enzyme, suitably diluted was further diluted into water and allowed to incubate for two minutes at room temperature before phosphate (100 μmoles) or phosphate and lipid were added. In each case, the activity was restored to a high level. The reconstitution in phosphate-lipid, at two different lipid concentrations, reaches higher values than in phosphate alone. The reconstitution of activity is immediate and the enzyme once activated follows a normal exponential decay.

In an attempt to further characterize the lipid effect, an examination of pH dependence, temperature and ionic strength was undertaken.

Figure 8 depicts the results of incubation of enzyme and lipid at various temperatures. Between 6°C and 37°C the activity in a lipid medium is greater by at least 60% than the activity remaining after incubation in a non-lipid

medium. On the other hand, at 0°C and 43°C, the stability in a lipid environment is less than in a non-lipid environment. The ability of lipid to stimulate enzyme activity shows no drastic temperature dependency between 6° and 37°C.

There is no apparent pH dependency for lipid stimulation of M-MDH activity (Figure 9). In both phosphate and Tris-acetate buffers, the highest activity for the reaction occurs in the physiological range of pH 7.3-7.5. The pH-activity curve found for each buffer with lipid included is similar in shape to that of buffer alone. The activity is higher, however, for the lipid-buffer system suggesting that both the ionic and lipid species play a role in the maintenance of enzymatic activity.

The enzyme was incubated in the presence of increasing concentrations of KCl (Figure 10) and the optimum activity obtained at 20-35 μmoles in the incubation system. At higher concentrations of KCl, the activity decreased. In the lipid system, with more than 30 μmoles KCl, the activity was always higher than that in the KCl alone and did not decrease substantially at 80 μmoles or even at 120 μmoles.

The influence of hydrophobic groups on the enzyme was studied using aqueous solutions of fatty acids. For every fatty acid examined stearic, palmitic, oleic, linoleic and linolenic addition of the fatty acid to the

assay system stimulated the activity. For the unsaturated acids, the peak activity was observed between 9 and 15 mμMolar in fatty acid whereas for palmitic and stearic acids, the peak was obtained between 18 and 32 mμMolar. In the peak range, the activity of the fatty acid system was 30-50% higher than the control activity.

Figure 1

The Stability of S and M-MDH in Serum Albumin

Legend

S and M-MDH (1.8×10^{-5}mg) were incubated in an aqueous medium consisting of 20 μmoles per ml Tris-acetate buffer, pH 7.4, or 20 μmoles per ml Tris-acetate buffer, pH 7.4 and 0.1 mg serum albumin.

Aliquots taken at appropriate time intervals were assayed using standard assay system #1 with 0.38 mM OAA.

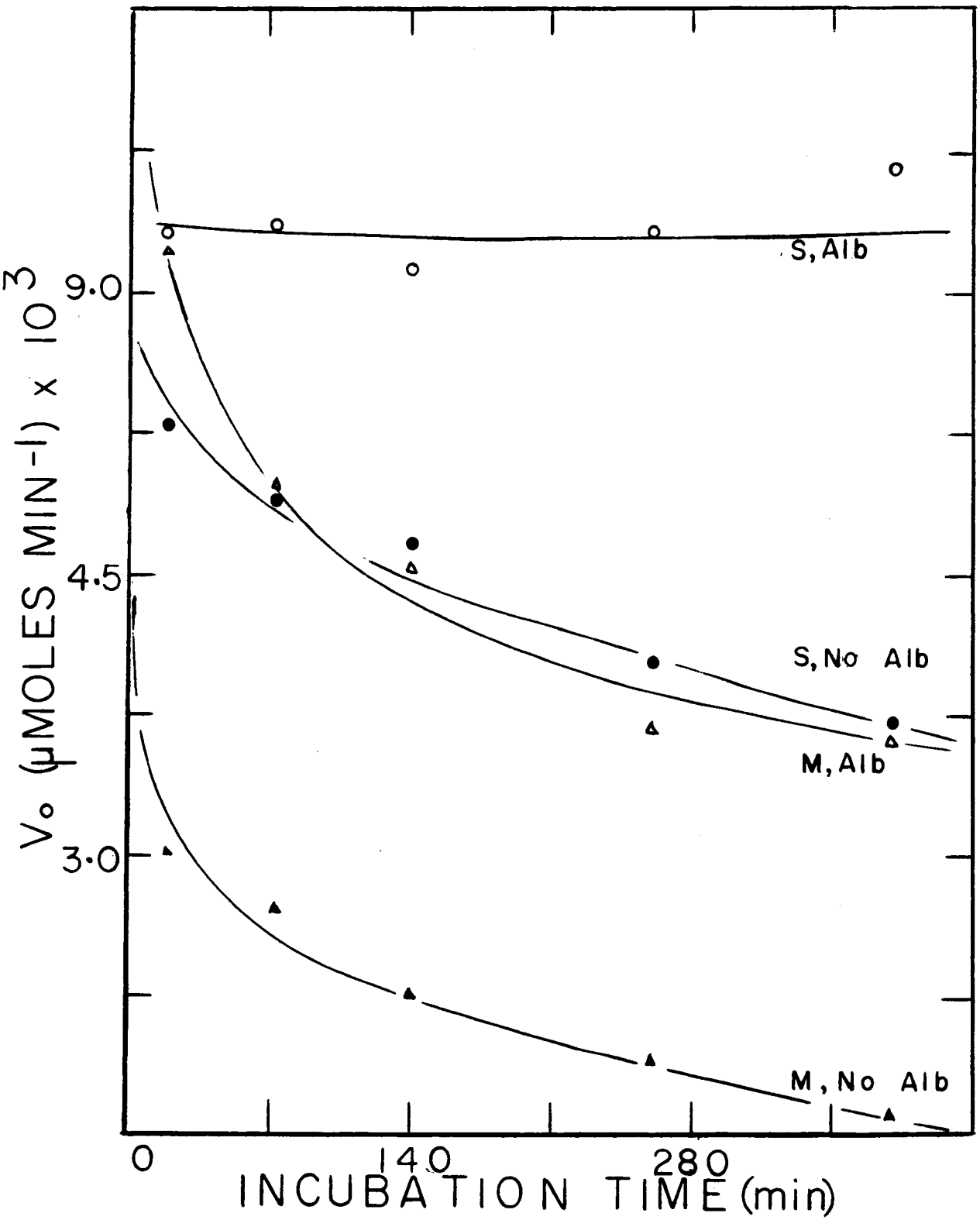

Figure 1

Figure 2

Stability of M-MDH in Mitochondrial Lipid at 25°C

Legend

M-MDH (1.8×10^{-5}mg) was incubated in an aqueous medium containing per ml : 0.2% (49 mmoles) DCA (adjusted to pH 7.4 with $NaHCO_3$), 20 µmoles Tris-acetate buffer, pH 7.4, 0.25 µg lipid-P of mitochondrial lipid added at time zero or 0.25 µg lipid-P added at the time indicated.

Aliquots taken at appropriate time intervals were assayed using standard assay #1 with 0.38 mM OAA.

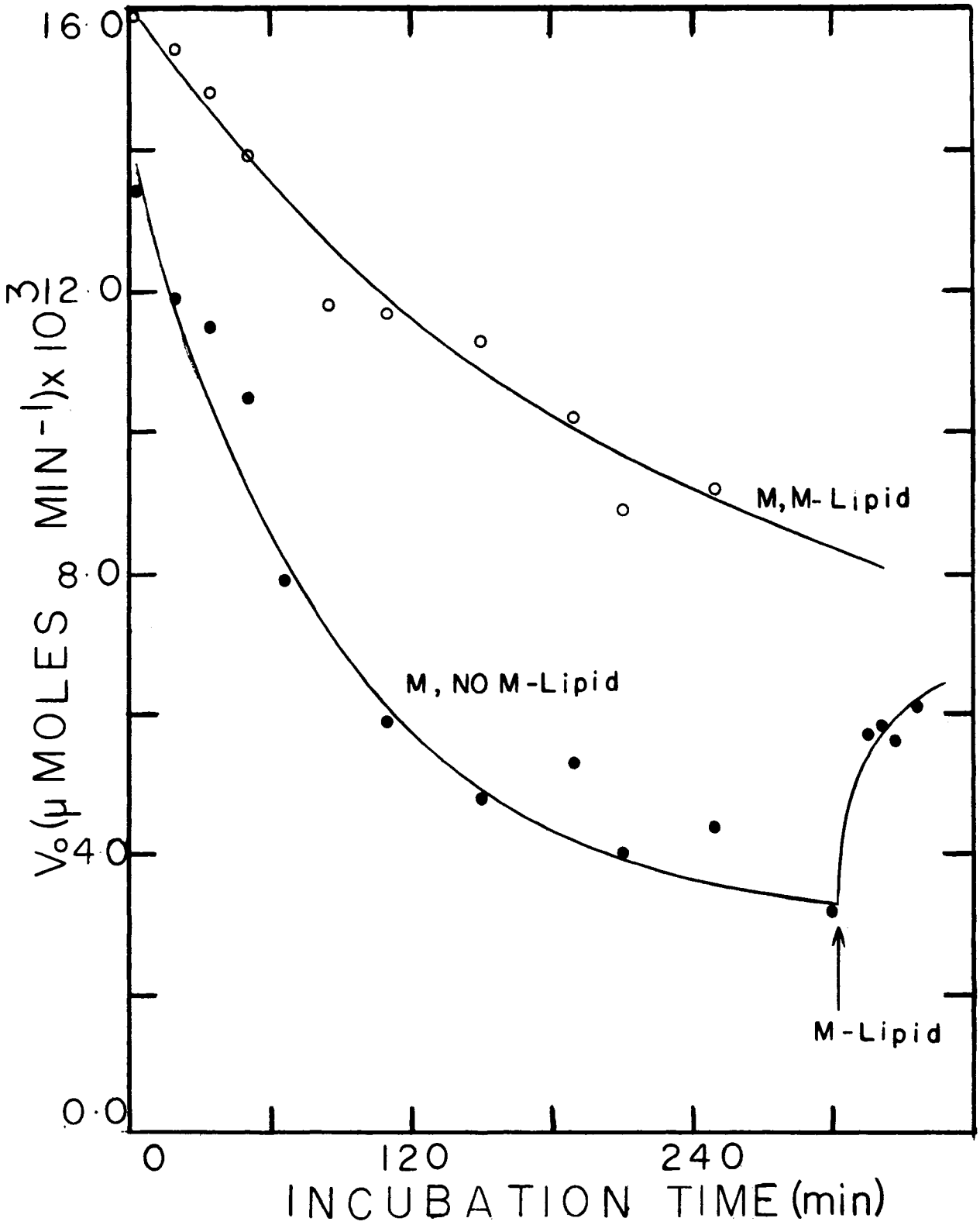

Figure 2

Figure 3

Stability of M-MDH in Mitochondrial Lipid in an Unbuffered Medium

Legend

Conditions the same as for Figure 2 except the Tris-acetate buffer was omitted. Water was adjusted to pH 7.4 with crystalline $NaHCO_3$.

Aliquots, taken at appropriate time intervals, were assayed using standard assay #1 with 0.38 mM OAA, except where indicated, 0.1 mg serum albumin added to the assay.

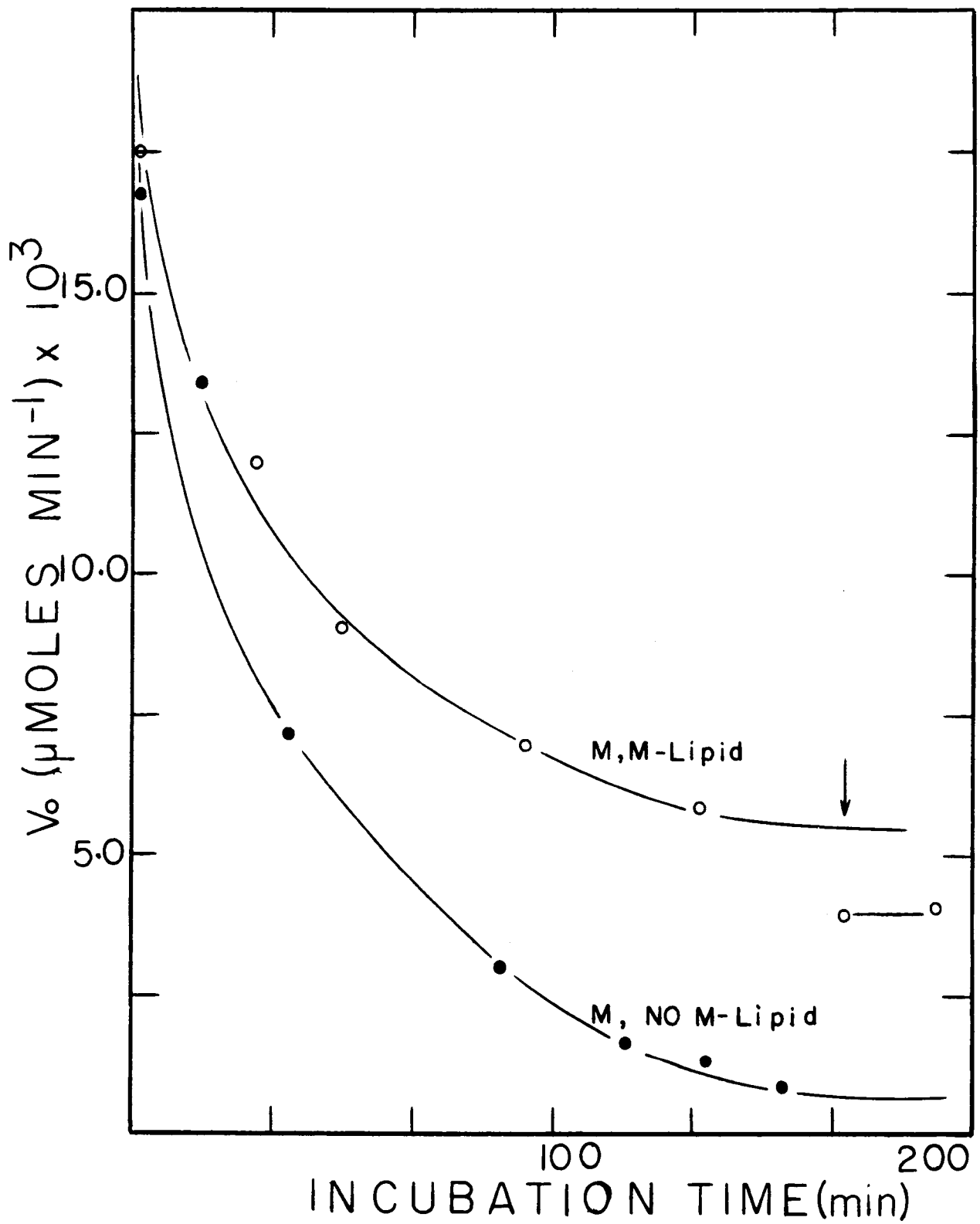

Figure 3

Table 1

Stability of M-MDH in Various Media after 120 Minutes Incubation at 25°C

Legend

M-MDH (1.8×10^{-5} mg) was diluted into each medium as indicated.

Aliquots were assayed after 120 minutes incubation using standard assay #1 with 0.38 mM OAA. The control was assayed at zero time using standard assay #1 with 0.38 mM OAA.

Table 1

Stability of M-MDH in Various Media after 120 minutes Incubation at 25°C

	Medium	Rate (μmoles min^{-1} x 10^3)	% Initial Velocity
1.	Control (100 μmoles Tris-acetate, pH 7.4)	4.34	100.0
2.	20 μmoles Tris-acetate, pH 7.4 + 0.08 mg serum albumin	0.98	22.8
3.	20 μmoles Tris-acetate, pH 7.4	0.29	6.8
4.	20 μmoles Tris-acetate, pH 7.4 at 0°C	2.86	65.9
5.	100 μmoles Tris-acetate, 0.2% DCA, M-lipid ($CHCl_3$)	1.83	42.3
6.	100 μmoles Tris-acetate, 0.2% DCA, no lipid	0.93	21.4
7.	100 μmoles Tris-acetate, 0.2% DCA, 10 mg brain lipid	2.82	64.9
8.	water, 0.2% DCA, M-lipid ($CHCl_3$), NaOH, pH 7.5	0.99	22.9
9.	water, 0.2% DCA, no lipid NaOH, pH 7.5	0.24	5.5

Table 2

Effect of Whole Pig Heart Lipid Micelles on M-MDH Activity

Legend

M-MDH (1.8×10^{-4} mg) was allowed to inactivate to a plateau of activity in 100 μmoles Tris-acetate, pH 7.4, at 0°C. Aliquots were incubated at 25°C for 10 minutes in 100 μmoles Tris-acetate, pH 7.4, to which was added:

i.) no addition (Tris-acetate control)

ii.) 0.1 mg serum albumin (Albumin control)

iii.) varying amounts of lipid micelles (Lipid)

Aliquots from the incubation media were removed and assayed with standard assay #1.

Table 2

The Effect of Whole Pig Heart Micelles on MDH Activity

State * of Enzyme	Tris-acetate Rate (μmoles $min.^{-1}$ x 10^3)	Albumin Rate (μmoles $min.^{-1}$ x 10^3)	Lipid added g lipid -P/mg MDH	$\frac{\text{Alb. rate}}{\text{Tris. rate}}$ x 10^2	$\frac{\text{Lipid rate}}{\text{Tris. rate}}$ x 10^2	$\frac{\text{Lipid rate}}{\text{Alb. rate}}$ x 10^2
109	2.52	3.86	2.15	1.53	0.86	0.55
119	2.27	3.74	1.46	1.65	0.64	0.40
261	1.78	3.55	2.14	1.99	1.19	0.60
303	1.53	3.91	2.31	2.55	1.51	0.60
332	1.09	3.33	2.04	3.05	1.87	0.61
396	1.49	3.60	2.17	2.41	1.45	0.60

* State of Enzyme: Refers to total elapsed time of incubation of Enzyme in 100 μmoles Tris-acetate buffer, pH 7.4 at 0°C.

Figure 4

Effect of Whole Heart Lipid Micelles on M-MDH Activity using Catalytic Amounts of Oxalacetate

Legend

Standard assay #1 was used except catalytic amounts of OAA was used. The enzyme was diluted to an appropriate level into 100 μmoles Tris-acetate buffer, pH 7.4. The initial rate was determined with no lipid, with 21.8 and 28 μg lipid -P per mg enzyme in the cuvette.

The control assay was standard assay #1 with 0.78 mM OAA.

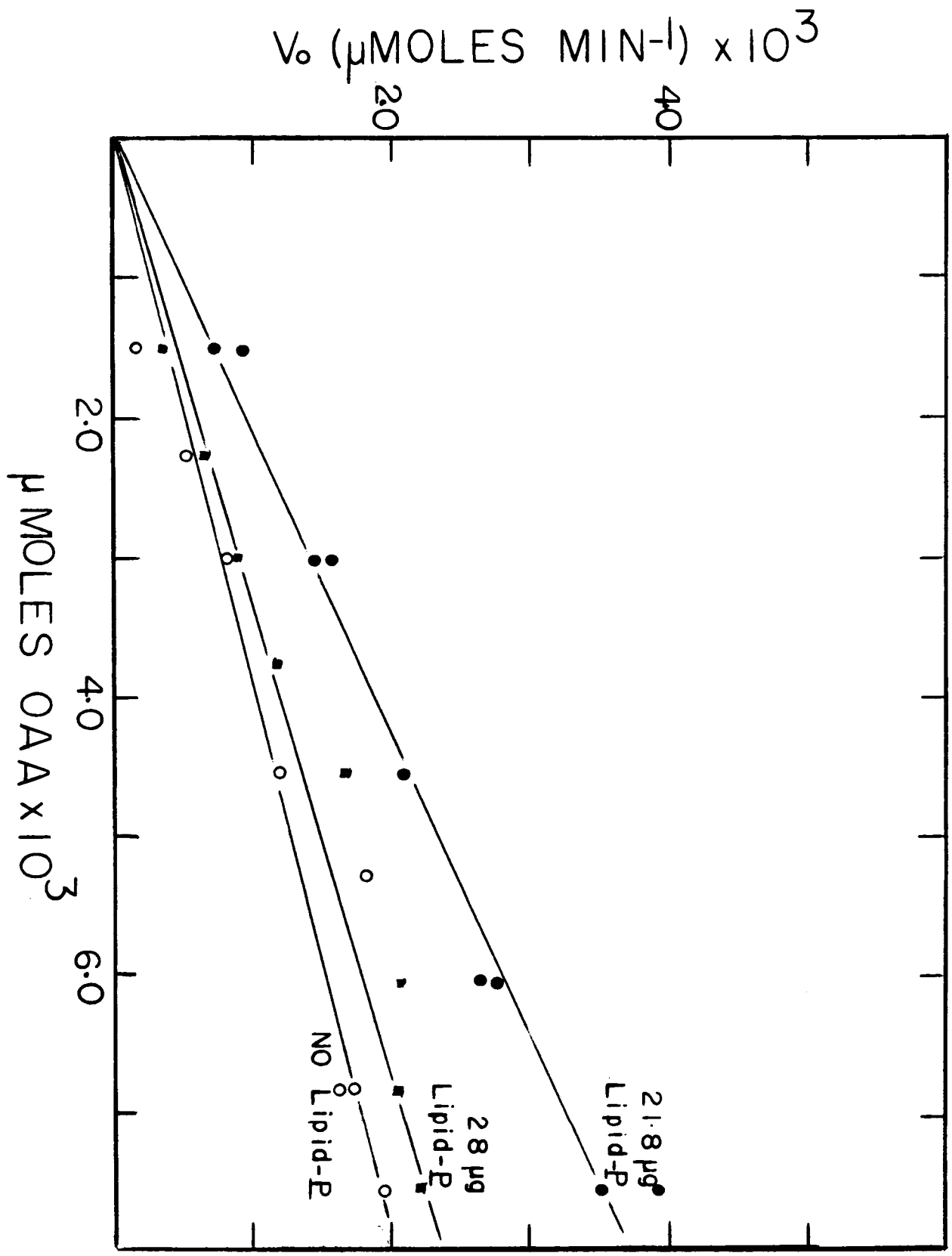

Figure 4

Table 3

The Effect of Order-of Addition of Assay Components on the Initial Rate of Oxidation of NADH by M-MDH with and without Lipid Micelles

Legend

Standard assay #1 was used. 4.5×10^{-5}mg of enzyme was used and 0.80 µg lipid-P added where indicated.

Table 3

The Effect of Order-of Addition of Assay Components On the Initial Rate of Oxidation of NADH by M-MDH with and without Lipid Micelles

Components in Order of Addition	% Initial Activity This Work	Previous Work (44)
H_2O Tris-acetate NADH OAA Enzyme	100	100
H_2O Tris-acetate OAA Enzyme NADH	90.2± 3.7 (3)*	85.3
H_2O Tris-acetate OAA Lipid Enzyme NADH	100.2±3.0 (3)	———
H_2O Tris-acetate NADH Enzyme OAA	94.8±1.0 (3)	98
H_2O Tris-acetate NADH Lipid Enzyme OAA	94.6±2.5 (3)	———

* The number of determinations are in brackets.

± is standard deviation about the mean.

Table 4

The Effect of Lipid on the Appearance of MDH Sulfhydryl Groups

Legend

Into each cuvette (1.0 cm light path) was added: 100 μmoles phosphate, pH 6.8, 1 μmole DTNB, 10 μmoles EDTA; urea was added where indicated, then a known amount of lipid micelles. This mixture was then allowed to equilibrate at 25°C. To this mixture, a known amount of enzyme was delivered through a lambda pipette.

The rate of appearance of thiol groups was determined by measuring the colour development at 412 mμ and the total final absorbance was measured after the reaction had attained equilibrium.

Table 4

The Effect of Lipid on the Appearance of MDH Sulfhydryl Groups

	Conditions	Rate Constant of -SH Reaction k (min^{-1})	Total Final Absorption 412 mμ
1.	5.6M urea, 0.036 mg MDH (incubated for 20 min. at 0°C, 5 min. at 25°C)	0.163	0.110
2.	as 1; except MDH incubated in the lipid (16 μg lipid-P)	0.189	0.117
3.	5.6M urea, 0.018 mg MDH incubated 15 min. at 25°C	0.157	0.049
4.	as 3; MDH incubated with 80 μg lipid-P	0.292	0.061
5.	4.0M urea, 0.018 mg MDH, no incubation, no lipid	0.057	0.045
6.	as 5; with 36 μg lipid-P	0.088	0.061
7.	as 5; with 0.29 mg egg yolk lecithins (32 μg lipid-P)	0.073	0.060
		Initial O.D. 412 mμ	Total Final Absorption
8.	no urea, 0.018 mg MDH, 36 μg lipid-P	0.025	0.034
9.	no urea, 0.018 mg MDH, no lipid	0.001	0.008

Figure 5

The Effect of Dilution into Various Media on the Activity of M-MDH

Legend

M-MDH (4.5×10^{-4}mg) was diluted into an aqueous medium. An aliquot was removed for assay. To the remaining solution was added the following:

i.) 100 μmoles KCl

ii.) 100 μmoles Tris-acetate, pH 7.4

iii.) 100 μmoles phosphate, pH 7.4

iv.) 158 μg lipid-P + 100 μmoles KCl

v.) H_2O

The total final volume of dilution medium is 1.0 ml.

Aliquots were removed at appropriate time intervals and assayed using the standard assay #1.

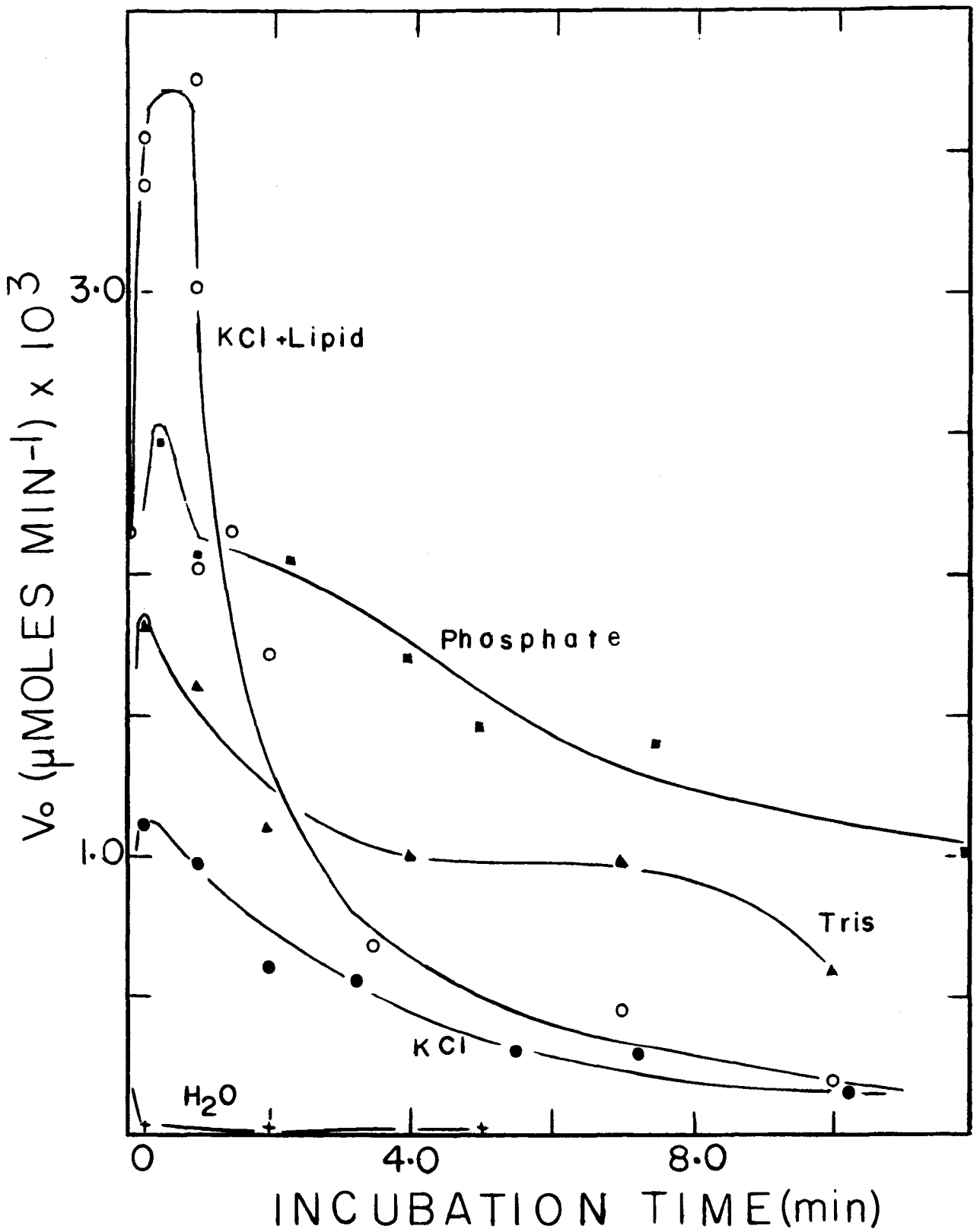

Figure 5

Figure 6

Effect of Lipid Micelles on M-MDH Activity when the Enzyme Pre-incubated at 25°C in the Presence of Various Salts

Legend

M-MDH (4.5×10^{-3}mg) was incubated in a medium containing 100 μmoles KCl per ml. Samples of lipid preparation #1 (8.0 μg lipid-P) and preparation #2 (6.1 μg lipid-P) were added at the time indicated.

Aliquots were assayed at appropriate time intervals with standard assay #1.

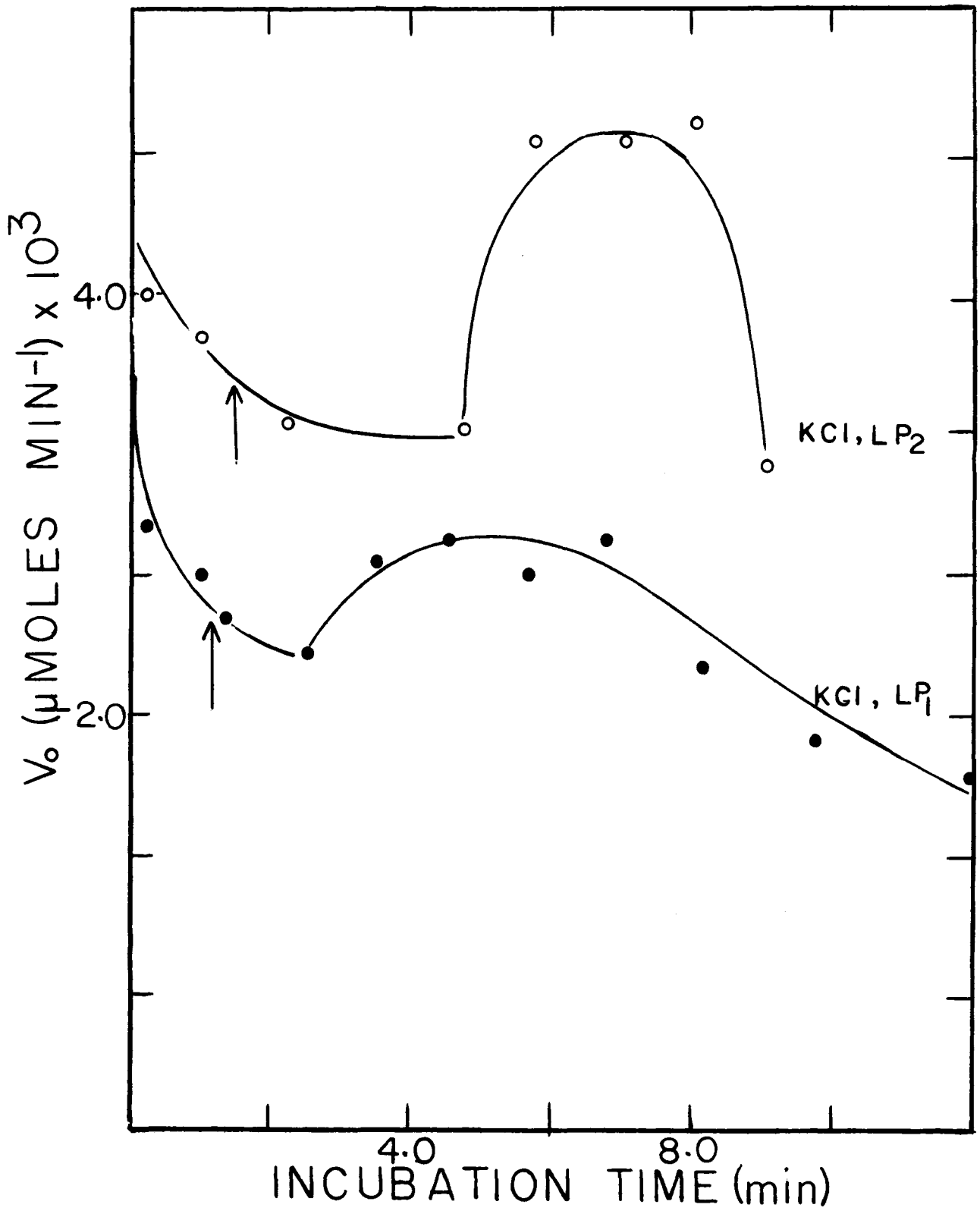

Figure 6

Figure 7

The Effect of Lipid on the Reconstitution of M-MDH

Legend

M-MDH (4.5×10^{-3}mg) was diluted to total final volume of 1.0 ml with H_2O. An aliquot was removed immediately and assayed. To the remaining solution, 100 μmoles phosphate buffer or 100 μmoles phosphate with 61.3 μg lipid-P were added.

Aliquots were removed at appropriate time intervals and assayed using standard assay #1. The rate was compared to a control system containing 0.1 mg serum albumin.

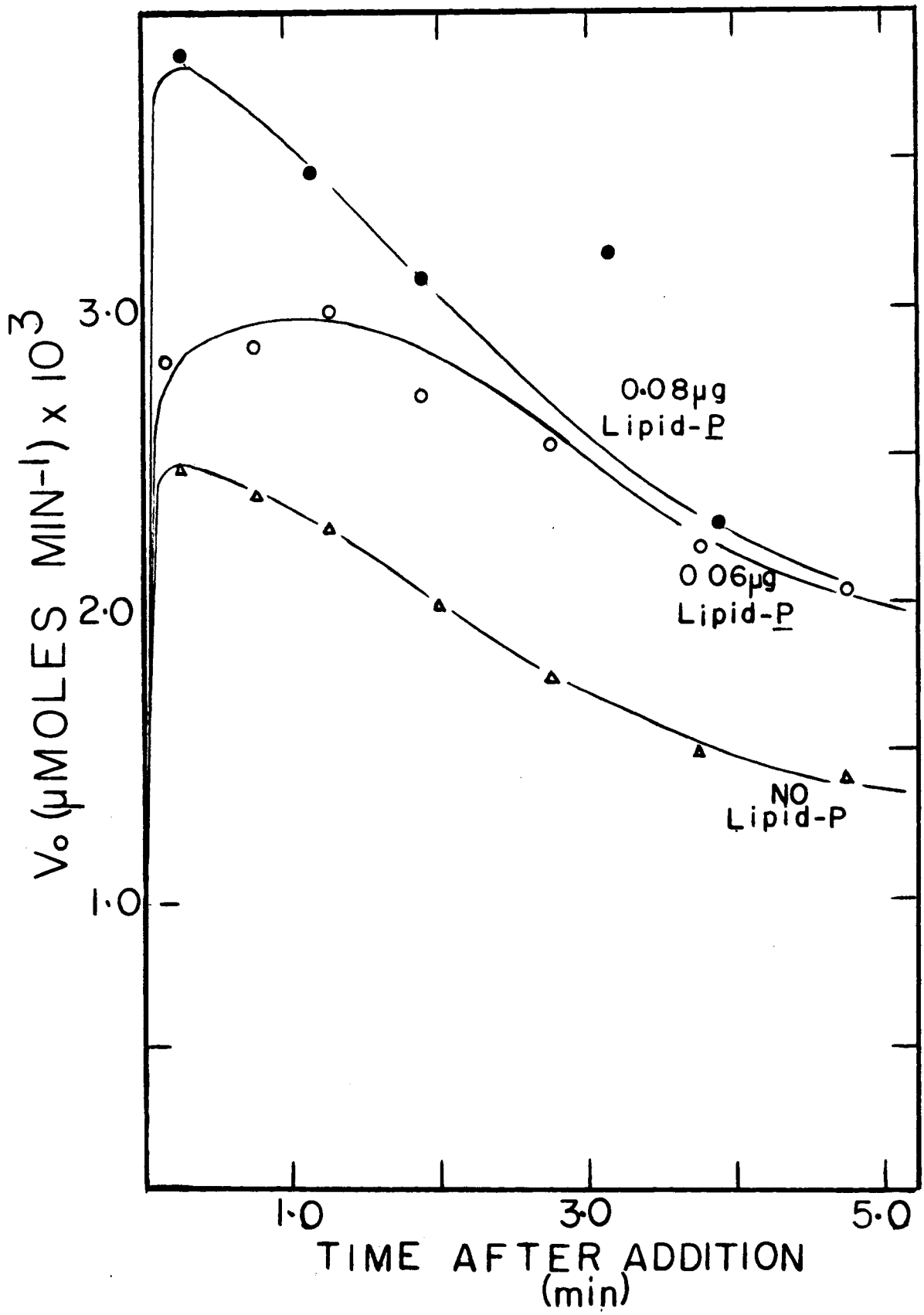

Figure 7

Figure 8

The Effect of Temperature on the Activity of M-MDH in the Presence of Lipid

Legend

M-MDH (4.5×10^{-3}mg) was incubated in the presence of 100 μmoles phosphate buffer, pH 7.4, at 0°, 6°, 15°, 26°, 37° and 43° for 5 minutes. At this time, a sample of water or 8.0 μg lipid-P was added. Incubation was continued for 4 more minutes, aliquots removed and assayed using the standard assay #1.

The control consisted of the same concentration of enzyme, incubated as above but in the presence of 0.1 mg of serum albumin and 20 μmoles Tris-acetate buffer, pH 7.4.

An aliquot from the control system was assayed after incubation for 5 minutes.

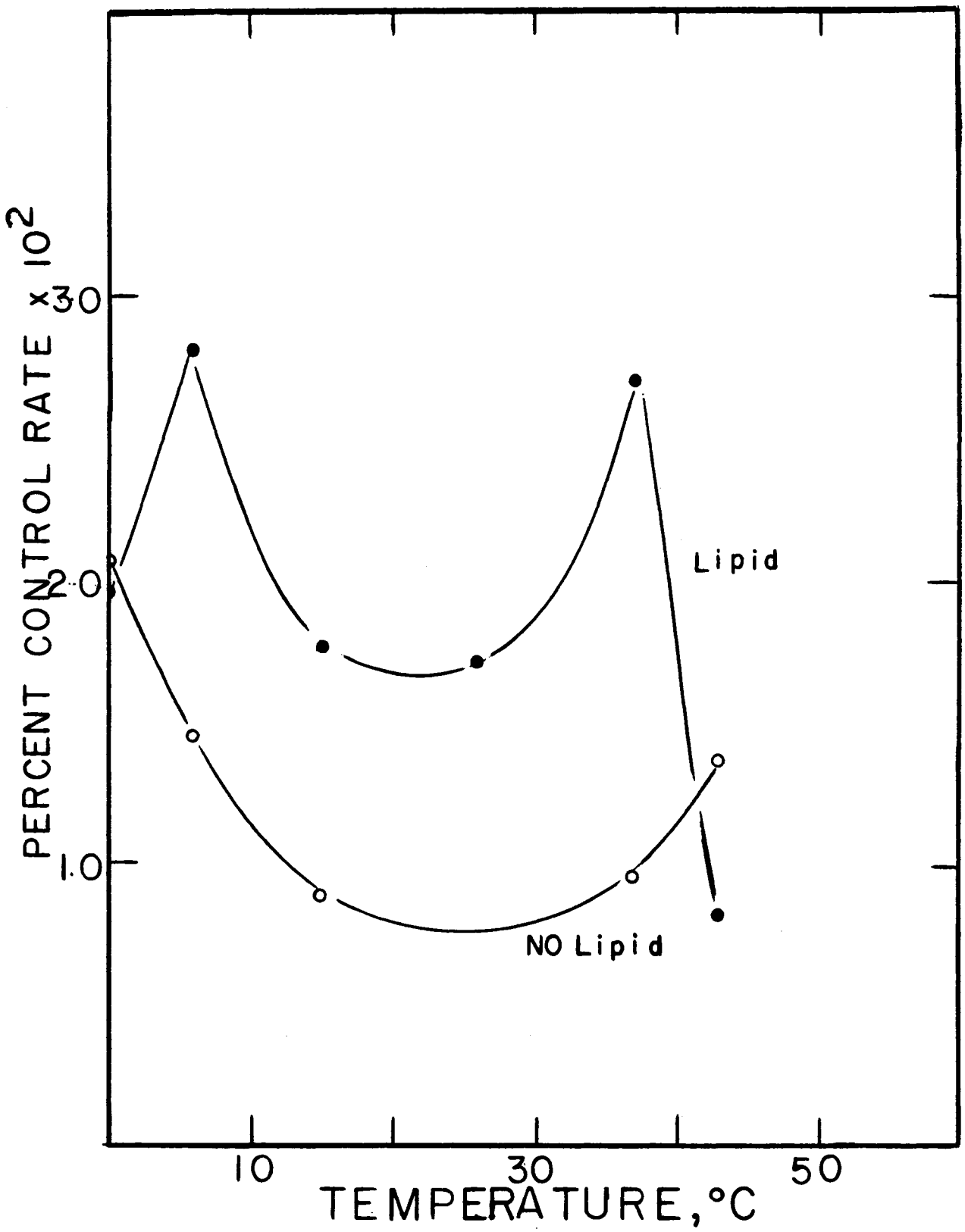

Figure 8

Figure 9

The Effect of pH on the Activity of M-MDH in the Presence of Lipid

Legend

M-MDH (4.5×10^{-3}mg) was incubated in the presence of 100 μmoles phosphate buffer, or 100 μmoles Tris-acetate, at various pH values for 5 minutes at 25°C. At this time a sample of water or 8.0 μg lipid-P was added. Incubation was continued for 4 more minutes, aliquots removed, and assayed using the standard assay #1.

The control consisted of the same concentration of MDH incubated as above in the presence of 0.1 mg serum albumin and 100 μmoles of the buffer at each pH studied.

An aliquot from the control system was assayed after incubation for 5 minutes.

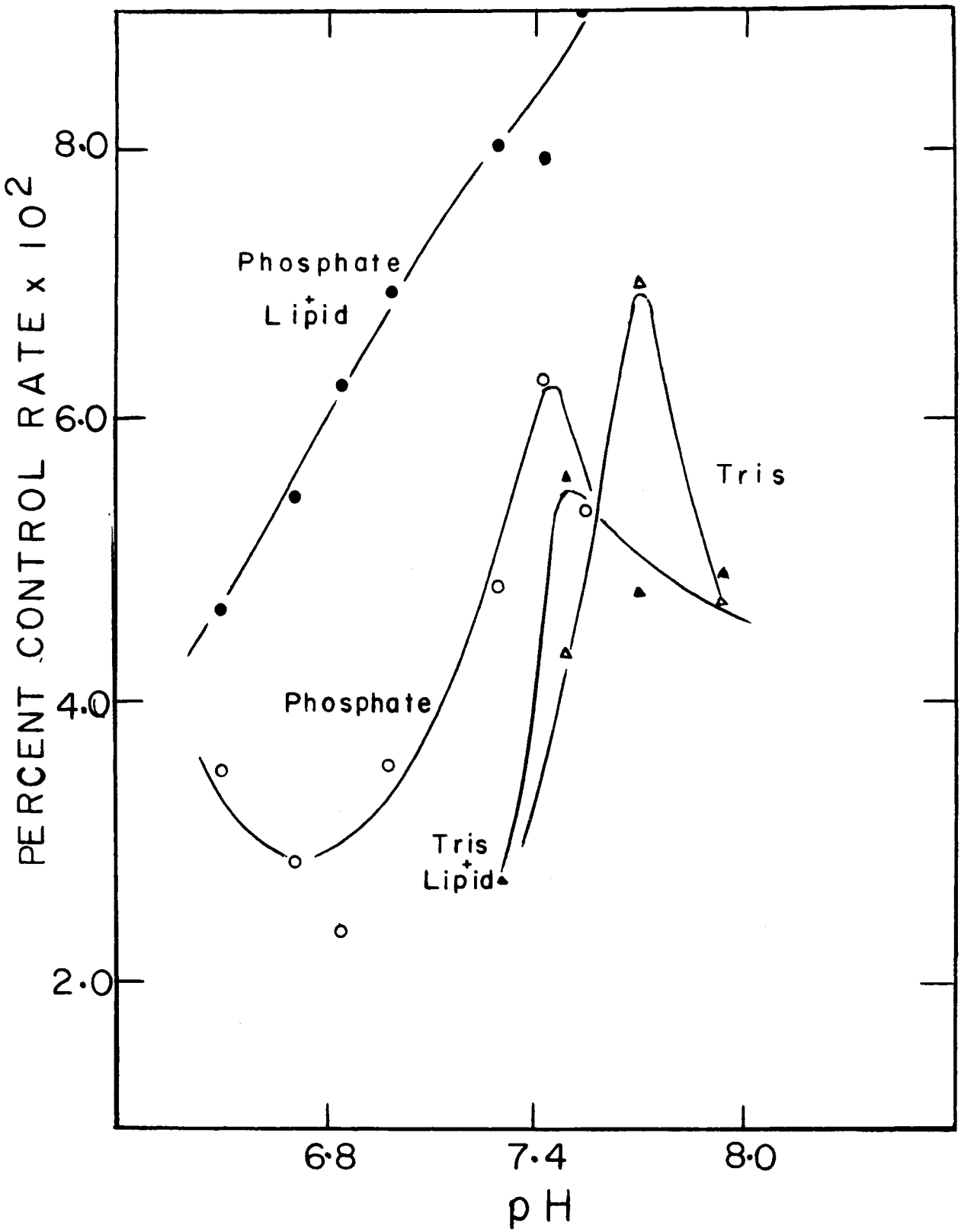

Figure 9

Figure 10

The Effect of KCl on the Activity of M-MDH in the Presence of Lipid

Legend

Conditions the same as for figures 8 and 9 except the enzyme was incubated in the presence of increasing concentrations of KCl, and 100 μmoles phosphate buffer, pH 7.4.

The standard assay #1 was used.

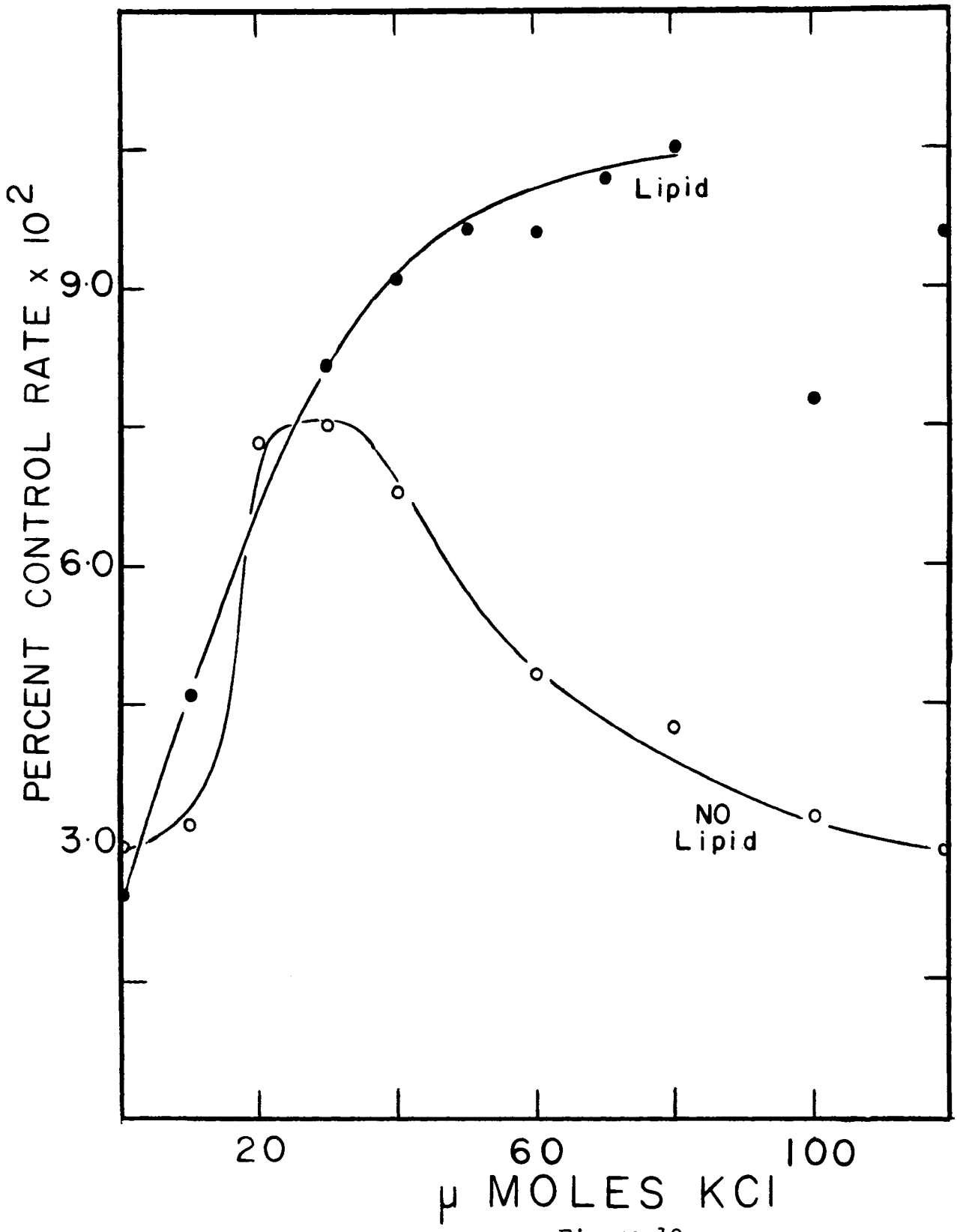

Figure 10

Figure 11

The Effect of Fatty Acid Micelles on M-MDH Activity

Legend

Varying amounts of fatty acid micelles (Na salts of sonicated fatty acid) were added to the standard assay #1 just prior to initiation of the reaction with the addition of MDH (approximately 4.5×10^{-4}mg).

The control assay was the standard assay #1 without fatty acid.

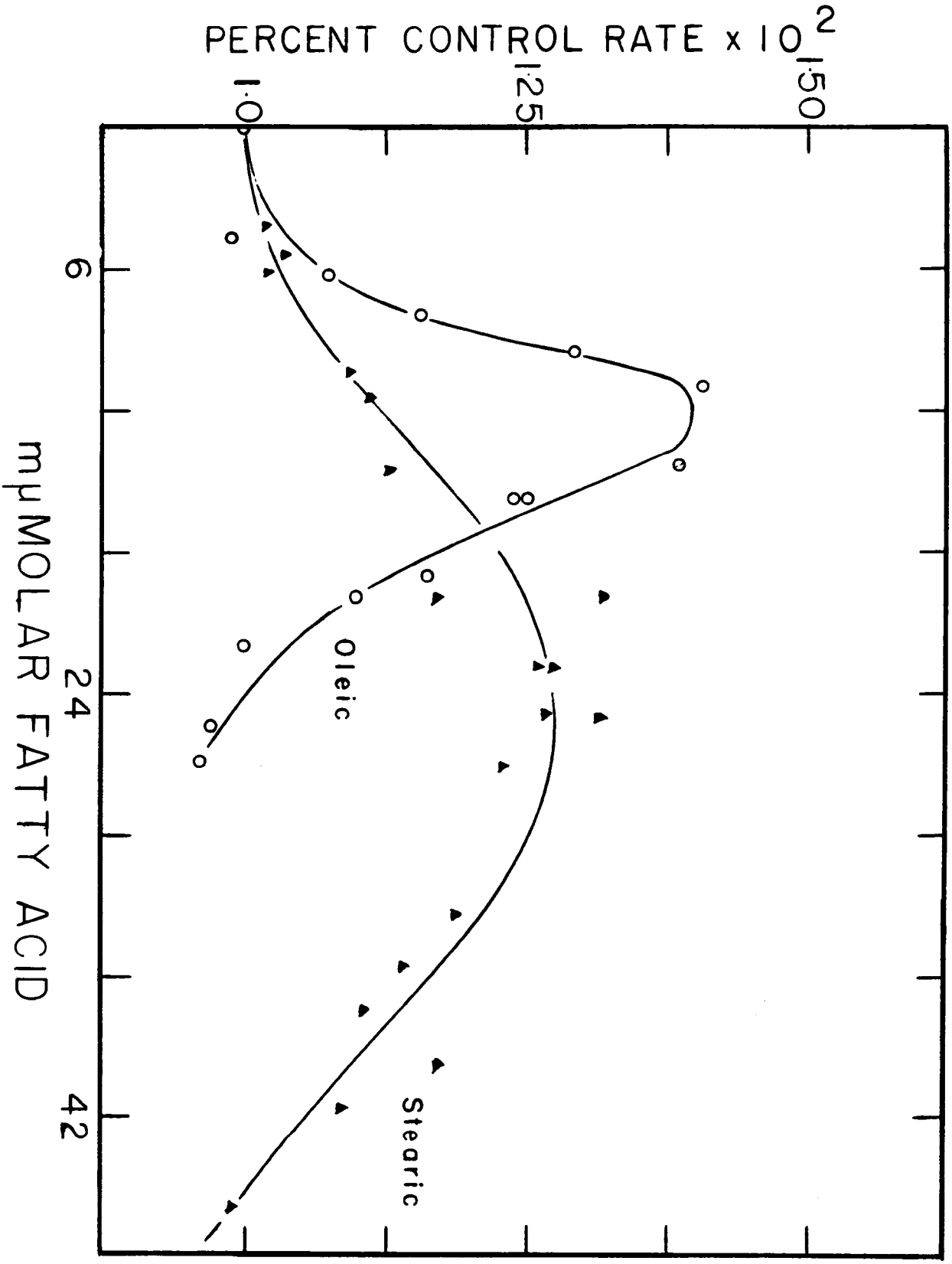

Figure 11

Discussion

The activity if M-MDH decays exponentially when it is incubated in an ionic environment. During such a decay process, the physical state of the enzyme can be assumed to change. The decay, however, depends on the type of ionic environment. The results can be interpreted in the light of recent work by Robinson and Jencks (45-47) who found that proteins in general, as well as the synthetic polymer acetyltetraglycine ethyl ester, are subject to changes in their physical state in an ionic environment. Phosphate increases the activity coefficient of the protein and in so doing preserves the tertiary structure of the enzyme. Tris and acetate change the activity coefficient to a lesser extent whereas the chlorides (KCl and NaCl) have no effect. A change in the activity coefficient means, in the case of phosphate, that the enzyme is more stable and can be salted out easily; its tertiary structure is thus more stable towards denaturation. These ionic effects have been attributed primarily to the anionic species (46,47). The order of binding of the anions studied to cationic sites of the protein is Cl^- Tris, acetate phosphate. While chloride is capable of a direct binding to the protein, Tris, acetate and phosphate binding abilities are less. The effect of Tris can be ascribed to the number of hydroxyl groups in the ion while for phosphate the number of charges as well as the size of the hydrated anion

determines its binding capacity. The tenacity of binding of anions to cationic protein sites is a reflection of the ability of the ion to inhibit the enzyme (47). Thus the decay of the enzyme activity can be attributed to ionic disruption of the water structuring around the enzyme. KCl disrupts this structure better than Tris and phosphate because of the greater binding capacity of chloride over Tris, acetate and phosphate. Thus, phosphate could enhance the stability of the active site better than could Tris or KCl by promoting group interaction without extensive loss of water structure.

During the exponential loss of catalytic ability, it is reasonable to suggest that the enzyme molecules are losing their active structure. Some molecules are expected to be in an intermediate stage between active and inactive conformations. It has been reported recently by Kaplan *et al.* (48, 49) that intermediate stages of active configurations of LDH are probable. Chilson *et al.* (50, 51) have found that an enzymically inactive molecule ($S_{20,w}$=6.0) forms on incubation of pig heart M-MDH. This higher sedimenting fraction is probably an aggregate molecule since the native enzyme has an S_{20w} of 4.0. Ionic environments especially those which decrease water structuring favour protein-protein interactions and aggregate formation. If aggregate molecules form during the decay of activity of M-MDH in the lipid-bile salt medium, the detergent character of the bile salt is capable of

breaking such an aggregate into its component molecules, thus exposing more protein surface to lipid interaction (18). This type of action may be advantageous since it could prevent inactive aggregate molecule formation while providing a micellar protein-lipid association found in mitochondria (17,18).

Under all conditions studied in this work, the lipid stimulation of the enzyme activity is observed whether the micelles be added to the enzyme after or before exposure to an ionic environment. The hydrophobic character of the lipid preparations is antagonistic to the ionic character of the salt solution. In the case of KCl, the chloride has been shown not to associate favourably with an apolar solute while Tris, acetate and phosphate can interact with an interphase. Thus with phosphate and Tris-acetate, the ionic character and the hydrophobic character complement each other to provide an increased stability of the enzyme while this is not the case for the stronger binding chloride ion.

When the enzyme is initially added to an ionic-lipid solution, there is an immediate appearance of thiol groups. These sulfhydryl groups may be in the active site region or in the rest of the molecule. The thiol groups are made more available to react by a lipid environment. In a non-lipid medium the NADH itself may induce a conformation change to expose the active site thiol groups, since it has been shown that active site groups, such as cysteine

and tyrosine (1,2,53), are found in the interior regions the tertiary structure. The lipid molecules, in general, can alter the conformation of the tertiary structure of the enzyme, and by such action may make the enzyme molecule not only more stable but also more reactive. In the presence of phosphate which stabilizes the active site of the enzyme, lipid alters the conformation in remote areas of the molecule which can further enhance the stability of the active site. In a Tris-acetate medium, the reconstitutive capacity is intermediate between that of phosphate and KCl; this is determined by the ionic character of the solution since the lipid effect is assumed to be the same in all cases.

Exposure of sulfhydryl groups by alteration of M-MDH tertiary structure could increase the hydrophobic character of the molecule and thus increase the water associated with it. Such an increase in water structure should compete favourably with the water breaking influence of the ionic species to maintain the enzymic activity for a longer period of time. In the case of enzyme molecules which have lost their active configurations, the lipids with active site protectors such as phosphate can reconstitute the catalytic activity to a higher level.

Exposure of the enzyme to lipid after prior solution of the enzyme in an ionic environment, presents a competition between lipid and ionic effects on the enzyme.

For the lipid to react it must remove ions from the surface of the enzyme, especially in the case of chloride ions. Thus reconstitution of activity in KCl requires a longer time of incubation than for Tris-acetate or phosphate which bind less actively to cationic sites.

Since the effect of lipid on the enzyme is probably due to hydrophobic interactions, no pH dependency to the lipid reaction is evident. However, a temperature dependency of the lipid reconstitutive power is noticed. At elevated temperatures (43°C), the water structure around the enzyme "melts". If the enzyme conformation has been changed by lipid to stabilize the active site at the expense of the structure in remote regions of the molecule it follows that the enzyme would be more unstable and lose activity faster than at lower temperatures (6°-37°C). At zero degrees the hydrophobic interactions between enzyme and lipid to increase water structuring provides an interference to the binding of OAA or NADH and thus reduce the reaction rate to a level lower than for the non-lipid system.

In summary, lipid may promote an increase in catalytic activity by altering the conformation of the enzyme. Also, it may be necessary to overcome any inhibitory or active tertiary structure degradative effect of ionic environments. The increased activity of M-MDH in the presence of lipid occurs regardless of lipid composition, ionic environment, or pH but it is dependent

on temperature.

The increased stability of the enzyme in a lipid medium may in part simulate the physiological environment of the enzyme from which it has been removed during the isolation procedures.

A further series of experiments should be conducted. The effect of "structural" protein on the stability of M-MDH has not been studied. Nor have there been attempts reported to determine the amount of lipid associated with particulate M-MDH or the enzyme isolated from mitochondria. It must be remembered, however, that the enzyme for such studies should be isolated from fresh heart minces which have not been extracted with organic solvents. Such studies may in part explain why M-MDH is easily lost on disintegration of intact mitochondria.

CHAPTER III

SUMMARY

Mitochondrial malate dehydrogenase undergoes an exponential decay of activity on incubation in ionic media. Lipid micelles, in amounts ranging up to 100 μg lipid-P per mg protein, decrease the rate of activity decay. The enzyme is more than twofold more active in a lipid-ionic environment than in an ionic environment alone.

Addition of lipid to the enzyme in the presence of catalytic amounts of OAA, results in a decrease of the K_m value for the reaction- - an apparent activation of the enzyme. Exposure of the desalted enzyme to an ionic environment protects against activity loss while in the presence of lipid-KCl, the activity increases to at least threefold higher than in the presence of KCl alone. Similar results were obtained for Tris-acetate and phosphate although the difference in the degree of activation is not as pronounced. Exposure of the enzyme to lipid after prior incubation in an ionic medium results in an increase of enzyme activity which remains for at least five minutes.

Water dilution of the enzyme inactivates the enzyme but the molecules can be reconstituted to activity with

phosphate, Tris-acetate and KCl. With lipid included in the reconstitution medium, the activation rises in all cases above the control. The ability of lipid to activate or to reconstitute activity is independent of the pH of incubation and the incubation temperature except for 0° and above 37°C. In lipid media of low ionic strength, the ionic effect predominates. At high ionic strength, the inhibitory effect of anionic species is overcome by the presence of lipid, resulting in threefold higher activity with lipid even in 80-120 μmoles KCl. The suggestion of hydrophobic interactions between lipid and protein to stabilize activity is strengthened by the ability of fatty acid emulsions to increase enzyme activity.

Preliminary evidence suggests that the lipid alters the conformation of the active or inactive molecules to expose sulfhydryl groups of the enzyme. This result may indicate that thiol appearance increases the degree of hydrophobicity of an area of the enzyme molecule and such a conformational change promotes reconstitution and stabilization of catalytic activity of the enzyme by means of a lipid-protein interaction.

APPENDIX I

Enzyme Isolation

Pig hearts were obtained from freshly killed animals and defatted and minced at 4°C. Acetone-dried powders of exhaustively washed minces were made by the method outlined by Wolfe and Neilands (9).

The initial purification steps were essentially the ammonium sulfate and zinc-ethanol fractionation steps of Wolfe and Neilands (9). These were followed by chromatography on carboxymethyl-cellulose and Sephadex G-100.

For carboxymethyl-cellulose chromatography, the resin was pre-treated as described by Srere and Kosicki (54). The enzyme extract from the alcohol fractionation was dialysed for 8 hours at 4°C against 0.005M potassium phosphate, pH 6.5, containing 0.001M EDTA. An 18 x 4 cm column of the resin was equilibrated in the same buffer and the dialysed enzyme applied to it. Any S-MDH contaminant was eluted in the first 550 mls of the starting buffer. Thereafter, M-MDH was eluted by gradient elution to 0.15M potassium phosphate- 0.001M EDTA, pH 6.5, as prescribed by Kitto (38).

Eluates were collected into 10 ml portions and assayed for protein and enzymatic activity. Flow rates were maintained between 100-120 mls per hour. The eluates

showing the highest specific activity were pooled and the protein precipitated by addition of crystalline ammonium sulfate to 77% saturation. The precipitate was collected by centrifugation at 23,000 x g for 20 minutes and dissolved into 0.05M sodium phosphate - 0.1M KCl, pH 7.4. This enzyme solution was applied to a Sephadex G-100 column, 23 x 2.5 cm, previously equilibrated in the same buffer. The protein was eluted into 15 ml portions by 0.05M sodium phosphate - 0.1M KCl, pH 7.4. The protein was eluted from the column in a distinct peak (60 mls in volume). To the pooled eluates, ammonium sulfate crystals were added to 77% saturation. The precipitate was collected by centrifugation at 23,000 x g for 20 minutes and redissolved into 77% ammonium sulfate. This suspension was stored at -10°C.

Chromatographic fractionations were carried out in cold at 4-6°C. Ammonium sulfate and ethanol fractionations were carried out according to Wolfe and Neilands (9) to -15°C. The specific activity of each step was compared to the preparation of Thorne (55). The yield obtained was 7% of the original activity while Thorne (55) obtained a 27% yield. The specific activity of the final preparation was 406 units/ml/A_{280} while Thorne reported a value of 655 units/ml/A_{280}.

APPENDIX II

Whole Pig Heart Lipid Isolation

Whole heart lipids were prepared according to the procedure of Entenman (52).

Fresh pig hearts were transported to the laboratory in cracked ice. The hearts were then defatted, diced, and minced in a hand grinder. Fifty gram quantities were homogenized in a Waring blendor in 500 mls of ethanol:ether (3:1,v/v) for 30 seconds and the homogenates were then extracted in a water bath for two hours at 60°C. The ethanol-ether soluble fraction was collected by suction while still warm and stored in a stoppered vessel overnight at 4°C. The precipitated lipids were collected and re-extracted with chloroform at 60°C for 10 minutes. The chloroform was removed by a steam jet and the gummy green residue was stored in a chloroform atmosphere in a stoppered vessel at -10°C until used.

A 60 mg amount of this preparation was weighed immediately and solubilized according to the procedure of Fleischer and Klouwen (28), as described in METHODS.

APPENDIX III

Pig Heart Mitochondrial Lipid Isolation

Fresh pig hearts, [less than one hour removed from the animal] were defatted, diced and ground in a hand grinder to a fine pulp. Mitochondria were then isolated: 50 g of pulp was added to 500 mls of a 0.25M sucrose solution and blended in a Waring blender for 30 seconds at low speed at 0°C. The sucrose homogenate, adjusted to pH 7.0 by crystals of $KHCO_3$, was centrifuged at 1,500-2,000 x g for 20 minutes at 0°C to remove cellular debris.

The strawberry-coloured supernatant fluid was collected and re-centrifuged at 15,000 x g for 10 minutes at 4°C. The brown pellet was suspended in a minimal amount of 0.15M KCl and homogenized using a Teflon ball homogenizer. The homogenate was centrifuged at 15,000 x g for 20 minutes at 4°C and the cream-coloured precipitate was collected (similar to the procedure of Marinetti et al. (20)).

This precipitate was then extracted three times with 20 ml portions of chloroform:methanol mixture (1:1,v/v) for 25 minutes at 60°C. The filtrates were collected over sintered glass and the solvent removed by vacuum evaporation. The residue was then extracted with 25 mls of chloroform and concentrated under a steam jet. The

temperature of the solution was held at or below 50°C. The green-yellow chloroform concentrate was stored in a sealed vessel at -10°C until used.

APPENDIX IV

Pig Brain Lipid Isolation

According to the procedure of Entenman (52), fresh pig brain was ground in a hand grinder and homogenized in 100 g in 500 mls of ethanol:ether (3:1,v/v) for 30 seconds at room temperature. The total homogenate was added to an Erlenmeyer flask in a water bath and extracted for two hours at 60°C. Immediately thereafter, the alcohol-ether extract was decanted through fat free filter paper into an Erlenmeyer flask. The residue was then re-extracted at 60°C for 14 hours with 500 mls of alcohol:ether (3:1,v/v). The residue was removed and the filtrate stored in the cold room in a stoppered vessel. White-coloured lipids precipitated within two hours at 4°C. A sample (10 mg) of these lipids was dried and weighed and dispersed into 0.2% (50 mmoles) DCA for use.

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