A CHARACTERIZATION OF CHICKEN HEART MITOCHONDRIAL MALATE DEHYDROGENASE

by

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

BDC-OH	4,4'-Bisdimethylaminodiphenylcarbinol
BME	2-Mercaptoethanol
CM	Carboxymethyl
DEAE	Diethylaminoethyl
DTNB	5,5'-Dithiobis-(2-nitrobenzoic acid)
EDTA	Ethylenediaminetetraacetic acid
gm/mol	Grams per mole
G3P DH	Glyceraldehyde-3-phosphate Dehydrogenase
Glut DH	Glutamate Dehydrogenase
IAA	Iodoacetic acid
IAM	Iodoacetamide
K_m	Michaelis constant
LDH	Lactate Dehydrogenase
MDH, m-MDH	Mitochondrial Malate Dehydrogenase(E.C.1.1.1.37)
c-MDH	Cytoplasmic Malate Dehydrogenase(E.C.1.1.1.37)
NAD^+, NADH	Oxidized and Reduced Nicotinamide Adenine Dinucleotide
NBT	Nitro Blue Tetrazolium
NEM	N-Ethyl Maleimide
nmol	Nanomoles
OAA	Oxalacetic acid
PLP	Pyridoxyl 5'-phosphate
PMS	Phenazinemethosulfate
SDS	Sodium lauryl(dodecyl) sulfate
-SH	Thiol, sulfhydryl

TCA	Tricarboxylic acid
Tris	Tris(hydroxymethyl)-aminomethane
U	Units(of activity)

LITERATURE REVIEW

Introduction

Malate dehydrogenase can be found in nearly all eukaryotic cells. The primary reaction catalyzed by this enzyme is reversible reduction of oxalacetate to L-malate in the presence of of NAD(H), with the apparent equilibrium at pH 7.0 lying in the direction of L-malate production(1).

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

Malate dehydrogenase also catalyzes several reactions of minor importance, including dehydrogenation of aspartate(2), along with oxidation of D-tartrate, tartronate, oxaloglycollate, and α-hydroxyglutarate(3). The class of enzymes described above are called simple malate dehydrogenases, in order to differentiate them from the decarboxylating malate dehydrogenases, or malic enzymes, which catalyze the conversion of L-malate to pyruvate and carbon dioxide(1).

$$\text{Pyruvate} + \text{CO}_2 + \text{NADPH} + \text{H}^+ \rightleftharpoons \text{L-Malate} + \text{NADP}^+$$

No further references will be made with regard to the decarboxylating malate dehydrogenases. All discussions will concern only the simple malate dehydrogenases.

Malate dehydrogenase(MDH) appears to be associated with both the inner membrane and the matrix of the eukaryotic mitochondria. There is evidence that the mitochondrial MDH possesses certain structural properties that are also found in other mitochondrial enzymes, such as aspartate aminotransferase, and that these properties can result in the association of the enzyme with the inner membrane itself. Very dilute

solutions of the types of lipids which comprise about 20% of the inner membrane affect the properties of mitochondrial MDH(4). Lysolecithin has been shown to reduce the rapid rate of thermal inactivation of the mitochondrial MDH upon binding to the enzyme(5). However, lysolecithin is only a minor component of mitochondrial lipids. On the other hand, lecithin, a major lipid component, produced no measurable stabilization upon binding(5). Palmitoyl CoA(6) and lipoic acid(7) are known inhibitors of malate dehydrogenase activity. There is evidence, therefore, that the enzyme can bind to lipids and become associated with the inner membrane.

The fact that mitochondrial MDH seems to be located in both the inner membrane fraction and the inner membrane matrix could be due to ionic effects. Increasing levels of succinate elicits the release of both mitochondrial malate dehydrogenase and mitochondrial aspartate aminotransferase from the inner membrane fraction of mitochondria(8). Increasing levels of either sucrose or certain monovalent and divalent cations produce the same effect as succinate(9). The molecular bases of these ionic- and lipid-related properties are unknown at the present.

Mitochondrial malate dehydrogenase catalyzes an important link in the tricarboxylic acid(TCA) cycle, which is found in nearly all aerobic organisms. The cycle is the common central pathway for the degradation of the two-carbon acetyl residues derived not only from carbohydrates but also from fatty acids and amino acids. The MDH reaction is one of several in the tricarboxylic acid cycle which generate hydride ions, and these, in turn, are transferred via the electron transport system for the reduction of molecular oxygen to water(10).

Most eukaryotic cells are known to possess two forms of malate dehydrogenase, the mitochondrial form(m-MDH) and a second form located in the extramitochondrial cytoplasm(c-MDH). A third type of MDH has been found in various plant tissues, primarily in the microbody or glyoxysome fraction, and is thought to function in the glyoxylate cycle or photorespiration(10).

Both the mitochondrial and cytoplasmic forms of MDH participate in the malate shuttle which transports hydride ions across the mitochondrial membrane. L-Malate in the presence of phosphate is permeable and thereby transfers a hydride ion when it is oxidized to oxalacetate by the mitochondrial MDH. Mitochondrial and cytoplasmic forms of aspartate aminotransferase act in a similar manner to transport hydride ions into the mitochondria via permeable glutamate and aspartate, which are co-transported across the membrane by a specific carrier protein. L-Malate is also co-transported with α-ketoglutarate by a specific transport system(10).

Mitochondrial and cytoplasmic forms of the same enzyme are examples of isozymes, whose differentiation is based not only on cellular or organelle origin, but also on distinguishing kinetic and mechanistic characteristics. When compared with the cytoplasmic isozyme, the mitochondrial MDH from chicken heart possessed higher specific activity, a lack of tryptophan, a more cationic charge at pH 7.0, and was less thermally stable(11). In addition, various studies indicate that each MDH isozyme can exist in multiple subforms with different isoelectric points. Although these subforms appear to be the result of genetic differences or structural alterations arising either intracellularly or

during preparative manipulations(e.g., deamination of asparaginyl and glutaminyl residues) the subforms, unlike the isozymes, do not normally show different kinetic or mechanistic characteristics(11). One study did indicate, however, that subforms which had undergone reversible denaturation possessed different thermal stabilities(12). Kitto and Kaplan(11) reported five subforms of the chicken mitochondrial MDH, while the cytoplasmic isozyme had three subforms. The physiological importance of these subforms is unknown, and may simply be artifacts resulting from purification procedures.

Purification

Malate dehydrogenase has been purified to homogeneity from a wide variety of sources. Porcine and bovine heart have been the sources of most studies concerning the enzyme, with Wolfe and Neilands(13) producing the first seemingly pure preparation of the porcine mitochondrial enzyme. Most methods of isolation involved combinations of some of the following preparative or purification procedures: acetone powders, ammonium sulfate or ethanol fractionations, anion and cation exchange columns, Sephadex columns, and crystallization(1). More recent advances involve affinity chromatography using Blue Dextran Sepharose(14-17) or Adenosine 5'-monophosphate-Sepharose(18), and hydrophobic interaction chromatography(19).

Simultaneous isolation of both isozymes from the same tissue can be achieved either by careful removal of the intact mitochondria in the crude extraction step or by anion and cation exchange chromatography of the initial homogenate. The latter method has been used to advantage(1). Kitto and Kaplan(11) devised a method for separation of the chicken heart

mitochondrial and cytoplasmic forms of the enzyme from the same homogenate, which netted final yields of 15% and 11% for the cytoplasmic and mitochondrial isozymes, respectively.

Physical Characteristics

The molecular weights of MDH from various species has been reported to be from 15,000-70,000 grams/mole(1). The values reported for the chicken heart enzyme alone have varied from 20,000 to 70,000 gm/mol(11). More recently, the molecular weights for both isozymes have been consistently reported to be in the 60,000-70,000 gm/mol range. Murphey(20) measured the molecular weights of eight different MDH species, including both chicken isozymes, and found them all to possess weights of 67,000 gm/mol.

All the experimental evidence indicated that animal malate dehydrogenase was composed of two identical or very similar polypeptide chains of molecular weight 33,000-35,000 gm/mol. The subunits of the dimer must be held together by noncovalent bonds, since an absence of disulfide bonds between the subunits has been reported(1).

There is a very distinct similarity in the amino acid composition of the mitochondrial isozyme among different species, while a corresponding similarity exists between the cytoplasmic isozyme from different sources. In addition, a noticeable similarity exists between the mitochondrial and cytoplasmic forms within the same species(1). Schellenberg(21-24) has claimed that tryptophan is present in both isozymes of MDH. However, Kitto and Kaplan(11), as well as Eberhart and Wolfe(25), have shown that tryptophan is present in the cytoplasmic form, but is totally lacking in

the mitochondrial isozyme.

Kitto and Kaplan(11) performed immunological tests to further study the similarities and differences between the chicken isozymes. Antibodies to mitochondrial malate dehydrogenase reacted strongly with the mitochondrial isozyme, but produced no detectable cross-reaction with the cytoplasmic form. Similarly, antibodies to cytoplasmic MDH reacted with the cytoplasmic isozyme, but failed to combine with the mitochondrial form. Antibodies to the chicken cytoplasmic and mitochondrial MDH do show cross-reactions with their respective isozymes from species other than chicken, and the amount of cross-reaction appears to be inversely related to the taxonomic distance between the species involved.

The mitochondrial isozyme in chicken has a slight cationic or neutral charge at pH 7.0, while the cytoplasmic subforms all have anionic charges at pH 7.0. Kitto and Kaplan(11) reported five subforms of the mitochondrial isozyme in chicken, while the cytoplasmic isozyme had three subforms. Porcine mitochondrial enzyme was reported to have six subforms by Thorne et al.(26), as determined by starch-gel electrophoresis. The distribution pattern of these porcine subforms was not affected by the age of the tissue of origin, the purification procedures used or by a number of degradative treatments, although methyl iodide and urea treatment had some effect on the preparations. After elution from the gel, the subforms of the porcine mitochondrial enzyme were shown to be relatively similar by some catalytic criteria, but to differ collectively from the porcine cytoplasmic isozyme.

Chilson et al.(27) studied the reversible dissociation of chicken mitochondrial MDH through the use of guanidine HCl, pH extremes, 8 M urea,

and 6 M lithium chloride. Buffers containing phosphate, citrate, or malate, salts, neutral pH, NADH, and the reducing agent β-mercaptoethanol were all found to promote the rate of reactivation and prevent heat denaturation of the reassociated enzyme, while the presence of halide ions had a deliterious effect on reassociation of the denatured MDH. Immunological studies and molecular weight determinations indicated no difference between the native and renatured enzyme. Chilson et al. (27) also used these reversible denaturation studies to produce interspecies hybrid forms of malate dehydrogenase(each subunit from a different species), including a hybrid of chicken mitochondrial and tuna mitochondrial isozymes, which possessed an electrophoretic mobility intermediate between the two parent isozymes.

Detailed molecular structural data has been reported for the cytoplasmic chicken heart isozyme only, and it has been found to possess a close structural homology to dogfish lactate dehydrogenase(28). Whether this homology and structural data can be extrapolated to the mitochondrial form of the enzyme is only speculative at this time. However, for the purposes of discussion of certain topics, the molecular model of cytoplasmic MDH shall be referenced occasionally.

The possibility of a stable and active monomer of the enzyme has generated much discussion but little data in recent years. The question of whether or not there is a structural basis for a stable and active monomer cannot be answered with the molecular model of cytoplasmic MDH. This model indicates that there are no known residues of one subunit that contribute to the active site on the opposite subunit. In addition, most of the chain segments that comprise the hydrophobic subunit interface

have many more nonbonding interactions with atoms in their own polypeptide chain than with the paired subunit. Therefore, upon dissociation, one might assume that it might take up an altered orientation with respect to the remainder of its polypeptide chain. Should this occur, a conformational difference between a polypeptide chain in the dimer form and one in the monomeric form would probably exist(1). Whether or not this change could allow or prevent the occurrence of an active monomer is unknown.

Since the dimer of MDH has been shown to be comprised of two identical, or close to identical, subunits, each dimer should possess two equivalent binding sites in both the cytoplasmic and mitochondrial isozymes. These two binding sites are thought to exist adjacent to the subunit interface, but on opposite sides of the dimer, with the monomers in a head-to-tail configuration(1). Holbrook and Wolfe(29) have shown the existence of two binding sites for the porcine isozymes, while Kitto and Kaplan(11) have reported two binding sites for the chicken isozymes. The only cases of a single binding site in the MDH dimer have been reported for the bovine isozymes. Gregory(30) and Siegel and Ellison(31) report one binding site for the bovine mitochondrial dimer, while Cassman and Englard(32) have found a single binding site in the bovine cytoplasmic isozyme.

There seems to be three basic models of enzyme types. Those enzymes which possess a crevice for an active site, among them MDH, act upon small molecules and chains in which the bond to be severed is relatively exposed. Examples of the other two enzyme models are chymotrypsin and carboxypeptidase. Chymotrypsin, thought to have a shallow depression

for an active site, appears to be designed to fit against a larger structure and to act on peptide bonds rather than to enfold a single strand. Carboxypeptidase possesses a pit, which seems logical since it acts to remove the end of a chain, which can easily fit into the pit-shaped active site(33).

Malate dehydrogenase is thought to possess a Y-shaped crevice for an active site, which will be considered equivalent to the substrate and coenzyme binding sites throughout this thesis, into which the pyridine nucleotide binds in an open or extended conformation. This open conformation of NAD^+ has been shown to be considerably different from the nucleotide's configuration in solution, where it takes on a folded or stacked conformation(34). The adenine end of the bound coenzyme is close to the solvent but still is located within a groove in the surface, while the pyridine ring is completely buried in the coenzyme binding cavity. The binding site for the substrate moiety has been proposed to be directly adjacent to the coenzyme binding site.

Kinetic Analysis

The mammalian malate dehydrogenase requires, as substrates, the dicarboxylic acid, the dinucleotide coenzyme, and a proton from the solvent. Most dehydrogenases are believed to operate via a compulsory ordered addition of substrates, as shown in Figure 1. An abortive ternary complex, MDH-NADH-L-malate, is shown in the scheme, since these are always possible whenever multiple substrates are involved. Although this abortive ternary complex, MDH-NAD^+-OAA, has yet to be reported(35). The presence of an anion in the substrate binding site, which has been suggested by crystallographic studies on cytoplasmic MDH, has not been

Figure 1: The Mechanism for the Malate Dehydrogenase Reaction

The catalytic pathway is an ordered reaction mechanism for malate dehydrogenase. The steps in the catalyzed oxidation for L-malate or reduction of oxalacetate are represented by a schematic set of ordered binding reactions. The substrates L-malate and oxalacetate are abbreviated MAL and OAA, respectively. The addition of a proton, a necessary step in the catalytic reaction, is indicated in dotted lines at three different positions in the pathway. This is to show that there are no data on where it participates in an ordered scheme. An example of an abortive ternary complex is also shown(1).

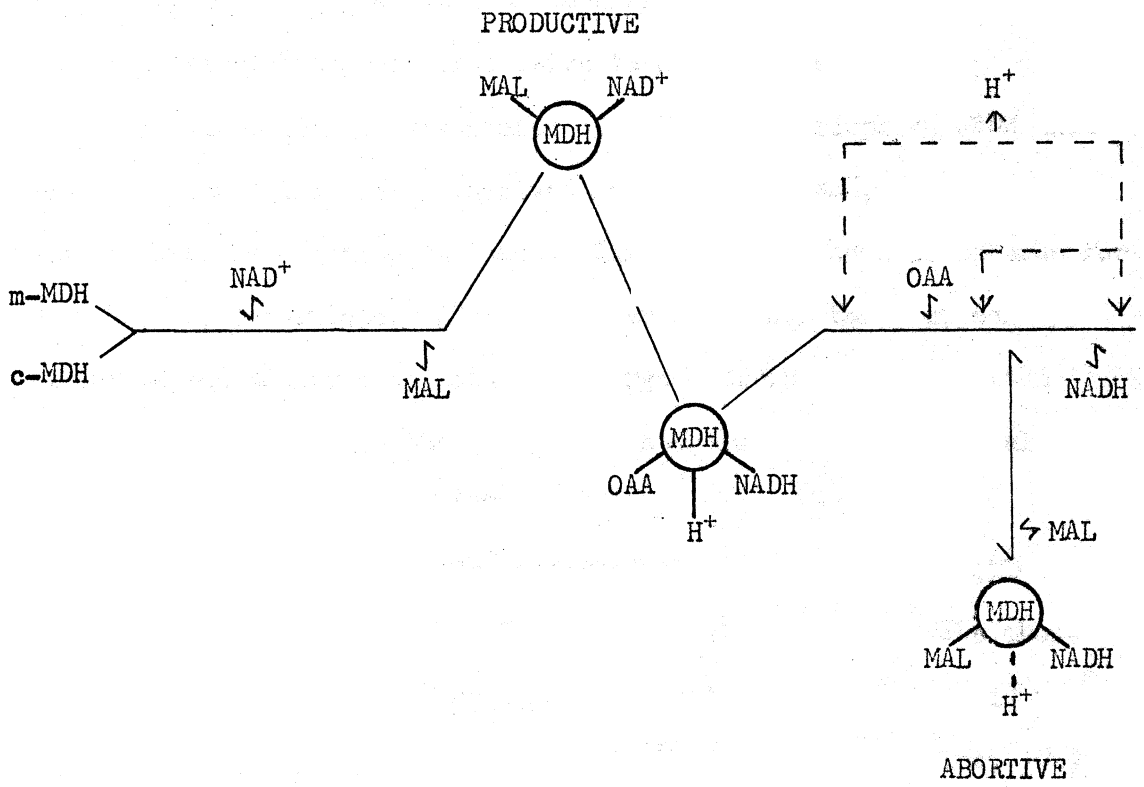

Figure 1

The Mechanism for the Malate Dehydrogenase Reaction

included in the reaction pathway(36). The pathway for the proton, which is necessary for the formation of the productive ternary complex, has also not been included. The rate-limiting step for both directions of the catalytic reaction appears to be dissociation of the coenzyme and not the interconversion of productive ternary complexes(1).

Since there are a large number of functional groups on NADH that have the potential of being involved in binding to MDH, fragments of this coenzyme should also be bound. This has been shown to be true for ADP, AMP, and ADP-ribose, with the latter substance being especially similar to the coenzyme in both the charge distribution around the pyrophosphate bridge and the number of potential groups that could be involved in hydrogen binding(37).

Cassman and Vetterlein(38) reported the bovine heart cytoplasmic MDH existed in phosphorylated and dephosphorylated forms. The phosphorylated species(1 mol of phosphate per mol of enzyme) is said to have a specific activity twice that of the dephosphorylated species. Differences in the behavior of the two forms with respect to the coenzyme and fructose-1,6-biphosphate was also reported. A significant increase in specific activity has been observed by Gregory for the bovine mitochondrial MDH upon addition of phosphate to the assay mixture.[1] The presence of these two forms may represent some type of metabolic control mechanism that is similar to that known for several regulatory enzymes(38).

Toxicology involves the study of how the body handles foreign compounds that have been introduced into the human organism, and the effect that these substances have on various biochemical reactions. A large

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[1]E. M. Gregory, unpublished data.

number of nonphysiological compounds which affect the activity of malate dehydrogenases have been demonstrated. Metal compounds such as platinum(II), which have been used in cancer chemotherapy, complex irreversibly with the enzyme(39). Phenols and substituted phenols(40) along with the insecticide Kepone(41) are inhibitors of MDH.

Chemical Modification

Specific chemical modification has been used in an attempt to identify the essential residues involved in the mechanism of the enzyme. A variety of amino acids have been investigated for their role as potential active site residues by using various modification reagents. Only histidine has been clearly implicated as a catalytic active residue, although modification of other amino acid residues can cause inactivation(1). Modification of sulfhydryl groups(30, 42-44), arginine(45), lysine(46, 47), tyrosine(31), and methionine(48) have all been reported to cause inactivation of the enzyme, and have been suggested to occupy a position in or near the active site. Since there is very little published data on the specific chemical modification of residues in the chicken heart enzyme, most of the references here involve studies done with other forms of MDH.

The reagent 5,5'-dithiobis-(2-nitrobenzoic acid)(DTNB) was found to react with three to four out of 10 to 11 thiol groups in the porcine mitochondrial isozyme(49). Similar studies with the porcine cytoplasmic (50) and ox kidney(49) enzymes demonstrated a reaction of two -SH residues with 5,5'-dithiobis-(2-nitrobenzoic acid). Three of six reactive cysteine groups were reported to combine with p-mercuribenzoate

when the bovine cytoplasmic form was used(51). None of these studies reported a loss of enzymatic activity upon modification.

Porcine mitochondrial MDH was activated when combined with mercurials in low concentrations but at higher concentrations of mercurials was inactivated(52). This phenomenon was examined with p-mercuribenzoate and it was found that maximum activation occurred after modification of three to four cysteine residues. Reaction of seven to eight residues produced greater but transient activation followed by inactivation(53). Stimulation by mercury(II) ions was reported by Kuramitsu for porcine mitochondrial MDH, but the activation was dependent on the presence of L-malate(52).

N-Ethyl maleimide(NEM) has been shown by Gregory to modify two essential thiol groups per molecule of enzyme for the porcine mitochondrial isozyme(42), and one essential thiol group per bovine mitochondrial dimer(30). Both reactions were inhibited by the presence of coenzyme. Similar results were obtained with the porcine enzyme by utilizing 4,4'-bisdimethylaminodiphenylcarbinol(BDC-OH), as reported by Humphries(54). It appears that the cytoplasmic form is not reactive with modifiers of -SH groups(1), whereas the mitochondrial isozyme sulfhydryl group is apparently necessary either for MDH activity or for a stable conformation of the active enzyme.

The modification of a histidine residue at each active site by iodoacetamide has been reported for both the porcine and bovine mitochondrial isozymes by Gregory(30, 55). The product of the inactivation reaction with the porcine heart enzyme was demonstrated to be a 3-carboxymethylhistidine. These modification reactions could be prevented by the

presence of coenzyme or substrate. Gregory(56) has isolated a labeled tripeptide from the porcine mitochondrial enzyme by the use of labeled iodoacetamide and a protease digest, and this tripeptide, 3-carboxymethylhistidine-glycine-glycine, is thought to be present in the active site.

Modification of the active center histidine group has also been reported involving the use of diethylpyrocarbonate(57), which acted upon the porcine cytoplasmic isozyme, and elicited inactivation even when the coenzyme was present. Reports of a histidyl modification by iodoacetic acid(58) in mitochondrial MDH probably involves a histidine residue that is different from the one mentioned above. No reaction with iodoacetate is reported by Gregory for either the porcine or bovine mitochondrial isozymes[1](56).

Yost and Harrison(59) have reported that porcine mitochondrial MDH is irreversibly inactivated by pyridoxal 5'-phosphate(PLP). In view of the usual reversibility of this reagent when reacted with the ϵ-amino group of lysine, and the unusual spectral properties of the derivative formed, it was concluded that the reaction with PLP proceeded by way of modification of lysine followed by the formation of a secondary stable complex. Such a complex might be a thiazolidine-like compound formed by the reaction of the Schiff base with a neighboring cysteinyl residue.

Foster and Harrison(60) demonstrated that 2.4 arginyl residues per dimer are modified by the reagent butanedione in the porcine mitochondrial enzyme. This inactivation can be prevented by the presence of coenzyme, suggesting that the modified residues are located near the coenzyme binding site. Bleile et al.(45) have reported a similar

modification by butanedione in the porcine cytoplasmic isozyme. They measured a total of four arginine residues modified, only two of which could be protected by the presence of coenzyme, and these two appeared to be essential.

Siegel and Ellison(31) have reported modification of a single tyrosine residue with acetylimidazole in the bovine heart mitochondrial isozyme with resultant loss in catalytic activity. A similar modification of tyrosine resulted from the presence of tetranitromethane. Both reactions could be prevented by the addition of coenzyme or substrate. The presence of a single essential tyrosine residue is in agreement with the single active site reported by Gregory(30) for the bovine enzyme.

Leskovac and Pfleiderer(48) claim to achieve modification of an essential methionine residue in the active site of porcine cytoplasmic MDH. The reagent used was iodoacetate, which carboxymethylated the residue to give a carboxymethylmethionine sulfonium salt. Concentrations of iodoacetate that were greater than 1000 molar excess over the enzyme concentration were required to elicit a reaction, and the presence of coenzyme offered only limited protection from inactivation.

EXPERIMENTAL PROCEDURE

Materials — L-Malic acid, oxalacetate(OAA), β-nicotinamide adenine dinucleotide(oxidized)(NAD^+), β-nicotinamide adenine dinucleotide (reduced)(NADH), iodoacetic acid(IAA), iodoacetamide(IAM), and 5,5'-dithiobis-(2-nitrobenzoic acid)(DTNB) were supplied by Sigma Co. Ponceau S, nitro blue tetrazolium(NBT), phenazinemethosulfate(PMS), L-cysteine, β-mercaptoethanol(BME), and ethylenediaminetetraacetic acid, disodium salt(EDTA) were also obtained from Sigma Chemical Co. N-Ethyl maleimide was a product of Aldrich Chemicals. Iodo-[C^{14}-1]acetamide was from New England Nuclear. Sephadex G-150, Blue Dextran Sepharose, and 10-Carboxydecyl Sepharose were purchased from Pharmacia Fine Chemicals. Bio-Rex 70 and Carboxymethyl cellulose(CM 32) were obtained from Bio-Rad Laboratories. DEAE-Cellulose was supplied by Whatman. All other chemicals were of reagent grade.

Protein Concentration Determinations — In the purification of the enzyme, protein was determined by the method of Lowry(61). Bovine serum albumin was used for calculation of a standard curve.

All other protein concentrations were determined spectrophotometrically by measuring the absorption of light at wavelengths of 280 and 260 nm, as described by Warburg and Christian(62). This method involved the use of a formula to correct for absorbance by non-protein substances.

Unless otherwise noted, the extinction coefficient reported by Kitto and Kaplan(11) for the homogeneous enzyme was used, which was a value of 3.0 ml mg^{-1} cm^{-1}. This extinction coefficient was verified for the homogeneous chicken heart MDH. The molar quantity of enzyme, determined from the extinction coefficient and molecular weight, acid hydrolyzed and

analyzed with the amino acid analyzer was identical to the molar quantity of amino acid residues recovered. Since the amount of amino acid residues is determined by a separate standard, these results verified that the extinction coefficient was correct.

All protein was dialyzed at 4°, and the buffers contained 1 mM EDTA.

Catalytic Properties Determinations — Enzyme assays were performed on a Unicam SP 800 at 25°. Except where noted, all assays were performed in 3 ml of 100 mM sodium pyrophosphate, pH 10.6, 4 mM NAD^+, and 33 mM sodium L-malate. The reaction was initiated by enzyme addition and the rate of absorbance increase at 340 nm was recorded.

The reduction of oxalacetate to L-malate was performed in 3 ml of 100 mM sodium phosphate, pH 7.4, 0.15 mM NADH, and 1.0 mM oxalacetate. The reaction was initiated by the addition of enzyme and the rate of absorbance decrease at 340 nm was recorded.

A millimolar extinction of 6.2 at 340 nm was assumed for NADH. One unit of MDH was defined as the amount of enzyme catalyzing the conversion of 1 micromole of substrate to product per minute under these conditions.

Enzyme Purification — The procedure for purification of the chicken heart mitochondrial MDH, as presented here, is summarized in Table I. The entire procedure was done at 4°.

Domestic chicken hearts, fresh or frozen ones that had been thawed, were used as the source of the enzyme. Into a chilled Waring blendor were placed 125 grams of chicken hearts. These hearts were then subjected to low speed blending until they were well minced. Five hundred milliliters of 25 mM sodium phosphate, 1 mM EDTA, 1 mM BME, pH 7.0, was added to the heart mince. The mixture was blended at high speed for one minute,

Table I: Purification of Chicken Heart Mitochondrial MDH

The purification scheme shown was used in isolating homogeneous enzyme for the experimentation performed for this thesis. The "Experimental Procedure" section contains a description of the buffers used in the purification of the enzyme, and also the storage conditions for the enzyme. Protein concentrations and catalytic activities were obtained according to the methods described in the experimental procedure section. The amount of recovery shown for the mitochondrial isozyme in "Total Percent Recovery" is low, since it is based on the activity of the crude extract, which contained both the mitochondrial and cytoplasmic isozymes. The "Corrected Percent Recovery" is the theoretical yield of only the mitochondrial isozyme, which assumes that the activity that did adhere to the DEAE-cellulose and did not bind to the Bio-Rex 70 column(26% of the initial activity) was exclusively all of the cytoplasmic isozyme. The figures shown are adjusted to represent the yield from 250 grams of tissue.

Table I: Purification of Chicken Heart Mitochondrial Malate Dehydrogenase

Step	Volume (ml)	Units/ml	Total Units	Total % Recovery	Corrected % Recovery[b]	Protein (mg/ml)	Total mg	Specific Activity (U/mg)	Purif. Factor
Crude Extract[a]	910	63	57,250	100	100	11.4	10,400	5.5	1
DEAE-Cellulose Effluent[a]	1025	53	53,800	94	100	3.1	3200	17	3
Bio-Rex 70 Effluent	1755	6.5	11,300	20	0	0.7	1250	9	-
Bio-Rex 70 Pool	138	220	30,600	54	72	1.85	255	120	22
Amicon Filtrate(1st)	154	0.5	100	-	-	-	-	-	-
CM 32 Pool(1st)	164	166	27,200	47.5	63	0.60	55	295	54
Amicon Filtrate(2nd)	147	0.5	100	-	-	-	-	-	-
CM 32 Pool(2nd)	127	187	23,700	42	56	0.35	45	340	62

[a] 10.0 ml mg^{-1} cm^{-1}; all others are 3.0 ml mg^{-1} cm^{-1}

[b] Theoretical recovery of mitochondrial isozyme. See text for explanation.

then cooled in ice for one minute, again blended at high speed for one minute, and allowed to cool in ice for five minutes. This procedure was repeated three times, for a total of eight one-minute treatments. The tissue slurry(250 grams of heart, 1000 milliliters of buffer) was centrifuged at 12,000 rpm(21,000 x g) for 20 minutes in the GSA rotor, and the pellets discarded. The supernatant fluid(Crude Extract) was retained and assayed for MDH activity.

DEAE-Cellulose was packed in a 5 x 55 cm column until a final bed volume of 950 ml had been achieved, and the resin was equilibrated in 25 mM sodium phosphate, 1 mM EDTA, 1 mM BME, pH 7.0. The crude extract was passed through this column, with 94% of the MDH activity passing unretarded through the DEAE-cellulose. The effluent changed from a reddish, opaque mixture to a clear red upon passage through the column. This step probably eliminated most of the lipids from the mixture, as evident from the increased clairity of the mixture. Even though mitochondrial MDH has a slight anionic charge at pH 7.0, it does not appreciably adhere to this anion-exchange column under these conditions. This is probably due to strong competition with the other substances in the crude extract(e.g., lipids) that apparently have a stronger affinity for the DEAE-cellulose. The column effluent was assayed for MDH activity.

Bio-Rex 70(200-400 mesh) was packed in a 3 x 60 cm column until a final bed volume of 230 ml had been attained, and the resin was equilibrated in 25 mM sodium phosphate, 1 mM EDTA, 1 mM BME, pH 7.0. The DEAE-cellulose effluent was pumped onto the column at a rate of 75 ml/hour, followed by the phosphate buffer until the absorbance at 280 nm of the column effluent was less than 0.1 absorbance units. Up to this point,

an extinction coefficient of 10.0 ml mg^{-1} cm^{-1} was used, while for the remainder of the purification scheme, an extinction coefficient of 3.0 ml mg^{-1} cm^{-1} was used. Following the Bio-Rex 70 chromatography, the enzyme solution became sufficiently pure to justify using the extinction coefficient reported by Kitto and Kaplan(11) for the homogeneous enzyme. Prior to the Bio-Rex 70 step, the enzyme mixture was too impure to use the smaller extinction coefficient. The MDH activity that does not adhere to the Bio-Rex 70(Bio-Rex 70 Effluent) was presumably the remainder of the cytoplasmic form that did not bind to the DEAE-cellulose column, since the cytoplasmic form is more anionic at pH 7.0 than the mitochondrial isozyme. Even though the Bio-Rex 70 is a cation exchanger, the mitochondrial isozyme remained bound to the column, possibly due to hydrophobic interactions with the acrylic polymer lattice. The enzyme was eluted from the column by an ionic strength gradient. The gradient consisted of 500 ml of 25 mM sodium phosphate, 1 mM EDTA, 1 mM BME, pH 7.0, and 500 ml of 25 mM sodium phosphate, 250 mM NaCl, 1 mM EDTA, 1 mM BME, pH 7.0. The effluent was collected in 8 ml fractions, and was assayed for MDH activity(Figure 2). Fractions with specific activities greater than 15 U/mg were combined(Bio-Rex 70 Pool).

The pooled fractions were concentrated to about 30 ml using an Amicon UM-10 ultrafilitration membrane with 70 psi of nitrogen pressure, and the Amicon Filtrate(1st) and retentate were assayed for activity. Carboxymethyl cellulose(CM 32) was packed into a 3 x 60 cm column until a final bed volume of 250 ml was attained, and the column was equilibrated in 5 mM sodium phosphate, 1 mM EDTA, 1 mM BME, pH 6.6. The concentrates from two separate Bio-Rex 70 Pools were combined and dialyzed against

Figure 2: Elution Profile from Bio-Rex 70 Chromatography

A column of 3 x 60 cm was packed with Bio-Rex 70(200-400 mesh) cation exchange resin and the enzyme sample, consisting of approximately 54,000 enzyme units with a specific activity of 17 units per milligram of protein, was pumped onto the column. Both the ion exchange column and the protein sample had been equilibrated with 25 mM sodium phosphate, 1 mM EDTA, 1 mM BME, pH 7.0. After adding the protein, the column was washed with the phosphate buffer for 12 hours. At this time, a linear ionic strength gradient as detailed in "Experimental Procedure" was applied for protein elution at a flow rate of 75 ml/hour. Protein was estimated in the collected fractions by absorbance at 280 nm, and enzyme activity was measured by the standard assay system. The conductivity of each tenth fraction was measured at 4° with a conductivity meter. The chromatography and all manipulations were performed at 4°.

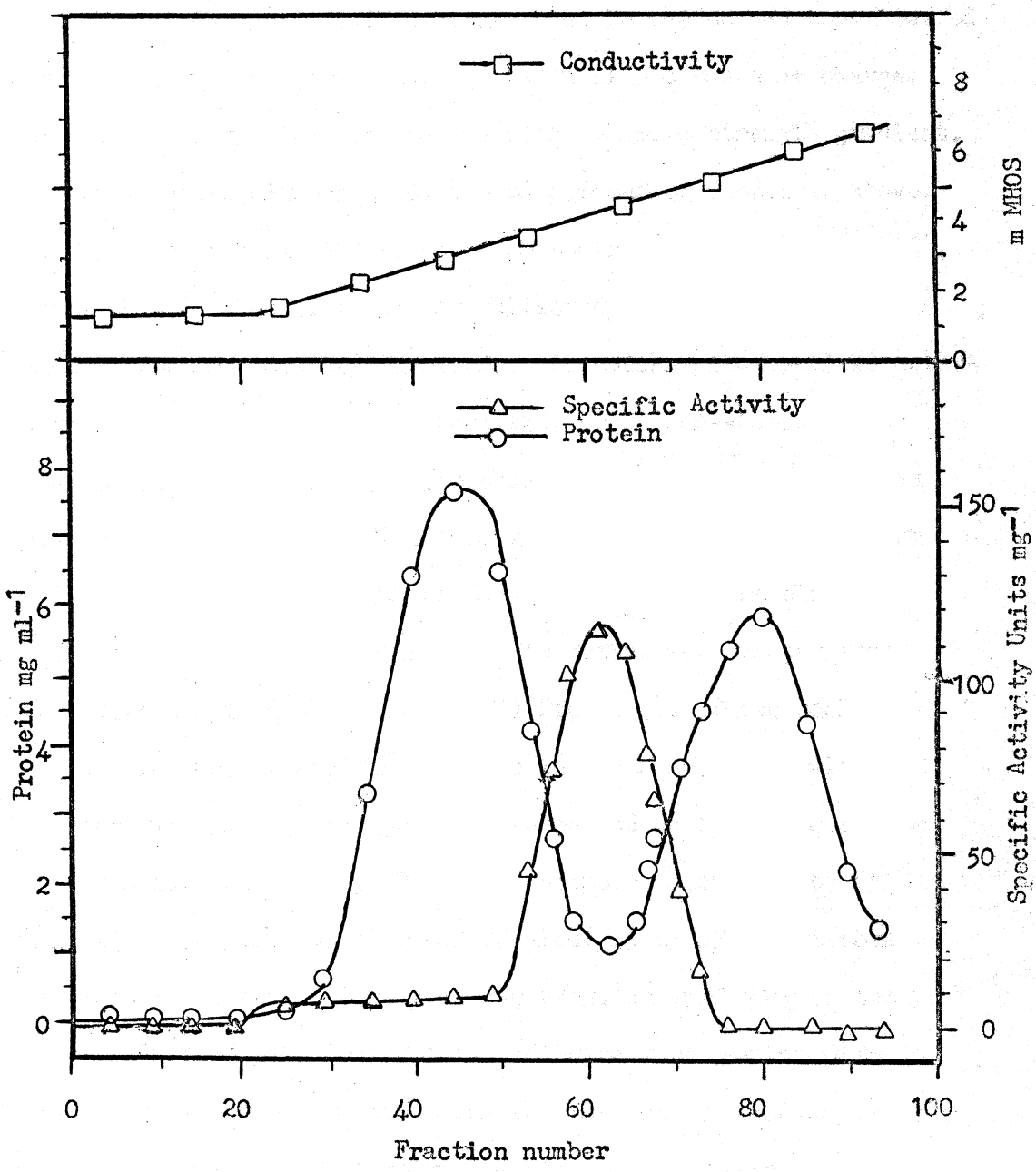


Figure 2

Elution Profile from Bio-Rex 70 Chromatography

three 4-liter volumes of the pH 6.6 buffer, and then pumped onto the CM 32 column. This was followed with phosphate buffer until the absorbance at 280 nm of the column effluent was less than 0.1 absorbance units. The enzyme bound to this column because the pH had been lowered to 6.6, and the mitochondrial MDH now had a slight positive charge. The enzyme was eluted from the column with an ionic strength gradient. The starting buffer was 500 ml of the pH 6.6 buffer described above, and the limit buffer was 500 ml of 60 mM sodium phosphate, 1 mM EDTA, 1 mM BME, pH 6.6. The effluent was collected in 8 ml fractions, and was assayed for MDH activity(Figure 3). Fractions with specific activities greater than 100 U/mg were combined(CM 32 Pool--1st).

The pooled fractions were concentrated by Amicon filtration as above, and pumped onto a CM 32 column equilibrated with 5 mM sodium phosphate, 1 mM EDTA, 1 mM BME, pH 6.6. The protein was eluted as described above. Fractions with specific activities greater than 330 U/mg were combined(CM 32 Pool--2nd)(Figure 4). These pooled fractions were concentrated by dialysis in 2.9 M ammonium sulfate, pH 7.0, centrifuged, and the pellet resuspended in a minimum volume of 2.9 M ammonium sulfate, pH 7.0, to a protein concentration of approximately 5 mg/ml. Final yield is about 45 mg of homogeneous enzyme, with a specific activity of 340 U/mg, or 1920 U/mg in the direction of oxalacetate to L-malate. Studies were performed to determine the best storage conditions for the homogeneous enzyme. Various concentrations of ammonium sulfate at pH 7.0 were prepared, and the MDH was dialyzed against them for three weeks. After this time, the enzyme was centrifuged and redialyzed against phosphate

Figure 3: Elution Profile from First CM 32 Chromatography

Approximately 54,000 enzyme units were placed on a 3 x 60 cm column of carboxymethylcellulose 32 cation exchange resin, which had been equilibrated with 5 mM sodium phosphate, 1 mM EDTA, 1 mM BME, pH 6.6. The specific activity was 120 units per milligram of protein. The enzyme had been equilibrated by dialysis in the sodium phosphate buffer described above. The protein was loaded onto the column, and was washed with the phosphate equilibration buffer for 12 hours. At this time, a linear ionic gradient as detailed in "Experimental Procedure" was applied for protein elution at a flow rate of 75 ml/hour. Protein was estimated by the absorbance of the collected fractions at 280 nm, and enzyme activity was measured by the standard assay system. The conductivity of each tenth fraction was measured at 4°. The chromatography and all manipulations were performed at 4°.

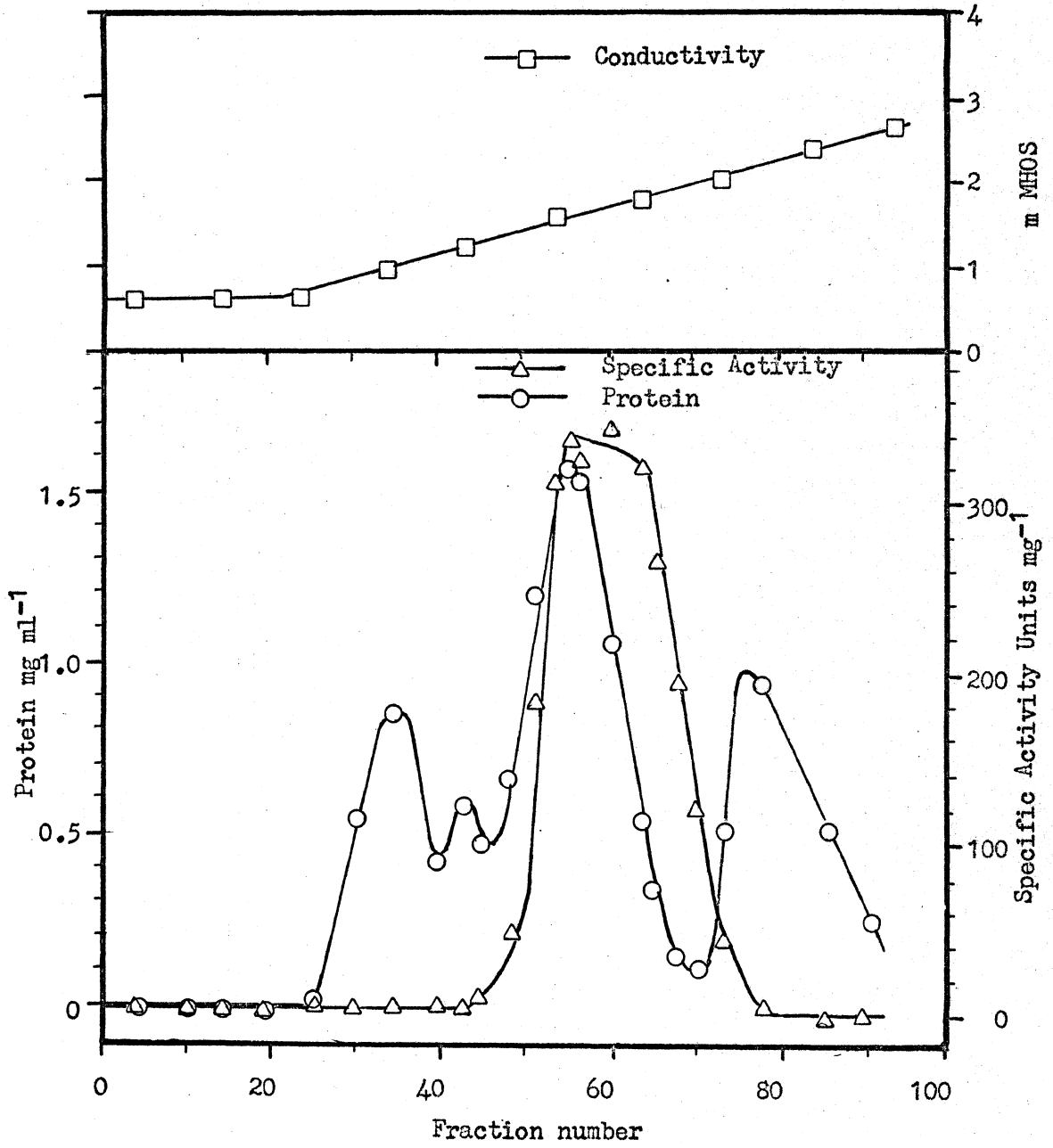


Figure 3

Elution Profile from First CM 32 Chromatography

Figure 4: Elution Profile from Second CM 32 Chromatography

Approximately 48,000 enzyme units were placed on a 3 x 60 cm column of carboxymethylcellulose 32 cation exchange resin, which had been equilibrated with 5 mM sodium phosphate, 1 mM EDTA, 1 mM BME, pH 6.6. The specific activity was 295 units per milligram of protein. The enzyme had been equilibrated by dialysis in the sodium phosphate buffer described above. The protein was loaded onto the column, and was washed with the phosphate equilibration buffer for 12 hours. At this time, a linear ionic gradient as detailed in "Experimental Procedure" was applied for protein elution at a flow rate of 75 ml/hour. Protein was estimated by the absorbance of the collected fractions at 280 nm, and enzyme activity was measured by the standard assay system. The conductivity of each tenth fraction was measured at 4°. The chromatography and all manipulations were performed at 4°.

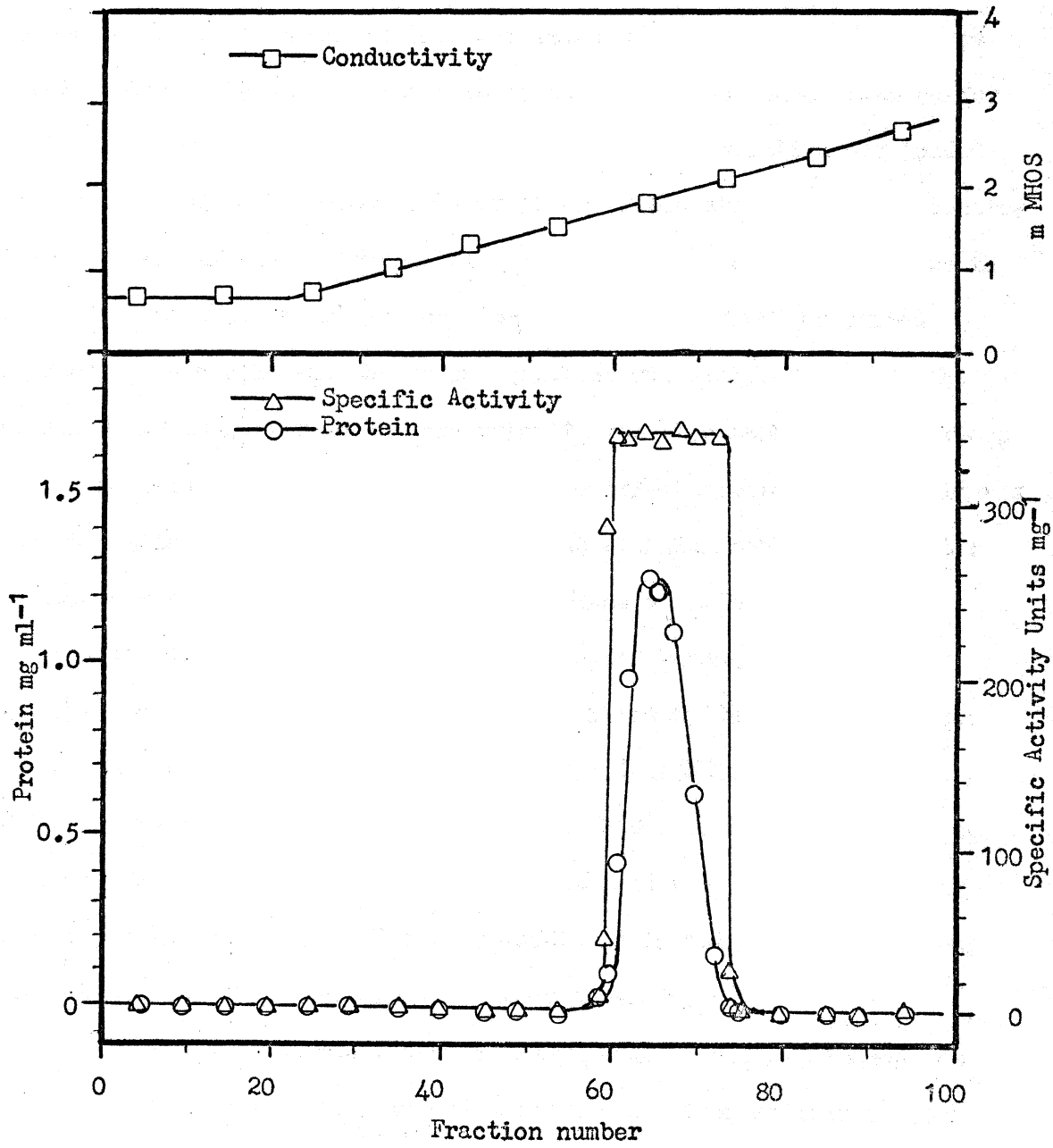


Figure 4

Elution Profile from Second CM 32 Chromatography

buffer at pH 7.0. The activity was measured in each fraction, and the results are shown in Table II.

Cellulose Acetate Electrophoresis -- Cellulose polyacetate strips were used to estimate the isoelectric point of the enzyme. The strips were pre-soaked in 1.4 mM sodium phosphate-8.6 mM sodium citrate buffer at 4°, and approximately 0.5 microliters of enzyme solution was loaded per strip. The tank buffer used was as described above, and the electrophoresis was allowed to proceed at a current of 1.5 mA/strip for two to four hours at 4°. The pH of the electrophoresis buffer was varied between 5.0 and 8.0, and the enzyme solution was dialyzed against the electrophoresis buffer to be used prior to the electrophoresis experiment.

A solution of 2 mg/ml Ponceau S in 30 mg/ml trichloroacetic acid was used to stain the strips for protein. The staining could be done at room temperature either in the presence or absence of light.

Malate dehydrogenase activity was located through the use of a specific tetrazolium staining mixture described by Dewey and Conklin(63). The buffer used was 0.11 M L-malate, 0.10 M Tris HCl, pH 9.0. Into 100 ml of this buffer were dissolved 70 mg NAD^+, 40 mg NBT, and 2.5 mg PMS. The strips were placed in a flat pan, and were flooded with the staining solution. The strips were developed in the dark at room temperature until the dark blue activity bands appeared.

Confirmation that the isolated homogeneous enzyme was indeed the mitochondrial isozyme was obtained through separation of intact mitochondria from the chicken heart tissue. Six chicken hearts were minced, mixed with 20 ml of 0.25 M sucrose, and homogenized with 20 strokes of a Potter homogenizer. The resulting extract was diluted to 80 ml with

Table II: Stability of MDH in Ammonium Sulfate(pH 7.0)

Homogeneous enzyme was stored at 4° in solutions of 1.2 M, 2.0 M, and 2.9 M ammonium sulfate at pH 7.0 for three weeks. The enzyme was then centrifuged and separated into precipitate and supernatant fractions, and redialyzed in phosphate buffer at pH 7.0 for 48 hours. Each fraction was then assayed to determine the amount of recoverable enzyme.

Table II: Stability of MDH in Ammonium Sulfate(pH 7.0)

	Amount of MDH recoverable from storage		
Ammonium Sulfate	Supernatant	Precipitate	Total
1.2 M	28%	0%[a]	28%
2.0 M	14%	35%	49%
2.9 M	1%	98%	99%

[a]No visible precipitate

0.25 M sucrose, and was centrifuged at 1000 x g. The pellet was discarded. The supernatant was further centrifuged at 5000 x g, and the pellet washed twice with 0.25 M sucrose. The resulting mixture was centrifuged at 11,000 x g, and the pellet washed twice with 0.15 M KCl. The pellet was resuspended in 25 mM sodium phosphate, 1 mM EDTA, 1 mM BME, pH 7.0. Sonification was performed at 60 watts for 2.5 minutes in an ice-salt bath, and the extract centrifuged at 11,000 x g. The supernatant was then assayed for malate dehydrogenase activity, and the electrophoresis was performed as described above.

Molecular Weight Determinations -- Ultracentrifugal analysis of the protein was carried out in a Beckman Model E ultracentrifuge equipped with a schlieren optical system. Sedimentation constants were determined by the Ehrenberg(64) procedure. Sedimentation velocity studies were performed at 59,780 rpm at 4° for 60 minutes, with an enzyme concentration of 40 micromolar. The solvent in each case was 50 mM sodium phosphate, 1 mM EDTA, except at pH 4.8 and pH 4.6, where the buffer was 50 mM sodium acetate, 1 mM EDTA.

Molecular weights were determined with a 2 x 100 cm column packed with Sephadex G-150 that had a final bed volume of 270 ml. The column was equilibrated either with 100 mM sodium phosphate(pH 7.0 or 5.0) or 50 mM sodium acetate(pH 4.8). The G-150 bed was protected by layering 5 ml of G-25 on top of the packed column. An elution profile of the column was determined by using the following standards: Blue Dextran (2,000,000 gm/mol), catalase(250,000 gm/mol), yeast alcohol dehydrogenase (140,000 gm/mol), bovine serum albumin(67,000 gm/mol), ovalbumin(45,000 gm/mol), and cytochrome c(13,000 gm/mol). The standards were divided into

two groups, with three substances in each group, and a separate elution study was performed for each standard group. Three milligrams of each standard within a group were dissolved into 1 ml of buffer, which was then centrifuged to remove the undissolved material. Sucrose was added to the standard solution to increase the density, and the solution was added to the top of the column. Elution was carried out using the same buffer under 15 cm of head pressure, and the effluent collected in 1.3 ml fractions. Samples of the native and modified MDH were prepared, placed on the column, and eluted in a similar manner.

Minimum molecular weight was also measured with SDS-polyacrylamide gel electrophoresis(65, 66). This was carried out in both the presence and absence of BME to determine if any disulfide bonds existed between the subunits. The standards used were as follows, with the minimum molecular weights given: Standard I — catalase(60,000 gm/mol), ovalbumin(43,000 gm/mol), and cytochrome c(13,000 gm/mol), and Standard II — bovine serum albumin(67,000 gm/mol), lactate dehydrogenase(36,000 gm/mol), and hemoglobin(16,000 gm/mol). Protein staining was carried out as described by Weber and Osborn(65) using 1.25 g of Coomassie brilliant blue dissolved in a mixture of 454 ml of 50% methanol and 46 ml of glacial acetic acid. The gels were destained using a solution of 75 ml of acetic acid, 50 ml of methanol, and 875 ml of water. Gels were destained until the discrete bands were clearly observable, which normally required about 30 minutes.

Amino Acid Analyses — The amino acid composition of the malate dehydrogenase, with the exception of tryptophan, was obtained according to the procedure of Spackman, Stein, and Moore(67) using a Beckman 120B

automatic amino acid analyzer. Samples were hydrolyzed in 6 N HCl for 24, 48, and 72 hours at 110°. All values were extrapolated to zero time hydrolysis, with the exception of leucine, isoleucine, and valine. Cysteine residues were determined as cysteic acid, and methionine residues were measured as methionine sulfone following performic acid oxidation (68). Tryptophan content was determined by the method of Edelhoch(69), with yeast alcohol dehydrogenase and lysozyme being used for positive controls. Partial specific volume was calculated according to the values published for the component amino acids(70). Hydrophobicity calculations were determined from the amino acid composition, using the procedure of Bigelow(71).

Enzyme that had been modified by iodoacetamide was analyzed by the same procedure for determination of the residue modified by the reagent. Iodoacetamide-modified enzyme and MDH that had been protected by coenzyme were hydrolyzed and their composition determined by the amino acid analyzer.

Kinetic Parameters — The buffers used for determining K_m in the absence of phosphate were 100 mM sodium glycinate, 1 mM EDTA, pH 10.6, and 100 mM Tris, 1 mM EDTA, pH 7.4. Buffers used for calculating K_m in the presence of phosphate were the same as those used in the normal assay solutions. The Michaelis constants were determined by varying individually the substrates in the assay mixture, and the assays were performed at 25° in the manner described. The data were subjected to linear regression analysis with the Wang 600 Programmable Calculator.

Inactivation Kinetics — The rates of inactivation were measured by incubating 100 to 1000-fold excess(0.34-3.4 mM) of the appropriate

modifying reagents with 10 nmol of malate dehydrogenase(3.3 micromolar) at 25° in either 50 mM sodium phosphate(pH 7.0 or 5.0) or 50 mM sodium acetate, 1 mM EDTA(pH 4.8). Reactions were carried out in 3 ml volumes in the absence of light until 95% inhibition had been achieved. The concentrations of enzyme and modifying reagent were increased 5-fold for use in amino acid analysis. The concentrations of enzyme and modifying reagents were increased 2.5-fold for use in quantitation of unmodified free sulfhydryl groups by the method of Ellman(72).

The effects of substrates and coenzymes on the rate of inactivation was determined by the addition of L-malate(5-10 molar excess, 20-37 micromolar), oxalacetate(5-10 molar excess, 20-37 micromolar), NAD^+(10-50 molar excess, 37-170 micromolar), or NADH(1-10 molar excess, 7-37 micromolar) to the incubation mixture. Controls of enzyme incubated under conditions identical with the test experiments, but with no modifying reagent present, were assayed in parallel experiments.

Incorporation of iodo-[C^{14}-1] acetamide was calculated from standard methods of isotope dilution. An internal standard of C^{14}-toluene (3.2 nanoCuries) was added to the vial after the initial readout was determined. The radioactivity was measured a second time and the efficiency of counting was determined. Ten nanomoles of malate dehydrogenase (3.3 micromolar) plus or minus 100 nmol of NADH(0.34 mM) were incubated with 5000 nmol of iodoacetamide(1.7 mM), a mixture of labeled and unlabeled reagent, at pH 7.0 as described above. When the nonprotected enzyme sample reached 95% inhibition, the reaction was quenched with the addition of 5000 nmol of β-mercaptoethanol(1.7 mM). After dialysis of the protein samples against three 4-liter volumes of 50 mM sodium phosphate, 1 mM EDTA,

pH 7.0, the samples were dissolved in scintillation fluid(2,5-diphenyl-oxazole-toluene-Triton X-100), and the amount of radioactivity per nmol of protein was determined by scintillation counting in a Beckman Model LS-133.

Other Instrumentation — The ISCO model 1200 PUP fraction collector was used in conjunction with the chromatography columns. Centrifugation was done in the Sorvall RC-5 centrifuge, using either the SS 34 or GSA rotor. Buffer preparation involved the use of the Corning pH meter model 12.

RESULTS AND DISCUSSION

Purification

Chicken heart mitochondrial malate dehydrogenase was not available from any commercial supplier, and had to be isolated and purified in the laboratory. Several purification schemes were tested before the final procedure, shown in Table I, was determined. That procedure gave the highest yield of homogeneous enzyme. The results in Table I have been calculated to demonstrate the yield of one batch of crude extract(250 g tissue), even though the actual values for the CM 32 columns were twice as large, since the yields of two Bio-Rex 70 columns were combined for each of the first CM 32 separations. A comparison of specific activity of the chicken isozyme with other MDH species is given in Table III. The chicken heart mitochondrial isozyme possessed a specific activity intermediate between those of the bovine and porcine mitochondrial forms, while all of the mitochondrial isozymes had specific activities that were 3 to 4-fold greater than those of the cytoplasmic isozymes.

The crude extract contained both cytoplasmic and mitochondrial forms of MDH. Therefore, the actual yield of purified isozyme was considerably higher than indicated by yields calculated on the total MDH activity present in the crude extract. This difference in yields is illustrated in Table I. The "Total Percent Recovery" column represents the amount of recoverable malate dehydrogenase activity in each of the purification steps, and includes activity from both isozymes. The theoretical yield of the mitochondrial isozyme, shown in the "Corrected Percent Recovery" column of Table I, was calculated assuming that the MDH which bound to the DEAE-cellulose column, as well as the MDH which

Table III: Specific Activities and Number of Active Sites in MDHs

The specific activity and number of active sites for the chicken mitochondrial isozyme are based on experimentally determined values. All other data shown are taken from values reported in the literature. All specific activities were assayed under the same conditions, except for the bovine and porcine cytoplasmic isozymes.

Table III: Specific Activities and Number of Active Sites in MDHs

Species of MDH (heart tissue)	Specific Activity(U/mg)	Number of Active Sites
Chicken Mito.	340	Two[a]
Bovine Mito.	310[b]	One[b]
Porcine Mito.	412[c]	Two[c]
Chicken Cyto.	65[a]	Two[a]
Bovine Cyto.	90*[d]	One[d]
Porcine Cyto.	110*[e]	Two[e]

*Assayed under different conditions

[a]See reference 11.

[b]See reference 30.

[c]See reference 42.

[d]See reference 32.

[e]See reference 48.

did not adhere to the Bio-Rex 70 column, was exclusively cytoplasmic MDH. Thus, twenty-six percent of the total initial activity in the crude extract was assumed to be cytoplasmic MDH. Further references to total yields and corrected yields are based on these assumptions.

The initial procedure was based on the method of Gregory(30) for purification of bovine heart mitochondrial malate dehydrogenase from acetone extracted heart muscle. No difference was observed between the total units recovered using the acetone powder procedure and using fresh or frozen heart tissue. Since no advantage was observed when the acetone extraction step was included in the purification procedure, the use of this procedure was eliminated.

The concentration of ammonium sulfate required to precipitate most of the MDH activity was determined by varying the amount of ammonium sulfate added to the crude extract, followed by assaying both the supernatnat and precipitate for MDH activity. The range of 1.5-2.7 M ammonium sulfate concentration was determined by Gregory(30) to be the optimal conditions for isolation of the bovine mitochondrial isozyme, and this range was used as a basis for the chicken heart enzyme. Further variation in these concentrations yielded the apparent optimal range for the chicken heart mitochondrial MDH. The crude extract was made 1.2 M in ammonium sulfate, and the precipitate discarded. The majority of MDH activity was then recoverable in the precipitate form upon the subsequent addition of enough ammonium sulfate to make the solution 2.9 M.

When the carboxymethyl cellulose column was used as the final chromatographic step in the purification procedure, the corrected yield of the isolated enzyme was 24%, while the specific activity obtained was 330 U/mg.

Various purification procedures were further used in attempting to increase both the yield and specific activity.

Blue Dextran Sepharose has been shown to be specific for the dinucleotide fold in proteins possessing this structure(14-17). Space-filling models of the moiety thought to be involved in binding to this site, reactive blue 2, show a remarkable similarity in configuration between the reactive moiety and the coenzyme, NAD^+. Inclusion of a chromatographic column of Blue Dextran Sepharose into the procedure in place of the carboxymethyl cellulose gave a corrected yield of 27%, but the specific activity of the enzyme decreased to 300 U/mg. Since the specific activity decreased in this isolated preparation involving Blue Dextran Sepharose, the use of this chromatographic resin in the purification scheme was eliminated. Cellulose polyacetate electrophoresis of an enzyme preparation that had passed through a Blue Dextran Sepharose column indicated a single subform with mobility corresponding to that of the solitary subform obtained using the final purification procedure, which is discussed below.

Nearly 49% of the MDH activity was lost in the 1.2-2.9 M ammonium sulfate fractionation steps. In addition, considerable activity was being lost when the chromatography column fractions were concentrated by dialysis against 3.9 M ammonium sulfate. Storage of homogeneous enzyme in a solution of 3.9 M ammonium sulfate at 4° was found to result in loss of nearly all MDH activity over a period of several weeks. This behavior indicated that the chicken heart isozyme was less stable under these conditions than other malate dehydrogenase species. For example, the bovine mitochondrial isozyme, which can be frozen while stored in

3.9 M ammonium sulfate, pH unadjusted, and still remain stable indefinitely.

Studies were undertaken to determine the optimal conditions for storage of the homogeneous enzyme. Solutions of 1.2 M, 2.0 M, and 2.9 M ammonium sulfate(pH 7.0) at 4° were utilized, and enzyme recovery following dialysis of the enzyme against those solutions are given in Table II. A 2.9 M ammonium sulfate solution(pH 7.0) was found to be optimal for the storage of the purified enzyme. The mitochondrial isozyme is completely stable under these conditions for at least six months. Apparently, the enzyme is not stable unless it is stored as the precipitate, since over half of the activity was lost in both 1.2 M and 2.0 M ammonium sulfate solutions, in which the enzyme remained unprecipitated. Approximately 99% of the activity was recovered in the 2.9 M ammonium sulfate precipitate fraction. It is not clear whether the instability of the enzyme in 3.9 M ammonium sulfate, pH unadjusted, is due to the higher concentration of salt, the more acidic pH, or to a combination of both of these factors. The dialysis of the homogeneous enzyme against 3.9 M ammonium sulfate, pH 7.0, may yield data indicating whether the salt concentration or pH was the primary factor involved in the loss of activity.

In an attempt to eliminate all procedures in the purification scheme that involved ammonium sulfate, other than storage of the homogeneous enzyme, the crude extract was passed through a DEAE-cellulose column and pumped directly onto the Bio-Rex 70 column, and thereby eliminating the need for the 1.2-2.9 M ammonium sulfate fractionation and precipitation steps. The substitution of the DEAE-cellulose anion exchange column for

the ammonium sulfate step increased the total yield of MDH activity that was added to the Bio-Rex 70 column from 51% to 94%, while at the same time eliminating the majority of lipids from the crude tissue extract. Passage through this column converted the dark red, opaque slurry into a clear red solution. The Bio-Rex 70 cation exchange column adsorbed the mitochondrial isozyme whereas the cytoplasmic isozyme failed to bind to the chromatographic column. The corrected yield of mitochondrial MDH from the clear yellow Bio-Rex 70 pooled fractions was 72%. While the DEAE-cellulose column provided only a 3-fold increase in specific activity, from 5.5 U/mg to 17 U/mg, the use of the Bio-Rex 70 column increased the specific activity to 120 U/mg, with a purification factor of 22 when compared with the crude extract. The use of a carboxymethyl cellulose column at a lower pH and with a phosphate buffer of lower ionic strength provided a further purification of the enzyme to a specific activity of 295 U/mg, with a purification factor of 54. The corrected yield of the CM 32 pooled fractions was 63%.

Previously, we had achieved a specific activity of 330 U/mg, although with much lower yield than the procedure discussed above, and felt that the enzyme obtained using this procedure could be purified further. Since the specific activity of the enzyme eluted from this carboxymethyl cellulose column was not quite constant, although nearly so, over the fractions collected(see Figure 3), it was felt that the nearly homogeneous preparation could be further purified by chromatographing the enzyme solution on the CM 32 column for a second time. Upon elution from this second CM 32 column, the enzyme had a specific activity of 340 U/mg in each protein-containing fraction. Thus, the enzyme was purified 62-fold

with an overall corrected yield of 56%.

One further attempt to improve the purification scheme involved hydrophobic interaction chromatography. The enzyme is fairly hydrophobic (see Table VIII), and one would predict some interaction with a hydrophobic matrix. A nine milliliter bed volume of 10-Carboxydecyl Sepharose was equilibrated in 1 M potassium phosphate, 1 mM EDTA, 1 mM BME, pH 8.5. Chicken heart was extracted into this buffer by using a Waring blendor in a manner identical to that described in "Experimental Procedure" for extraction of the heart muscle. When this mixture was washed through the column, only about 30% of the initial activity remained bound to the 10-Carboxydecyl Sepharose. Elution of the bound enzyme from the column was attempted with the phosphate buffer plus 5% ethanol, but only 6% of the enzymatic activity was recovered. Further elution with 0.05 M potassium phosphate, 1 mM EDTA, 1 mM BME, pH 8.5, recovered no further MDH activity. The low yields of enzyme activity resulting from incorporation of the 10-Carboxydecyl Sepharose into the purification scheme rendered this step impractical.

The specific activity of 340 U/mg is much higher than the value of 260 U/mg previously reported by Kitto and Kaplan(11) for the mitochondrial isozyme. Their reported yield was 11% as compared to the total yield of 42% obtained from our studies. The homogeneity of the preparation of Kitto and Kaplan was illustrated by crystallization, molecular weight studies, and sedimentation velocity experiments, while the criteria for the purity of our enzyme preparation are discussed below.

Gregory reported a total yield of 40% for both the porcine mitochondrial isozyme(72) and the bovine mitochondrial form(30). The homogeneity

of these enzyme preparations was determined by the same criteria listed below for the chicken isozyme.

Criteria of Purity:

1) Constant specific activity -- On the second chromatography through the CM 32 column, the enzyme had a constant specific activity of 340 U/mg in the fractions pooled(see Figure 4).

2) Molecular weight determinations -- The enzyme preparation eluted as a single, sharp peak of malate dehydrogenase activity when chromatographed on a Sephadex G-150 column. In addition, SDS-polyacrylamide gels gave a single band when stained for protein following electrophoresis. Therefore, any contaminant must have the same monomeric and multimeric molecular weights as malate dehydrogenase, and the chances of this occurring are rather remote.

3) Cellulose acetate electrophoresis -- Single, corresponding bands of both protein and MDH activity were observed when the enzyme was subjected to electrophoresis on cellulose polyacetate strips. The electrophoresis on polyacetate strips was repeated at several different pH values and a single coincident protein and activity band was observed in each case. Similar electrophoresis of the mitochondrial extract, described in the "Experimental Procedure" section, yielded bands of MDH activity that were identical in electrophoresis characteristics, including pI, to that of the purified enzyme. This indicated that the isolated enzyme was indeed the mitochondrial isozyme, and not the cytosolic form.

4) Sedimentation velocity and sedimentation equilibrium -- Whenever the enzyme preparation was analyzed for sedimentation constants,

the schlieren pattern obtained was a single, sharp peak(see Figure 5).

These results are listed in approximate decreasing order of their acceptance as reliable criteria of purity. They clearly indicate that this enzyme preparation is homogeneous by these criteria, and that the enzyme isolated is mitochondrial malate dehydrogenase.

The final purification scheme appears to be a very efficient and fairly simple system for isolating the chicken heart mitochondrial malate dehydrogenase. The total time required for one complete purification is usually 10-14 days. The total yield and final specific activity is higher than any previously reported figures for the chicken heart mitochondrial malate dehydrogenase.

Physical and Chemical Characteristics

Insight into the mechanism of an enzyme and the various properties of the protein *in vivo* may be gained through *in vitro* studies of the physical, chemical, and catalytic properties of the homogeneous enzyme. A number of techniques and instrumentation are available for the performance of such studies, and a thorough physical and chemical analysis is essential for understanding the enzyme function.

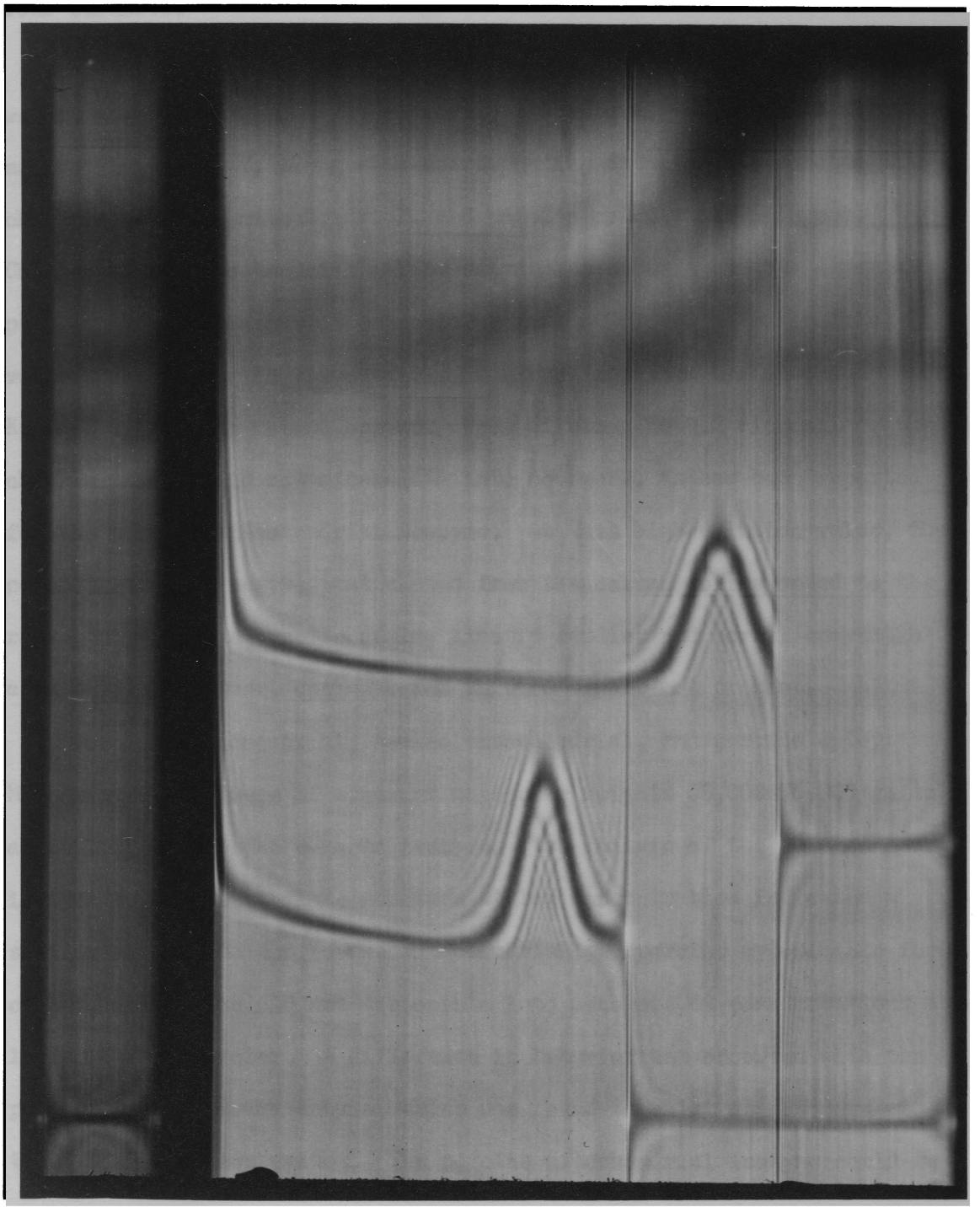
Molecular weight — Electrophoresis of the chicken enzyme on SDS-polyacrylamide gels yielded a single, sharp band that corresponded to 33,000 gm/mol. The same results were obtained in the presence and absence of BME, indicating that no inter-chain disulfide bonds existed between the subunits. This technique indicated that chicken heart mitochondrial MDH is composed of two subunits of approximately equal weight. All mitochondrial and cytoplasmic forms of MDH that have been

Figure 5: Sedimentation Velocity of Chicken Heart Mitochondrial MDH

The enzyme was dialyzed in 50 mM sodium phosphate, 1 mM EDTA at either pH 7.0 or pH 5.0, and the final protein concentration of 40 micromolar. The protein was centrifuged at 59,780 rpm at 4° for 60 minutes. The photograph shown here was taken at 24 minutes after the ultracentrifugation began. The upper pattern is the native enzyme at pH 7.0, while the lower pattern is the native enzyme at pH 5.0.

Figure 5

Sedimentation Velocity of Chicken Heart Mitochondrial MDH



studied are reported to be dimeric at pH 7.0. There have been several reports of microbial MDHs that possessed multimeric structures larger than dimers. An example is B. subtilis MDH(74), which apparently has four subunits.

The effects of variations in pH and enzyme concentration on molecular weight were studied using a Sephadex G-150 column. The results are summarized in Table IV, along with corresponding data for the porcine mitochondrial, bovine mitochondrial, and porcine cytoplasmic isozymes. Chicken heart mitochondrial malate dehydrogenase existed as a dimer at pH 7.0, giving an apparent molecular weight of 67,000 gm/mol. This value agreed with the value of 67,000 gm/mol reported by Kitto and Kaplan(11) for the native enzyme. Upon dilution to 0.2 micromolar, the chicken isozyme did not dissociate into monomers, as has been reported for the porcine mitochondrial isozyme. At this high dilution value, the concentration of enzyme that eluted from the column corresponded to the range found in the cuvette during kinetic studies and normal enzymatic assays(75). However, coenzyme and substrate were not present.

Porcine mitochondrial, bovine mitochondrial, and porcine cytoplasmic MDH existed as dimers of apparent molecular weights 68,000-76,000 gm/mol at pH 7.0, as did the chicken isozyme. The failure of the chicken heart isozyme to dissociate into monomers at low concentration indicated a similarity to both the bovine mitochondrial and porcine cytoplasmic forms of MDH, which also did not dissociate into monomers at concentrations as low as 0.2 micromolar. A difference in behavior was observed with the porcine mitochondrial enzyme, which was found to exist as monomers at these high dilution values. The porcine mitochondrial isozyme could be

Table IV: Molecular Weights by Sephadex Chromatography

The molecular weight values shown for the chicken heart mitochondrial MDH were obtained with the use of an appropriately calibrated Sephadex G-150 column. The values shown for the other isozymes were obtained by Sephadex G-100 chromatography, and were reported in the literature. The pH and enzyme concentrations were as shown, and the buffers used are described in the "Experimental Procedure" section.

Table IV: Molecular Weights by Sephadex Chromatography

Sample	Concentration	Molecular Weight			
		Chicken Heart m-MDH	Porcine Heart m-MDH(75)	Bovine Heart m-MDH(75)	Porcine Heart c-MDH(75)
Native Enzyme, pH 7.0	30 micromolar	66,000	68,000	68,000	76,000
Native Enzyme, pH 7.0	0.2 micromolar	62,000	36,000	66,000	78,000
Native Enzyme, pH 5.0	30 micromolar	46,000	34,000	33,000	76,000
Native Enzyme, pH 4.8	30 micromolar	33,000	-	-	-

reassociated into the dimeric form by the addition of NADH.

The purpose of measuring the molecular weight of these isozymes at this low concentration was to determine if the monomer could exist at cuvette concentrations. Even though the porcine mitochondrial MDH did exist as a monomer at cuvette concentrations, this does not mean that the monomer is active, since the presence of coenzyme caused reassociation of the active form. Although the concentrations of coenzyme in the assay mixture may not be large enough to elicit all of the MDH monomers to reassociate into dimers, there may be sufficient coenzyme to cause transient dimers, or monomers that exist as dimers for short periods of time, which would catalyze a reaction and result in a change in the absorbance at 340 nm, indication of enzymatic activity. Studies involving active enzyme ultracentrifugation, which determines the sedimentation coefficient of the enzyme while it is actively undergoing a reaction catalysis, would probably yield invaluable data concerning the existence of an active monomer.

A molecular weight of 46,000 gm/mol was measured for the chicken mitochondrial MDH at pH 5.0. That value is intermediate between the molecular weights for the dimer and monomer configurations, which were 67,000 and 33,000 gm/mol, respectively. Upon dialysis of the enzyme at pH 4.8, the chicken isozyme eluted from the G-150 column at a position indicative of the monomer. Decreasing the pH to 5.0 caused dissociation into monomers in both the porcine and bovine mitochondrial isozymes, while the porcine cytoplasmic MDH remained in the dimeric state. Information was not available on the acid dissociation of the porcine cytoplasmic species at pH values less than 5.0, if any such dissociation

did occur. The chicken isozyme appeared to be slightly more resistant to pH-dependent-dissociation into monomers than the bovine and porcine mitochondrial forms, but was not as stable as porcine cytoplasmic MDH.

Sedimentation equilibrium studies were also used to determine the molecular weight of the native enzyme. Protein concentrations of 1.0 and 0.5 mg/ml gave calculated molecular weights of 69,800 and 72,200 gm/mol, respectively. The average of these two values, 71,000 gm/mol, actually deviates only 7% from the value of 67,000 gm/mol obtained elsewhere, and this figure is well within the normal range of error for such studies.

Kitto and Kaplan(11) reported two coenzyme-substrate binding sites in chicken heart mitochondrial MDH, one active site being on each subunit. Incorporation of C^{14}-labeled iodoacetamide, discussed in "Chemical Modification", further indicated the presence of two active sites per chicken isozyme dimer. A comparison of the number of binding sites in the chicken isozyme and other species of MDH is given in Table III. Nearly all mitochondrial and cytoplasmic forms of MDH have been reported to possess one active site per subunit, with the exceptions of the bovine mitochondrial(30) and bovine cytoplasmic(38) isozymes. The two bovine isozymes have been shown to contain only one active binding site per dimer, although it is not clear whether only one subunit contained an active site, or whether two active sites were present and only one was reactive.

Cellulose polyacetate electrophoresis -- The technique of electrophoresis on cellulose acetate strips was chosen to study the electrophoretic properties of the chicken isozyme. Electrophoresis of the

native enzyme was attempted on polyacrylamide gels, but the MDH did not migrate into the 5% gels, or resulted in bands that were smeared. The somewhat hydrophobic nature of the enzyme may be part of the reason for this behavior with polyacrylamide gel electrophoresis.

The isolation of chicken heart mitochondria was described in the "Experimental Procedure" section. This mitochondrial extract was studied for MDH activity, and was found to possess, upon electrophoresis, a single band of MDH activity which corresponded to that of the homogeneous enzyme preparation, indicating that the purified enzyme was of mitochondrial origin.

Chicken heart mitochondrial MDH possessed a slight negative charge at pH 7.0. The isoelectric point(pI) of 6.5-7.0 was estimated by varying the pH of the electrophoresis buffer. Compared with other malate dehydrogenases(see Table V), the chicken heart mitochondrial isozyme had a pI that did not differ significantly from those of other species. Cytoplasmic forms of MDH, in general, have a lower isoelectric point than the mitochondrial species.

Amino Acid Analysis -- Analysis of the amino acid composition of the enzyme was carried out, with the results shown in Table VI. Numbers are given as total residues per molecule of enzyme dimer. The data obtained agreed very closely with those previously reported by Kitto and Kaplan(11), which are also listed in Table VI. The amino acid composition of other malate dehydrogenases is shown in Table VII, which indicates that most MDH forms are quite similar in amino acid composition, especially when the same isozyme is compared between species. The most noticeable difference between isozymes is the absence of tryptophan in the

Table V: Isoelectric Point of Malate Dehydrogenases

The isoelectric point of the chicken heart mitochondrial MDH was estimated using cellulose acetate strip electrophoresis by varying the pH of the electrophoresis buffer. Conditions for the strip electrophoresis are described in the "Experimental Procedure" section. The values shown for the other isozymes were reported in the literature, and were derived from experimental schemes that were quite similar to that used for the chicken enzyme.

Table V: Isoelectric Points of Malate Dehydrogenases

Species	pI
Chicken heart m-MDH	6.5-7.0
Bovine heart m-MDH	6.2(76)
Ox heart m-MDH	>6.9(76)
Porcine heart m-MDH	6.1-6.4(76)
Spinach microbody MDH	5.7(77)

Table VI: Amino Acid Analysis of Chicken Heart Mitochondrial MDH

The amino acid composition of the enzyme was determined after 24, 48, and 72 hour hydrolysis in 6 N HCl. Values for all residues were extrapolated to zero time of hydrolysis except leucine, isoleucine, and valine. Methionine and cysteine residues were analyzed as methionine sulfone and cysteic acid after performic acid oxidation. Tryptophan content was determined by the method of Edelhoch(69). The literature values reported by Kitto and Kaplan(11) are shown for comparison. Numbers are given as total residues per enzyme dimer.

Table VI: Amino Acid Analysis of Chicken Heart Mitochondrial MDH

Residue	Experimental	Kitto and Kaplan(11)	Difference
Lys	52	49	+3
His	12	11	+1
Arg	21	20	+1
Asp	51	52	-1
Thr	43	44	-1
Ser	44	42	+2
Glu	56	60	-4
Pro	48	41	+7
Gly	58	61	-3
Ala	63	66	-3
Val	49	48	+1
Met[a]	14	13	+1
Ileu	35	40	-5
Leu	54	57	-3
Tyr	9	9	0
Phe	26	30	-4
Cys[a]	16	14	+2
Trp	0[b]	0	0
Total residues	651	657	-6

[a]Determined by performic acid oxidation

[b]Determined by Edelhoch method

Table VII: Amino Acid Composition of Various Malate Dehydrogenases

The malate dehydrogenases listed were analyzed for amino acid content in a manner similar to the methods described in "Experimental Procedure". All of these values were reported in the literature, and are expressed as total residues per enzyme dimer.

Table VII: Amino Acid Composition of Various Malate Dehydrogenases

Amino Acid	Porcine Heart Mitochondrial(1)	Bovine Heart Mitochondrial(1)	Chicken Heart Cytoplasmic(1)	Porcine Heart Cytoplasmic(1)	Bovine Heart Cytoplasmic(1)
Lys	52	48	56	62	70
His	10	10	12	8	8
Arg	16	16	20	20	20
Asp	50	46	68	72	78
Thr	42	38	30	32	24
Ser	36	32	36	44	32
Glu	50	48	60	54	58
Pro	46	40	28	24	22
Gly	58	50	60	46	44
Ala	66	60	60	64	58
Cys	14	16	8	10	10
Val	54	52	52	52	50
Met	12	12	14	16	16
Ileu	42	42	42	38	36
Leu	56	52	42	38	36
Tyr	10	12	16	16	12
Phe	22	22	24	22	20
Trp	0	0	12	10	12

mitochondrial isozymes.

Tryptophan content in the chicken mitochondrial MDH was analyzed using the Edelhoch method(69), and no tryptophan residues were observed for the enzyme. Positive controls for tryptophan content were yeast alcohol dehydrogenase and egg white lysozyme, and values of 22.7 and 5.9, respectively, were obtained for these proteins. These figures agreed very well with the tryptophan content previously reported for these proteins, which are 23 residues(21) and six residues(69), respectively. The lack of tryptophan in the chicken isozyme, in addition to agreeing with the absence of the residues as reported by Kitto and Kaplan(11), is consistent with the lack of tryptophan as reported for most other mitochondrial malate dehydrogenases. The majority of cytoplasmic malate dehydrogenases, in contrast, are reported to possess some tryptophan residues. The fact that tryptophan is not present in the mitochondrial isozyme is in direct contrast to the findings of Schellenberg(21-24) with the porcine mitochondrial isozyme. Schellenberg also proposed a general mechanism for all malate dehydrogenases that involved the participation of tryptophan as an intermediate hydride donor/acceptor on the enzyme.

Using the experimentally obtained values for the amino acid composition of chicken mitochondrial MDH, the partial specific volume was calculated by the method of Cohn and Edsall(70), and a value of 0.74 ml/gm was computed. This value is quite similar to the value determined for the majority of proteins.

The data from the amino acid analyzer provided verification of the extinction coefficient reported for chicken mitochondrial MDH by Kitto and Kaplan(11). The results verified that the reported value of

3.0 ml mg^{-1} cm^{-1} was correct. The molar quantity of enzyme, determined from the extinction coefficient and molecular weight, and which had been acid hydrolyzed and analyzed with the amino acid analyzer was identical to the molar quantity of amino acid residues recovered. Multiplying the total number of residues obtained per enzyme dimer, 651 residues, by the average molecular weight of an amino acid residue, 103 gm/mol, resulted in a calculated molecular weight of 67,000 gm/mol for the native enzyme. Since this value was identical to the reported molecular weight, and since the amount of amino acid residues obtained from the analyzer is determined by a separate standard, these results verified that the extinction coefficient was correct. One of the main reasons for the low extinction coefficient of the enzyme is the absence of tryptophan and the small number of tyrosine residues, both of which absorb very strongly at 280 nm.

The hydrophobicity of chicken MDH was calculated to be 1123 cal/residue, which is very similar to the calculated hydrophobicities for other MDH species, as shown in Table VIII. Malate dehydrogenases, as well as other dehydrogenases, possess slightly above average hydrophobicities, with the median value for most proteins being approximately 1050 cal/residue. Examples of non-hydrophobic and quite hydrophobic proteins, by the criteria discussed here, are fibroin and Gramicidin S, respectively.

The hydrophobicity of a protein molecule can be calculated by the method of Bigelow(71) from the amino acid composition and a list of weighted factors describing the hydrophobicity of each amino acid residue. These factors are based on the free energy involved in transferring an amino acid from an aqueous solution to an ethanolic solution. The total hydrophobicity of a protein is a measure of the stabilization that a

Table VIII: Hydrophobicities of Dehydrogenases and other Proteins

The average hydrophobicities were calculated from the amino acid compositions of the various proteins through the method of Bigelow(71). The NPS values(non-polar side chain frequencies) were calculated according to the method of Waugh as described by Bigelow(71). Both values are designed to indicate the hydrophobic or non-polar content of proteins based on the number of non-polar residues versus polar residues in the amino acid composition of the protein.

Table VIII: Hydrophobicities of Dehydrogenases and Other Proteins

Protein	Average Hydrophobicity(cal/residue)	NPS(71)
Chicken Heart m-MDH	1123	0.34
Porcine Heart m-MDH(71)	1171	0.36
Bovine Heart m-MDH(71)	1174	0.36
Horse Heart m-MDH(71)	1124	0.36
Tuna Heart m-MDH(71)	1108	0.35
Porcine Heart c-MDH(71)	1139	0.34
Bovine Heart c-MDH(71)	1151	0.34
Chicken Heart c-MDH(71)	1152	0.36
Salmon AA MDH(71)	1092	0.34
Salmon BB MDH(71)	1074	0.32
B. subtilis MDH(71)	1200	0.40
E. coli MDH(71)	1131	0.37
Chicken heart LDH(74)	1044	0.34
Chicken muscle LDH(74)	1053	0.33
Porcine G3P DH(79)	1090	0.33
Chicken liver Glut. DH(80)	1097	0.33
Bovine ribonuclease(71)	870	0.23
Anaphe malonye fibroin(71)	440	0.02
Bovine pepsin(71)	1080	0.38
Catalase(71)	1200	0.37
Gramicidin S(71)	2020	0.80

molecule could achieve if all of its non-polar residues were buried inside the polar shell or surface(71). The average hydrophobicities of many malate dehydrogenases and other proteins, as shown in Table VIII, can be compared to another method for measuring the hydrophobicity of a protein. This method, called the NPS value, appears to be quite compatable with the method of Bigelow, since we see from Table VIII that the two methods yield consistently similar values. The NPS value is a ratio of the total number of non-polar residues, defined by Bigelow(71), and the total number of residues present in the protein.

Kinetics — The Michaelis constant(K_m) is defined as the substrate concentration at which the reaction velocity is half maximal. The Michaelis constants for the substrates and coenzymes of chicken heart mitochondrial MDH were determined in the presence and absence of phosphate or pyrophosphate. The K_m of a substrate was determined by varying the concentration of that substrate in the assay mixture. The data were analyzed on Lineweaver-Burk plots, and the Michaelis constants were calculated from linear regression of the data. Results are listed in Table IX. The K_m values obtained for L-malate and oxalacetate(in the presence of either pyrophosphate or phosphate), which were 0.90 mM and 0.03 mM, respectively, agreed quite well with the reported values(11) of 0.9 mM and 0.038 mM, respectively. The presence of pyrophosphate or phosphate caused a decrease in K_m of about 38% for the cofactor and around 21% for the substrate. Enzyme used was dialyzed in 5 mM sodium phosphate, 1 mM EDTA, pH 7.0.

The presence of either pyrophosphate or phosphate caused a 35% increase in the activity of the enzyme. Phosphate-dependent activation

Table IX: Michaelis constants(K_m) and V_{max}

Assay mixtures and conditions are described in the "Experimental Procedure" section. The substrate whose K_m was being measured was varied in concentration in the assay mixture. The presence of pyrophosphate or phosphate appeared to have a marked effect on decreasing the K_m for both the substrate and the coenzyme, and the relative change in the K_m upon addition of the phosphate moiety is indicated in the last column. The apparent V_{max} effect in the presence of a phosphate moiety is also shown.

Table IX: Michaelis Constants(K_m) and V_{max}

Substrate	Conditions	K_m(mM)	Decrease in K_m
NAD^+	no pyrophosphate, pH 10.6	1.2	
NAD^+	pyrophosphate, pH 10.6	0.75	37%
L-Malate	no pyrophosphate, pH 10.6	1.15	
L-Malate	pyrophosphate, pH 10.6	0.90	22%
NADH	no phosphate, pH 7.4	0.030	
NADH	phosphate, pH 7.4	0.020	33%
OAA	no phosphate, pH 7.4	0.040	
OAA	phosphate, pH 7.4	0.030	25%

V_{max} = 340 U/mg (pyrophosphate present)
V_{max} = 250 U/mg (pyrophosphate absent)

has also been observed by Gregory with the bovine mitochondrial isozyme[1], by Yost with the porcine mitochondrial isozyme(78), and by Cassman and Vetterlein for bovine cytoplasmic MDH(38). The amount of pyrophosphate or phosphate needed to elicit this activation was quantified. Michaelis constants for phosphate or pyrophosphate were determined in a manner similar to that described above. These were called K_p values, since they were not true Michaelis constants. It was noted that the larger the concentration of phosphate that the enzyme dialysis buffer contained, the less pyrophosphate or phosphate was required to bring about this activation. This pre-incubation-activation was found to occur in less than 15 seconds. A graph of the effect of this pre-incubation-activation on the K_p of the phosphate moiety is shown in Figure 6. An apparent saturation results in the range of 50 mM sodium phosphate incubation buffer, with no significant further decrease in K_p values being observed when higher concentrations of phosphate were used in the incubation buffer.

Apparently the pyrophosphate or phosphate in the assay mixture is binding or complexing with the enzyme, either at or near the active site or some other site on the molecule. Cassman and Vetterlein(38) reported that one mol of phosphate was bound per mol of enzyme in the phosphorylated form of the bovine cytosolic isozyme. The phosphorylated form reportedly contained covalently bound phosphate, while the phosphate moiety in the chicken enzyme was not covalently bound to the enzyme. This associated phosphate is thought to bring about a conformational change which either increased the turnover rate or increased the affinity of the enzyme for the substrate and/or coenzyme. Incubation

Figure 6: Activation of Pyrophosphate and Phosphate

The activity of the enzyme was significantly increased when pyrophosphate or phosphate was added to the assay mixture. The amount of pyrophosphate or phosphate moiety needed to elicit this increased activity was determined as the K_p value. Further studies revealed that the amount of moiety required to bring about this activity increase decreased with the amount of phosphate present in the buffer that the enzyme was dialyzed against before being assayed. Each line represents enzyme that was dialyzed against a different concentration of phosphate. While this graph illustrates the effect of pyrophosphate at pH 10.6, a graph of the effect of phosphate at pH 7.4 would look identical with the exception of smaller K_p values.

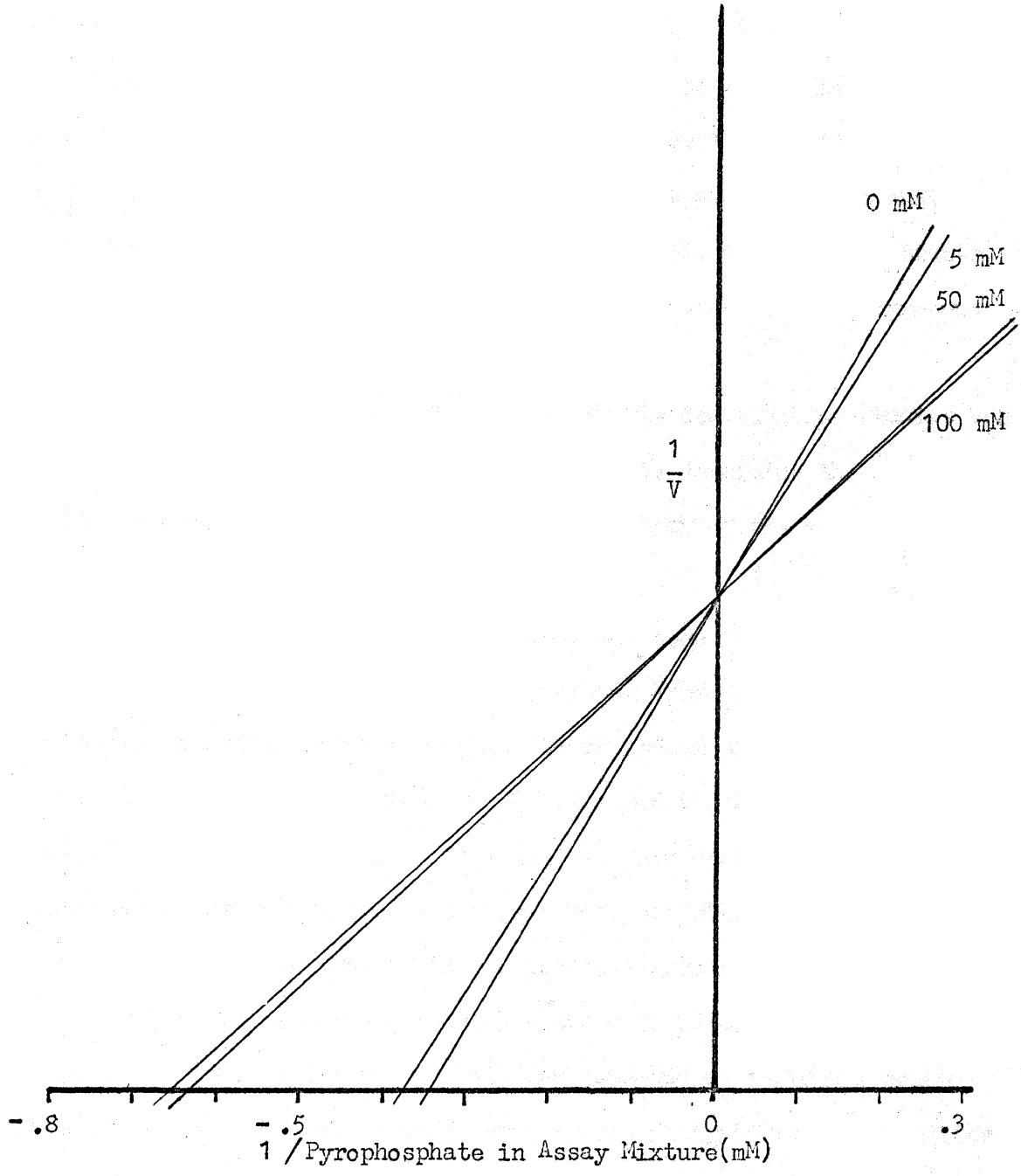


Figure 6

Activation of Pyrophosphate and Phosphate

of the enzyme with concentrations of phosphate prior to the catalytic studies reduced the amount of pyrophosphate or phosphate required in the assay mixture to bring about this activation.

A second possibility is that the phosphate moiety could simply have been eliciting a stabilization of the enzyme structure against activity loss under the conditions of dialysis. A slight change in the quaternary structure of the enzyme may have resulted in a slight loss of activity. Upon addition of the phosphate moiety, the structure of the enzyme was stabilized and the full activity was restored.

Reversible acid denaturation — Reversible denaturation studies were performed on the homogeneous enzyme in order to determine the stability of MDH to lower pH values, and the recoverability of the enzyme that had been exposed to acidic conditions.

Chicken heart mitochondrial MDH that had been exposed to pH 5.0 for 48 hours possessed a specific activity of 192 U/mg when assayed at pH 10.6. The enzyme was then dialyzed against 100 mM sodium phosphate, 1 mM EDTA, pH 7.0 for 24 hours, whereupon the MDH regained all of its activity when assayed at pH 10.6(340 U/mg). Cellulose acetate electrophoresis of this reactivated enzyme resulted in a single band of activity which corresponded in mobility to that of the homogeneous enzyme preparation.

Similarly, enzyme that had been exposed to pH 4.8 for 48 hours, and possessed a specific activity of 185 U/mg(assayed at pH 10.6), could likewise regain complete activity upon simple dialysis in pH 7.0 buffer. Strip electrophoresis revealed a single band of activity which also corresponded to that of the homogeneous enzyme.

These data indicated that the acid-exposed enzyme could indeed be

reactivated, if the enzyme were dialyzed to pH 7.0 and stored in 2.9 M ammonium sulfate(pH 7.0). Other studies have reported the reversible acid denaturation of chicken heart MDH using pH values as low as pH 2.0. These same studies also reported the reversible acid denaturation of tuna mitochondrial, porcine mitochondrial, and porcine cytoplasmic MDH, with similar success. Chicken heart mitochondrial MDH appears to be very resistant to extremes of pH, both acidic and basic, especially when certain buffers are present to aid in reactivation(27).

Chemical Modification

In theory, selective modification of specific residue types will result in a change in the catalytic or regulatory activity of the enzyme. If such a change does occur upon modification, the residue that has reacted could be essential for the activity of the protein, although this alone does not prove such an assumption. The presence of the modifying groups on the residue could block the binding site or evoke a conformational change that alters the behavior of the protein. In the case of enzymes, inactivation upon modification implies either the location of the modified residues near the active site, or the importance of the residue in maintaining a stable and active structure for the enzyme. If part or all of the modified residues are protected from modification by the presence of an excess of substrate or coenzyme during the modification procedure, the number of those essential residue(s) that are modified can be determined by differential labeling with radioactive-modifying reagent. To increase the probability of a specific reaction with only a limited number of residue(s) being modified, modifying

reagents should be reasonably specific for the residue being modified, and used in sufficiently low concentration to ensure modification of only the most reactive residues.

The reactivity of a group in a protein with a particular reagent depends, among other things, upon the effect of the environment on the group, and upon the ability of the reagent to enter that environment. Because proteins are quite large, many of their constitutive amino acid residues, especially those of a hydrophobic nature, are partially shielded from solvent by being buried beneath the protein surface, and therefore are relatively unreactive with reagents present in the solvent. Some residues possess unusually high reactivities as a result of their presence in a catalytic center and their being near the active site(81). More explicitly, the increased reactivity of an active site residue may be due to the proximity of a charged group, a basic or nucleophilic group, or by virtue of possessing an ionic charge within a hydrophobic region(73). Relative reactivities of groups can be affected either positively or negatively by neighboring groups. Enhanced reactivities are in many cases, but not always, associated with the unique chemistry resulting from the presence of a group in a catalytic site(81). Lower concentrations of modifying reagents will minimize the reactions of residues on the enzyme surface that are not in the vicinity of the active site. Differentiation between active center and non-active center reactive residues can often be accomplished by using coenzyme or substrate to protect active site residues from modification.

Chemical modification of the chicken heart mitochondrial malate dehydrogenase was attempted using four modifying reagents. Iodoacetate

(IAA), iodoacetamide(IAM), 5,5'-dithiobis-(2-nitrobenzoic acid)(DTNB), and N-ethyl maleimide(NEM) were tested for their ability to alter the catalytic activity of MDH. If enzyme inactivation occurred, the residues that had been modified were identified and quantified. Unless otherwise specified, all reactions were carried out in a total volume of 3.0 ml of buffer at 25° with 10 nmol of enzyme(3.3 micromolar) present. Structures of the modifying reagents and the resulting reactions are shown in Figure 7.

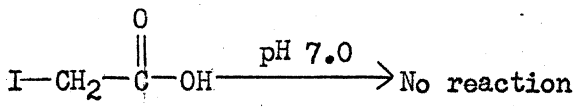
Reaction of the four modifying reagents with the native enzyme at pH 7.0 resulted in appreciable inactivation by the iodoacetamide only, which was chosen for further studies. Reaction of 5,5'-dithiobis-(2-nitrobenzoic acid), or Ellman's reagent, and N-ethyl maleimide with the native enzyme at pH values of 5.0 and 4.8, respectively, gave rapid modification of the enzyme, and N-ethyl maleimide was chosen for further studies. Each reaction was performed using a control sample of enzyme with no modifying reagent present. These results are discussed below, and are illustrated in Figure 8.

Iodoacetate(IAA) and Iodoacetamide(IAM) — The alkylating reagent iodoacetate reacts primarily with sulfhydryl groups, although it also undergoes modifications of both imidazole and amino groups, depending upon the pH and the availability of these groups(81). At pH 7.0 with 2000 nmol(0.7 mM) of iodoacetate, no appreciable reaction occurred with chicken heart mitochondrial malate dehydrogenase. The reaction half-life was 640 minutes, with a rate constant of 1.1×10^{-3} min^{-1}. These results are given in Table X and Figure 8. Gregory similarly reported no enzyme inactivation by iodoacetate with either porcine mitochondrial(56) or

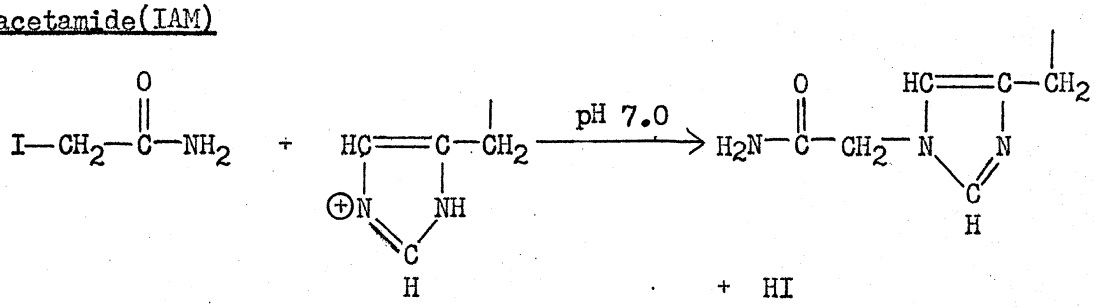
Figure 7: Modification Reagents Used and Their Reactions

The four modification reagents that were used in studying chicken heart mitochondrial MDH are illustrated here. If the reagents reacted with the enzyme, the reaction conditions and products are also shown.

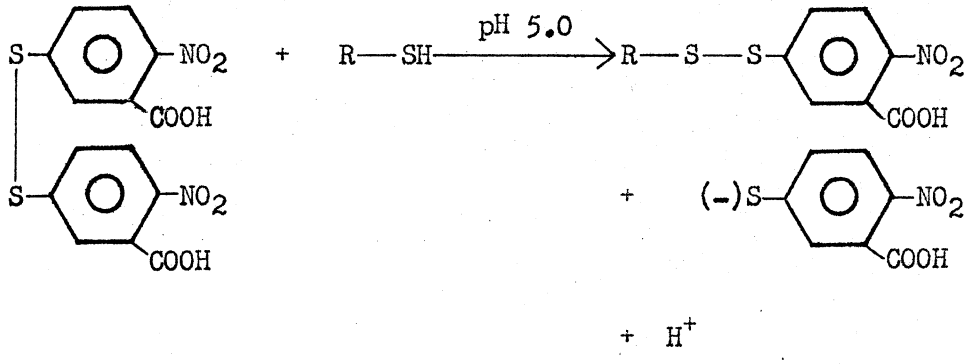
Iodoacetate(IAA)



Iodoacetamide(IAM)



5,5'-dithiobis-(2-nitrobenzoic acid)(DTNB)



N-Ethyl Maleimide(NEM)

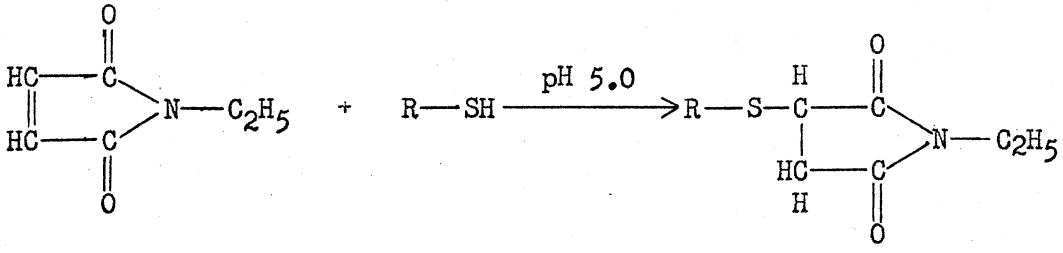


Figure 7

Modification Reagents Used and Their Reactions

Figure 8: Inhibition of Mitochondrial MDH with Modifying Reagents

The modification reactions were performed with 10 nmol of enzyme (3.3 micromolar) in a volume of 3 ml at 25°. The buffers used were 50 mM sodium phosphate, 1 mM EDTA at pH 7.0 or 5.0, and 50 mM sodium acetate, 1 mM EDTA, pH 4.8. Native enzyme was dialyzed in the reaction buffer prior to the modification reaction. All the reactions were allowed to proceed until 95% inhibition had been achieved, except the case of iodoacetate(IAA) reaction, which was continued for over two hours and the results extrapolated. The legend for the graph is as follows: ——○—— iodoacetate(IAA)200 molar excess(0.7 mM) at pH 7.0, ——□—— iodoacetamide(IAM)500 molar excess(1.7 mM) at pH 7.0, ——×—— 5,5'-dithiobis-(2-nitrobenzoic acid)(DTNB)200 molar excess (0.7 mM) at pH 5.0, and ——△—— N-ethyl maleimide(NEM) 1000 molar excess(3.4 mM) at pH 4.8.

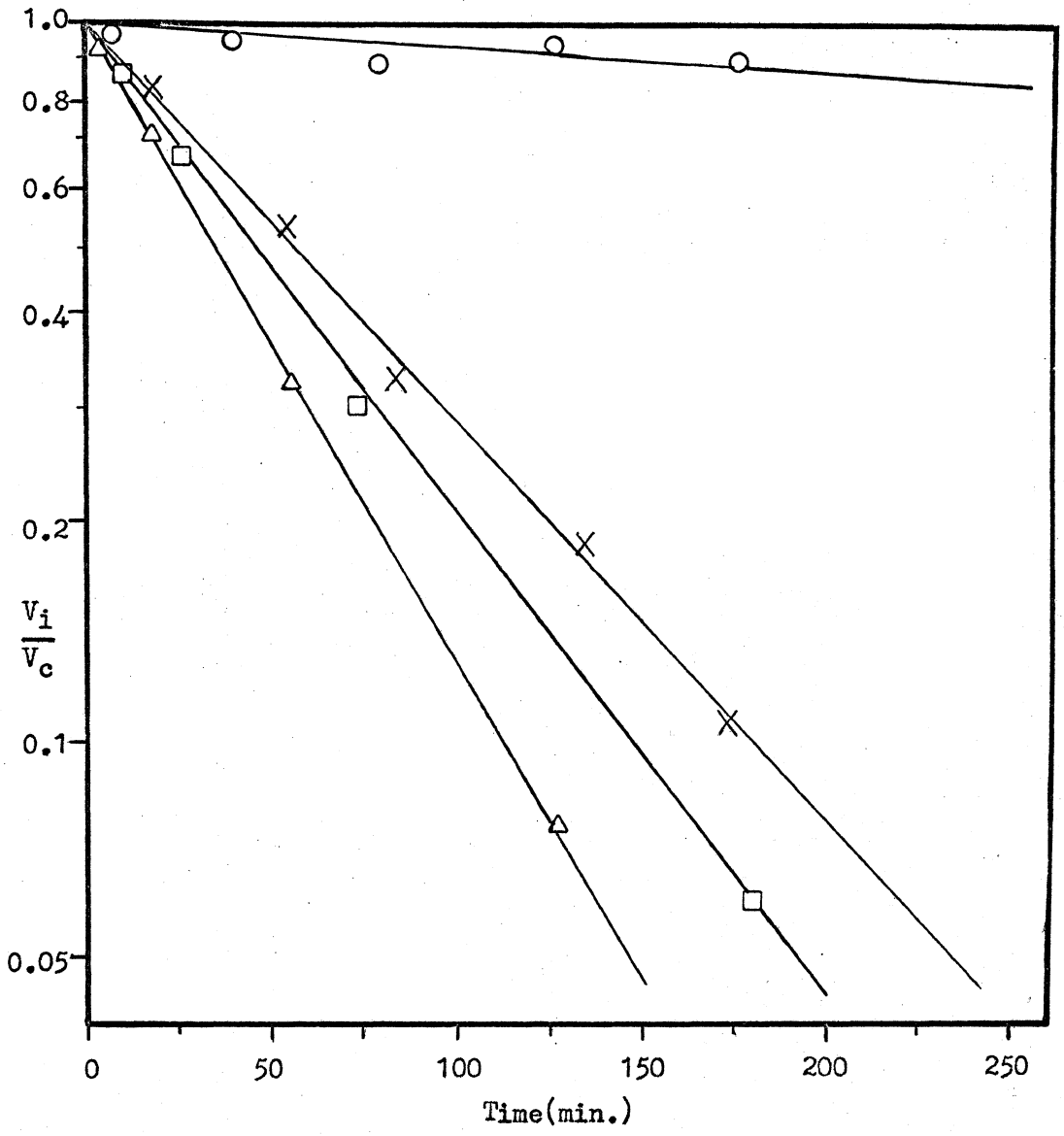


Figure 8

Inhibition of Mitochondrial MDH with Modifying Reagents

Table X: Modification of MDH by Iodoacetate(IAA) and 5,5'-dithiobis-(2-nitrobenzoic acid)(DTNB)

The rates of inactivation by the modifying reagents IAA and DTNB are indicated. Each reaction was carried out with 10 nmol of enzyme (3.3 micromolar) in a volume of 3 ml at 25°. The iodoacetate modification was allowed to proceed for three hours, and the rates were extrapolated to give the half-life of the modification. The DTNB reaction was allowed to continue until 95% inhibition had been achieved. Molar excess refers to the number of moles of modifying reagent present as compared with the number of moles of enzyme present. Some of these data are in Figure 8.

Table X: Modification by Iodoacetate and Ellman's Reagent

Reagent	pH	molar excess	reagent concentration	$t_{\frac{1}{2}}$(min.)
Iodoacetate	7.0	200	0.7 mM	640
DTNB	5.0	200	0.7 mM	49
DTNB	5.0	500	1.7 mM	< 5[a]

[a]Most of modified enzyme precipitated from the solution

bovine mitochondrial(30) malate dehydrogenases.

Iodoacetamide is also an alkylating reagent that modifies the same groups as iodoacetate. Very rapid inactivation resulted at pH 7.0 when 5000 nmol(1.7 mM) of iodoacetamide was present, with a reaction half-life of 42 min., and a rate constant of 0.017 min^{-1}. Studies were undertaken to determine the effects of reagent concentration and pH on the rates of inactivation. These results are summarized in Table XI. A greater than 9-fold difference in the reaction rate was observed between iodoacetamide concentrations of 0.34 mM and 1.7 mM, with reaction half-lives of 317 min. and 35 min., respectively. The latter concentration was used in all further experiments. An increase in reaction rate was observed upon increasing the pH from 6.2 to 7.0. A half-life of 89 minutes was observed for the reaction at pH 6.2, while the reaction half-life decreased to 45 min. for the iodoacetamide reaction at pH 7.0. Conditions of 1.7 mM iodoacetamide at pH 7.0 were chosen for use in all further experimentation.

Studies of substrate and coenzyme protection from modification showed that 10 molar excess(35 micromolar) of NADH slowed the inactivation to a half-life of 710 min., yet similar and greater concentrations of NAD^+, L-malate, and oxalacetate gave very little protection from modification. The results from these studies are given in Table XI and Figures 8-10.

The increased protection from modification found with NADH when compared with NAD^+, and with oxalacetate when compared with L-malate, can be explained by the K_m values that each of these coenzymes or substrates possesses. The increased protection from modification observed

Table XI: Modification of MDH by Iodoacetamide(IAM)

Each reaction, unless otherwise indicated, was carried out with 10 nmol of enzyme(3.3 micromolar) in a volume of 3 ml at 25°. The first set of data indicates the pH profile of the iodoacetamide modification, while the second set illustrates the rate effects of varying both the iodoacetamide and enzyme concentrations at pH 7.0. The last set of data shows the amount of protection afforded by the coenzymes and substrates under conditions of 500 molar excess(1.7 mM) of IAM at pH 7.0. Molar excess in the first two sets of data refers to the modifying reagent, while in the last set it refers to the protecting reagent. Some of these data are graphed in Figures 8-10.

Table XI: Modification of MDH by Iodoacetamide(IAM)

pH	molar excess	reagent concentration	$t_{\frac{1}{2}}$(min.)
6.20	500	1.7 mM	89
6.55	500	1.7 mM	64
6.95	500	1.7 mM	50
7.35	500	1.7 mM	40
7.75	500	1.7 mM	25

pH = 7.0

MDH	enzyme concentration	reagent molar excess	reagent concentration	$t_{\frac{1}{2}}$(min.)
10 nmol	3.3 micromolar	100	0.34 mM	317
10 nmol	3.3 micromolar	200	0.67 mM	113
10 nmol	3.3 micromolar	500	1.70 mM	35
50 nmol	17 micromolar	500	8.40 mM	7.5

500 molar excess IAM(1.7 mM) at pH 7.0 conditions	$t_{\frac{1}{2}}$(min.)
control(no protection)	43
1 molar excess NADH(7 micromolar)	72
5 molar excess NADH(20 micromolar)	208
10 molar excess NADH(37 micromolar)	711
5 molar excess NAD^+(20 micromolar)	52
10 molar excess NAD^+(37 micromolar)	55
50 molar excess NAD^+(170 micromolar)	75
5 molar excess L-malate(20 micromolar)	46
10 molar excess L-malate(37 micromolar)	54
5 molar excess OAA(20 micromolar)	45
10 molar excess OAA(37 micromolar)	53

Figure 9: Iodoacetamide Modification of MDH

The modification reactions were performed with 10 nmol of enzyme (3.3 micromolar) in a volume of 3 ml at 25°. The buffer used was 50 mM sodium phosphate, 1 mM EDTA, pH 7.0. Native enzyme was dialyzed in the reaction buffer prior to the modification reaction. All the reactions were allowed to proceed until the 1.7 mM IAM-treated sample had reached 95% inhibition of catalytic activity. Concentrations of the modifying reagent iodoacetamide(IAM) were as follows: ——○—— 100 molar excess(0.34 mM), ——□—— 200 molar excess(0.7 mM), and ——△——500 molar excess(1.7 mM).

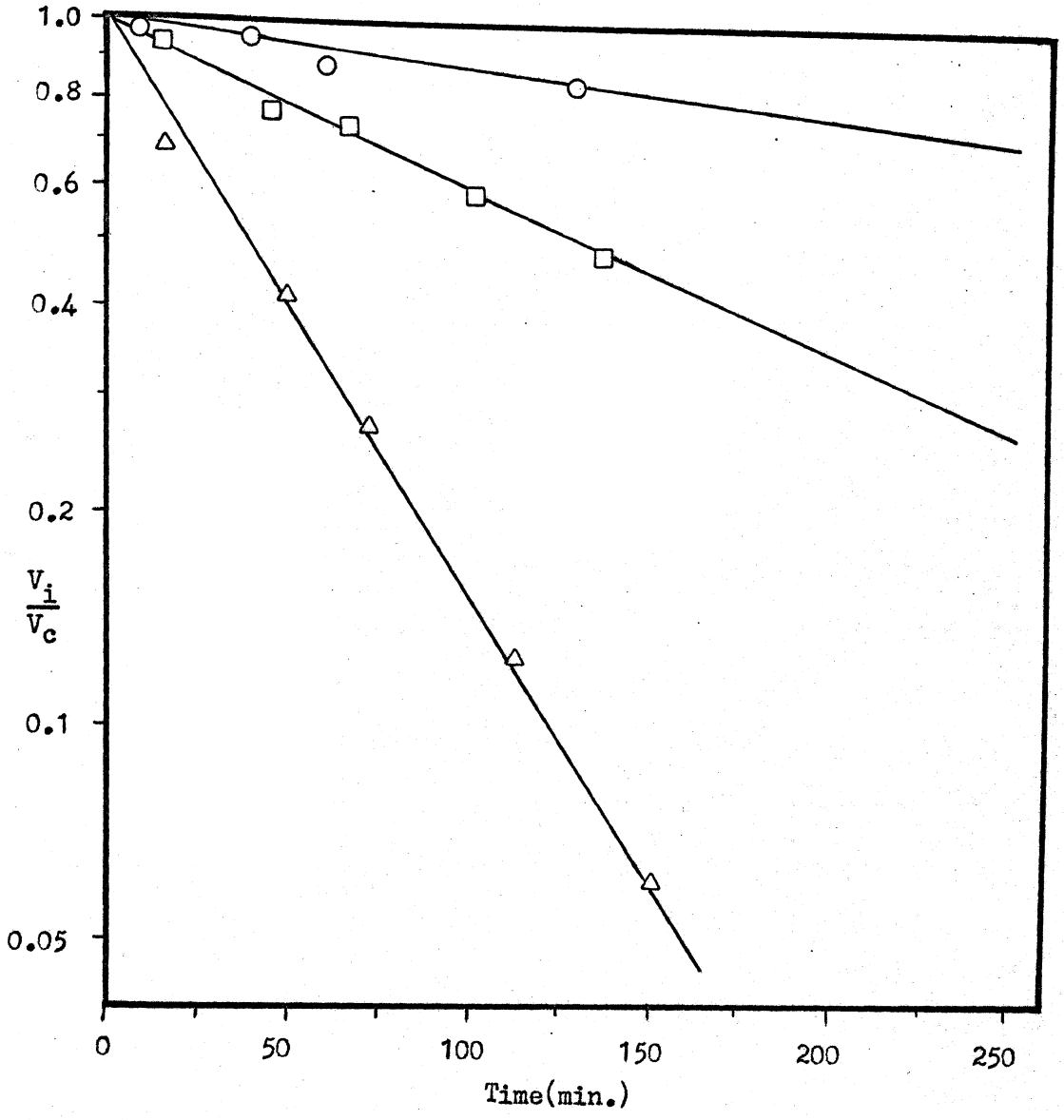


Figure 9

Iodoacetamide Modification of MDH

Figure 10: Protection of MDH from Iodoacetamide Modification

The modification reactions were performed with 10 nmol of enzyme (3.3 micromolar) and 500 molar excess iodoacetamide(IAM)(1.7 mM) in a volume of 3 ml at 25°. The buffer used was 50 mM sodium phosphate, 1 mM EDTA, pH 7.0. Native enzyme was dialyzed in the reaction buffer prior to the modification reaction. Various concentrations of either coenzyme or substrate were added to the reaction mixture to determine their value as protection reagents. All the reactions were allowed to proceed until the positive control(no protecting substances) reached 95% inhibition. Concentrations of the protecting substances were as follows: ——○—— 500 molar excess(1.7 mM) iodoacetamide with no protecting substances, ——□—— IAM + 10 molar excess(35 micromola NADH, ——✕—— IAM + 50 molar excess(170 micromolar) NAD^+, ——●—— IAM + 10 molar excess(35 micromolar) L-malate, and ——△—— IAM + 10 molar excess(35 micromolar) oxalacetate.

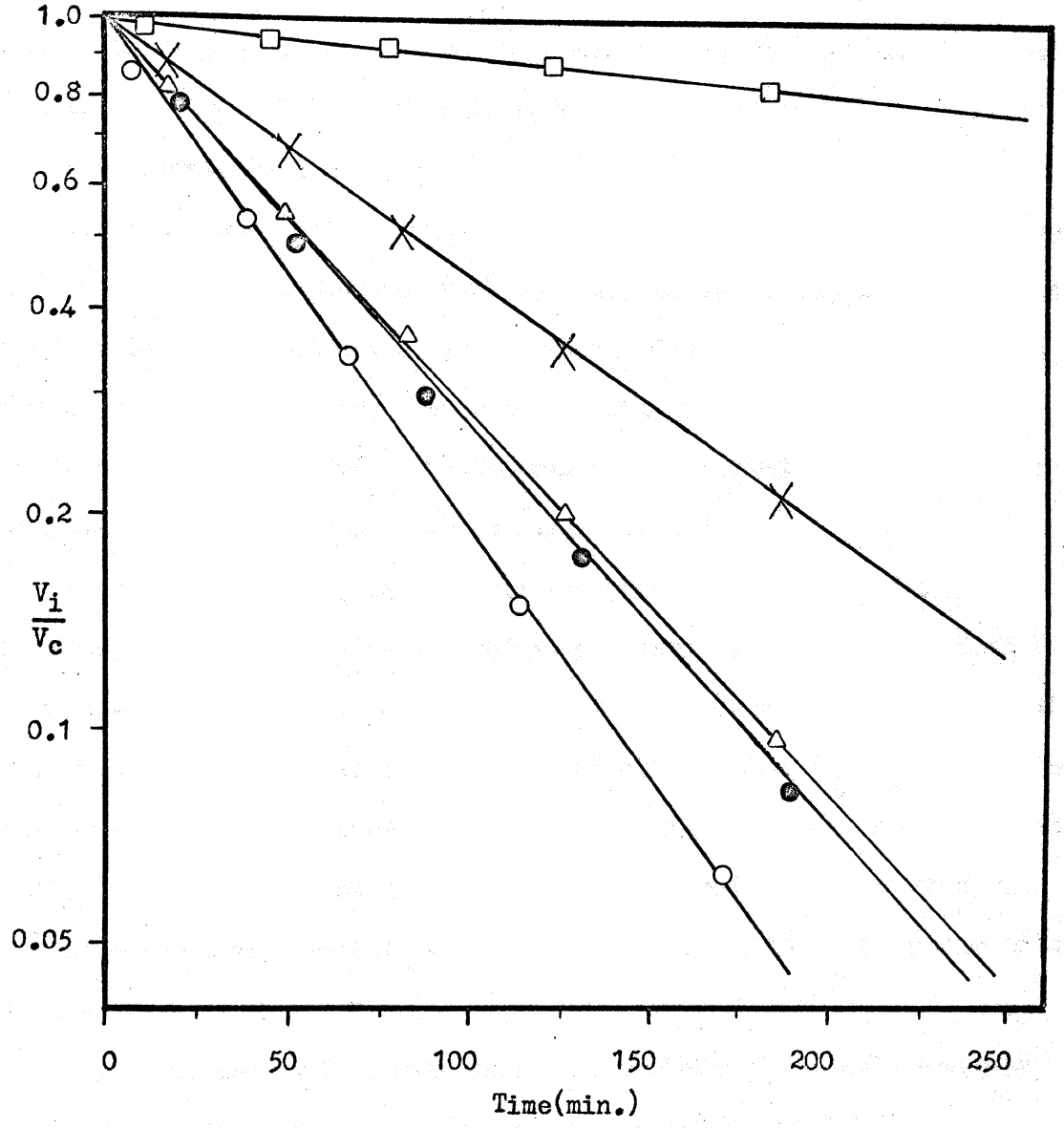


Figure 10

Protection of MDH from Iodoacetamide Modification

with the coenzymes when compared with the substrates corresponds to the ordered binding mechanism for dehydrogenases, in which the coenzyme must bind to the enzyme before the substrate.

Less iodoacetamide(0.7 mM) was required to give the same rate of inactivation, which was a half-life of around 45 min., with both porcine (73) and bovine mitochondrial MDH(30), but the concentration of coenzyme required for full protection was the same. Gregory was able to achieve partial protection of the porcine enzyme with the use of high concentrations of substrate, with the minimum concentrations being 117 mM for L-malate and greater than 16 mM for oxalacetate(73).

Ellman's method(72) was used to quantify the cysteine content of the NADH-protected and IAM-modified enzyme samples. The results indicated that no -SH groups had reacted with the reagent iodoacetamide. No sulfhydryl groups were found to react with iodoacetamide in either the porcine (73) or bovine(30) mitochondrial isozymes. Values of the number of free sulfhydryl groups present in the native enzyme would indicate that no disulfide bonds are present in the chicken isozyme, as is also true with the bovine species.

Molecular weight determinations on a Sephadex G-150 column showed the iodoacetamide-modified enzyme to have an apparent molecular weight of 60,000 gm/mol. The sedimentation velocity experiments with both the modified enzyme and protected samples indicated that both species remained in the dimeric state. Iodoacetamide-modified species of both the porcine and bovine mitochondrial enzymes similarly remained as dimers. Sedimentation velocity experiments of the protected chicken enzyme indicate that it possessed a tighter subunit configuration than the modified enzyme.

These data are summarized in Table XII.

Two moles of carbon-14 label from iodo-[C^{14}-1]acetamide were bound per mole of enzyme. Amino acid analysis of the modified enzyme showed the appearance of two carboxymethylhistidine residues per mol of enzyme, and a concurrent decrease of two histidine residues per mol of enzyme. Since it has been documented that there are two active sites per chicken heart mitochondrial MDH dimer(11), we can argue that two histidine residues have been modified per mole of enzyme. The location of these histidine residues is probably in the active sites, with one residue being in each active site. Analysis of NADH-protected enzyme indicated no radioactive incorporation and the absence of any carboxymethylhistidine residues. The results for these studies are also found in Table XII. Gregory has reported iodoacetamide-modification of a histidyl residue in each active site for both the porcine and bovine mitochondrial isozymes(56, 30).

The reaction of the enzyme with iodoacetamide and not iodoacetate is not what one would predict. Both reagents normally alkylate histidine or cysteine. The $-NH_2$ group of the amide is only slightly better as an electron donating group than the -OH moiety of the carboxyl group, and this minute difference does not explain the tremendous difference in reactivity of the two reagents with respect to the enzyme. Steric effects could also not explain the difference rates, since both reagents are approximately the same size. The only observable difference between the two molecules is that at neutral pH, the carboxylate group is negatively charged, while the amide group is neutral. The presence of a negatively charged residue in the proximity of the active site histidyl

Table XII: Properties of the Iodoacetamide-modified Enzyme

The table summarizes the properties of iodoacetamide-modified MDH. Each reaction was performed at pH 7.0 in 3 ml volume at 25°. Reactions were performed with 10 nmol enzyme(3.3 micromolar) and 500 molar excess of IAM(1.7 mM), while the protected samples also contained 10 molar excess of NADH(37 micromolar). Reactions analyzed with Ellman's reagent contained a 2.5-fold increase in enzyme and reagents, while amino acid analyzed samples contained a 5-fold increase in enzyme and reagents. The number of free sulfhydryl groups was determined with Ellman's reagent. The quantity of histidine, carboxymethylhistidine, and methionine residues was determined using the amino acid analyzer. The determination of incorporated reagent was performed with a mixture of labeled and unlabeled IAM using scintillation counting as described in the "Experimental Procedure" section. The molecular weights of the samples were determined by the use of a calibrated Sephadex G-150 column.

Table XII: Properties of Iodoacetamide-modified Enzyme

Property	Native MDH	IAM-modified MDH	NADH-protected MDH	Difference
Free sulfhydryl groups	15	15	15	0
Activity	100%	5%	100%	95%
Histidines	12	10	12	2
Carboxymethylhistidines	0	2	0	2
Methionines	14	14	14	0
Mol C^{14}-IAM/mol enzyme	0	2	0	2
Molecular weight	66,000	60,000	66,000	-

group could prevent the iodoacetate anion from approaching the histidine residue, but would have no effect upon the neutral amide group. The presence of an anionic group in the substrate binding site has been suggested by crystallographic studies on porcine cytoplasmic MDH(36), indicating a possible molecular basis for this theory of charge repulsion.

5,5'-Dithiobis-(2-nitrobenzoic acid)(DTNB) and N-Ethyl Maleimide (NEM) — Ellman's reagent, 5,5'-dithiobis-(2-nitrobenzoic acid), combines with free sulfhydryl groups to form a thionitrobenzoate-substituted protein. One mole of thionitrobenzoate anion is released for each mole of -SH group modified. This anion possesses a strong absorbance at 412 nm, and can be used to quantify the number of sulfhydryl groups modified(81).

Ellman's reagent gave no apparent reaction with the chicken isozyme at pH 7.0, based on catalytic activity. However, it combined very rapidly with native mitochondrial MDH at pH 5.0. After 20 minutes incubation of 10 nmol(3.3 micromolar) of enzyme with 5000 nmol(1.7 mM) of DTNB, the protein precipitated from the solution, indicating a very rapid rate of reaction. Modification of nearly all sulfhydryl groups of the enzyme may have occurred under these conditions, or at least enough of the -SH groups to result in the modified enzyme precipitating out of solution. A rapid reaction apparently occurred, in which modification of one sulfhydryl group may have brought about a conformational change that exposed one or more unmodified -SH groups, although no data was available as to whether this actually occurred. It is uncertain as to the number of sulfhydryl groups that were actually

modified by Ellman's reagent under these conditions. The data for these modifications are shown in Table X and Figure 8.

The reaction of Ellman's reagent with free sulfhydryl groups of proteins normally occurrs at pH 7.0 and above. This is due to the pK' of the sulfhydryl group being approximately 8.3. At more basic pH values, the sulfhydryl anion is present and reacts with DTNB more rapidly than does the protonated form. However, this reagent could not react with the chicken enzyme until either the dimer had dissociated or a conformational change had resulted from the lower pH conditions.

Sulfhydryl groups add across the double bond of N-ethyl maleimide in a manner quite like the Michael reaction. This modifying reagent could not react with the dimeric enzyme, but did react when the enzyme was dissociated(see Table XV). Even though the mercaptide ion mentioned as the nucleophile for these two modifications does not normally exist to a significant degree at pH 5.0 and below, the presence of strong hydrogen bonding from the solvent and with near-by residues may have given the thiol group enough anionic character to elicit the reaction. The protonated form of sulfhydryl groups normally do not possess enough nucleophilic character to elicit such a reaction.

Although the reaction of 1.7 mM N-ethyl maleimide with chicken heart mitochondrial MDH at pH 7.0 and 6.0 was negligible, rapid inactivation occurred upon dissociation of the dimer at pH 5.0 and below. With a concentration of 3.4 mM N-ethyl maleimide, modification took place over twice as fast at pH 4.8 than at pH 5.0. The half-life at pH 4.8 was 32 min., while at pH 5.0 the half-life was 68 min. Rate constants were 2.2×10^{-2} min^{-1} and 1.0×10^{-2} min^{-1}, respectively.

The addition of 10 molar excess(35 micromolar) of NADH offered nearly full protection of the enzyme from inactivation. These data are shown in Figures 8, 11-13.

The dependence of the inactivation rate upon N-ethyl maleimide concentration was tested to determine the effects of reagent concentration upon inactivation kinetics at pH 4.8. Reagent concentrations of 340 micromolar gave a half-life of 115 min., whereas concentrations of 3.4 mM N-ethyl maleimide gave half-lives of 32 min. . Nearly a 4-fold difference in reaction rates was observed for these two concentrations of modifying reagent. The latter concentration of reagent at pH 4.8 was chosen for all further experiments involving N-ethyl maleimide.

An equivalent rate of inactivation observed at pH 4.8 with the chicken isozyme could be observed with half the reagent concentration (1.7 mM) and at a higher pH of 5.0 for both the porcine(73) and bovine (30) mitochondrial isozymes. The chicken enzyme was fully protected from both N-ethyl maleimide and iodoacetamide inactivation by a 10 molar excess (35 micromolar) of NADH. This concentration of NADH was also used to protect the bovine mitochondrial and porcine mitochondrial MDH from modification by N-ethyl maleimide and iodoacetamide(30, 73).

Estimates of molecular weight by chromatography on a calibrated Sephadex G-150 column indicated that the N-ethyl maleimide-modified enzyme was dissociated into 33,000 gm/mol monomers at pH 4.8. Sedimentation velocity experiments indicated that MDH that had been modified by N-ethyl maleimide at pH 5.0 remained in the monomeric state even when the pH was increased to 7.0. If a 5 molar excess(18 micromolar) of NADH was then added to the modified enzyme at pH 7.0, the chicken isozyme

Figure 11: Effects of pH on Modification of MDH by N-Ethyl Maleimide

The modification reactions were performed with 10 nmol of enzyme (3.3 micromolar) in a volume of 3 ml at 25°. The buffers used were 50 mM sodium phosphate, 1 mM EDTA at pH 7.0, 6.0, and 5.0, and 50 mM sodium acetate, 1 mM EDTA, pH 4.8. Native enzyme was dialyzed in the reaction buffer prior to the modification reaction. The reactions at pH 4.8 and pH 5.0 were allowed to proceed until 95% inhibition had be achieved, while the other two reactions continued for slightly over t hours, and the results extrapolated. Concentrations of the N-ethyl maleimide(NEM) were as follows: ——○—— 500 molar excess(1.7 mM at pH 7.0, ——□——1000 molar excess(3.4 mM) at pH 6.0,——●— 1000 molar excess(3.4 mM) at pH 5.0, and ——△——1000 molar exce (3.4 mM) at pH 4.8.

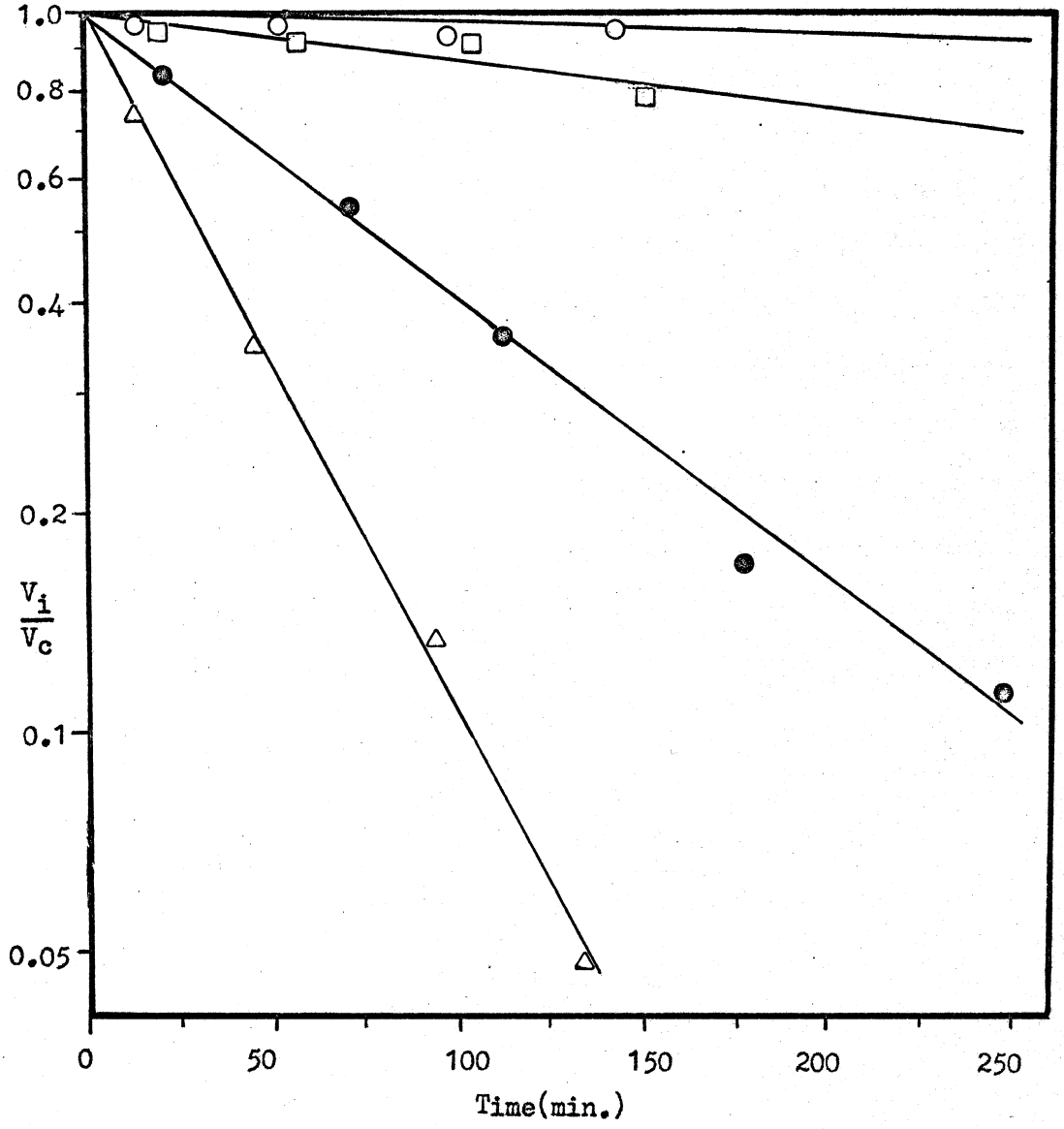


Figure 11

Effect of pH on Modification of MDH by N-Ethyl Maleimide

Figure 12: Effects of Reagent Concentration on Modification of MDH by N-Ethyl Maleimide

The modification reactions were performed with 10 nmol of enzyme (3.3 micromolar) in a volume of 3 ml at 25°. The buffer used was 50 mM sodium acetate, 1 mM EDTA, pH 4.8. Native enzyme was dialyzed i the reaction buffer prior to the modification reaction. All the reactions were allowed to proceed until the 1000 molar excess sample h reached 95% inhibition. Concentrations of the N-ethyl maleimide were follows: ——○—— 100 molar excess(0.34 mM), ——□—— 200 mol excess(0.7 mM), ——◎—— 500 molar excess(1.7 mM), and ——△—— 1000 molar excess(3.4 mM).

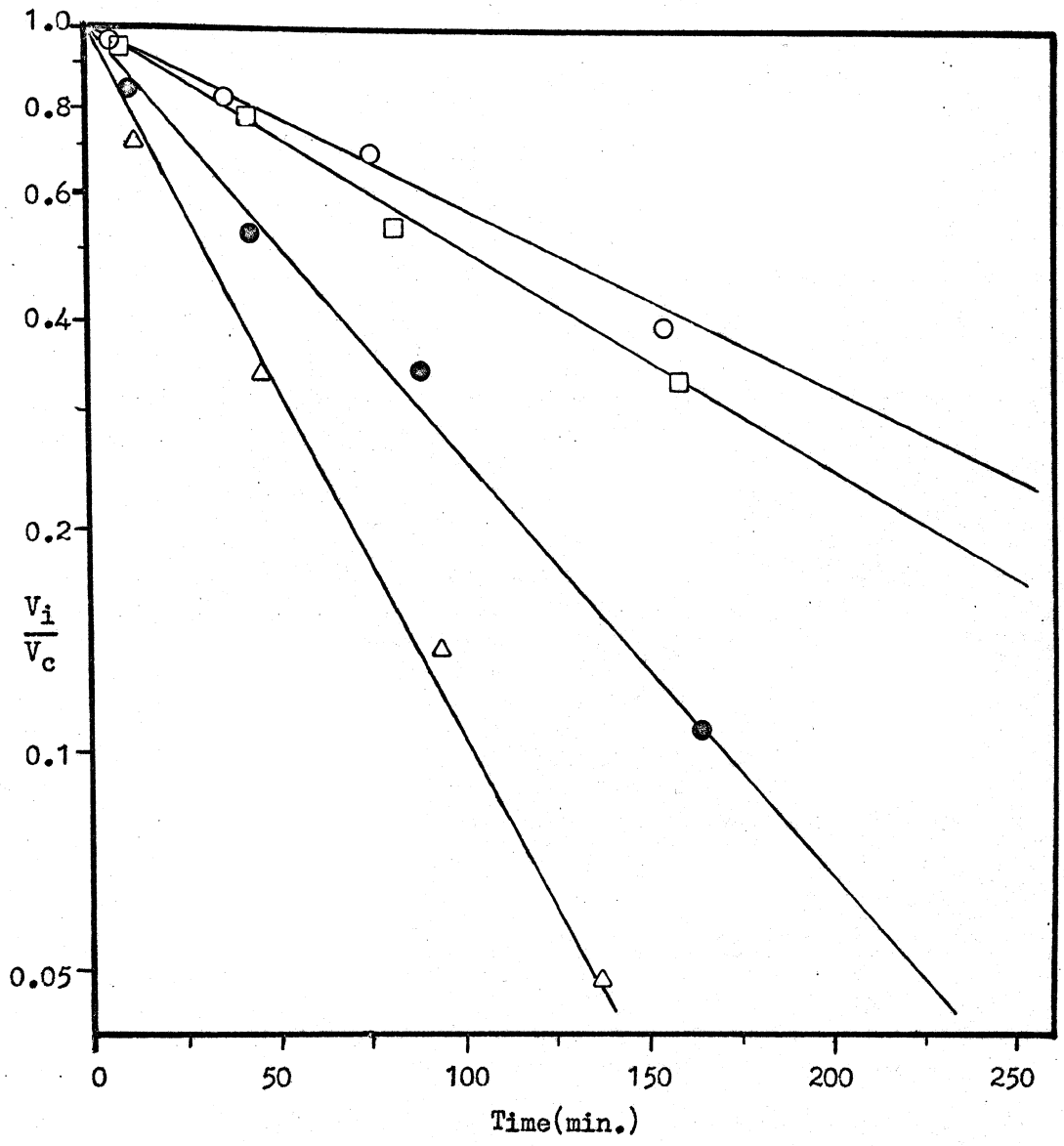


Figure 12

Effects of Reagent Concentration on

Modification of MDH by N-Ethyl Maleimide

Figure 13: Protection of MDH from Modification by N-Ethyl Maleimide

The modification reactions were performed with 10 nmol of enzyme (3.3 micromolar) and 1000 molar excess of N-ethyl maleimide(NEM)(3.4 mM in a volume of 3 ml at 25°. The buffer used was 50 mM sodium acetate, 1 mM EDTA, pH 4.8. Native enzyme was dialyzed in the reaction buffer prior to the modification reaction. Various concentrations of NADH were added to the reaction mixtures to determine the value of the coenzyme as a protection reagent. The concentrations of the coenzyme were as follows: ——○—— 1000 molar excess(3.4 mM) NEM with no protecting substances, ——□—— NEM + 1 molar excess(7 micromolar) NADH, ——●—— NEM + 5 molar excess(20 micromolar) NADH, and ——△—— NEM + 10 molar excess(37 micromolar) NADH. All of the reactions were allowed to proceed until the modified enzyme at pH 4.8, at which the enzyme was entirely in the monomeric state, was 95% inhibited. Even though the NADH-protected samples exhibited increased initial rates of activity, with over a 4-fold increase in the 10 molar excess sample when compared with the unprotected sample, this graph does not reflect this difference in activity because each sample posses a separate control that was deficient in modifying reagent but possesse the same amount of enzyme and NADH.

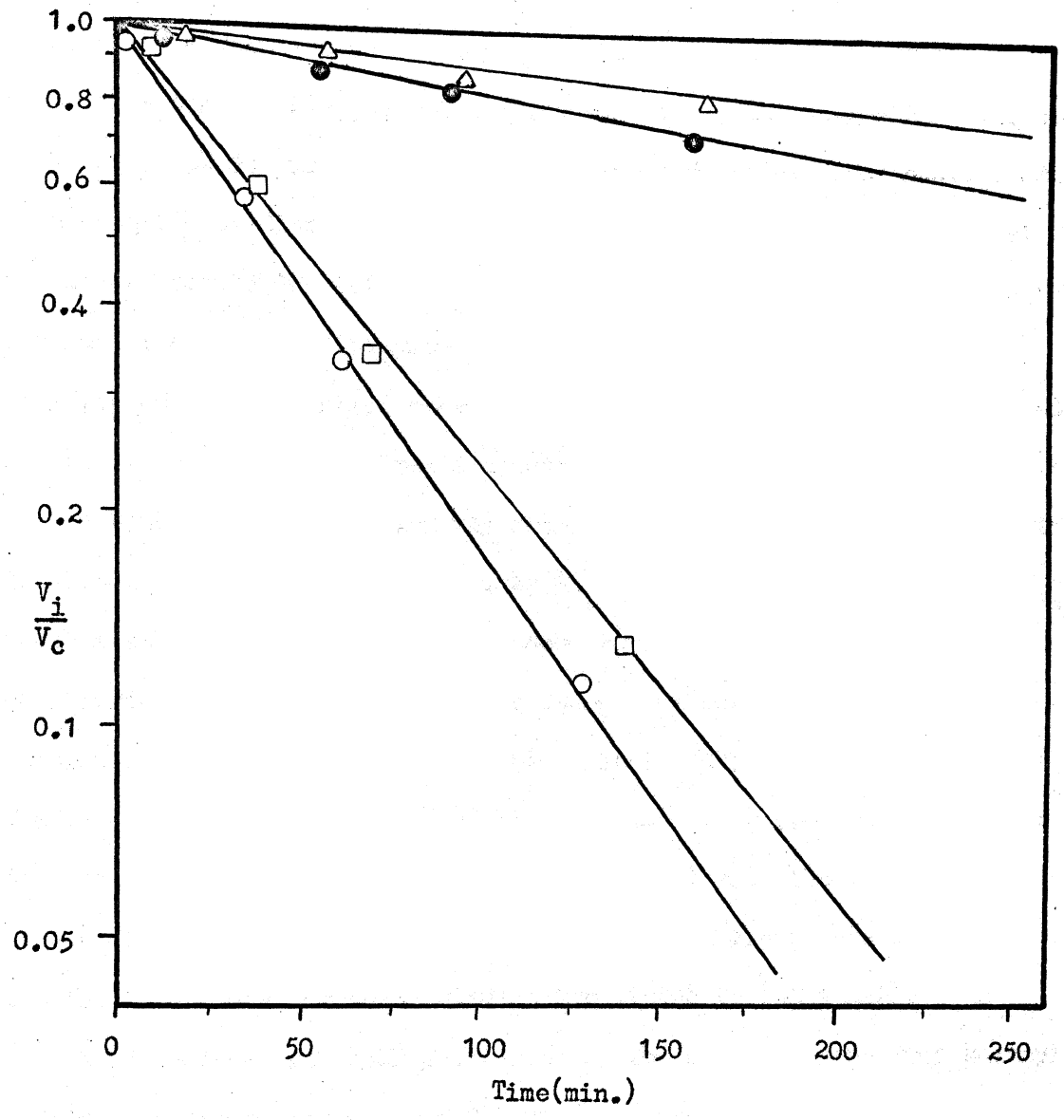


Figure 13

Protection of MDH from Modification by N-Ethyl Maleimide

reassociated into the dimer. Enzyme that had been modified at pH 4.8, followed by a decrease in acidity to pH 7.0 and subsequent addition of an equal amount of NADH, would not reassociate to the degree that enzyme modified at pH 5.0 would. Both the porcine and bovine mitochondrial MDHs were observed to dissociate into monomers upon modification by N-ethyl maleimide, as was the chicken isozyme. Once modified, the bovine and porcine mitochondrial isozymes would reassociate only in the presence of large concentrations(90 micromolar) of NADH(75), whereas the modified chicken isozyme only required a concentration of 35 micromolar NADH.

N-ethyl maleimide apparently reacted at a reasonable rate only with the monomeric form of the enzyme, as did Ellman's reagent. At pH 5.0, N-ethyl maleimide reacted with the monomer and apparently shifted the equilibrium in the direction of the monomer, since the modified enzyme existed entirely in the monomeric state in the absence of coenzyme. The rate of inactivation by N-ethyl maleimide at pH 4.8 was much faster than at pH 5.0. This observation is consistent with the model of the enzyme dissociating into monomers before the -SH groups are exposed to the solvent. Data for these studies are listed in Table XIII.

Studies involving NADH protection of the native enzyme from N-ethyl maleimide modification at pH 5.0 and pH 4.8 revealed that increased concentrations of NADH resulted in higher initial rates of enzymatic activity, with nearly a 4-fold difference between the unprotected sample and the 10 molar excess(35 micromolar) NADH sample at pH 4.8. Gregory has also observed these differences in enzymatic activity in modification studies of the bovine mitochondrial isozyme at low pH[1]. Since sedimentation velocity experiments(see Table XV) indicated that

Table XIII: Properties of N-Ethyl Maleimide Modified MDH

The molecular weight of the modified enzyme was determined using a calibrated Sephadex G-150 column at pH 7.0. The modified enzyme was obtained using 10 nmol(3.3 micromolar) of enzyme at pH 5.0 with 1000 molar excess(3.4 mM) NEM in 3 ml at 25°.

The sedimentation velocity samples were obtained using 40 nmol (13 micromolar) of enzyme at either pH 5.0 or 4.8 with 1000 molar excess (13.4 mM) NEM in 3 ml at 25°. The modified enzyme was then redialyzed to pH 7.0, whereupon the coenzyme was then added to determine its reassociation properties with respect to the modified enzyme. The protected sample had a 10 molar excess(150 micromolar) of NADH. The sedimentation coefficient for the native enzyme at pH 7.0 is 4.2-4.3.

Table XIII: Properties of N-Ethyl Maleimide-modified Enzyme

Molecular Weight by Sephadex G-150 column at pH 7.0 --- 33,000 gm/mol (monomer)

Sedimentation Velocity

pH of reaction	reassociation reagent	reassociation reagent concentration	value
5.0	None	0 micromolar	2.81
5.0	5 molar excess NADH	240 micromolar	3.80
4.8	1 molar excess NADH	80 micromolar	2.93
4.8	5 molar excess NADH	240 micromolar	3.23

Enzyme protected with NADH at pH 5.0 had a value of 4.35s after increasing the pH to 7.0.

excess coenzyme could reassociate the native enzyme into dimers at low pH, these protection studies may indicate that the monomer is much less active, if active at all, than the dimer state.

Assays for modified sulfhydryl groups by Ellman's method(72) indicated that the number of -SH groups that reacted with N-ethyl maleimide depended both on the relative concentrations of enzyme and reagent, and also on the pH. If 25 nmol of enzyme(9 micromolar) and 4.2 mM N-ethyl maleimide were allowed to react at pH 5.0, a total of six -SH groups were modified. The same reaction at pH 4.8 resulted in the modification of 11 sulfhydryl groups. By increasing the enzyme and reagent concentrations 2.5-fold at pH 5.0, the number of sulfhydryl groups reacting increased to at least 13, with most of the modified protein precipitating out of solution. Final data are shown in Table XIV. The number of groups modified increased with either a lowering of the pH or an increase in the enzyme and reagent concentrations. Reaction of one -SH group may have caused a conformational change that exposed one or more previously hidden groups, making them available for subsequent modification. The essential sulfhydryl groups are apparently among the six groups modified during the first set of conditions described above, and the actual number of essential sulfhydryl groups is probably less than six. However, there are no data to support these hypotheses.

Gregory reported a single essential sulfhydryl residue modified with N-ethyl maleimide per active site in both the porcine mitochondrial isozyme(73), which contains two active sites, and bovine mitochondrial MDH (30), which contains one active site per dimer. None of the essential sulfhydryl groups in the chicken isozyme, which may be as many as six,

Table XIV: Ellman's Assays of N-Ethyl Modified MDH

The reaction volume was 3 ml at 25°. The effects of varying both the pH and the enzyme and reagent concentrations on the number of -SH groups modified were studied. The concentrations used are shown in the table. Enzyme protected with 10 molar excess(90 micromolar) of NADH at both pH 4.8 and 5.0 showed no modified sulfhydryl groups.

Table XIV: Ellman's Assays of N-Ethyl Maleimide-modified Enzyme

MDH	MDH concentration	molar excess NEM	reagent concentration	pH	unmodified -SH groups	modified -SH groups
25 nmol	8 micromolar	0	0 mM	5.0	15	0
25 nmol	8 micromolar	0	0 mM	4.8	15	0
25 nmol	8 micromolar	500	4.2 mM	5.0	9	6
25 nmol	8 micromolar	500	4.2 mM	4.8	4	11
60 nmol	20 micromolar	1000	20 mM	5.0	2	13[a]

[a]Modified enzyme precipitated from solution

Enzyme protected with NADH at both pH 5.0 and 4.8 had 15 unmodified sulfhydryl groups.

do not appear to be located at or near the active site, but are probably exposed by the dissociation of the enzyme into monomers. Alterations in the quaternary structure are clearly implicated upon modification with either Ellman's reagent or N-ethyl maleimide. Since the coenzyme NADH has been shown to reassociate the dissociated enzyme into the active dimeric state, the ability of coenzyme to completely prevent any modification of the enzyme by N-ethyl maleimide indicated that the initial modification of a sulfhydryl group probably occurred at the subunit interface. Since the modified enzyme could not readily be reassociated by the presence of coenzyme, the modified sulfhydryl groups probably were more closely associated with maintaining an enzymatic structure that was catalyticly active than with the actual catalytic process itself.

The type of modification by N-ethyl maleimide is obviously very different for the chicken isozyme when compared with the porcine and bovine systems, since the modification is non-specific with respect to the essential residues, and prevents complete reassociation by coenzyme into the complete dimeric form, with the amount of reassociation depending upon the number of groups modified.

Subunit Dissociation and Reassociation

Molecular weight determinations and ultracentrifugation studies are two of the more common methods for determining the dissociation and reassociation of subunits in multimeric molecules. Most of the studies to determine monomer-dimer transitions in the native enzyme under various conditions were performed with sedimentation velocity experiments, and the results are given in Table XV. An additional listing

Table XV: Sedimentation Coefficients of Chicken Heart Mitochondrial MDH

The enzyme sample was subjected to centrifugation at 59,780 rpm for 60 minutes at 4°. Sedimentation coefficients were determined from the time-dependent migration of the enzyme in the ultracentrifugal field. Each sample contained 40 micromolar concentrations of native enzyme, and the buffer used was as indicated. The data are arranged to indicate the effects on sedimentation coefficients of various pHs, various coenzyme concentrations, and different buffers. Molar excess refers to the number of moles of coenzyme present in the sample compared to the number of moles of the enzyme.

Table XV: Sedimentation Coefficients of Chicken Mitochondrial MDH

Sample	Value
50 mM phosphate, pH 7.0	4.12, 4.22, 4.20
50 mM phosphate, pH 5.5	4.16
50 mM phosphate, pH 5.0	4.00, 3.75, 3.54
50 mM phosphate, pH 4.8	2.91, 2.79 (monomer)
50 mM phosphate, pH 4.6	2.83 (monomer)
50 mM phosphate, pH 5.0	
1 molar excess NADH(80 micromolar)	4.16
5 molar excess NADH(240 micromolar)	4.48
10 molar excess NADH(440 micromolar)	4.47
10 molar excess NAD^+(440 micromolar)	4.47
50 molar excess NAD^+(2.0 mM)	4.51
50 mM phosphate, pH 4.8	
1 molar excess NADH(80 micromolar)	3.61
5 molar excess NADH(240 micromolar)	4.37
10 molar excess NADH(440 micromolar)	4.46
10 molar excess NAD^+(440 micromolar)	3.19
50 molar excess NAD^+(2.0 mM)	3.58
25 mM phosphate, pH 7.0	4.39
25 mM Tris, pH 7.0	4.43
25 mM Tris + 100 mM phosphate, pH 7.0	3.75

for comparison with values reported for other native forms of malate dehydrogenase can be found in Table XVI. Studies of subunit dissociation and reassociation in the chemically modified enzyme were discussed in their respective sections in "Chemical Modification". Unless otherwise stated, all experiments were performed in 50 mM sodium phosphate, 1 mM EDTA when the pH was 5.0 or above, and 50 mM sodium acetate, 1 mM EDTA when the pH was below 5.0.

Effect of enzyme concentration -- The lack of dissociation in the chicken isozyme upon dilution to 0.2 micromolar has previously been discussed in the "Physical and Chemical Characteristics" section.

Effect of pH -- The effects of pH on the quaternary structure of chicken heart mitochondrial MDH were evaluated by determining molecular weight or sedimentation velocity as a function of pH. The results are summarized in Tables IV and XV. These techniques indicate that the chicken isozyme existed as a dimer at pH 7.0. The s value of 4.2 for the native enzyme agrees closely with the value of 4.3 reported by Kitto and Kaplan(11). Between pH 7.0 and pH 5.5, the chicken isozyme remained in a dimeric state, but dissociated into the monomer at pH 4.8 and below. Chicken heart mitochondrial MDH gave molecular weight and sedimentation coefficient values at pH 5.0 that were intermediate between those of the dimer and monomer. An apparent equilibrium existed between the monomer and dimer forms in the chicken isozyme at pH 5.0, which shifted completely to the monomer upon an increase in acidity to pH 4.8.

Porcine mitochondrial, porcine cytoplasmic, and bovine mitochondrial MDH all existed as dimers at pH 7.0, as did the chicken heart enzyme. Both the porcine mitochondrial and bovine mitochondrial isozymes

Table XVI: Sedimentation Coefficients of Various Malate Dehydrogenases

The sedimentation coefficient for the native chicken enzyme was obtained in a manner described elsewhere. All of the other values have been reported in the literature, and were obtained in a manner similar to that described in the "Experimental Procedure" section.

Table XVI: Sedimentation Coefficients of Various Malate Dehydrogenases

Source of enzyme	Sedimentation Coefficient(pH 7.0)
Chicken mitochondrial	4.2
Porcine mitochondrial(75)	4.2
Bovine mitochondrial(75)	4.4
Porcine cytoplasmic(75)	4.4
Bovine cytoplasmic(38)	4.6
Chicken cytoplasmic(11)	4.3
E. coli(74)	4.4
B. subtilis(74)	6.7

dissociated into monomers upon lowering the pH to 5.0, while the porcine cytoplasmic form remained in the dimeric state at this pH. Although the chicken isozyme required a slightly lower pH of 4.8 before converting to the monomeric state, the similarity of the chicken mitochondrial isozyme was much greater with the porcine and bovine mitochondrial MDH, in this respect, than with the porcine cytoplasmic form, which did not dissociate into monomers under any of the conditions reported.

Effect of pyridine nucleotides -- The effects of coenzyme on the quaternary structure of chicken heart MDH were evaluated by determining sedimentation velocity as a function of coenzyme concentration. Various concentrations of NADH and NAD^+ caused partial or complete reassociation of the monomers into the dimeric state at pH 5.0 and pH 4.8. Since the affinity of the enzyme for NADH was higher than for NAD^+, smaller quantities of the NADH moiety were required to achieve the dimeric state under these conditions. Concentrations of 80 micromolar and 440 micromolar of NADH and NAD^+, respectively, were required to elicit complete reassociation of the chicken enzyme to the dimer at pH 5.0, when the enzyme was present at a concentration of 40 micromolar. The porcine mitochondrial and bovine mitochondrial isozymes, when present at the same concentration as the chicken isozyme, could also be reassociated at pH 5.0, but required slightly higher concentrations of coenzyme, 240 micromolar NADH and 4.0 mM NAD^+(75).

The fact that NADH binding favored reassociation of subunits is in direct contrast to observations made by Shore and Chakrabarti(82). They reported dissimilar effects for the reduced and oxidized coenzyme forms, with the NADH causing dissociation of the enzyme.

Larger quantities of NADH and NAD^+ were required to bring about the reassociated state at pH 4.8 than at pH 5.0 in the chicken isozyme. For chicken heart mitochondrial MDH at a concentration of 40 micromolar, NADH was required at a concentration of 240 micromolar, while the amount of NAD^+ needed was some quantity much larger than 2.0 mM, probably in the neighborhood of 4.0 mM. Those quantities of coenzyme correspond almost exactly to those required to reassociate the monomers of porcine and bovine mitochondrial MDH at pH 5.0. Since the chicken enzyme has completely dissociated into monomers at pH 4.8, this would indicate that the chicken isozyme required the same amount of coenzyme to reassociate complete monomer to complete dimer as does the other two isozymes.

Apparently, the amount of oxidized coenzyme required to elicit the dimer at pH 4.8 compared to pH 5.0 is disproportionally larger than the amount of NADH required. This could be due to a simple binding effect.

SUMMARY

A composite of the subunit interactions of the chicken isozyme in the native and modified forms is given in Figure 14. This should be compared with the composite diagram for the porcine and bovine mitochondrial isozymes, shown in Figure 15. The main difference between the two schemes is the absence of dissociation at high dilution values, the monomer-dimer equilibrium state at pH 5.0, and the failure of the NEM-modified enzyme to completely reassociate into the dimer when coenzyme is present in the chicken isozyme.

The porcine, bovine, and chicken heart mitochondrial isozymes all existed as dimers at pH 7.0 at concentrations of 30 micromolar. When the enzyme was diluted to 0.2 micromolar, only the porcine enzyme dissociated into monomers, while the bovine and chicken isozymes remained as dimers. This dissociation of the porcine MDH could be reversed by the addition of NADH to the enzyme solution.

Upon lowering of the pH of the enzyme to 5.0, the porcine and bovine enzymes dissociated into monomers. The chicken isozyme existed in a monomer-dimer equilibrium at pH 5.0, and required a more acidic pH of 4.8 before being completely converted to the monomeric state. Either the addition of coenzyme or an increase in the pH to 7.0 reassociated all three enzyme forms into dimers.

Iodoacetamide reacted with all three isozymes at pH 7.0 to give modified products. The modifying reagent converted a histidine residue in each active site to a carboxymethylhistidine residue. The product in each case was an inactive dimer. Modification of the isozymes by iodoacetamide could be prevented by a ten to one ratio of NADH to enzyme.

Figure 14: Subunit Interactions of Chicken Heart Mitochondrial MDH

The subunit interaction scheme for chicken heart mitochondrial malate dehydrogenase is shown. This figure is based on both molecular weight and sedimentation velocity studies of both the native and modified enzyme. At pH 5.0, an equilibrium exists between the monomeric and dimeric forms, with the predominant form being the dimer. The symbol [E]–[E] indicates two monomers that have reassociated to a certain degree, but not to the extent of existing as a dimer.

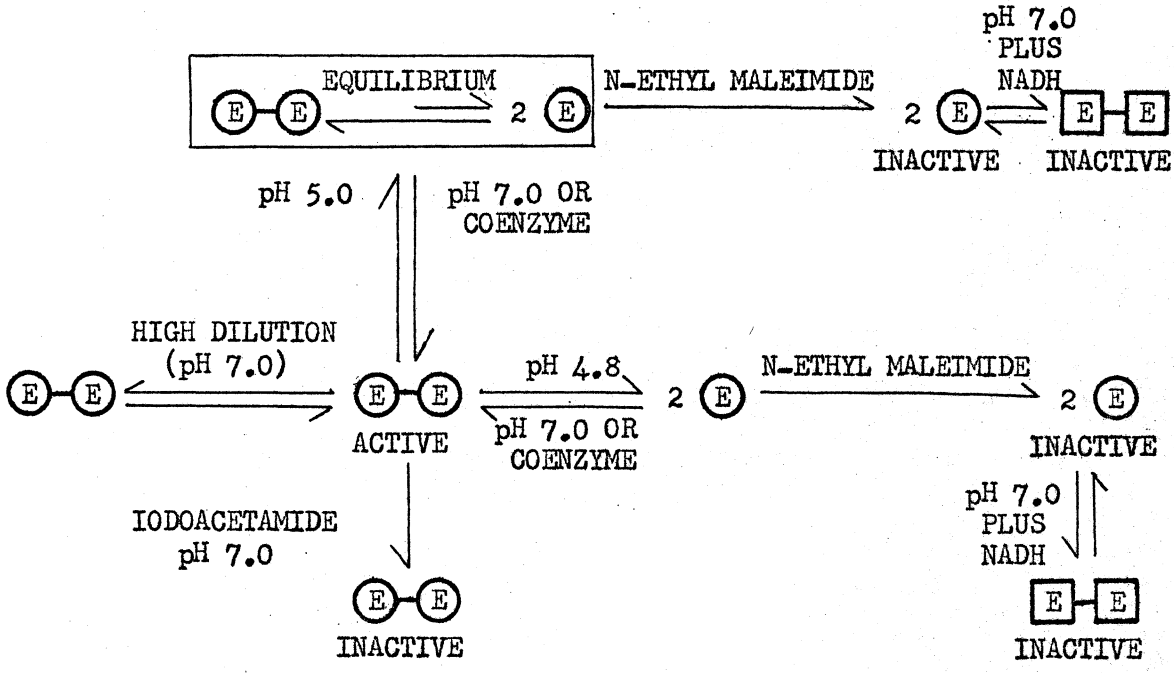


Figure 14

Subunit Interactions of Chicken Heart Mitochondrial MDH

Figure 15: Subunit Interactions of Porcine and Bovine Mitochondrial MDH

The scheme for the subunit interactions of porcine mitochondrial malate dehydrogenase is shown. The bovine mitochondrial isozyme follows the same pattern, except that the bovine enzyme does not dissociate at high dilution. The diagram is based on both molecular weight and sedimentation velocity studies of both native and modified enzyme(75).

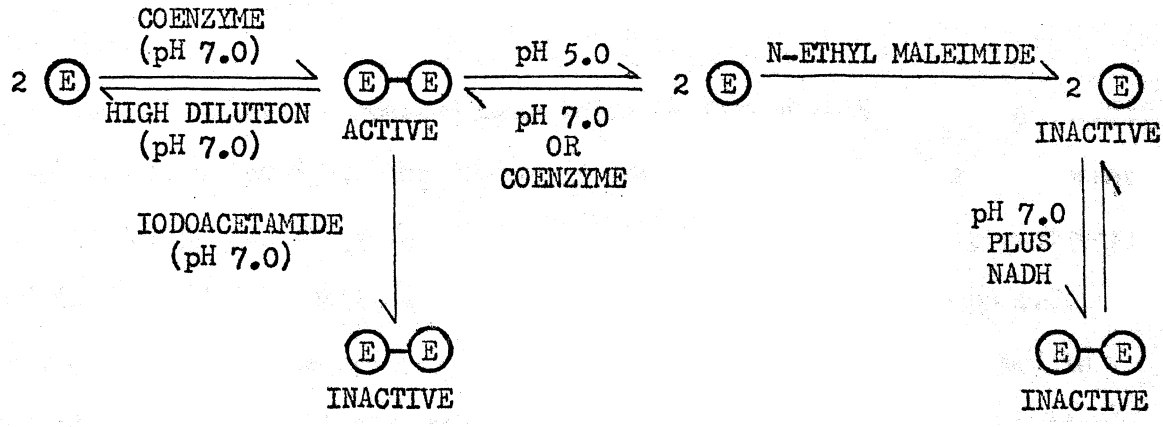


Figure 15

Subunit Interactions of Porcine and Bovine Heart Mitochondrial MDH

N-Ethyl maleimide modified an essential sulfhydryl group in both the porcine and bovine isozymes at pH 5.0. The product of this modification was inactive monomers, which could be completely reassociated into inactive dimers by the addition of NADH at pH 7.0. This modification by N-ethyl maleimide could be prevented by the presence of coenzyme.

N-Ethyl maleimide reacted over twice as fast with the chicken isozyme at pH 4.8 as at pH 5.0. The number of sulfhydryl groups modified depended on both the pH and the reagent concentration. Six sulfhydryl groups were modified at pH 5.0, while eleven sulfhydryl groups reacted at pH 4.8. Increasing the enzyme and reagent concentrations at pH 5.0 resulted in the modification of 13 sulfhydryl groups. The product of each reaction was inactive monomers. The modified enzyme would not reassociate to the degree observed with the porcine and bovine enzymes upon the addition of NADH at pH 7.0, with the amount of reassociation depending on the number of sulfhydryl groups modified. The modification reactions at both pH 5.0 and pH 4.8 could be prevented by the presence of coenzyme.

The characterization of the chicken isozyme as either a porcine-like or a bovine-like system simply is not possible, since there are certain properties possessed by chicken heart mitochondrial MDH that could place it in either category. One of the properties that the chicken isozyme shared with the bovine-system only was a failure to dissociate at high dilution. One of the properties shared with the porcine-system only was the number of active sites. Most of the properties of the chicken heart enzyme were shared in common with both porcine and bovine mitochondrial isozymes, but there were some characteristics which only the

chicken isozyme possessed, such as the failure to dissociate completely at pH 5.0, and the non-specific modification of the sulfhydryl groups by N-ethyl maleimide. As we are attempting to compare an avian system with two mammalian systems, the remarkable similarity between the three systems is rather amazing.

Investigation of other oligomeric enzymes by means of the techniques cited above could provide significant information concerning the quaternary structures of such enzymes in solution. This information could be used to gain insight into the mechanisms and possible regulation of oligomeric enzymes at physiological concentrations and pH values.

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A CHARACTERIZATION OF CHICKEN HEART MITOCHONDRIAL MALATE DEHYDROGENASE

by

James Stuart Nichols

Chicken heart mitochondrial malate dehydrogenase has been purified by an improved isolation method to give 56% of the initial mitochondrial enzyme. The purified enzyme has a specific activity of 340 U/mg. This homogeneous enzyme has been shown to be pure by several criteria.

The enzyme has been shown to be a dimer with a molecular weight of 67,000 gm/mol. Upon exposure of MDH to pH 4.8, the enzyme dissociates into 33,000 gm/mol monomers. At pH 5.0, an apparent equilibrium exists between the monomeric and dimeric states.

If pyrophosphate or phosphate were present, stabilization of the enzyme occurred, causing an increase in the enzymatic activity and a decrease in the K_m values for both substrate and coenzyme, probably by an induced conformational change.

Iodoacetamide was found to modify two histidine residues, one group in each active site, per enzyme dimer at pH 7.0. Ellman's reagent reacted with enzyme at pH 5.0, possibly modifying most of the sulfhydryl groups. N-Ethyl maleimide modification of the enzyme's sulfhydryl groups at pH 4.8 was found to be slightly more specific in its modification than Ellman's reagent. The number of sulfhydryl groups modified by N-ethyl maleimide increased with either lowering of the pH or an increased reagent concentration. Six -SH groups were modified at pH 5.0, while eleven sulfhydryl groups reacted at pH 4.8. The presence of NADH was

found to prevent modification entirely when either N-ethyl maleimide or iodoacetamide was present.

A model of subunit interactions in the native and modified chicken isozyme is presented, and is compared with the model of the porcine and bovine isozymes.