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THE EFFECTS OF IONS AND FREEZE-THAWING ON THE ACTIVITY OF MITOCHONDRIAL AND SUPERNATANT MALATE DEHYDROGENASE

BY

DENNIS J. BLONDE

A Thesis

Submitted to the Faculty of Graduate Studies through the
Department of Chemistry in Partial Fulfillment
of the Requirements for the Degree of
Master of Science at the
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Windsor, Ontario

1966

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ABSTRACT

The effects of various ions added to the assay system for purified mitochondrial and supernatant malate dehydrogenase have been studied. Phosphate, arsenate and zinc ions strongly stimulate the mitochondrial enzyme. Phosphate and arsenate ions have little effect on supernatant malate dehydrogenase whereas zinc inhibits this enzyme. Potassium chloride, magnesium and manganese ions stimulate both the mitochondrial and supernatant enzymes at concentrations less than 60 mM, and inhibit at higher concentrations. Citrate, sulfate and calcium ions inhibit both enzymes at all concentrations tested. Tris-acetate buffer stimulates both the mitochondrial and supernatant enzymes, having a greater effect on the supernatant form. The stimulation by phosphate is relieved by the addition of magesium ion to the assay system. Magnesium ion (15 mM) causes a 50% drop in stimulation.

Supernatant and mitochondrial malate dehydrogenases were subjected to freezing and thawing under a variety of conditions and the effects on enzymatic activity were studied. In general, rapid freezing results in greater activity losses than slow freezing. Consecutive freeze-thaws of the mitochondrial and supernatant enzymes in Tris-acetate and citrate buffers in the presence or absence of mercaptoethanol result in small activity losses. Supernatant malate dehydrogenase displays a marked

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loss in activity when frozen quickly in a medium containing both phosphate buffer and mercaptoethanol. NADH, OAA, NAD, malate, potassium chloride and zinc ion, in order of decreasing effectiveness, protect against activity loss in this system. If mercaptoethanol is omitted, activity loss is small. The mitochondrial enzyme loses little activity when frozen and thawed quickly in phosphate buffer with or without mercapto-ethanol. Repeated freeze-thawing of mitochondrial malate dehydrogenase in the presence of both phosphate and magnesium ion results in extensive losses in activity. If Tris-acetate buffer is substituted for phosphate buffer in this system, little activity loss occurs. The effects of freeze-thawing on the supernatant enzyme are not greatly altered by the addition of magnesium ion. The magnesium ion - phosphate effect suggests that these moieties may be factors in a cellular mechanism to regulate the activity of mitochondrial malate dehydrogenase. Generally, the results indicate the behavioral individuality of the mitochondrial and supernatant forms of malate dehydrogenase.

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TABLE OF NOMENCLATURE

Tris	Tris(hydroxymethyl)aminomethane buffer, adjusted to a given pH with acetic or hydrochloric acid
OAA	Oxalacetic acid
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide (reduced)
NADP	Nicotinamide adenine dinucleotide phosphate
M-MDH	Mitochondrial malate dehydrogen-ase
S-MDH	Supernatant malate dehydrogenase
DEAE	Diethylaminomethyl cellulose (Cellex-D)
PMB	p-mercuribenzoate
SH	Sulfhydryl
LDH	Lactate dehydrogenase
EDTA	Ethyenediamine tetraacetic acid
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)

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INTRODUCTION

A. General Introduction

Malate dehydrogenase (L-malate : NAD oxidoreductase EC 1.1.1.37), a key enzyme in the tricarboxylic acid cycle, is an L-hydroxydicarboxylic acid dehydrogenase (1) which catalyzes the following reversible reaction:

$$\text{L-malate} + \text{NAD} \rightleftharpoons \text{Oxalacetate} + \text{NADH} + \text{H}^+$$

It is distinct from the so-called "malic" enzyme that catalyzes the decarboxylation of malate. NADP can substitute for NAD as coenzyme, but is less effective. MDH can catalyze the dehydrogenation of other related compounds, but oxalacetate and malate are its main substrates (2).

The enzyme was discovered independently by Batelli and Stern (3) and Thunberg (4), and subsequently preparations were described by Green (5), Straub (6) and Ochoa _et al_. (7). The preparation of Wolfe and Neilands (8) from pig heart was the first with acceptable purity for molecular weight determination. Davies and Kun (1) isolated and purified MDH from ox heart mitochondria. They suggested that there might be more than one molecular species of MDH in a cell, pointing to earlier chromatographic analyses by Wolfe (9) that revealed two distinct peaks of MDH activity on elution from carboxymethyl-cellulose.

Delbruck _et al_. (10,11) and Wieland _et al_. (12) were the first to demonstrate a multiplicity of malate dehydrogenases in cells.

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Subsequently, Thorne (13) separated and studied two forms of rat liver MDH from mitochondrial and supernatant fractions. Siegel, Englard and Breiger (14,15,16,17,18) isolated and characterized mitochondrial and supernatant forms from beef heart, and similar enzymes were isolated from pig, horse and pigeon heart (19,20). Two MDH species have been isolated from rat brain (21), six from human and sheep brain (22), three from human serum (23), four from _Neurospora_ (24), two from potato tubers (25), two from pea seedlings (26,27), and two from _Euglena_ (27). In a number of cases where two forms were found it was shown that one was of mitochondrial origin, and that the other originated in the non-particulate portion of the cell. These and subsequent works emphasized the clear-cut distinction between the supernatant and mitochondrial forms of MDH with respect to extractability, physical properties, and kinetic behavior.

As is evident in the extraction procedures, S-MDH is easily extracted, requiring only homogenation of the tissue in buffer. On the other hand, isolation of M-MDH requires acetone treatment prior to disruption of the mitochondria in a homogenizer. The mitochondrial enzyme is more resistant to the effects of organic solvents (i.e., acetone, alcohol) than is the supernatant enzyme. Delbruck _et al._ (10,11) first described a characteristic inhibition of the mitochondrial enzyme by high concentrations of OAA. This inhibition has been demonstrated for M-MDH from several species, and it has been shown that S-MDH is not sensitive to high OAA concentrations (14,16).

As soon as workers succeeded in purifying these enzymes molecular weight determinations became feasible. Wolfe and

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Neilands (8) reported a value of 40,000 ± 5000 for pig heart M-MDH. Thorne (28) reported 70,000 for the same enzyme. A value of 15,000 to 20,000 was given by Davies and Kun (1) for ox heart MDH of mitochondrial origin. Values of 62,000 and 65,000 have been reported for beef heart M-MDH, and a value of 52,000 was given for beef heart S-MDH (10,17).

The two enzymes exhibit distinct chromatographic behavior. Englard et al.(14) found that beef heart S-MDH adsorbs to a DEAE-cellulose column, whereas M-MDH is not retained. Thorne (13) showed that rat liver M-MDH is retained by an Amberlite IRC 50 column while S-MDH passes through, and that the reverse is true when a DEAE-cellulose is used. Thorne et al.(29) found that during electrophoresis on starch gel S-MDH moves as a single band and is strongly anodal. On the other hand, M-MDH separates into several bands, some of which remain near the origin while others migrate toward the cathode. Recently Kulick and Barnes (30) have succeeded in resolving the pig heart S-MDH band into four entities which migrate slowly toward the anode.

The amino acid compositions of M- and S-MDH show considerable differences. Siegel and Englard (18) compared beef heart M- and S-MDH and found that S-MDH has more lysine, arginine, tyrosine, methionine, aspartic acid, and tryptophan, whereas M-MDH has more phenylalanine, glycine, proline, and threonine. Thorne (19,20) reported that pig heart S-MDH has a higher proportion of arginine, aspartic acid, and tryptophan than M-MDH, but a lower proportion of threonine, proline, and glycine.

The sulfur chemistry of M- and S-MDH serves as a disting-

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uishing feature for these enzymes. For beef heart MDH, Siegel and Englard (18) reported a value of twelve SH groups per mole for M-MDH and six SH groups per mole for S-MDH. All the SH groups in undenatured M-MDH are titratable with PMB, whereas in S-MDH only one-half react even in the presence of excess PMB. Titration of M-MDH with three equivalents of PMB results in activity loss. In contrast, titration of one-half of the SH groups in S-MDH does not affect the activity. Their data indicate that both enzymes lack disulfide linkages. Thorne and Kaplan (28) determined a value of fourteen SH groups per mole in pig heart M-MDH and their results point to a lack of disulfide bridges. A greater than six-fold molar excess of mercurial is required to cause activity loss. These workers showed that M-MDH binds two NADH molecules per mole, that the enzyme has a thirty to forty per cent helical content, and that it lacks tryptophan. Siegel and Englard (31) found one tryptophan residue per mole in beef heart M-MDH, and eight tryptophan residues per mole in the supernatant enzyme.

The persistant kinetic and molecular differences between supernatant and mitochondrial forms of MDH indicate that they are structurally different proteins.

B. The Effects of Ions on Malate Dehydrogenase

Englard _et al._ (14,18) mentioned several ionic effects on M- and S-MDH. M-MDH from both pig and beef heart showed increased activity in the presence of added phosphate ion. A similar effect was noted when sulfate, arsenate, maleate, and EDTA were added to the assay medium. They concluded that the

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effect was due to an increase in the ionic strength of the assay medium. The stimulation was not observed with the supernatant enzyme.

During purification procedures for pig heart M-MDH, Joyce and Grisolia (32,33) found a considerable variance in activity and stability depending on whether the enzyme was diluted in water or ammonium sulfate solution. They also noted that inorganic phosphate markedly stimulated malate formation from oxalacetate in the presence of MDH. Replacement of Tris buffer with phosphate buffer resulted in a four-fold increase in activity. Pre-incubation with Tris-HCl buffer, ammonium sulfate, or NADH stimulated the enzyme to an extent that depended on its purity, and its exposure to dialysis or lyophilization. Activity losses due to the latter two procedures appeared to be restored on incubation with these agents.

Kresack (34) found that addition of magnesium ion to a Tris-acetate buffered assay system resulted in increased activity for commercial (mitochondrial) MDH from pig heart. The effects of KCl on this enzyme suggested that the stimulation was not an ionic strength effect. If phosphate buffer was substituted for Tris in the assay system, addition of increasing amounts of magnesium ion caused an inhibition.

Work by Vallee _et al._ (35) on crude preparations of MDH suggested that zinc was a component of the MDH molecule. He achieved a fifty per cent inhibition of the enzyme by incubation with 1,10-o-phenanthroline, a chelating agent. Pfleiderer and Hohnholz (36) were unable to confirm these results. However, Harrison (37) analyzed MDH and found one zinc molecule

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per molecule of MDH. He showed that o-phenanthroline inactivated the enzyme by removing zinc. Zinc ion and NADH but not oxalacetate protected against this inactivation. Zinc was suggested to be the binding site for NADH.

C. Reversible Denaturation of Malate Dehydrogenase

Early attempts to reactivate MDH after inactivation were variously successful. Thorne and Kaplan (28) raised the pH of an MDH preparation to 12 and recorded a loss of activity and a quenching of fluorescence. Lowering the pH to 7 with acid did not restore activity. Harrison (37) was unable to reactivate MDH with zinc ion after incubation with o-phenanthroline. However, Joyce and Grisolia (32) showed that activity lost through dialysis or lyophilization could be restored by ammonium sulfate or NADH. Ammonium sulfate was particularly effective and greater activation was achieved with purer preparations.

Lactate dehydrogenase, an enzyme composed of four subunits, has been successfully inactivated and reactivated by several workers (38,39,40,41). Deal _et al._(42) dissociated rabbit muscle aldolase with acid into unfolded subunits, and reassociated the enzyme by neutralization with a substantial recovery of activity. He described a general procedure for dissociation and reactivation of enzymes with acid and base (43). The inclusion of mercaptoethanol in his system is worthy of note. Other multi-chain enzymes have been reversibly inactivated in urea, guanidine hydrochloride, acid, and sodium dodecyl sulfate, i.e. aldolase (44), fumarase (45), alkaline phosphatase (46),

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and α-glycerophosphate dehydrogenase (47).

Burton and Morita (48) demonstrated that crude preparations (cell-free extracts) of MDH from a marine psychrophile could be inactivated by heat and reactivated by cooling in a medium containing mercaptoethanol, NADH, and malate. Munkres (49) denatured Neurospora MDH reversibly with acid and urea.

Recently, Chilson et al. (40,41) succeeded in reversibly inactivating chicken, tuna, and pig heart M-MDH and pig heart S-MDH, using urea, guanidine hydrochloride, lithium chloride, and acid. In all cases, after the enzyme was completely inactivated, dilution or neutralization brought about a substantial restoration of activity under experimental conditions. Their work suggested that previous attempts to reversibly denature MDH failed because of a lack of a suitable reducing agent. The presence of such an agent was absolutely required in the dissociation step; yields of active enzyme were greatly increased if it was included in the reactivation mixture also. Ultracentrifugation studies indicated that the presence or absence of a reducing agent had a profound effect on the state of aggregation of the enzyme during inactivation.

The reactivated enzymes displayed the same physical and kinetic characteristics as the native enzymes. The inactivated enzymes, on the contrary, showed extensive changes in these properties.

Both the rate and extent of reactivation were dependent upon the buffer used. For a mixture containing 0.1 M sodium salt plus mercaptoethanol, the order of effectiveness for re-

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activation of pig heart M-MDH was as follows:

citrate > malate >> phosphate > sulfate > Tris > acetate

The order was the same for protection against heat inactivation. Halide ions inhibited reactivation.

NADH and NAD have been shown to protect a number of dehydrogenases against inactivation by various agents (50,51,52,53). Chilson *et al.*(40,41) found that NADH enhanced both the rate and the extent of reactivation of MDH whereas NAD had no effect.

By mixing aliquants from inactivated enzyme preparations and then reactivating the mixture by dilution, the authors demonstrated through starch gel electrophoresis the formation of hybrids between MDH from different species and between S-and M-MDH. The hybridization was inhibited by NADH and the hybrids exhibited properties intermediate between those of the parent enzymes. This is the first demonstration that vertebrate malate dehydrogenases can be reversibly dissociated into independently-acting subunits. The results show that the supernatant and mitochondrial forms, while displaying a large number of distinguishing properties, possess enough similarity to permit hybrid formation.

D. The Effects of Freeze-thawing

Low temperatures or freezing and thawing often has a drastic effect on the activity of an enzyme in solution. Kirkman and Hendrickson (54) showed that freezing and thawing caused the dissociation of glucose-6-phosphate dehydrogenase. Markert (55) used the process to investigate hybridization of LDH in 1 M NaCl. He found that if two electrophoretically distinct

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forms were frozen together in NaCl, hybrids were formed by random recombination of subunits that had presumably dissociated during the freezing step. Van Eys et al.(56) pointed out the importance of protein concentration and dilution effects in obtaining good quantitative data from freeze-thaw studies.

Chilson et al.(57,58) has investigated thoroughly the effects of freeze-thawing on LDH and other dehydrogenases, and his experiments provide the basis for approaching the problem of low temperature effects on proteins.

Previous workers (59,60,61) demonstrated that a slow rate of freezing and thawing had a greater effect on the properties of proteins than a fast rate. With LDH, rapid freezing in a dry ice - methanol bath and thawing at room temperature resulted in no hybridization and little activity loss whereas slow freezing and thawing produced hybridization and caused greater loss in activity. These effects are apparently due to different periods of exposure to the critical conditions of the frozen mixture.

The effects of repeated freezing and thawing vary greatly with different enzymes. Chilson and his fellow workers found that often there was a large initial loss in activity followed by a gradual drop-off that was not proportional to the number of times the enzyme preparation was frozen.

They found that the components of the medium played a major role in the freeze-thaw effect. The presence of both phosphate and NaCl was required for hybridization of LDH. The halides influenced activity loss in order of their effectiveness as denaturants. In all cases the enzymes were stable in

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the phosphate-halide mixture before freezing. This suggests that the large increase in salt concentration on freezing plays a major role in affecting enzymatic activity. Chicken heart M-MDH, in contrast to the other dehydrogenases tested, showed remarkable stability toward freeze-thawing.

A marked difference in the rate of hybridization and activity loss with repeated freeze-thawing in sodium and potassium phosphate was observed. Freeze-thawing in sodium phosphate produced more extensive losses in activity and a faster rate of hybridization than was observed when potassium phosphate was used. Van den Berg and Rose (62,63) have studied the properties of phosphate solutions on freezing, and have observed extensive changes in pH and concentration, particularly with sodium phosphate. The pH can drop as low as 3.5, and concentrations have been observed to increase from 0.02 M to 3 M. In general, the pH and concentration changes of potassium phosphate on freezing are of a lesser degree and pH can increase or decrease depending on the ratio of the monobasic to the dibasic salt. Freezing of a water solution results in the freezing out of ice and crystallization of salts present depending on their solubility. The data of Chilson et al.(57,58) on LDH suggest that the denaturation of enzymes caused by freezing is mainly the result of pH change rather than ice formation, concentration changes, and low temperature.

Coenzymes have been shown to cause reduced dissociation of various enzymes in the presence of a number of denaturing agents (50,51,52,53). Lea and Hawke (59) showed that NAD reduces activity loss caused by freezing. The work of Chilson

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et al.(57,58) demonstrated that coenzymes reduce hybridization and activity loss on freeze-thawing of a number of dehydrogenases. The reduced form was the most effective in most cases. The relative protective efficiency is related to the binding constant of the enzyme and coenzyme.

Freezing and thawing increased the activity of the SH groups of LDH toward mercurials; this suggests that some of the inactivation resulting from the process is due to oxidation of essential SH groups. Levitt (64) proposed that freezing of cells allows protein chains to come into physical contact permitting formation of stable intermolecular disulfide bridges. On thawing the enzyme cannot re-form its native conformation and hence remains inactive. Chilson et al.(57,58) found that mercaptoethanol protected LDH against inactivation but not against hybridization.

It appears that freezing causes a dissociation into subunits and thawing causes a reassociation. Failure to reassociate properly results in hybrid formation and activity loss.

Freeze-thaw studies on MDH have been sparse. Joyce and Grisolia (32) reported that concentrated crude preparations of M-MDH lost 27% activity on an initial freeze, but were stable to further freezes. Kresack (34) reported a 43% loss in activity for commercial MDH when this enzyme was frozen and thawed overnight. If magnesium ion was included in the storage mixture, all activity was lost on freezing.

The present study was undertaken in order to determine more precisely and to compare the effects of various ions on purified S-and M-MDH. The effects of freeze-thawing on these

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enzymes were investigated also and the results were related to the ionic effects.

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THE EFFECTS OF IONS ON THE ACTIVITY OF MITOCHONDRIAL AND SUPERNATANT MALATE DEYDROGENASE

A. Methods

Primary standard grade Tris(hydroxymethyl)aminomethane was obtained from the Fisher Scientific Company. NADH and NAD were obtained from Boehringer Mannheim and Son. EDTA (tetrasodium dihydrate), oxalacetic acid, bovine serum albumin, and DEAE-cellulose were purchased from the California Corporation for Biochemical Research. Zinc sulfate and citric acid were purchased from British Drug Houses Ltd. 2-mercaptoethanol was from Eastman Organic Chemicals, and 1,10-o-phenanthroline, Hyflo Super Cel, sucrose, and ammonium sulfate were obtained from Fisher Scientific Co. as were the reagent grade chemicals used for the ionic studies.

For the assays, oxalacetate and NADH were prepared fresh daily in deionized water and were kept at 0°C. An inhibitor forms in NADH solutions when they are stored in the frozen state (65).

Supernatant MDH was purified from whole pig heart according to the procedure of Englard and Breiger (16) as described in the APPENDIX. Mitochondrial MDH was purified according to the procedure of Wolfe and Neilands (8) with modifications as described in the APPENDIX. For the studies of the effects of ions added to the assay system, the mitochondrial and superna-

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tant enzymes were diluted one part to thirty in 20 mM Tris-acetate buffer, pH 7.4, and serum albumin was added (1 mg per ml). These preparations were dialyzed four times against 20 mM Tris-acetate buffer, pH 7.4, to remove extraneous ions - ammonium sulfate, in particular. The dialyzed samples were divided into 0.5 ml portions and stored frozen in the deep freeze. For each experiment, a sample was thawed and diluted with a 1 mg per ml solution of serum albumin. A second dilution was made with 20 mM Tris-acetate buffer, pH 7.4, such that a suitable reaction rate was achieved when a 0.1 ml sample was assayed. For the freeze-thaw studies the enzyme preparations were not dialyzed.

Spectrophotometric assays were made at 340 mμ with a Beckman model DU spectrophotometer coupled to a Gilford 2000 absorbance recorder. The temperature of the cuvette chamber was maintained at 25.0 ± 0.5°C with a Haake circulating water bath.

Enzymatic activity was determined by measuring the rate of conversion of NADH to NAD. For the control assay, the quartz cuvette (1 ml volume, 1 cm light path) contained, in order of addition, 100 μmoles Tris-acetate buffer, pH 7.4, 0.76 μmoles OAA, 0.38 μmoles NADH, and deionized water to give a total volume of 0.9 ml. Lastly, 0.1 ml of suitably diluted enzyme preparation was added after the rest of the ingredients had been mixed. This will henceforth be referred to as the "standard assay system". Activity was expressed as μmoles NADH oxidized per minute, using the molar absorptivity of 6.22×10^3 for NADH.

For the freeze-thaw studies a mixture was prepared con-

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taining, in order of addition, buffer, serum albumin, mercaptoethanol, ionic species, and deionized water. Enzyme was added last with a lambda pipette to give a protein concentration of 0.053 mg per ml. The mixture was divided into two equal portions in thick-walled test-tubes. One part was stored on ice for the duration of the experiment, to serve as a control; the other was subjected to either of two methods of freezing. The "fast freeze" involved immersion in a dry ice - acetone bath (-35°C, approximately) for two minutes. For the "slow freeze" the preparation was placed in the deep freeze (-10 to -13°C) for one hour or more. After the freezing period, the sample was allowed to thaw at room temperature in a closed chamber. As soon as thawing was complete, the sample was placed on ice and a suitably diluted sample was assayed as described above.

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B. Results

1. The Effects of Ions Added to the Assay System

The effects of a number of divalent cations added to the assay system for M-and S-MDH were investigated. The chlorides of magnesium, manganese and calcium generally had an inhibitory effect on both enzymes at concentrations greater than 50 to 60 mM. Figure 1 depicts these results. Low concentrations (5 to 30 mM) of manganese stimulated both enzymes to a maximum of 110% over control activity. Magnesium ion had a similar effect on S-MDH but not on M-MDH.

To determine if the observed low concentration stimulation was an ionic strength effect, increasing concentrations of KCl were added to the assay mixture. Figure 2 shows that stimulation to a maximum of 110% over the control level was achieved with the greatest stimulation occurring at KCl concentrations in the range of 20 to 40 mM. Inhibition is observed at concentrations greater than 80 mM.

An attempt was made to determine the effects of Tris on the activity of M-and S-MDH. An assay mixture without Tris and enzyme was neutralized at room temperature by titration with NaOH to a pH of 7.4. To this mixture KCl was added to bring the ionic strength to the level of the standard mixture, and the enzyme was assayed. For both M-and S-MDH the activity recorded when Tris was absent was approximately 20 to 30 % lower

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than that obtained using the standard assay system (100 mM Tris). However, the absorbance of the neutralized mixture was lower than that of the standard assay mixture. It is likely that some NADH was broken down (probably by hydrolysis of the phosphate bond) by exposure to room temperature and/or sudden increases in pH. With only 10 µmoles of Tris-HCl buffer in the neutralized mixture the activity was close to that observed with the standard mixture, and no drop in the absorbance was noted. With 30 to 40 µmoles of Tris-HCl buffer in the system, greater activity than achieved with the standard system was observed. This may have been due to a pH effect. Tris-HCl concentrations greater than 100 µmoles inhibited both M- and S-MDH slightly. (See Figure 3)

Figure 4 shows the effects of low concentrations of zinc sulfate on the activity of M- and S-MDH. For M-MDH maximum stimulation of about 180% over the control level was found. Precipitation of zinc sulfate at concentrations greater than 18 mM prevented investigations at higher concentrations. The decrease in stimulation by the addition of greater than 6 µmoles of zinc ion was probably due to a lowering of the pH of the assay mixture, since the zinc sulfate was made up in dilute acid. Addition of zinc ion inhibited S-MDH at all concentrations investigated. Addition of various amounts of sodium sulfate to the assay mixture revealed that sulfate ion had little effect on either enzyme.

The effect of increasing concentrations of potassium phosphate on the activity of M- and S-MDH is shown in Figure 5. M-MDH is stimulated strongly with a maximum of 150% over the con-

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trol level occurring at 100 mM phosphate. Sodium arsenate had a very similar effect on M-MDH. S-MDH was influenced very slightly by arsenate and phosphate.

Figure 6 illustrates the effects of magnesium ion on the activity of phosphate-stimulated M-MDH. Magnesium chloride (15 mM) reduced the phosphate-produced stimulation by 50%. Precipitation at magnesium concentrations greater than 60 mM made it impossible to determine the amount of magnesium ion required to completely abolish phosphate activation. The data suggest that a one-to-one magnesium-to-phosphate ratio would be required. The addition of magnesium to the assay mixture containing added phosphate and S-MDH gave results resembling those obtained when magnesium ion alone was added to the assay system for S-MDH.

ii. The Effects of Freeze-thawing

Tables 1 and 2 show the effects of two consecutive fast freezes on the activity of M- and S-MDH. Part C of the APPENDIX presents more detailed data. In the standard mixture (potassium phosphate buffer, serum albumin, and mercaptoethanol), the supernatant enzyme proved remarkably unstable. However, omission of mercaptoethanol or replacement of phosphate buffer with Tris-acetate or citrate buffer results in a relatively small loss of activity after two fast freezes. NADH, OAA, NAD, malate, 1 M KCl, and zinc chloride, in order of decreasing effectiveness, protect S-MDH against activity loss when fast frozen in the standard mixture. Potassium chloride (1 M) increases loss of activity in the phosphate system without mer-

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captoethanol. In direct contrast to the effect of fast-freezing S-MDH in the standard mixture, consecutive slow-freezes result in either stimulation or in small losses in activity.

M-MDH was stable when fast-frozen in the standard mixture, and some stimulation was observed. Omission of mercaptoethanol resulted in greater loss of activity and addition of KCl had a similar effect.

Addition of magnesium ion to the standard mixture containing either S-or M-MDH resulted in increased activity loss. When the standard mixture was made 30 mM in magnesium ion, fast-freezing caused drastic loss of activity for the mitochondrial enzyme. If phosphate buffer was replaced by Tris buffer in this system, activity loss was slight. Freeze-thawing M-MDH in the standard mixture containing zinc ion resulted in small activity loss. Substitution of sodium phosphate buffer for potassium phosphate buffer in the standard mixture resulted in little change in activity on freezing and thawing.

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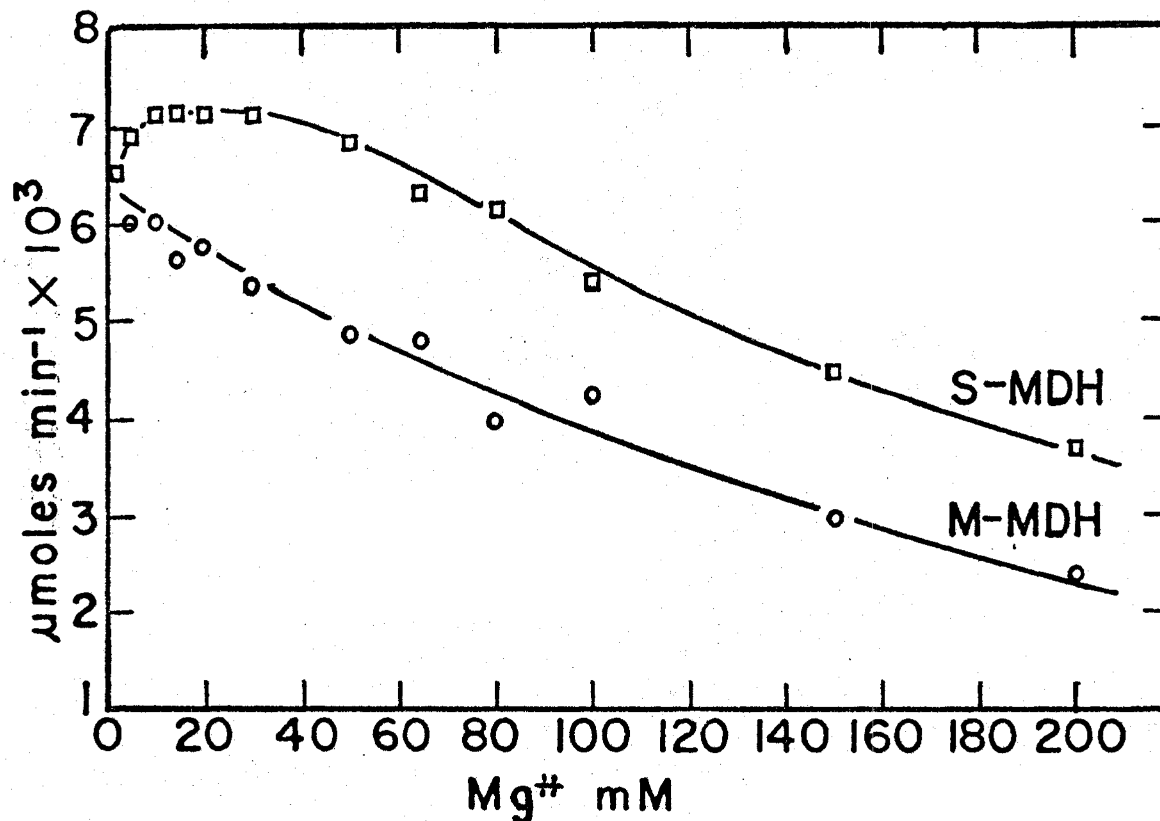
Figure 1

The Effect of Magnesium Ion on the Activity of M-and S-MDH



Legend

Various amounts of magnesium chloride were added to the standard assay system (0 - 200 µmoles). The assays were carried out immediately after addition of enzyme. The activity (µmoles per min) of the enzyme in the presence of various amounts of ion was expressed as follows:

$$\frac{\text{activity of enzyme sample at time t}}{\text{activity of enzyme sample in the presence of ion at time t}} \times 6.23 \times 10^{-3} \ \mu\text{moles min}^{-1}$$

The average activity of the enzyme samples at zero time was 6.23×10^{-3} µmoles min^{-1}.

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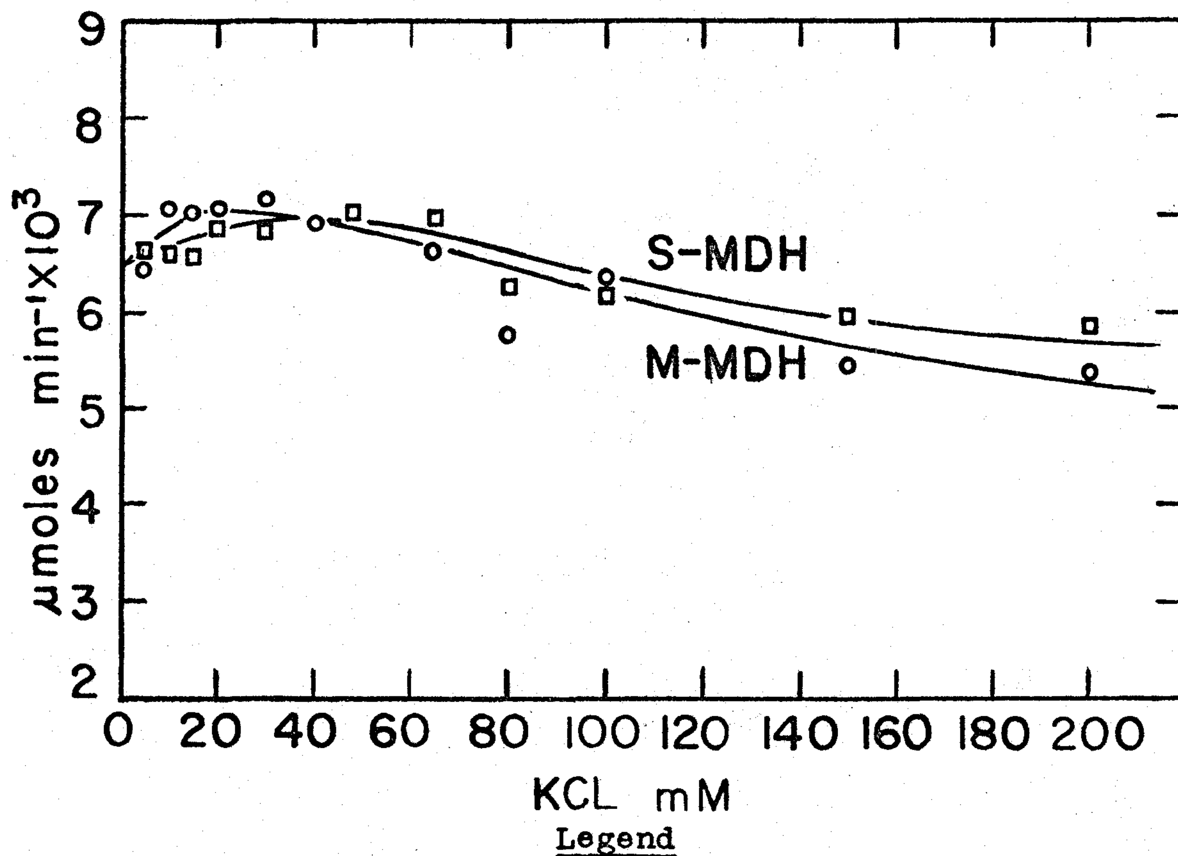
Figure 2

The Effect of Ionic Strength on the Activity of M- and S-MDH



Legend

Various amounts of KCl were added to the standard assay system (0 - 200 umoles). Assays were carried out immediately after addition of enzyme. Data were expressed as in Figure 1.

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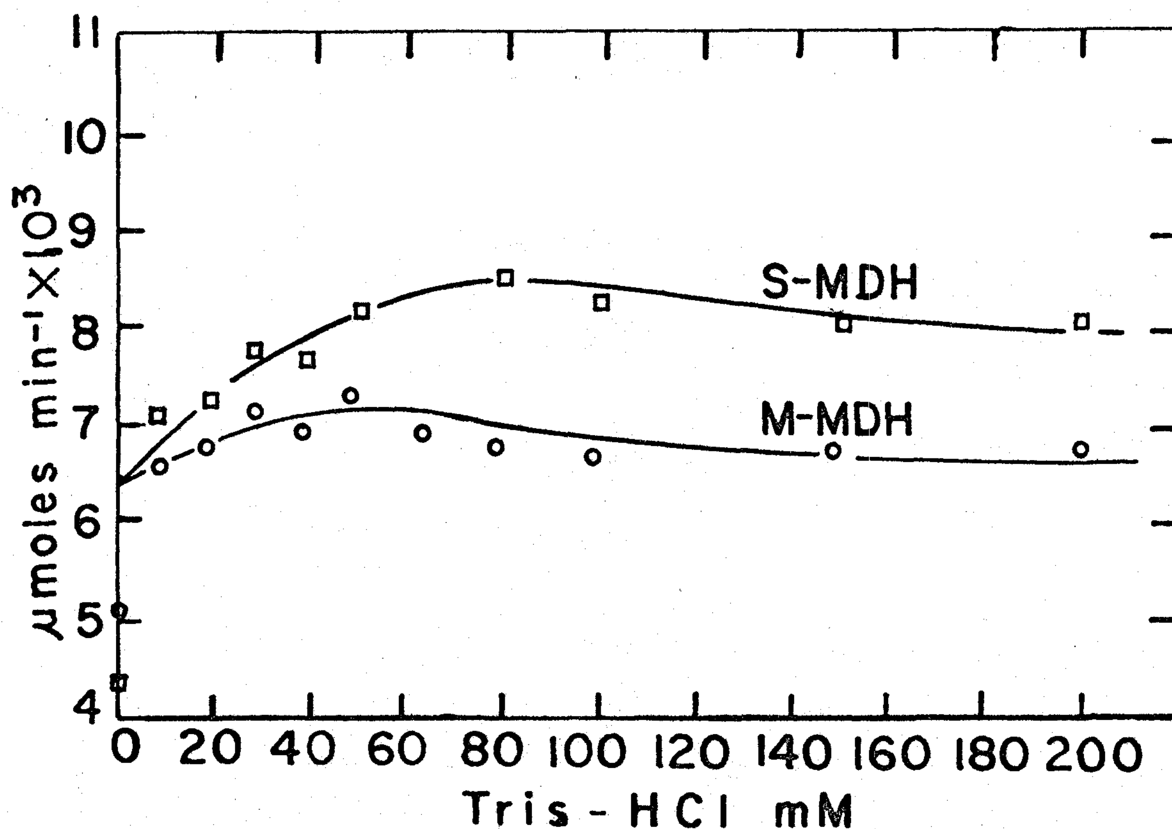
Figure 3

The Effect of Tris-HCl on the Activity
of M- and S-MDH



Legend

A mixture containing 22.8 µmoles OAA, 0.56 µmoles NADH, and various amounts of Tris-HCl buffer, pH 7.4, was prepared and was neutralized with 0.025 N NaOH to a pH of 7.4. Suitably diluted enzyme was added and the assay was carried out immediately. Data were expressed as in Figure 1.

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Figure 4

The Effect of Zinc Sulfate on the Activity of M- and S-MDH

Legend

Various amounts of zinc sulfate dissolved in dilute HCl were added to the standard assay system. Assays were carried out immediately after addition of enzyme. Data were expressed as in Figure 1.

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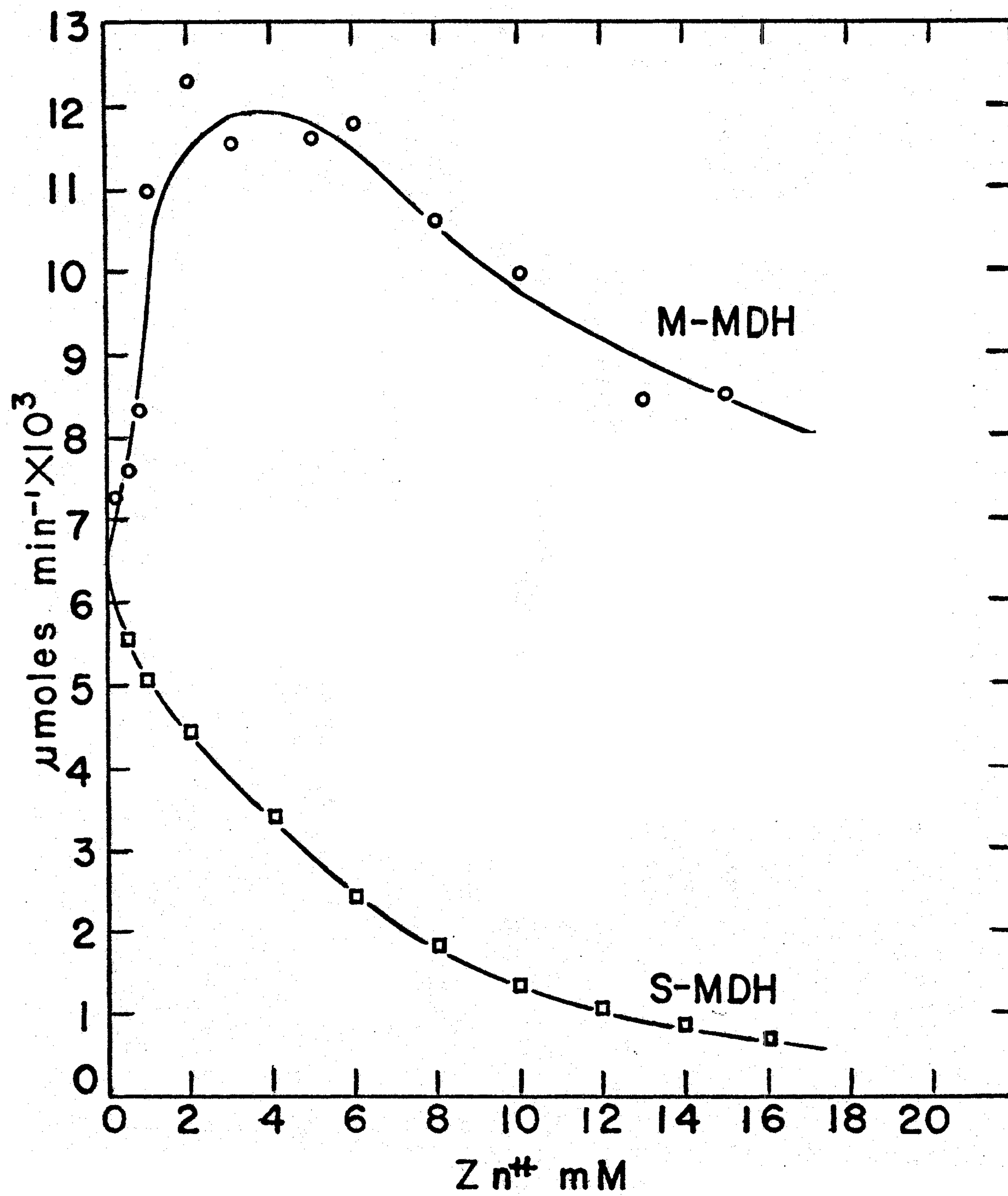

Figure 4

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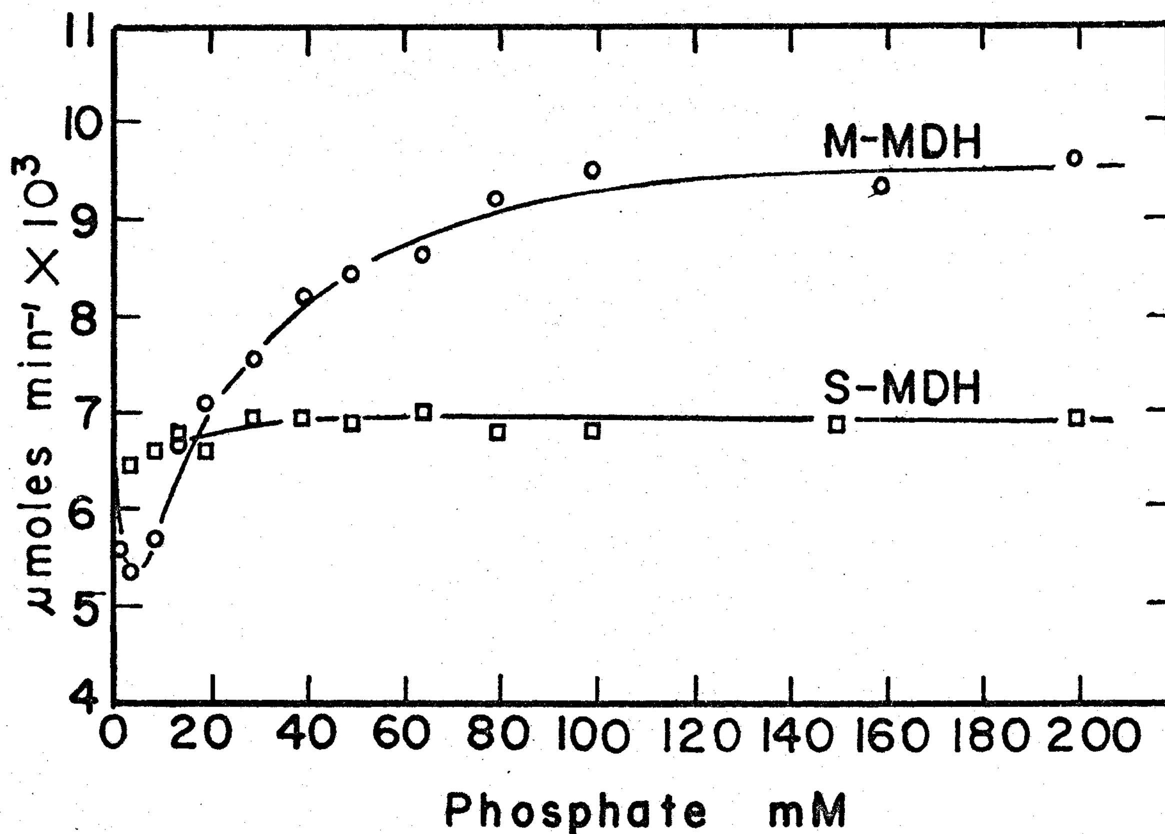
Figure 5

The Effect of Phosphate on the Activity of M- and S-MDH



Legend

Various amounts of potassium phosphate buffer, pH 7.4, were added to the standard assay system. Assays were carried out immediately after addition of enzyme. Data were expressed as in Figure 1.

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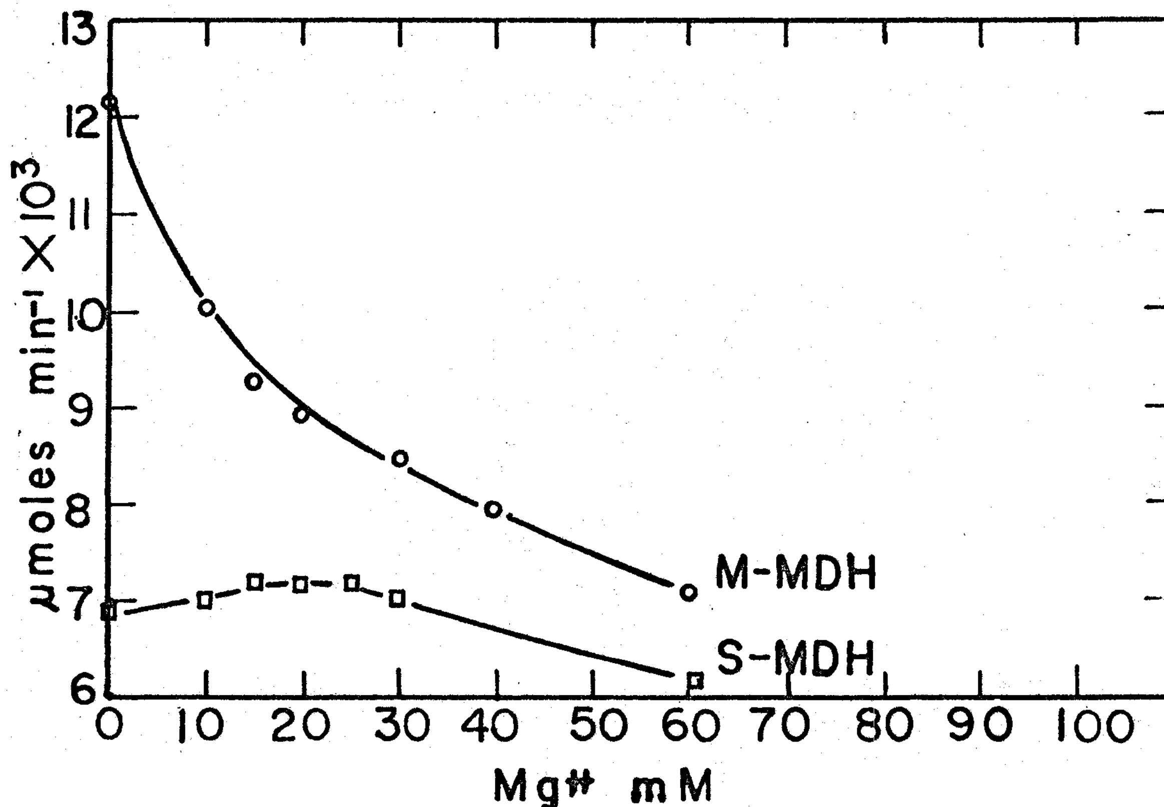
Figure 6

The Effect of Magnesium Ion on the Activity of M- and S-MDH Assayed in the Presence of Phosphate



Legend

Potassium phosphate buffer, pH 7.4, (100 μmoles) was included in the standard Tris-acetate system and various amounts of magnesium chloride were added. Assays were carried out immediately after addition of enzyme. Data were expressed as in Figure 1.

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Table 1a, 1b

The Effects of Freeze-thawing on
the Activity of M- and S-MDH

Legend

S-MDH and M-MDH mixtures prepared as described on page 14 were fast-frozen and thawed twice in succession and a suitably diluted sample was assayed.

i. The standard mixture (1 ml) contained 0.053 mg per ml M or S-MDH, 100 µmoles potassium phosphate buffer, pH 7.4, 0.1 mg per ml bovine serum albumin, and 10 µmoles mercaptoethanol.

ii. Other mixtures were similar to the standard mixture except as noted in column one of the table. The notation of a buffer indicates that this buffer replaces the potassium phosphate buffer used in the standard mixture.

The concentrations of the various additions are as follows:

Sodium phosphate or Tris-acetate buffer	100 mM
KCl	1 M
NADH	753 uM
NAD	752 uM
OAA	7.57 mM
Malic acid	7.45 mM
Zinc sulfate	2 mM
Magnesium chloride	10-30 mM

For detailed freeze-thaw experiments see APPENDIX C.

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Table 1a - S-MDH

System	% Activity Remaining
a. Tris-acetate buffer	94.3
b. Standard mixture	3.0
c. Sodium phosphate buffer	2.2
d. Without mercaptoethanol	88.8
e. Tris-acetate buffer plus KCl	99.0
f. Without mercaptoethanol plus 10 μmoles Mg^{++}	98.0
g. Tris-acetate buffer plus 20 μmoles Mg^{++}	93.4
h. Plus 2 μmoles Zn^{++}	15.0
i. Plus NADH	76.5
j. Plus NAD	30.6
k. Plus OAA	61.0
l. Plus malic acid	29.6
m. Plus KCl	14.8

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Table 1b - M-MDH

System	% Activity Remaining
a. Tris-acetate buffer	89.3
b. Standard mixture	108.9
c. Without mercaptoethanol	86.6
d. Plus KCl	84.4
e. Plus 10 µmoles Mg^{++}	80.3
f. Plus 30 µmoles Mg^{++}	17.5
g. Tris-acetate buffer plus 20 µmoles Mg^{++}	102.8
h. Tris-acetate buffer plus 2 µmoles Zn^{++}	122.5
i. Tris-acetate buffer plus KCl	91.7
j. Plus 2 µmoles Zn^{++}	97.7

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C. Discussion

The slight stimulation of M-and S-MDH observed for low concentrations of magnesium and manganese ions appears to be the result of non-specific ionic strength effects. Concentrations of KCl giving a similar ionic strength cause a similar stimulation. The inhibiting effects at concentrations of magnesium, calcium and manganese greater than 60 mM also appear to be due to ionic strength changes.

In contrast, the stimulation of M-MDH by zinc ion is a specific effect. Vallee _et al._ (35) and Harrison (37) have suggested that zinc is an integral part of the MDH molecule. Harrison has suggested that it represents the binding site for NADH. The stimulation observed when zinc ion is included in the assay medium may be a reactivation of inactive enzyme since zinc may be lost during the preparation procedure. Zinc may bind specifically to some region of the protein molecule and cause a slight alteration in conformation favouring increased turn-over of substrate.

An attempt was made to remove zinc from M-MDH by incubation with 10 mM o-phenanthroline. The presence of this chelating agent, however, caused no greater activity loss with time than was observed in a control preparation. Addition of o-phenanthroline to the assay mixture had a negligible effect on activity. When the incubation mixtures with and without o-phen-

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anthroline were assayed with the standard assay mixture plus 2 μmoles zinc ion, greater activiation was observed for the preparation incubated with o-phenanthroline. The role of zinc in the structure of M-MDH remains unclear.

Phosphate and arsenate ions stimulate M-MDH to approximately the same degree as zinc ion. As with zinc ion, the stimulation could be a reactivation or the result of binding to specific sites on the protein. Together with the evidence that phosphate adheres to the surface of the enzyme, these observations suggest that phosphate plays a very specific role in the structure of M-MDH.

Neither phosphate nor arsenate brings about any significant stimulation of S-MDH. Zinc ion inhibits the supernatant enzyme strongly at low concentrations. The inhibition is greater than that caused by high concentrations of other divalent metal ions such as magnesium or calcium, suggesting that more than an ionic strength effect is involved. Zinc may bind irreversibly to some group at the active center of the enzyme and thus produce a poisoning effect similar to that produced by heavy metal ions on a number of enzymes. The inhibition also suggests that zinc is not a part of the supernatant enzyme.

The degree of activation of M-MDH produced by zinc and phosphate is dependent on the history of the enzyme preparation; that is, exposure to temperatures above 0°C, or to freezing and thawing. After standing on ice for 4 hours a dilute sample of M-MDH was stimulated only 15% over the control level. The maximum stimulation produced by zinc and phosphate ions varies considerably with different enzyme dilutions. However,

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there is a consistant activation.

Figure 6 shows that M-MDH activated with phosphate is very sensitive to the presence of magnesium ion. Only 15 μmoles of magnesium chloride were required to abolish 50% of the phosphate-produced stimulation. This effect is similar to one described by Kosicki and Lee (67). They found that divalent metal ions relieved inhibition of citrate synthase by ATP and other nucleotides, presumably by binding polyphosphate chains on these molecules. Urea - DTNB studies (68) have shown that phosphate protects M-MDH against opening by urea, as evidenced by decreased availability of SH groups. Also, addition of magnesium ion to the phosphate-containing system results in a faster unfolding of the protein. The data for M-MDH indicates that magnesium ion binds the phosphate involved in the stimulation. These effects suggest a control system for the mitochondrial enzyme. By regulating phosphate and magnesium concentrations in the mitochondria the cell could control the activity of MDH. If such a system exists for the mitochondrial enzyme, it is apparently lacking for the supernatant enzyme, since phosphate and magnesium ion have little effect on S-MDH. In Figure 6, the slight peak observed at 20 mM magnesium is probably the non-specific effect produced when magnesium is added to the assay system buffered by Tris.

S-and M-MDH exhibit distinct behavior when frozen and thawed in different media. Several factors must be taken into account when studying freeze-thaw effects: changes in pH and in salt and protein concentration, temperature effects, time of exposure to freezing, removal of "structured" water, and

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specific and non-specific ion effects.

It is known that water can form ordered crystalline structures around apolar molecules if the concentration of the apolar substance in water is high enough (69,70,71). Klotz (69) has proposed that similar structured water should exist around apolar groups projecting from the protein molecule since the local concentration of such groups is high; this should stabilize apolar bonds between groups on the protein. Kauzman (74) has proposed that hydrophobic bonds, the tendency for apolar groups to associate in water solution, are a source of stabilization for the protein molecule.

Removal of water as ice would play havoc with the stabilization of conformation provided by apolar or hydrophobic bonding, and together with pH effects and ionic effects could either facilitate dissociation of the protein into subunits or cause local changes in conformation.

Chilson <u>et</u> <u>al</u>.(57,58) suggested that pH changes account for much of the inactivation produced by freeze-thawing. Sodium phosphate solutions, which change pH with freezing more drastically than potassium phosphate solutions, produced the greater activity losses with LDH. The data in Tables 1a and 1b show that replacing potassium phosphate with sodium phosphate buffer results in little change in the amount of activity lost. M-MDH appears to be more stable to freeze-thawing in sodium phosphate than in potassium phosphate when mercaptoethanol is absent. These results suggest that pH is not the major factor contributing to activity loss of MDH with freezing.

The effects of the rate of freezing and thawing on MDH

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activity differ from those observed for LDH. Chilson et al. (58) found that longer exposure to low temperatures (slow freezes) produced a greater amount of hybridization and more extensive losses in activity. The opposite effect was observed for MDH. In a number of instances slow freezing produces a stimulation. Slow freezing restores activity lost by several fast freezes in many cases. It appears that sudden freezing effects irreversible changes in enzyme structure, whereas the slow procedure produces either small changes or changes that are restored on thawing. The large differential in the temperatures of the fast and slow-frozen mixtures may be an important factor.

It should be pointed out that while freeze-thawing produced dissociation of LDH into subunits, it may not have the same effect on MDH. The evidence presented in this paper is insufficient to determine whether dissociation or merely local changes in conformation occur. Both effects probably exist, but the small activity losses produced in many cases suggest that the amount of dissociation taking place is small.

Freezing and thawing in the presence and absence of mercaptoethanol produces some unexpected results. Previous failures to reversibly inactivate LDH and MDH were attributed by Chilson et al. to be due to the lack of a reducing agent (41). Acid inactivation of MDH in the absence of mercaptoethanol produced an initial increase in sedimentation coefficient followed by conversion to a slower sedimenting form which could not be reactivated by neutralization (41). Some of the inactivation could be due to oxidation of SH groups and to the formation of intermolecular disulfide bonds as proposed by Levitt (58,64).

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In phosphate buffer, mercaptoethanol appears to protect M-MDH from the effects of freeze-thawing. The proposals mentioned above may apply here. However, consecutive fast freezes of an S-MDH mixture containing both phosphate and mercaptoethanol cause a drastic loss in enzymatic activity. If mercaptoethanol is excluded fom the phosphate - S-MDH system, the enzyme loses little activity.

On the basis of available data it is difficult to explain this phenomenon. It has not been shown that pig heart S-MDH lacks disulfide bridges, although beef heart S-MDH has none (18). If indeed such bridges are present, it is possible that freezing in phosphate buffer results in a cleavage of these bonds. On thawing, the SH groups produced by the cleavage would be maintained as such in the presence of excess reducing agent and the native conformation would not be re-established. On the other hand, fast freezing in phosphate may expose essential SH groups which may react with mercaptoethanol in the environment existing in the frozen mixture. Fast freezing in citrate or Tris-acetate may not expose important SH groups. The presence of mercaptoethanol makes little difference when either S-or M-MDH is frozen and thawed in these buffers. Little difference in activity loss is noted whether the enzymes are frozen in Tris-acetate or citrate buffer. However, Chilson et al.(40) reported that pig heart M-MDH can be reactivated to a much greater extent in citrate buffer than in Tris buffer. This suggests that little dissociation occurs when S-or M-MDH is frozen in citrate or Tris buffer.

Substrates are very effective protective agents against

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activity loss of S-MDH when fast frozen in the standard mixture. By binding to the active site they may prevent phosphate and/or mercaptoethanol from interacting with critical groups on the protein molecule, by steric hindrance or by stabilization of conformation. If dissociation is involved a substrate could bridge the subunits and hold them together.

In view of the role of phosphate in the structure and activity of M-MDH, it is significant that freeze-thawing in the presence of phosphate and mercaptoethanol causes little activity loss for this enzyme. Phosphate appears to protect M-MDH by some unknown mechanism against the effects of freeze-thawing in the same way that it does against the effect of urea denaturation (68).

Addition of magnesium ion to the mixture containing phosphate and M-MDH results in extensive activity loss on freeze-thawing. This correlates with the observation that magnesium relieves phosphate activation when added to the assay mixture. Whether phosphate exerts a special protective influence over M-MDH against freeze-thawing effects cannot be positively affirmed since the enzyme loses little activity when frozen in Tris or citrate buffer. Frozen mixtures of Tris and citrate may provide different environments than frozen phosphate solutions. It is noteworthy that magnesium ion has little effect in a mixture buffered by Tris-acetate, and that it does not cause increased activity loss in S-MDH mixtures buffered with phosphate or Tris buffer.

High salt concentration, such as that produced when a salt solution is frozen, is known to exert a number of effects on

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enzymes or model proteins leading to dissociation and/or inactivation. Effects on pK's of ionizable groups and on the activity coefficients of peptide bonds, modification of solvent (water) structure and protein-solvent interactions, and specific binding of ions must be taken into account. The activity losses of an M-MDH mixture containing KCl (1M) differ little from those observed for a mixture containing no added KCl. It would appear that the effects of inclusion of magnesium ion are not due to non-specific effects such as changes in water structure (69,73), but rather to a specific interaction between the ion and groups on the protein molecule.

KCl causes increased activity loss when a S-MDH mixture containing phosphate but no mercaptoethanol is frozen and thawed. This may be the result of ionic strength effects on groups exposed by phosphate. On the other hand, KCl protects S-MDH against loss of activity in the standard mixture. In this case there may be a competition between KCl and mercaptoethanol for a group on the protein. This might also be the case with zinc ion which also protects S-MDH.

With the exception of the standard S-MDH mixture (plus or minus mercaptoethanol) the presence of 1 M KCl in a freeze-thaw mixture makes little difference in the amount of activity lost by S- or M-MDH. This is in contrast to the results of Chilson _et al._ (58) for lactate dehydrogenase, triosephosphate dehydrogenase, and alcohol dehydrogenase, all of which showed extensive losses in activity when frozen in the presence of NaCl and phosphate. However, chicken heart M-MDH shows stability of the same order as pig heart M- and S-MDH.

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The results described in this paper point out the need for further experimentation. The role of phosphate and zinc ion in the structure and behavior of M-MDH is by no means clear. Further studies are required to confirm and define the possible control system for M-MDH. The difficulty in interpreting the results of the freeze-thaw studies suggests more detailed investigations. Determination of the effects of freezing on the properties of phosphate-mercaptoethanol solutions and the electrophoretic characteristics of the enzymes after freezing and thawing would resolve many problems. Also it would be interesting to determine the role of sulfhydryl groups in the structure of MDH.

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SUMMARY

The supernatant and mitochondrial forms of MDH behave distinctly in the presence of a number of cations and anions. M-MDH is stimulated strongly by phosphate, arsenate and zinc ions. The effect of these ions is not due to increases in ionic strength in the assay medium but is probably related to their presence in the structure of the enzyme. S-MDH is affected only slightly by phosphate and arsenate but is inhibited strongly by zinc ion. The latter effect suggests that zinc is not a part of the S-MDH structure.

Increases in ionic strength stimulate both M-and S-MDH at low levels and inhibit at higher levels. A similar effect is observed for magnesium, manganese and calcium ions.

Magnesium ion relieves the activation of M-MDH by phosphate through a binding of the phosphate. This observation and others suggests that the activity of M-MDH may be regulated in the mitochondria by control of magnesium and phosphate concentrations.

M-and S-MDH are stable to repeated freezing and thawing in citrate and Tris-acetate buffers, with or without mercaptoethanol. S-MDH loses activity rapidly when fast-frozen in a mixture containing both phosphate and mercaptoethanol. Omission of mercaptoethanol from this mixture eliminates much of the inactivation. A specific interaction of phosphate, mercapto-

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ethanol and S-MDH at low temperature is apparent; however, the nature of this phenomenon is not clear. The substrates NADH, OAA, NAD, and malate protect against activity loss in this system, as do zinc ion and KCl.

Fast freezing generally results in greater losses of activity than does slow freezing. The data suggest that freezing does not cause extensive dissociation of MDH into subunits and that pH is not a major factor in causing loss of activity.

Increased activity losses are observed when M-MDH is fast-frozen in a mixture containing phosphate and magnesium ion. If phosphate is replaced by Tris buffer, activity losses on freezing are small. High ionic strength has little effect on the activity of M- and S-MDH when these are frozen and thawed; thus the effect of magnesium ion is not due to increased ionic strength at temperatures near freezing. The data suggest that a protective influence of phosphate against the effects of freeze-thawing exists which is relieved by magnesium ion.

In general, the results point to an extensive behavioral individuality of these enzymes.

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APPENDIX

A. Preparation of Supernatant MDH

Pig heart S-MDH was prepared using the procedure of Englard and Breiger (16). All operations except the heat fractionation were carried out in the cold room at 4°C.

Fresh pig hearts were freed from fat and connective tissue, diced and passed through a mechanical grinder. One kilogram of mince was stirred for 15 minutes in 5 liters of ice cold 0.25 M sucrose buffered with 0.01 M Tris-acetate, pH 7.6, and the suspension was passed through cheesecloth. The filtrate that was collected had a pH of 6.1.

The pH of this extract was raised to 7.2 with ammonium hydroxide, and ammonium sulfate was added to 40% saturation. Hyflo Super Cel (1 gm per 100 ml solution) was added and the preparation was allowed to filter overnight. The clear filtrate was brought to 82% saturation with ammonium sulfate and was centrifuged at 13,000 $\times$ g at 5°C. The resulting precipitate was dissolved in 0.05 M potassium phosphate buffer, pH 7.4, and was adjusted to 400 ml. This solution was brought to 35% saturation with ammonium sulfate and was centrifuged for 1 hour.

The supernatant was heated quickly to 60°C and was held at that temperature for 20 minutes. The resulting brown precipitate was removed by centrifugation and discarded. The

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supernatant was fractionated with ammonium sulfate and the precipitate between 43% and 72% saturation was removed by centrifugation and dissolved in 0.01 M potassium phosphate buffer, pH 6.9, to a total volume of 75 ml. This solution was dialyzed three times against 0.01 M potassium phosphate, pH 6.9, for a total of 24 hours and then centrifuged.

The clear dialyzed preparation was applied to a 25 × 4 cm column of DEAE-cellulose which had been equilibrated with 0.01 M potassium phosphate buffer, pH 6.9. A large dark band moved rapidly down the column on the application of 750 ml buffer, leaving behind a lighter coloured band. A gradient elution was performed, using buffer concentrations of 0.01 M to 0.05 M. The eluate was collected in 13 ml fractions at the rate of 30 to 40 ml per hour. Samples containing the majority of the activity were pooled to yield 1 liter of pale yellow solution which was then brought to 72% saturation with respect to ammonium sulfate. The precipitate was collected by centrifugation and dissolved in 20 ml potassium phosphate buffer, pH 7.4. This preparation was dialyzed against increasing concentrations of ammonium sulfate ranging from 55% to 72% saturation. Precipitates were removed at 55%, 58%, 60%, 65%, 70%, and 72% saturation and were dissolved in 5 ml deionized water. The 70% sample, which had the highest specific activity was used for all experiments. It was stored in the deep freeze at -13°C.

The total yield was 17% and the 70% sample represented a 160-fold purification. Englard and Breiger achieved a 14% yield after recrystallization and a 63-fold maximum purification (for beef heart S-MDH).

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B. Preparation of Mitochondrial MDH

Pig heart M-MDH was prepared with modifications according to the procedure of Wolfe and Neilands (8). All operations except the ethanol fractionation were carried out in the cold at 4°C.

Fresh whole pig hearts were defatted and minced in a mechanical grinder. The mince was washed five times with 25 liter portions of ice water and was strained on cheesecloth. The tissue was treated with a 90% acetone-water mixture for 30 minutes and then twice with pure acetone. It was collected on cheesecloth and was dried in a vacuum desiccator.

MDH was extracted from the acetone powder by homogenation in a Waring blendor with 0.05 M sodium phosphate buffer, pH 7.0. After removal of the insoluble debris, the milky solution was brought to 30% saturation with respect to ammonium sulfate and the precipitate removed by centrifugation and discarded. Finally, the solution was made 65% saturated with respect to ammonium sulfate, and the precipitate that formed was recovered by centrifugation and dissolved in 0.01 M maleate buffer, pH 6.2, containing 0.2 M NaCl. The preparation was dialyzed against this buffer for 15 hours, and then against 0.01 M maleate containing 1×10^{-4} M $ZnCl_2$ for 12 hours. The precipitate that formed was recovered by centrifugation and discarded.

After fractionation with ethanol in the deep freeze at -10 to -13°C, the precipitate between 50% and 70% saturation was removed by centrifugation and dissolved immediately in cold 0.05 M phosphate buffer, pH 7.0.

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This preparation was dialyzed against 0.005 M potassium phosphate, pH 6.5, containing 0.001 M EDTA for 8 hours. The solution was then applied to a 25 × 4 cm column of carboxymethyl-cellulose previously equilibrated in the same buffer. Elution with 550 ml buffer removed S-MDH contaminant. The M-MDH was eluted with a gradient of 0.001 M to 0.15 M potassium phosphate, pH 6.5, containing 0.001 M EDTA (7). Fractions (10 ml) were collected at 100 to 120 ml per hour. Those with the highest specific activity were pooled and the protein precipitated by adding ammonium sulfate to 77% saturation. The precipitate was removed by centrifugation and was dissolved in 0.05 M sodium phosphate, pH 6.5, containing 0.1 M KCl, and the resulting preparation was applied to a 23 × 2.5 cm column of Sephadex G-100 previously equilibrated in 0.05 M sodium phosphate. The protein was eluted with the same buffer and 15 ml samples were collected. The fractions with highest specific activity were pooled and the preparation was brought to 77% saturation with ammonium sulfate. The precipitate was collected by centrifugation then dissolved in a 77% saturated solution of ammonium sulfate. The suspension was stored at -10°C to -13°C in the deep freeze.

A yield of 6% and a 200-fold purification were achieved. Thorne (19) achieved a 15% yield and a 120-fold purification for the same enzyme.

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C. The Effects of Freeze-thawing on the Activity of S-and M-MDH

Legend

Each section represents a series of consecutive freeze-thaws. A "fast freeze" (f) was performed by immersion of the mixture into a dry ice - acetone bath for the time noted. A "slow freeze" (s) was carried out in a deep freeze at -10°C to -13°C for the time noted. Following either a fast or a slow freeze the mixture was allowed to thaw at room temperature (25°C) then placed on ice as soon as thawing was completed. A suitably diluted sample was assayed immediately. A control sample of each mixture was kept on ice for the duration of the experiment and was assayed periodically. The per cent activity is given by:

$$\frac{\text{activity of freeze-thaw mixture at time t}}{\text{activity of control mixture at time t}} \times 100$$

The concentrations of the components of the mixtures are as noted on page 27.

Tables 1a,b,c,d,e,f,g .... The Effects of Freeze-thawing in the Presence of Various Buffers on the Activity of M- and S-MDH With or Without Mercaptoethanol

Tables 2a,b,c .... The Effects of Freeze-thawing in the Presence of 1 M KCl on the Activity of M-and S-MDH With or Without Mercaptoethanol

Tables 3a,b,c .... The Effects of Freeze-thawing in the Presence of Magnesium Ion on the Activity of M-and S-MDH

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Table 4 .... The Effects of Freeze-thawing in the Presence of Zinc Ion on the Activity of M- and S-MDH

Table 5 .... The Effects of Freeze-thawing in the Presence of Substrates on the Activity of S-MDH in the Standard Mixture

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Table 1a

No. Times Frozen and Thawed	Time Allowed for Freezing (min)	Time for Thawing (min)	% Activity Remaining	System
i. 1f	2	20	96.2	S-MDH, Tris-acetate, serum albumin, mercaptoethanol
2f	2	20	94.2	
3f	8	21	89.6	
4f	20	25	79,6	
5s	60	20	172.9	
6s	60	20	128.4	
7s	60	21	86.7	
ii. 1f	3	15	98.3	M-MDH, Tris-acetate, serum albumin, mercaptoethanol
2f	2.5	14	89.3	
3f	2	14	115.1	
4f	5	14	100.0	
5s	60	10	109.5	

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Table 1b

No. Times Frozen and Thawed	Time Allowed for Freezing (min)	Time for Thawing (min)	% Activity Remaining	System
i. 1f	2	20	95.2	S-MDH, Tris-acetate, serum albumin
2f	2	18	97.6	
3f	2	19	92.6	
4s	168	11	104.4	

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Table 1c

No. Times Frozen and Thawed	Time Allowed for Freezing (min)	Time for Thawing (min)	% Activity Remaining	System
i. 1f	2	20	88.7	S-MDH, citric acid, serum albumin, mercaptoethanol
2f	2	18	89.7	
3f	2.5	20	81.0	
4f	2	17	74.8	
5s	60	20	76.0	
ii. 1f	2	15	96.6	M-MDH, citric acid, serum albumin, mercaptoethanol
2f	2	16	104.7	
3f	2	17	98.5	
4f	2.5	14	113.3	
5s	56	11	104.0	

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Table 1d

No. Times Frozen and Thawed	Time Allowed for Freezing (min)	Time for Thawing (min)	% Activity Remaining	System
i. 1f	2	20	8.7	S-MDH, potassium phosphate, pH 7.4, serum albumin, mercaptoethanol
2f	2	22	3.0	
3f	2	20	1.4	
4s	66	18	3.6	
ii. 1s	60	19	192.5	S-MDH, potassium phosphate, pH 7.4, serum albumin, mercaptoethanol
2s	60	18	98.1	
3s	60	14	80.6	
4s	268	18	359.3	
iii. 1f	2	14	99.1	M-MDH, potassium phosphate, pH 7.4, serum albumin, mercaptoethanol
2f	2.5	15	108.9	
3f	3	13	145.1	
4f	5	12	115.2	
5s	65	13	114.6	
6f	2	2	89.8	

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Table 1e

No. Times Frozen and Thawed	Time Allowed for Freezing (min)	Time for Thawing (min)	% Activity Remaining	System
i. 1f	2	22	9.8	S-MDH, sodium phosphate, pH 7.4, serum albumin, mercaptoethanol
2f	5	20	2.2	
3s	129	20	2.6	
ii. 1f	2.5	20	91.8	M-MDH, sodium phosphate, pH 7.4, serum albumin, mercaptoethanol
2f	2	20	107.6	
3f	2	19	107.6	
4s	219	17	113.0	

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Table 1f

No. Times Frozen and Thawed	Time Allowed for Freezing (min)	Time for Thawing (min)	% Activity Remaining	System
i. 1f	2	20	90.7	S-MDH, potassium phosphate, pH 7.4, serum albumin
2f	2	24	88.8	
3f	2.5	21	88.7	
4s	131	17	110.1	
ii. 1f	2	18	91.3	M-MDH, potassium phosphate, pH 7.4, serum albumin
2f	5	18	86.6	
3f	3	14	80.4	
4f	2	15	80.8	
5f	2	14	96.0	
6s	62	10	78.6	

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Table 1g

No. Times Frozen and Thawed	Time Allowed for Freezing (min)	Time for Thawing (min)	% Activity Remaining	System
i. 1f	3.5	21	82.1	S-MDH, sodium phosphate, pH 7.4, serum albumin
2f	2	21	78.8	
3f	2	22	76.9	
ii. 1f	2	21	100.6	M-MDH, sodium phosphate, pH 7.4, serum albumin
2f	2	22	114.0	
3f	2	22	114.6	

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Table 2a

No. Times Frozen and Thawed	Time Allowed for Freezing (min)	Time for Thawing (min)	% Activity Remaining	System
i. 1f	2	22	30.8	S-MDH, potassium phosphate, pH 7.4, serum albumin, mercaptoethanol, 1 M KCl
2f	2	22	14.8	
3f	2	17	7.7	
ii. 1f	4	16	98.8	M-MDH, potassium phosphate, pH 7.4, serum albumin, mercaptoethanol, 1 M KCl
2f	2	13	84.4	
3f	2	15	82.5	

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Table 2b

No. Times Frozen and Thawed	Time Allowed for Freezing (min)	Time for Thawing (min)	% Activity Remaining	System
i. 1f	2	17	110.8	S-MDH, Tris-acetate, serum albumin, mercaptoethanol, 1 M KCl
2f	2	17	99.0	
3f	2	17	113.7	
ii. 1f	2	19	90.2	M-MDH, Tris-acetate, serum albumin, mercaptoethanol, 1 M KCl
2f	2	14	91.7	
3f	2	16	112.9	

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Table 2c

No. Times Frozen and Thawed	Time Allowed for Freezing (min)	Time for Thawing (min)	% Activity Remaining	System
1. 1f	2	18	87.2	S-MDH, potassium phosphate, pH 7.4, serum albumin, 1 M KCl
2f	2	17	73.2	
3f	2	16	61.2	
4s	296	8	63.4	

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Table 3a

No. Times Frozen and Thawed	Time Allowed for Freezing (min)	Time for Thawing (min)	% Activity Remaining	System
i. 1f	2	18	4.3	S-MDH, potassium phosphate, pH 7.4, serum albumin, mercaptoethanol, 10 μmoles Mg^{++}
2f	2	19	1.1	
3f	62	14	1.8	
ii. 1f	2	20	88.9	M-MDH, potassium phosphate, pH 7.4, serum albumin, mercaptoethanol, 10 μmoles Mg^{++}
2f	2	19	80.3	
3f	2	20	83.8	
4s	386	20	88.9	
iii. 1f	2	20	45.6	M-MDH, potassium phosphate, pH 7.4, serum albumin, mercaptoethanol, 30 μmoles Mg^{++}
2f	2	20	17.5	
3f	4.5	18	1.6	

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Table 3b

No. Times Frozen and Thawed	Time Allowed for Freezing (min)	Time for Thawing (min)	% Activity Remaining	System
i. 1f	2	19	93.4	S-MDH, potassium phosphate, pH 7.4, serum albumin, 10 μmoles Mg^{++}
2f	2	18	98.0	
3f	2	23	91.1	
4s	159	11	89.5	

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Table 3c

No. Times Frozen and Thawed	Time Allowed for Freezing (min)	Time for Thawing (min)	% Activity Remaining	System
i. 1f	2	21	91.7	S-MDH, Tris-acetate, serum albumin, mercaptoethanol, 20 μmoles Mg^{++}
2f	2	20	102.8	
3f	2	19	68.7	
4s	246	14	57.0	
ii. 1f	2	21	95.0	M-MDH, Tris-acetate, serum albumin, mercaptoethanol, 20 μmoles Mg^{++}
2f	2	19	93.4	
3f	2	20	116.0	

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Table 4

No. Times Frozen and Thawed	Time Allowed for Freezing (min)	Time for Thawing (min)	% Activity Remaining	System
i. 1f	2	20	29.4	S-MDH, potassium phosphate, pH 7.4, serum albumin, mercaptoethanol, 2 μmoles Zn^{++}
2f	6	16	15.0	
3f	2	16	10.7	
4s	120	14	11.9	
ii. 1f	2	18	101.9	M-MDH, potassium phosphate, pH 7.4, serum albumin, mercaptoethanol, 2 μmoles Zn^{++}
2f	2	18	97.7	
3f	2	18	93.2	
4s	43	10	98.2	

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Table 5

No. Times Frozen and Thawed	Time Allowed for Freezing (min)	Time for Thawing (min)	% Activity Remaining	System
i. 1f	3	16	76.1	S-MDH, potassium phosphate, pH 7.4, serum albumin, mercaptoethanol, NADH
2f	2	17	76.5	
3f	2	17	68.9	
4f	2	16	59.1	
5s	121	11	67.6	
ii. 1f	2	17	47.5	S-MDH, potassium phosphate, pH 7.4, serum albumin, mercaptoethanol, NAD
2f	2	19	30.6	
3f	2	17	26.4	
4s	120	21	11.7	
iii. 1f	2	18	63.3	S-MDH, potassium phosphate, pH 7.4, serum albumin, mercaptoethanol, OAA
2f	2	18	61.0	
3f	2	17	38.4	
4f	2	16	38.9	
5s	62	12	39.3	
iv. 1f	2	15	42.4	S-MDH, potassium phosphate, pH 7.4, serum albumin, mercaptoethanol malic acid
2f	2	19	29.6	
3s	62	14	29.4	

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