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Abstract approved:

The cytoplasmic isozyme of malate dehydrogenase (s-MDH) has been isolated from brine shrimp nauplii to a state of high purity with a protocol involving ammonium sulfate fractionation and chromatography on DEAE-cellulose, followed by sequential passes over cellulose phosphate columns at pH 5.8 then 5.0. The purified enzyme is free of glutamate-oxaloacetate transaminase and lactate dehydrogenase activities and has specific activities of 550-570 uM NAD^+/min/mg protein (oxaloacetate reduction) or 115 uM NADH/min/mg protein (malate oxidation). Holoenzyme polyacrylamide gel electrophoresis resolved at least 3 catalytically active s-MDH subforms with no observable non-MDH protein present on the gels. The enzyme displays a single uniform boundary in sedimentation velocity centrifugation which yields a $S_{20,w}$ value of 4.4. From sedimentation equilibrium a molecular weight of 75,000 is calculated, and a break in the plot of ln C versus r^2 is evident for the low protein concentration portion of the boundary, suggesting a dimer-monomer dissociation and/or a low molecular weight microcontaminant. Sodium

dodecyl sulfate polyacrylamide gels give an apparent molecular weight of 36,000-38,000 for the s-MDH subunit. The cytoplasmic isozyme has a pH optimum of 8.0 for oxaloacetate reduction and exhibits low susceptibility to thermal denaturation, with less than 10% loss of catalytic activity observed at 48^{o}C in 1 h. Michaelis constants in 0.05 M tris buffer, pH 8.0, at 25^{o}C are 4.2 X 10^{-5} M for oxaloacetate and 1.5 X 10^{-5} M for NADH, and the molecular activity is 41,000. Substrate inhibition by oxaloacetate or malate is not observed at low concentrations, but begins at 7-10 mM and 20-30 mM, respectively.

Monospecific rabbit antiserum was produced against purified s-MDH and used for rocket IEP as a quantitative assay for the cytoplasmic isozyme. Since cross-reactivity is not observed against the mitochondrial MDH (m-MDH), the assay allows specific measurement of the s-MDH in crude naupliar supernatants in the presence of contaminating m-MDH. The assay has a sensitivity of approximately 100 nanograms s-MDH protein with a 4% standard deviation using either the purified enzyme or supernatant preparations. Catalytic inhibition studies using the monospecific antiserum gave 85% inhibition of s-MDH but no significant inhibition of the brine shrimp m-MDH. Lack of complete s-MDH inhibition by the antiserum suggests a difference between the enzyme active site and immunological binding site. Porcine s-and m-MDH, and beef and pigeon m-MDH were also tried as antigens against the brine shrimp s-MDH antiserum, and reactivity was not achieved with the enzymes during rocket IEP or catalytic inhibition experiments.

Brine shirmp cytoplasmic MDH is a component of an energy-yielding glycolytic pathway which may play a role in meeting the energy demands

imposed on nauplii in high salt environments. To investigate this possibility, it is necessary to quantitatively measure s-MDH levels under varying developmental and environmental conditions. Brine shrimp nauplii challenged with fortified sea water (2.5 M NaCl) maintain significantly higher levels of cytoplasmic malate dehydrogenase (s-MDH) than larvae incubated in sea water. Eight to ten hours after emergence of free-swimming nauplii in sea water, s-MDH exhibits a steady decline for 20 to 40 hours; the decrease is smaller and stabilizes sooner in nauplii incubated in fortified sea water. Incorporation of $H^{14}CO_3$ into s-MDH protein was assayed using quantitative rocket immunoelectrophoresis (IEP) with monospecific antiserum prepared against purified brine shrimp s-MDH. A 40% faster rate of enzyme biosynthesis is observed in high salt, and together with the rapid s-MDH turnover (half-life of approximately one day), probably accounts for the difference in level between salt treatments. In contrast, $H^{14}CO_3$ incorporation into total TCA-precipitable protein in supernatant preparations decreases slightly in high salt, indicating a preferential synthesis of s-MDH. The results are discussed in relation to the bioenergetics and temporal development of water and electrolyte regulation in hypersaline environments.

Studies on the Cytoplasmic Malate Dehydrogenase from a Larval Halophilic Crustacean, *Artemia salina*

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Isolation and Partial Characterization of the Cytoplasmic Malate Dehydrogenase from a Larval Halophilic Crustacean, *Artemia salina*

Abstract

The cytoplasmic isozyme of malate dehydrogenase (s-MDH) has been isolated from brine shrimp nauplii to a state of high purity with a protocol involving ammonium sulfate fractionation and chromatography on DEAE-cellulose, followed by sequential passes over cellulose phosphate columns at pH 5.8 then 5.0. The purification can be completed in 6-8 days with a recovery of 11-15%. The purified enzyme is free of glutamate-oxaloacetate transaminase and lactate dehydrogenase activities and has specific activities of 550-570 μM NAD^+/min/mg protein (oxaloacetate reduction) or 115 μM NADH/min/mg protein (malate oxidation). Holoenzyme polyacrylamide gel electrophoresis resolved at least 3 catalytically active s-MDH subforms with no observable non-MDH protein present on the gels. The enzyme displays a single uniform boundary in sedimentation velocity centrifugation which yields a $S_{20,w}$ value of 4.4. From sedimentation equilibrium a molecular weight of 75,300 is calculated, and a break in the plot of ln C versus r^2 is evident for the low protein concentration portion of the boundary, suggesting a dimer-monomer dissociation and/or a low molecular weight microcontaminant. Sodium dodecyl sulfate polyacrylamide gels give an apparent molecular weight of 36,000-38,000 for the s-MDH subunit. The cytoplasmic isozyme has a pH optimum of 8.0 for oxaloacetate reduction and exhibits low susceptibility to thermal denaturation, with less than 10% loss of catalytic activity observed at 48°C in 1 h. An Arrhenius

plot gives a linear relationship with no transitions and an estimated molar activation energy of 9.75 Kcal/mole for the overall catalytic step. Michaelis constants in 0.05 M tris buffer, pH 8.0, at 25°C are 4.2×10^{-5} M for oxaloacetate and 1.5×10^{-5} M for NADH, and the molecular activity is 41,000. Substrate inhibition by oxaloacetate or malate is not observed at low concentrations, but begins at 7-10 mM and 20-30 mM, respectively.

Introduction

In eukaryotic cells malate dehydrogenase[1] is a ubiquitous enzyme generally occurring as two isozymes, one compartmentalized in the mitochondrial matrix[2] and the other in the extramitochondrial cytosol. This intracellular segregation of the two isozymes was first suggested in 1954 for plant tissue (55,56) and 5 years later for animal tissues (16,17). The mitochondrial enzyme (m-MDH) is a well-known, essential component of the tricarboxylic acid cycle. The cytosolic or soluble isozyme (s-MDH) is normally considered to take part in the cytoplasmic side of the "malate shuttle" providing a means of transporting NADH equivalents, in the form of malate, across the mitochondrial membranes (3). The s-MDH has been purified to apparent homogeneity from several vertebrate tissues (20,25,26,29,38,39), an invertebrate source (32), a fungus (40) and yeast (30).

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[1]The abbreviations used are: malate dehydrogenase (s-MDH, m-MDH), L-malate: NAD^+ oxidoreductase (EC1.1.37); Glutamate-oxaloacetate transaminase (GOT), L-aspartate: 2-oxoglutarate aminotransferase (EC2.6.1.1); Lactate dehydrogenase (LDH), L-lactate: NAD^+ oxidoreductase (EC1.1.1.27); HM, homogenizing medium; SDS, sodium dodecyl sulfate; SDS-PAGE, SDS polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid; Tris, tris (hydroxymethyl) aminomethane.

[2]There are reports that under certain conditions m-MDH may associate with the inner mitochondrial membrane (58,67).

In the larval brine shrimp, Artemia salina, s-MDH is a component of a facultative glycolytic shunt located in the cytosol which fixes exogenous HCO^-_3 into malate and eventually other organic and amino acids (10,11). The pathway produces metabolic energy in the form of GTP and provides NAD^+ to maintain glycolytic redox balance. Our interest is to investigate the possible role of s-MDH and this glycolytic shunt in meeting the large metabolic demands of the brine shrimp sodium-potassium transport system (12,21,53,54) in high salt environments. In order to achieve this purpose and for the cytochemical localization of the s-MDH within the chloride cell, it is necessary to purify the enzyme from this source.

The work presented will describe the purification of s-MDH from halophilic brine shrimp nauplii. Physical properties of the purified enzyme are reported along with partial kinetic characterization of the oxaloacetate reduction reaction and compared to values from other sources. A preliminary report of this study has been published (31).

Materials and Methods

Materials: DEAE-cellulose and cellulose phosphate were obtained from Schleicher and Schuell. Sephadex G-25 and G-100, L-malic acid, oxaloacetic acid, NADH (Grade III), NAD^+ (Grade III), nitro blue tetrazolium, phenozine methosulfate, malate dehydrogenase (pig heart mitochondrial) and lactate dehydrogenase (type V) were purchased from Sigma Chemical Company. Ultra-pure ammonium sulfate was obtained from Schwartz-Mann Company. Materials used in polyacrylamide disc gel electrophoresis were all products of Bio-Rad Laboratories. All other chemicals were reagent grade.

Source and rearing of brine shrimp nauplii. Encysted brine shrimp gastrulae were purchased from San Francisco Bay Brand Company, San Francisco, CA, and stored in vacuo at 20°C. Three hundred grams of dried cysts were hydrated in tap water at 5°C for 4 hours with occasional stirring. Cysts which settled from suspension were separated from floating cysts and debris by decanting and washing several times with tap water. Washed cysts were transferred to 36 2800-ml Fernbach flasks (15 g hydrated cysts/flask) containing 500 mls of artificial seawater (Instant Ocean Sea Salts, East Lake, Ohio) and incubated 27 hours at 30°C with continuous shaking. Nauplii were separated from empty shells and most unhatched cysts by the method of Finamore and Clegg (22), which utilizes the phototactic response exhibited by nauplii. A yield of 190 to 230 g wet weight of nauplii was obtained in this manner.

Enzyme assays: (A) Malate dehydrogenase. Based on characterization experiments malate dehydrogenase (oxaloacetate reduction reaction) was assayed using the following 3 ml reaction mixture: 50 mM Tris HCL buffer, 0.33 mM oxaloacetic acid, 0.14 mM NADH and 0.1 ml of enzyme solution. Oxaloacetate (neutralized) was prepared freshly and kept on ice to minimize spontaneous decarboxylation or pyruvate. In this manner oxaloacetate decarboxylation was less than 1% over a 6 to 8 hour period, as estimated by measuring pyruvate accumulation with lactate dehydrogenase. NADH was dissolved in neutralized distilled water and kept in the dark to reduce oxidation. During characterization experiments fresh aliquots of purified MDH were kept on ice and diluted hourly to appropriate assay concentrations with 50 mM Tris-HCL buffer (pH 8.0) containing 0.2 mg/ml BSA, 1.0 mM dithiothrietol, 10% glycerol (v/v) and 0.5 mM EDTA and kept on ice. Enzymatic oxidation of NADH was followed at 340 nm using a Varian-Cary 219 dual beam recording spectrophotometer at a temperature of 25°C ± 0.1°C maintained with thermostatable cuvette holders. Under these conditions the reaction was linear for 1-2 minutes at a rate of 0.030 O.D. units/minute. The malate oxidation reaction was optimized for substrate concentration and pH and assayed in the following reaction mixture: 0.1 M glycine-NaOH buffer (pH 10) 2.5 mM NAD^+, 16.5 mM malate and 0.1 ml enzyme solution. Appearance of NADH was followed spectrophotometrically at 340 nm.

(B) Lactate dehydrogenase. LDH activity was measured in the following 3 ml reaction mixture optimized for pH and substrate concentration: 95 mM potassium phosphate buffer (pH 7.0), 7.6 mM sodium pyruvate, 0.2

mM NADH and 0.1 ml enzyme mixture in a total volume of 3 mls. The reaction was initiated by addition of enzyme and the decrease in A_{340} followed spectrophotometrically.

(C) Glutamate-oxaloacetate transaminase. GOT activity was optimized for substrate concentration and pH and measured under the following reaction conditions: 94 mM potassium phosphate (pH 7.4), 180 mM potassium aspartate, 19 mM α-ketoglutarate, 0.2 mM NADH, 7.5 units MDH (porcine) and 0.1 ml of enzyme sample in a final volume of 3.3 mls. Since this GOT assay was coupled to MDH reduction of oxaloacetate and significant GOT contamination exists in commercial MDH preparations, the rate of a blank reaction mixture (without GOT sample) must be subtracted from sample reaction rates. Enzymatic oxidation of NADH was followed at 340 nm and initiated by addition of enzyme preparation. This coupled-reaction assay was similar to that originally described by Karmen (37). Other assays based on the direct spectrophotometric measurement of oxaloacetate appearance or disappearance at 260-280 nm are more suitable for kinetic work but are relatively insensitive due to the low absorbancy of oxaloacetate (65).

Protein Determinations. Total TCA-precipitable protein was determined by the method of Lowry et al. (47), as modified recently by Peterson (52). TCA precipitation was nececssary since buffers either interfered directly with color development of the assay or contained interfering additives (e.g., EDTA, dithiothreitol). Additionally, ammonium sulfate used in the purification protocol reduces Lowry color development at concentrations as low as 0.15% (45).

Chromatography. All chromatographic procedures were performed at 4-5°C. Column flow rates were controlled by hydrostatic head, and fractions were collected with Gilson LBI fraction collectors. Buffers for chromatographic procedures were pH adjusted at 4°C.

(A) Gel Filtration. Sephadex G-25 beaded-dextran gel was used for routine desalting of enzyme preparations stored in ammonium sulfate. The initial desalting step (Table I) required a column 5 X 39 cm, run with a 4 ml/minute flow rate. Maximum sample volume applied was 60 mls. Subsequent desalting steps utilized a smaller column 1.2 X 37 cm with a maximum volume of 6 mls and a flow rate of 1.7 ml/minute.

(B) DEAE-Cellulose. Dry resin was hydrated and cleaned by allowing the material to sink into 1.0 N NaOH and then washing with 0.5 N HCl and 1.0 N NaOH (rinsed to neutrality with distilled H_2O after each step). Two columns (2.0 X 45 cm) were equilibrated with buffer A (50 mM tris-HCl buffer, pH 8.0, containing 0.5 mM EDTA, 1.0 mM DTT and 10% glycerol, v/v) at a flow rate of 2.0 ml/minute. Applied sample volume was approximately 50 mls per column.

(C) Cellulose phosphate. Dry resin was washed (0.1 N HCl, 0.1 N NaOH, 0.1 N HCl) and equilibrated with either 20 mM potassium phosphate buffer at pH 5.8 (buffer B) or 20 mM potassium acetate buffer at pH 5.00 (buffer C), both containing the same additives as buffer A. Two pH 5.8 columns and one pH 5.00 column (1.2 cm X 45 cm) were pH equilibrated with a flow rate of 1.0 ml/minute overnight. Maximum sample volume was 23 mls.

Polyacrylamide Disc Gel Electrophoresis. (A) Holoenzyme Gels. The procedure for non-denaturing gels described by Davis (15) and more recently reviewed by Gabriel (24) was followed, utilizing a 2.5% acrylamide stacking gel and a 7% running gel. Running gels were pre-electrophoresed (2 hrs, 3 mM/tube) before application of stacking gels. Bromophenol blue (0.1%, 5µl) was added to the top of each stack as a tracking dye. Gel samples were stacked at 2 mA/tube and run at 3 mA/tube, then stained for either total protein with Coomassie Brilliant Blue R-250 or enzymatic activity using the tetrazolium salt-formazan derivative technique as used by Chen (9). During activity staining, control gels were incubated in a reaction mixture lacking malate to insure that the observed staining was indeed substrate dependent.

(B) Sodium Dodecyl Sulfate Gels. The method of SDS gel electrophoresis was taken from the Burgess modification of the Laemmli method (44) and used as described by Hokin et al. (33). Running gels of 8.75% and 15% acrylamide were employed, both with a 3% stack. Holoenzyme and SDS gels stained with Coomassie Brilliant Blue R-250 were destained electrophoretically in 7.5% acetic acid at 4 mA/gel.

Analytical Ultracentrifugation. Studies were performed using a Beckman Model E analytical ultracentrifuge equipped with scanning optics. Malate dehydrogenase samples were prepared by extensive dialysis against 0.1 M potassium phosphate buffer, pH 7.4, containing 0.1 M NaCl. Velocity centrifugation (An-F rotor) was performed with rotor speed of

45,000 rpm at 3.2°C with a monochromator setting of 280 nm. Positions of the sedimenting boundary were determined from recordings of optical density versus distance from the center of rotation using the half-height method. Observed sedimentation coefficients were corrected for viscosity and temperature to standard $S_{20,w}$ values. The partial specific volume of MDH was assumed to be 0.74 as reported by Kitto and Kaplan (39) and Gerding and Wolfe (25) for chicken and pig heart enzymes, respectively.

Sedimentation equilibrium experiments were performed with the same protein concentration and solvent as in velocity runs at 2.5°C (An-D rotor). Rotor speed was 44,000 rpm for the initial 3 hours and then reduced to 30,000 rpm for 48 hours after which boundary measurements were made. To obtain a meniscus optical density baseline corresponding to zero protein concentration, rotor speed was increased to 60,000 rpm. The slope of the plot ln A versus $(\text{radius})^2$ was derived from a least-squares regression line and used to calculate the s-MDH molecular weight.

Temperature Effects on Enzyme Activity and Stability. To obtain a temperature profile of catalytic activity, small aliquots (0.2 ml) of enzyme were equilibrated to experimental temperature for 1.0 minute in a circulating water bath. The enzyme sample (0.1 ml) was immediately transferred into cuvettes containing 2.9 ml of reaction mixture at temperature in thermostatable cuvette turrets and the change in A_{340} recorded. Reaction mixture temperature was measured before and after each assay, and variation was no more than ± 0.1°C. Substrates were saturating at all assay temperatures.

For thermal inactivation studies enzyme samples in the previously described assay buffer were incubated at 48°C and 55°C. At time intervals during the one hour incubation, samples were withdrawn and diluted 30-fold into the reaction mixture at 25°C and assayed.

Kinetic Studies. Being a two-substrate enzyme the true Michaelis constants and initial velocity maximum for s-MDH must be determined by extrapolation to infinite substrate concentration for both substrates (conditions which are zero order). Following the method initially described by Dalziel (13), double reciprocal "primary plots" were generated by adjusting the concentration of one substrate ("fixed" substrate) to a constant value and varying the concentration of the second substrate over a suitable range. The experiment was repeated several times using 5 different concentrations of fixed substrate. Then the fixed and variable substrates were reversed, and the series repeated. From the primary plots, two types of "secondary plots" can be made by graphing either the slopes or y-intercepts versus the [fixed substrate]$^{-1}$. All kinetic constants can be evaluated in this manner.

Results

Isolation of Cytoplasmic Malate Dehydrogenase

Homogenation and Centrifugation. Twenty-seven hour nauplii (200-230 g wet weight) were homogenized with six strokes of a Potter-Elvehjem teflon-glass homogenizer at 2500 rpm (1 g nauplii per 2 ml ice-cold HM[3]). Cell debris was removed by low speed (1000 X g) centrifugation for 15 minutes in an RC-5B refrigerated centrifuge with GSA rotor. The pellet was washed with an equal volume of HM and recentrifuged. Combined supernatants (4.4-5.0 units MDH/mg protein) were filtered through miracloth to remove unhatched cysts and centrifuged 30 minutes at 39,000 X g in a Sorvall SS-34 rotor. The high-speed supernatant was carefully decanted leaving the mitochondrial pellet overlayed with a lighter-colored membranous layer. Ammonium sulfate fractionation was immediately initiated on the post-mitochondrial supernatant (PMS).

Ammonium Sulfate Fractionation. Optimization of ammonium sulfate fractions revealed that a majority of mitochondrial MDH isozyme could be separated from cytoplasmic MDH isozyme at this initial stage. In an ice bath crystalline ammounium sulfate was slowly added to the PMS with continuous stirring over a period of 30 minutes, to achieve 45% saturation[4]. The solution was left unstirred for an additional 30

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[3]Homogenizing medium consisted of 50 mM tris-HCl buffer, 0.5 mM EDTA, 1.0 mM dithiothreitol, 0.25 M sucrose, pH 7.5.

minutes and then centrifuged 15 minutes at 16,000 rpm. The supernatant, containing 87-90% of the total MDH activity found in the PMS, was salted to 60% saturation and centrifuged. This pellet from the 45-60% AS fraction was enriched in s-MDH and was the fraction used in further purification steps. The rationale for choosing a 45-60% AS fraction is not immediately obvious, since only 65% of the MDH activity was precipitated, leaving behind 35% in the supernatant (with a 2-fold higher specific activity). However, the majority of the precipitated MDH was the cytoplasmic isozyme, while most of the mitochondrial enzyme remained unprecipitated. Therefore, a significant separation of the two isozymes was achieved.

Identification of the two isozymes (each with multiple subforms) was based on migration differences in holoenzyme polyacrylamide gels stained for enzymatic activity. The 45-60% AS fraction, resuspended in buffer A and salted to 70% AS, can be stored for several months at -20°C with negligible loss of MDH activity.

DEAE-Cellulose Chromatography. Thirty milliliters of the AS fractionated preparation (500-600 mg protein) was diluted 1:1 with buffer A and desalted by Sephadex G-25 chromatography. The break-through peak containing the MDH was pooled and applied to 2 DEAE-cellulose columns. Although s-MDH did not adhere to the columns,

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[4]Percent saturation of ammonium sulfate was based on values from Green and Hughes (26) at 25°C.

80-85% of the non-MDH protein was bound, resulting in a 6-fold MDH enrichment. The DEAE-cellulose columns also adsorbed all carotenoid pigment, which bound irreversibly to the column matrix. Catalytic activity of the desalted enzyme preparation was significantly more stable after the DEAE treatment. Pre-DEAE preparations can lose 90% of the MDH activity by standing at 10°C in buffer A overnight, as compared to 10% loss with post-DEAE enzyme under similar conditions. Normally the enzyme was stored at this step as a 70% AS precipitate at -20°C, resulting in no loss of activity for several weeks.

Cellulose PO_4 pH 5.8. Post-DEAE material was centrifuged for 30 minutes at 16,000 rpm and the 70% AS supernatant discarded. The pellet was resuspended in buffer B and desalted to remove AS by gel filtration. The sample (50-80 mg protein) was chromatographed on two cellulose phosphate columns at pH 5.8, producing a 2-3 fold MDH enrichment by negative absorption, as in the DEAE step. The breakthrough peak containing MDH activity (75-100 units/mg proteins) was pooled and stored as described above.

Cellulose Phosphate pH 5.00. Accurate pH equilibration of this column was essential in order to achieve proper binding of s-MDH to the column and optimal separation of s-MDH from the enzyme GOT. The sample to be rechromatographed at the lowered pH was prepared as before, and the desalted protein (15-25 mg protein) applied in buffer C to one column. After the break-through peak eluted, a 300 ml linear buffer gradient was initiated (20 to 300 mM potassium acetate, pH 5.00), which eluted the

s-MDH after 200-250 ml (Fig. 1). The center of the peak was pooled while leading and trailing edges were discarded.

A typical purification gave a 70-90 fold enrichment of the enzyme over the 70% AS starting material (100-130X over crude homogenates[5]), resulting in a final specific activity of 550-570 units/mg protein. Percent recovery of MDH units was normally 11-15%, representing 1-2 mg of purified protein, which was stable for at least 6 months stored at -20°C as a 70% AS precipitate. A summary of the purification procedure is given in Table I.

Elimination of GOT and LDH. Glutamate-oxaloacetate transaminase, and to a lesser extent LDH, were common contaminants of purified s-MDH. Therefore, the activities of these enzymes were monitored at all stages of the purification procedure. The initial ratio of MDH:GOT:LDH activities present in the PMS was 58:7:1, the specific activities being 7.6, 0.93 and 0.13 units/mg protein, respectively. LDH was completely eliminated from the s-MDH preparation with the DEAE-cellulose chromatographic procecure, while GOT was paritally removed by adsorption to the cellulose phosphate pH 5.8 column. On the subsequent cellulose phosphate pH 5.0 column, the remaining GOT was adsorbed and eluted in a very small gradient peak well separated from the later s-MDH peak. No

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[5]Fold enrichments are underestimates, since the since the mitochondrial isozyme contributes significantly to the total MDH units measured in homogenates.

Table 1. Summary of s-MDH purification steps.

Step	Volume (ml)	Protein mg/ml	Protein Total (mg)	Activity u /ml	Activity Total u	Activity % Recovery	Specific Activity (u /mg)	Fold Enrichment
70% $(NH_4)_2SO_4$ precipitate	30	28.8	865	213	6400	100	7.4	0
Sephadex G25	126	5.2	655	333	4196	66	6.4	0
DEAE	272	0.40	109	14.9	4052	63	37	5
Sephadex G25	15.5	5.3	83	197	3054	48	37	5
Cellulose PO_4 (pH 5.8)	235	0.13	30	9.3	2210	34	74	12
Sephadex G25	20.5	1.02	21	75	1538	24	73	10
Cellulose PO_4 (pH 5.0)	47.5	0.030	1.42	16.9	803	13	565	76

LDH or GOT catalytic activity could be detected in the purified s-MDH preparation.

Polyacrilamide Disc Gel Electrophoresis. At pH 8.3 holoenzyme PAGE revealed three distinct protein bands that coincided exactly with triplet bands visualized by MDH-specific activity staining (Fig. 2). Other protein bands were not observable in the gels. Therefore, non-denaturing electrophoresis suggests the s-MDH from Artemia salina nauplii was apparently homogeneous. The s-MDH triplet had a greater anodal mobility than the three slower-moving m-MDH bands which were eliminated during the purification. The relative anodal mobilities of the two isozymes were charcteristic of MDHs from many sources (Table II). Since both cytoplasmic and mitochondrial isozymes of pig heart MDH are commerically available, they were routinely used during PAGE experiments for comparison with Artemia MDH isozyme mobilities. Migrations of respective isozymes from each source matched closely, lending support for the cytoplasmic origin of the purified Artemia MDH. Additionally, rabbit antiserum prepared against the purified s-MDH did not show cross-reactivity to m-MDH extracted from washed mitochondrial preparations (to be reported elsewhere).

SDS-PAGE revealed that the subunit molecular weight of brine shrimp s-MDH was similar to pig heart m-MDH, which was used as a 35,000 mw standard (Fig. 2). An apparent molecular weight of 36,000-38,000 was estimated from SDS-PAGE for the brine shrimp subunit, supporting a dimer structure for the holoenzyme. A faint secondary band of lower molecular weight (30,000) has sometimes been observed in both brine shrimp and

Table II. Comparison of s-MDH molecular and kinetic charcteristics from various organisms.

Source	$S_{20,w}$	Molecular Weight		Electrophoretic Pattern of Holoenzyme		Specific Activity[a] of Purified Enzyme		Kinetic Properties of Reduction Reaction			Reference
		Holoenzyme	Subunit	Number of Bands (s-MDH)	Greater anodal mobility (s- vs. m-MDH)	Oxaloacetate Reduction	Malate Oxidation	K_m Oxalo. (molar)	K_m NADH (molar)	pH optimum	
Chicken (Heart)	4.27[2]	66,500[1]		1[4]	S		65	5×10^{-5}		7.6	39,42
Yeast	4.45[1]	75,000[1]	39,000[3]	3[5]		840					30
Fruit Fly		68,000[7]		1[4]	S			4×10^{-5}		8.5	48
Ostrich (Heart)	3.8[2]	67,000±5,000[7]		1[4]	S	375 (25°C)	75 (25°C)	3×10^{-5}			38
Beef (Heart)	5.1[2]	72,000[1] 80,000[1]	37,000[1] 36,000[3] 40,000[1]	1[3]	S	675 (28-30°C) 817 (25-27°C)	118 (25°C)	4.2×10^{-5} 5.1×10^{-5}	2.7×10^{-5} 3.8×10^{-5}	9.2 8.0	5,7,20,28 29,60,69
Pig (Heart)	4.53[2] 4.1[2] 4.4[2]	73,900[1]	37,000[1]	3[3]	S	556 (25°C)	100 (25°C) 110 (25°C)	3.3×10^{-5}	2.4×10^{-5}	7.0-7.5	4,23,25,26,50,64,66
Brown Shrimp	4.94[2]	75,800[1]		3[3]	M		49 (25°C)	5.8×10^{-5}		7.5	32
Brine Shrimp	4.40[2]	75,300[1]	36,000-38,000[3]	3[3]	S	550-570(25°C)	115 (25°C)	4.2×10^{-5}	1.5×10^{-5}	8.0	This paper

[1]Sedimentation equilibrium centrifugation
[2]Boundary sedimentation velocity
[3]Polyacrylamide gel electrophoresis
[4]Starch gel electrophoresis
[5]Isoelectric focusing

[a]Units are μM NADH oxidized (or NAD^+ reduced)/min/mg protein. Temperature of assay where available given in parentheses. Assay conditions vary significantly among reports.

commerical pig heart preparations, which may represent a microcontaminant. Reports exist of a low molecular weight microcontaminant impairing complete crystallization of chicken s-MDH, and chromatography on G-100 Sephadex can apparently remove the component (39).

Analytical Ultracentrifugation. Purified brine shrimp s-MDH (0.45 mg/ml) displayed a single uniform boundary in sedimentation velocity ultracentrifugation ($S_{20,w}$=4.40). At the protein concentration used in the experiments, extrapolation to zero concentration would not produce a significantly different S value, based on the concentration dependence of sedimentation coefficients reported with chicken supernatant MDH (39). As a more sensitive test for heterogeneity and better molecular weight determination, sedimentation equilibrium centrifugation was performed. From the slope of the plot ln absorbance versus $(\text{radius})^2$, a molecular weight of 75,300 was calculated (Fig. 3). Using the method outlined by Tanford (62), the frictional ratio (f/fo) and diffusional coefficient ($D_{20,w}$) for s-MDH were 1.35 and 5.64×10^{-7} cm^2/second respectively. A distinct break in the ln A versus r^2 curve was evident in the region corresponding to the low protein concentration portion of the boundary. Reports exist of s-MDH displaying a dimer-monomer dissociation system in sedimentation equilibrium analysis at low protein concentration (6,25). A rough estimate of the secondary component's molecular weight was 33,000, approximately that of a subunit. However, it is possible that subunit dissociation was not completely (or partially) responsible for the above observation, and that a 33,000 mw

microcontaminant exists. Additional work is in progress to distinguish between these 2 possibilities.

Buffer Concentration and pH Optimum. Several buffer systems at concentrations between 0.025 M and 1.0 M (pH 7.5) were examined to determine the buffer species and strength promoting greatest s-MDH catalytic activity (Fig. 4a). Highest activity (oxaloacetate reduction) was achieved in 50 mM tris-HCl buffer, although at high concentrations this buffer was the most inhibitory. Potassum phosphate gave very similar activities at 50 mM, and therefore tris-HCl and potassium phosphate were chosen for spanning a pH range of 5.5 to 9.5 to arrive at a pH optimum for s-MDH. Figure 4b shows a pH optimum of 8.0 at 25°C for the oxaloacetate reduction reaction. For the malate oxidation reaction, the optimum was more alkaline (pH 10).

Temperature Effects on Enzyme Activity and Stability. Maximal catalytic activity was achieved at 45°C (Fig. 5a), but evidence of significant protein denaturation was observed at these higher temperatures with longer incubation times (Fig. 5b). The first order decay of s-MDH catalytic activity at 48°C and 55°C is presented in Figure 5b. An Arrhenius plot of the temperature data gave a linear relationship with no transitions in enzyme activity observable until the sudden drop at high temperatures (45°C) due to denaturation (Fig. 5c). An estimate of the molar activation energy (E_a^*) for the overall catalytic step calculated from the Arrhenius equation was 9.75 Kcal/mole.

Kinetic Parameters for the Oxaloacetate Reduction Reaction. Primary double reciprocal plots for oxaloacetate and NADH at 5 different concentrations of fixed substrate are presented in Figures 6 and 7. From the secondary plots in Figure 8 (intercepts versus reciprocal of fixed substrate concentrations), the Michaelis constant for oxaloacetate is 4.2×10^{-5} M and for NADH was 1.5×10^{-5}. Values for maximal velocity (μM NAD^+ produced/min/mg protein) computed from the two secondary plots agreed closely, 552 (oxaloacetate plot) or 558 (NADH plot). Using a molecular weight of 75,300 the average molecular activity for the oxaloacetate reduction reaction was 41,200.

Effects of increasing substrate concentrations on the reaction velocity were examined for both oxaloacetate reduction and malate oxidation. Neither oxaloacetate or malate were inhibitory until very high levels, inhibition beginning at 7-10 mM and 20-30 mM, respectively (Fig. 9).

Discussion

Isolation of Brine Shrimp s-MDH.

The cytoplasmic isozyme of malate dehydrogenase has been isolated from brine shrimp nauplii by a procedure which yields 1-2 mg protein from 200 g of nauplii with a recovery of 11-15%. The purification can be completed in 6-8 days and has been repeated no less than 10 times with reproducible results. The resulting enzyme preparation is free from m-MDH protein (judged by PAGE) and GOT and LDH catalytic activity. The ability to partially separate the two MDH isozymes by AS fractionation early in the purification is probably due to large differences in molecular charge; distinctly different isoelectric points have been reported for pig heart isozymes - 5.1 for s-MDH, 10.0 for m-MDH (19). GOT is a particularly difficult contaminant to remove from MDH preparations (26,39,40) due in part to the very similar isoelectric points of the two soluble (acidic) enzymes (65). The low pH of the cellulose phosphate columns causes significant loss of GOT activity, but adequate activity remains to establish that its elution profile is well separated from the s-MDH peak. The irreversible reduction in GOT activity is presumably due to loss of the two molecules of tightly bound pyridoxal phosphate per molecule enzyme in the acidic conditions (26,35). Catalytic activity of the s-MDH is depressed at low pH, but the reduction in activity is reversible when brought to a higher pH. In this regard Bleile, et al. (4) recently reported that porcine s-MDH does not demonstrate any pH-dependent subunit dissociation when compared at pH 5.0 and 7.0 by gel filtration and velocity centrifugation at 2 and 8 mg protein/ml, respectively.

Subforms of Brine Shrimp s-MDH.

The multiple conformational forms of purified Artemia s-MDH revealed with native-protein PAGE is a commonly observed characteristic of purified MDH (both soluble and mitochondrial isozymes) from a variety of sources. With a few exceptions (32,41,49,51) the cytoplasmic isozyme has greater anodal mobility (more acidic protein) than m-MDH, which is consistent with observations for the brine shrimp enzyme. In addition to the purified preparations listed in Table II, multiple conformations of s-MDH homogenates and enriched preparations have been demonstrated for fungi (40), a parasitic roundworm (70), a marine snail (49), sea urchins (51), a horseshoe crab (59), several teleost fish (1,2), turtles (36) and birds (36,42). Up to five electrophoretically separable subforms of s-MDH from pig heart have been reported by Kulick and Barnes (43).

Since the initial description of pig heart m-MDH subforms by Thorne (63), the nature of these subforms has not been clarified. Certain studies (1,2) present evidence for these subforms being distinct genetic isozymes (differing in the primary, covalent protein structure), but numerous reports suggest post-translational modification and preparation artifacts resulting from conformational changes (42), proteolytic degradation (7), varying amounts of covalently bound phosphate (8) and deamination of glutamine and asparagine residues (30,46). A few studies demonstrate that indeed many of these subforms are interconvertible, implying conformational changes. In the case of pig heart m-MDH, conversion of an alkaline subform to more acidic subforms can accompany

purification under certain isolation conditions (3). Kitto et al. (42) separated 2 of the 5 subforms of chicken m-MDH with carboxmethyl cellulose chromatography. A single subform was then subjected to acid inactiviation (dissociation into subunits) followed by reassociation of the active holoenzyme. The single subform when treated in this manner, migrated not as a single band, but as several bands on starch gel electrophoresis, matching 3 of the more acidic subforms from which it was initially isolated. From these data and subunit iodination studies, Kitto and coworkers concluded the m-MDH subforms were interconvertible "conformers". Dialysis against saturated AS solutions produced similar interconversions. Similarly, Meizel and Markert (49) using s-MDH of the marine snail Ilyanassa obsoleta were able to cause apparent interconversion between subforms by exposure to 2-mercaptoethanol (10-80 mM) for 4 to 20 hours.

Several recent papers (3,30,46) have suggested that the electrophoretic splitting observed for both s-MDH and m-MDH may be caused by in vivo or in vitro deamination of particular protein residues, resulting in a sufficient change in overall net charge to affect mobility. However, no direct evidence yet exists to prove or disprove this hypothesis, although in the case of yeast MDH, deamination appeared likely since several alternate explanations could be eliminated (30).

The present investigation demonstrated at least 3 subforms for brine shrimp s-MDH on polyacrylamide gel electrophoresis. The least acidic form was generally more abundant as judged by relative protein-staining intensity, with intensity decreasing with increasing acidity of

the subforms (Fig. 2). This pattern is consistent with those shown for chicken and tuna m-MDH (42), and snail (49) and pig (43) s-MDH. Banaszak has suggested that the more acidic bands may be derived from the more basic band in pig m-MDH (3). However, further evidence is required before a definitive molecular explanation of the subforms will emerge. One approach would be to isolate reasonable quantities of individual s-MDH subforms with a large scale procedure followed by protein sequencing of the subforms, although conflicting reports exist concerning blockage of the subunit N-terminal position with an N-acetyl group (25,66). This method could distinguish between conformation- or deamination-derived differences and primary structural differences.

At this time, the nomenclature suggested by Banaszak (3) referring to the cytoplasmic and mitochondrial MDHs as true isozymes, and the electrophoretic variants of these isozymes as subforms seems appropriate. Numerous investigations have reported genetic differences among species and animal populations based on MDH "isozyme" patterns observed in electropherograms of crude tissue homogenates. Some reports are accompanied by sufficient biochemical and genetic data to warrant these conclusions (e.g. 1,2), but in many studies interpretations should be offered cautiously until the molecular basis for MDH electrophoretic subforms is determined.

Subunit Characterization of Brine Shrimp s-MDH.

SDS-PAGE suggests that brine shrimp s-MDH is a dimer of very similar if not equal subunits of 36,000-38,000 molecular weight, since separation or spreading of the single band could not be detected by

electrophoresing nearly the full gel length. Similar results were obtained in parallel experiments with commerical pig heart m-MDH. In agreement with the findings for brine shrimp, SDS electrophoresis of purified pig heart s-MDH by Lodola, Spragg and Holbrook (46) revealed a single band, while the enzyme exhibited 3 bands under non-denaturing electrophoretic conditions. The s-MDH from the penaeid shrimp Penaeus aztecus also showed triplet banding under non-dissociating conditions (32), but in contrast displayed two widely-separated bands under denaturing conditions (6 M urea). The bands were interpreted as representing two non-identical subunits. Respective molecular weights for the two bands were not calculated from the 7.5% polyacrylamide gels, but size differences appeared to be very large. We have on occasion noticed during SDS electrophoresis a faint, rapid migrating component (best observed on 15% acrylamide gels) in commercially purified porcine m-MDH and sometimes in brine shrimp preparations corresponding to about 30,000 mw, but in light of data reviewed earlier it is probably a microcontaminant.

Ultracentrifugation Studies on Brine Shrimp s-MDH.

As seen in Table II, the $S_{20,w}$ value of 4.40 for brine shrimp s-MDH is in close agreement with sedimentation coefficients obtained from other sources, the majority being 4 to 5 S. Molecular weight determinations for s-MDH have varied between 52,000 (20) and 80,000 (7), but the more recent determinations average 68,000-76,000. Values as low as 15,000-20,000 have been reported for m-MDH (14) with 68,000-70,000 being the current range (for review see 3,69). These data suggest a

slightly smaller molecular weight for the mitochondrial as compared to the cytoplasmic isozyme. The value of 75,300 calculated from sedimentation equilibrium centrifugation for the brine shrimp s-MDH is comparable to reported values. As mentioned earlier, the observed break in the plot ln A versus r^2 suggests a dimer-monomer dissociation system and/or a low molecular weight microcontaminant for the s-MDH preparation from brine shrimp. Subunit dissociation has been suggested for MDH at low protein concentrations (6,25), but Bleile et al. (4), when contrasting beef and pig MDH, showed the dissociation is dependent on the source and the particular isozyme used.

Kinetic Properties of Brine Shrimp s-MDH.

Specific activities of MDH from different sources are somewhat difficult to compare due to differences in assay conditions, but values for brine shrimp MDH based on either the forward or reverse reaction are approximately the same or higher than the literature values (Table II). The pH optimum of 8.0 agrees with the slightly alkaline conditions normally reported as optimal for the oxaloacetate reduction reaction. Though not studied in detail, the malate oxidation reaction of brine shrimp s-MDH has a more basic pH optimum (pH 10), presumably because the coenzyme-enzyme dissociation can be the rate limiting step (23) and is greatly enhanced for NADH in alkaline conditions (34,57).

One characteristic of s-MDH that aids in distinguishing it from m-MDH is the lower susceptibility to thermal denaturation of the cytoplasmic isozyme. The sensitivity of brine shrimp s-MDH catalytic activity to long incubation at 55°C is far less than that reported for

m-MDH (chicken heart) at the same temperature, but similar to the chicken cytoplasmic isozyme (39). The same trend is observed by comparison to tuna s- and m-MDH at 48°C (41); gulf shrimp MDHs exhibited comparable patterns (32).

Michaelis constants for oxaloacetate and NADH at 25°C, pH 8.0, in tris buffer were 4.2×10^{-5} M and 1.5×10^{-5}, respectively, for the brine shrimp enzyme. In addition to values reported in Table II for purified enzymes, apparent Michaelis constants for ox kidney (18) are 2×10^{-5} M (oxal.) and 1×10^{-5} M (NADH), for the fungus Phycomyces blankesleeanus (61) 9.2×10^{-5} M (oxal.), and for the sea urchin Strongylocentrotus purpuratus (51) 1.0×10^{-5} M (oxal.). When comparing malate dehydrogenase K_m values, the significant pH dependence of the constants should be noted (68). For example, the oxaloacetate k_m for beef heart s-MDH increases 5-fold as the pH is elevated from 6.4 to 8.3 (5). Recently, significantly lower k_m values have been obtained by using very low substrate concentrations with stopped-flow, full time course studies (23) or 10 cm-light path cuvettes (5).

Although stable at concentrations above 20 µg/ml, the brine shrimp s-MDH was very unstable when diluted to assay concentrations (below 1 µg/ml). Consequently, bovine serum albumin (0.2 mg/ml) was added to the diluent under these conditions to prevent loss of catalytic activity. The added BSA did not appear to affect the kinetic parameters. Frieden and Fernandez-Sousa (23) reported similar irreversible inactivation of pig heart s-MDH at concentrations below 5 µg/ml and suggested the loss of activity might reflect dissociation of the active enzyme to inactive subunits. Another probable cause could be protein adherence to glass test tubes at the dilute concentrations.

In most cases MDH isozymes can be distinguished by their substrate inhibition characteristics. The mitochondrial isozyme is generally inhibited by oxaloacetate at a threshold of 0.1 to 0.5 mM, while s-MDH shows no such inhibition until much higher concentrations (14,20,38,41,60,63). As shown in Figure 9, brine shrimp s-MDH exhibits no oxaloacetate substrate inhibition up to 7-10 mM. Chicken isozymes appear to be the primary exception to the rule with both s- and m-MDH inhibited above 0.1 mM (39). The reverse condition is generally true of substrate inhibition by malate, when the supernatant isozyme is the more sensitive. Inhibition begins at approximately 10-40 mM malate for the supernatant enzyme (20,50,51,64) while m-MDH generally shows no inhibition until 0.1 M or above (39,31,51). Malate inhibition begins at 20-30 mM for brine shrimp s-MDH in accordance with the above generalization.

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Figure 1. Elution profile of s-MDH from the cellulose phosphate column with linear gradient of potassium acetate buffer, pH 5.0.

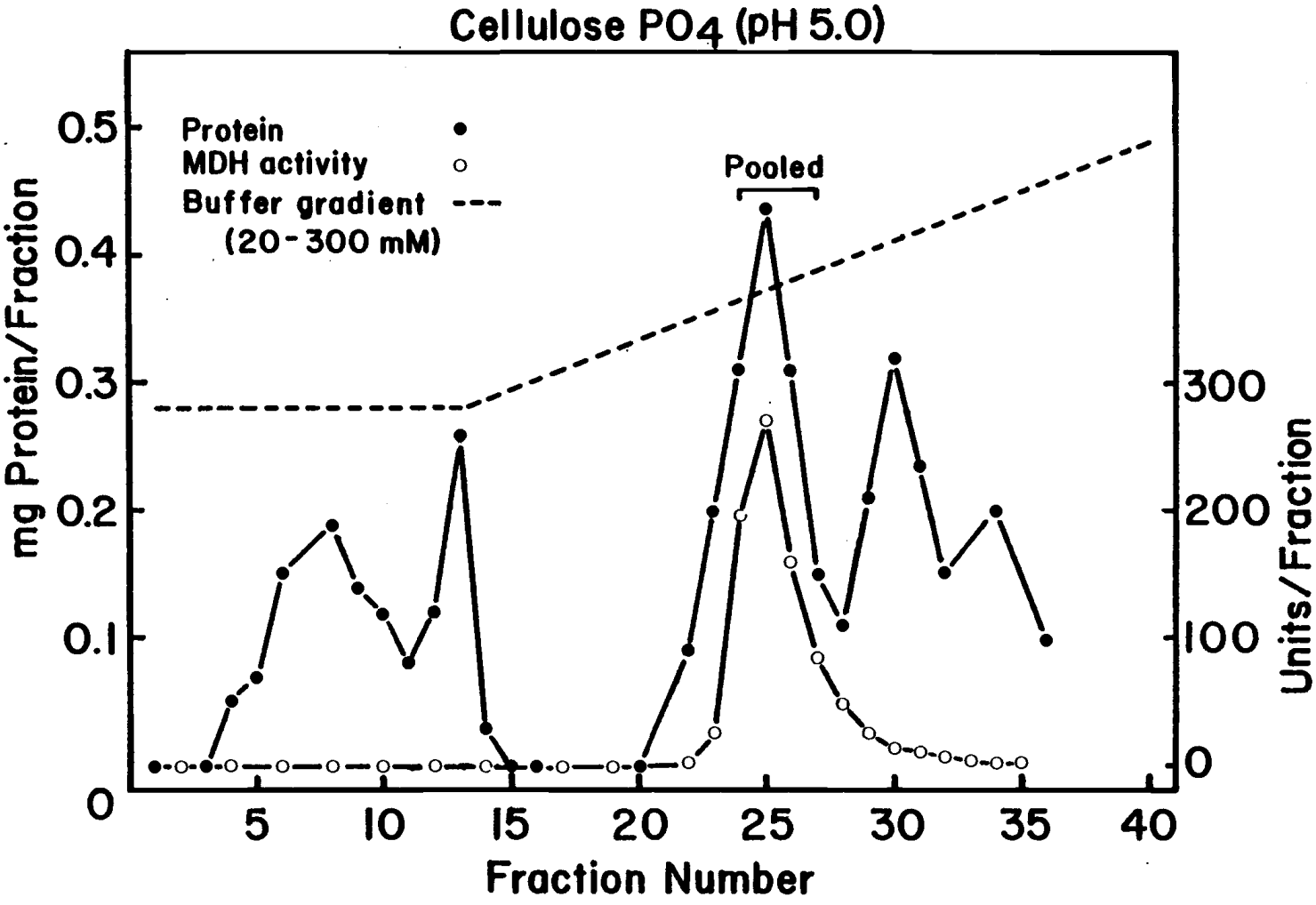
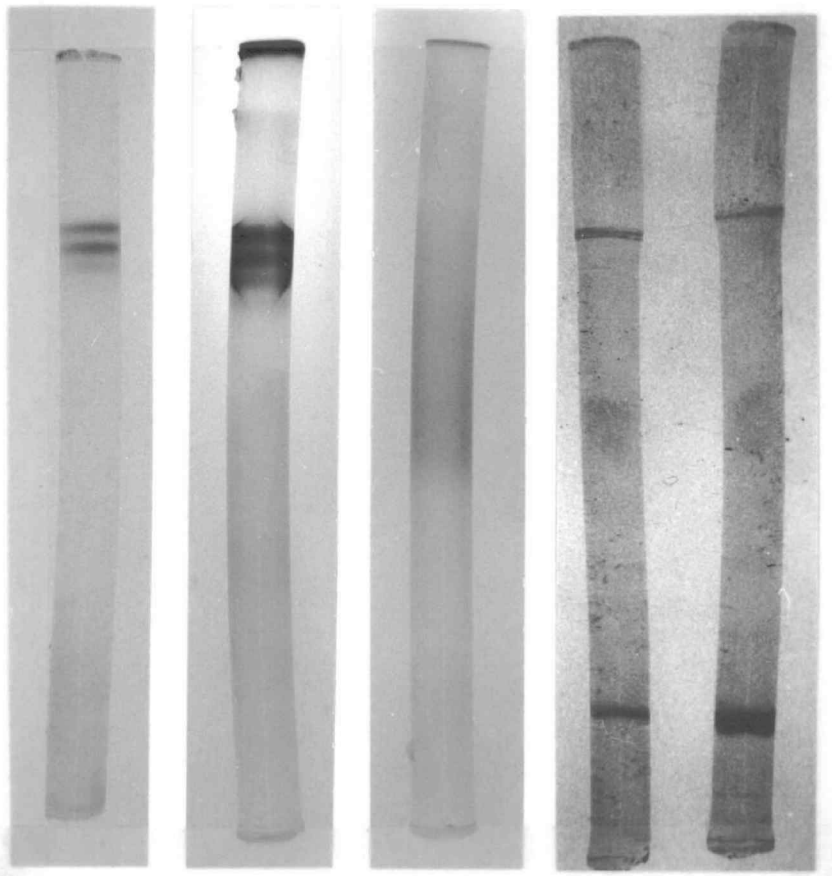

Cellulose PO4 (pH 5.0)
Protein
MDH activity
Buffer gradient
(20-300 mM)
Pooled
mg Protein/Fraction
Units/Fraction
Fraction Number
0
0.1
0.2
0.3
0.4
0.5
100
200
300
5
10
15
20
25
30
35
40

Figure 2. Non-denaturing 7% polyacrylamide gels (A-C) of the purified brine shrimp s-MDH protein. Gel A is stained for total protein with Coomassie Brilliant Blue. Gels B and C are identical to gel A but stained for MDH activity using the tetrazolium salt-formazan derivative technique. Gel C (control, no malate present) demonstrates the substrate specificity of the staining reaction. An SDS gel (7.5% polyacrylamide) of brine shrimp s-MDH (gel D) is compared to porcine heart m-MDH (gel E). Both are stained with Coomassie Brilliant Blue.



A B C D E

Figure 3. Plot of ln C versus r^2 for the data from the sedimentation equilibrium experiment with brine shrimp s-MDH at 0.45 mg/ml. Regression lines above and below the break are calculated by the method of least squares.

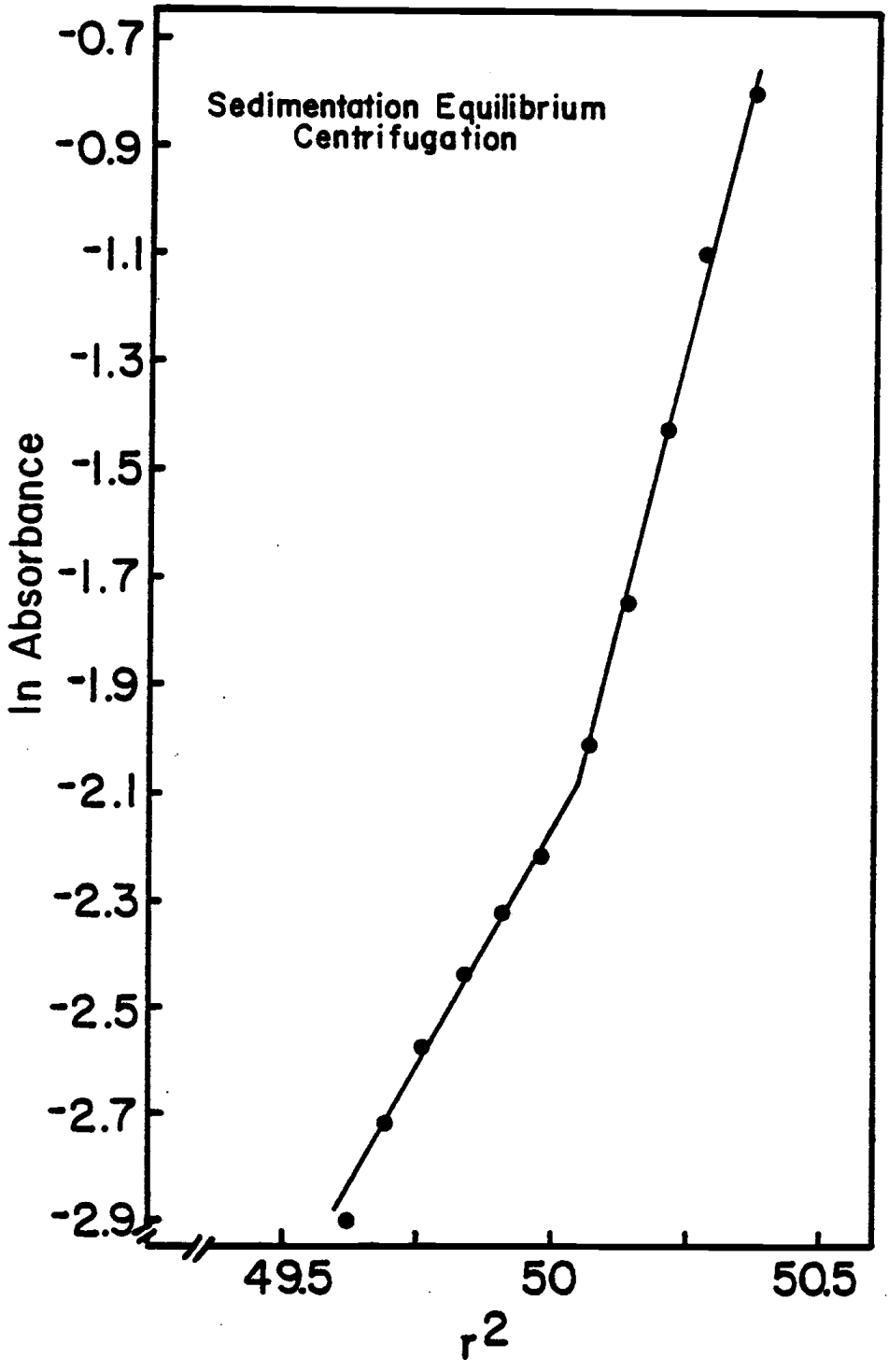
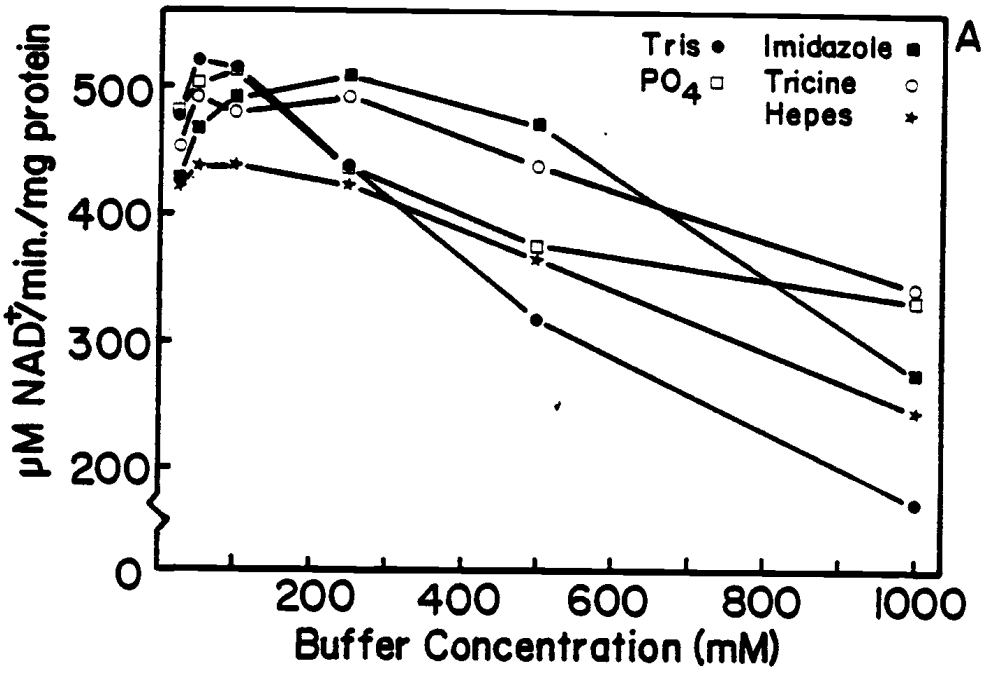

Sedimentation Equilibrium
Centrifugation
-0.7
-0.9
-1.1
-1.3
-1.5
-1.7
-1.9
-2.1
-2.3
-2.5
-2.7
-2.9
ln Absorbance
49.5
50
50.5
r2

Figure 4. (a) Effect of buffer species and concentration on s-MDH catalytic activity. (b) Maximal activity as a function of pH in 0.05 M buffer.


A
Tris •
PO4 □
Imidazole ■
Tricine ○
Hepes ★
μM NAD+/min./mg protein
0
200
300
400
500
200
400
600
800
1000
Buffer Concentration (mM)

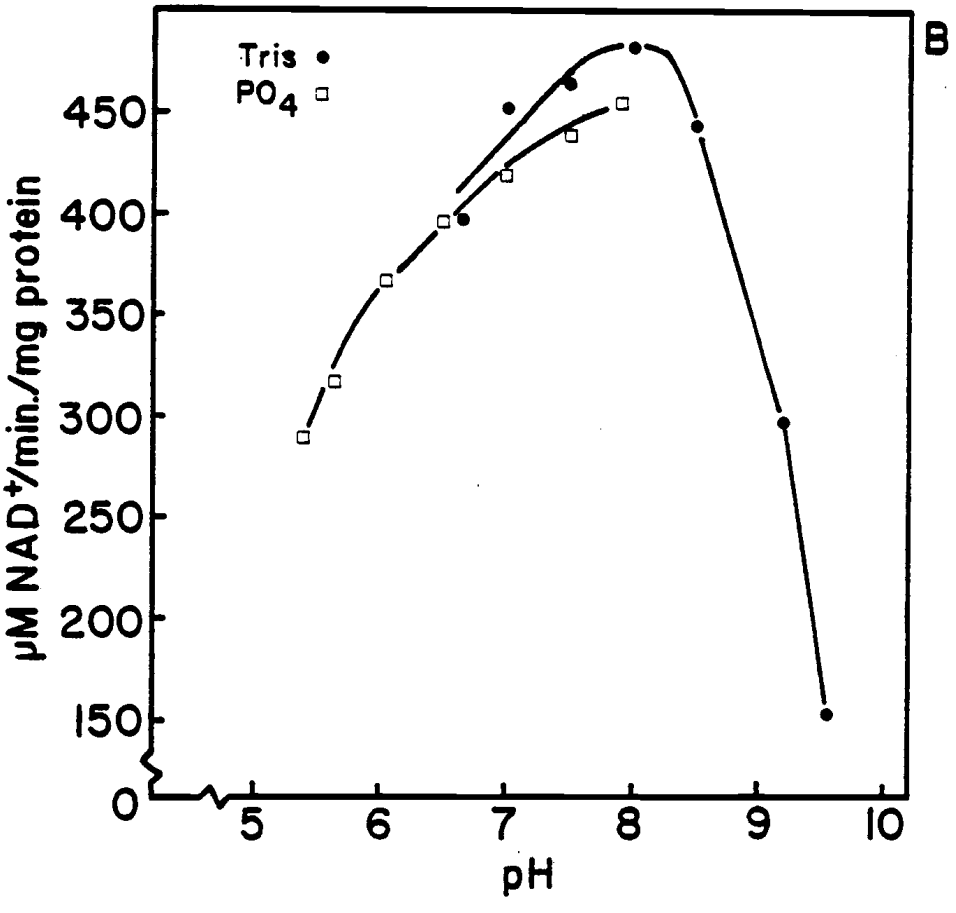

B
Tris •
PO4 □
μM NAD+/min./mg protein
0
150
200
250
300
350
400
450
5
6
7
8
9
10
pH

Figure. 5. (a) Catalytic activity (μM NAD^+/min/ml) of brine shrimp s-MDH after equilibration to indicated temperatures for 1.0 min. (b) First order decay of enzymatic activity with time at 48°C and 55°C. (c) Arrhenius plot of temperature data from (a). Regression line calculated by least squares method. Velocity expressed as molecular activity.

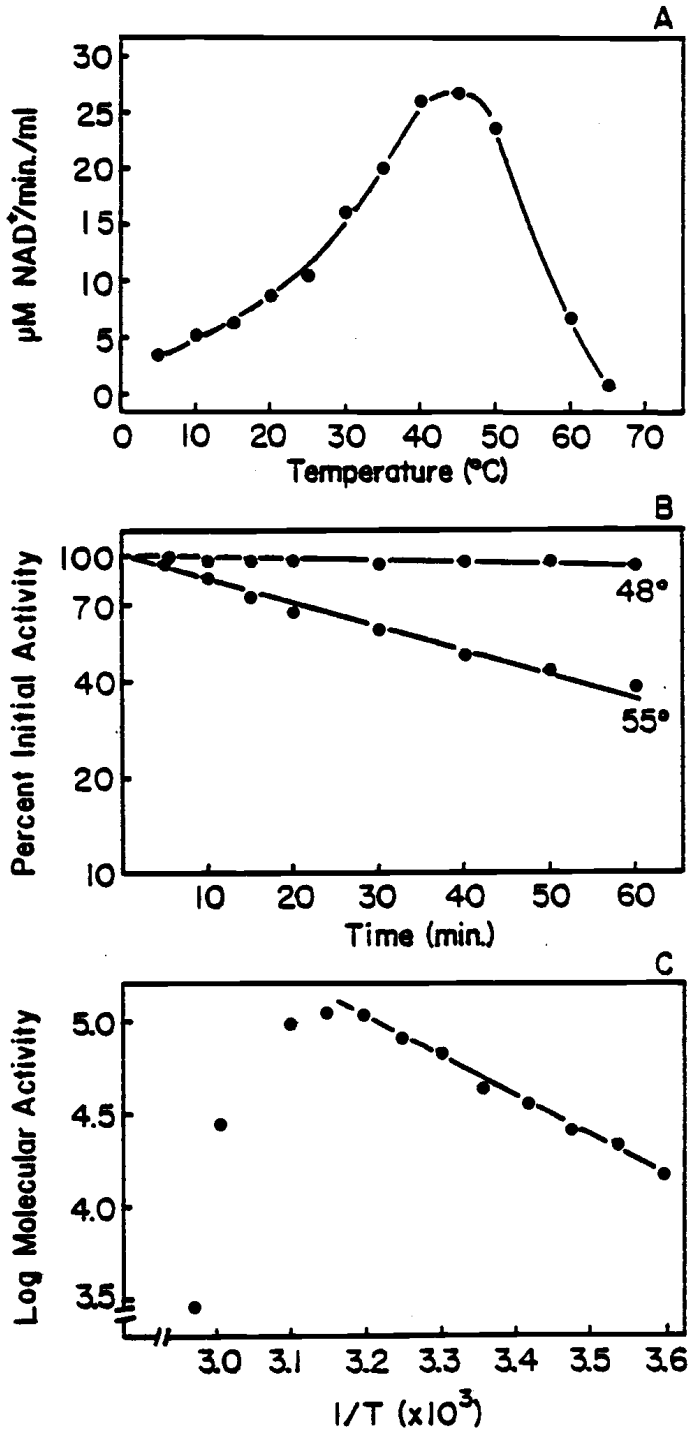

A
μM NAD*/min./ml
30
25
20
15
10
5
0
0 10 20 30 40 50 60 70
Temperature (°C)
B
Percent Initial Activity
100
70
40
20
10
48°
55°
10 20 30 40 50 60
Time (min.)
C
Log Molecular Activity
5.0
4.5
4.0
3.5
3.0 3.1 3.2 3.3 3.4 3.5 3.6
1/T (x10³)

Figure 6. Primary plots of reciprocal initial velocity versus reciprocal NADH concentration. Oxaloacetate concentrations (fixed substrate) are indicated near appropriate curve. Reaction conditions are 0.05 M tris-HCl buffer, pH 8.0, at 25°C. Initial velocities expressed as μM NAD^+/min/ml.

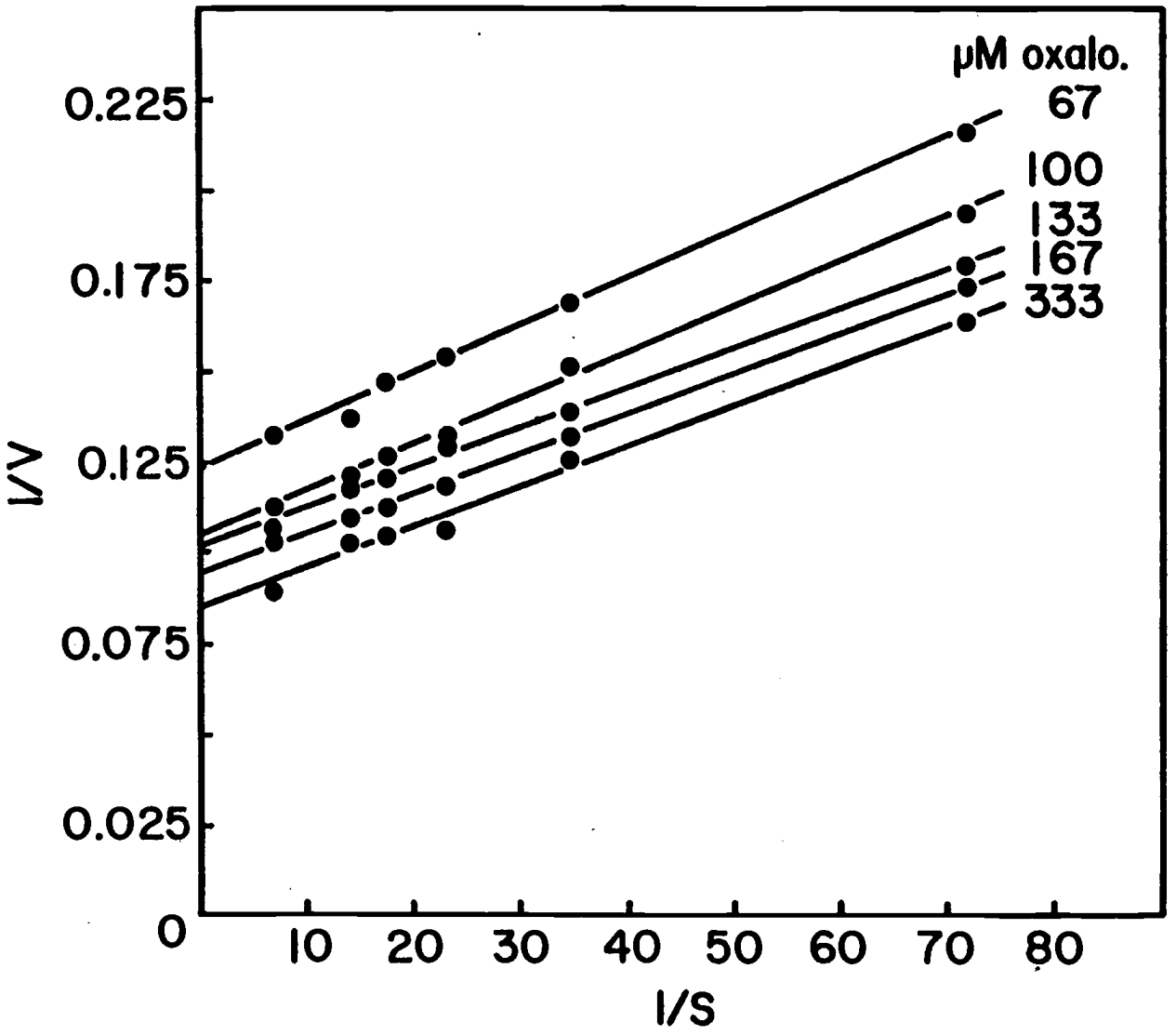

μM oxalo.
67
100
133
167
333
0.225
0.175
0.125
0.075
0.025
0
1/V
10
20
30
40
50
60
70
80
1/S

Figure 7. Primary plots of reciprocal initial velocity versus reciprocal oxaloacetate concentrations. NADH concentrations (fixed substrate) are indicated near appropriate curve. Reaction conditions are the same as in Figure 6. Initial velocities expressed as μM NAD^+/min/ml.

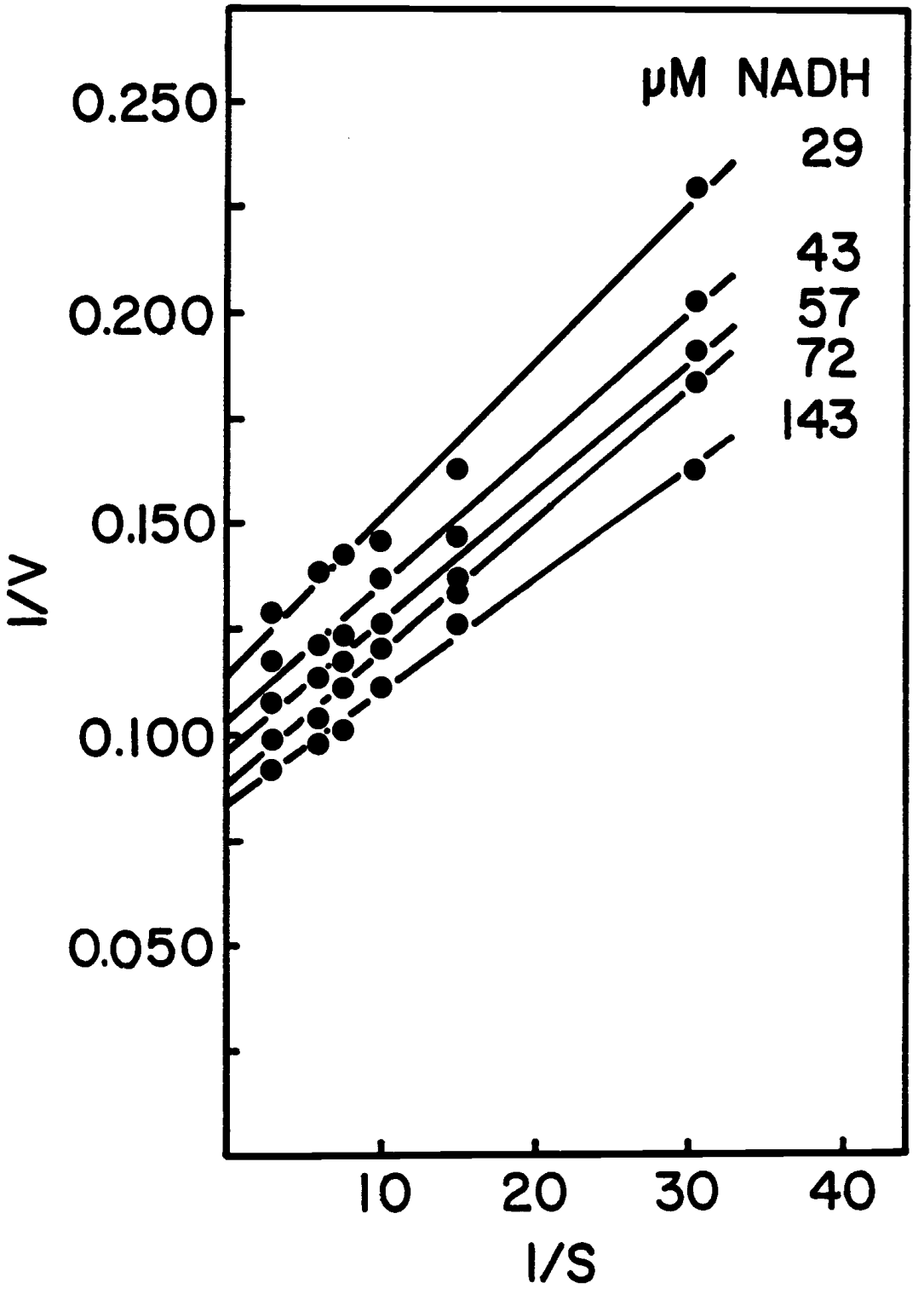

μM NADH
29
43
57
72
143
0.250
0.200
0.150
0.100
0.050
1/V
10
20
30
40
1/S

Figure 8. Secondary plots of ordinate intercepts versus reciprocal of fixed substrate concentrations from Figures 6 and 7.

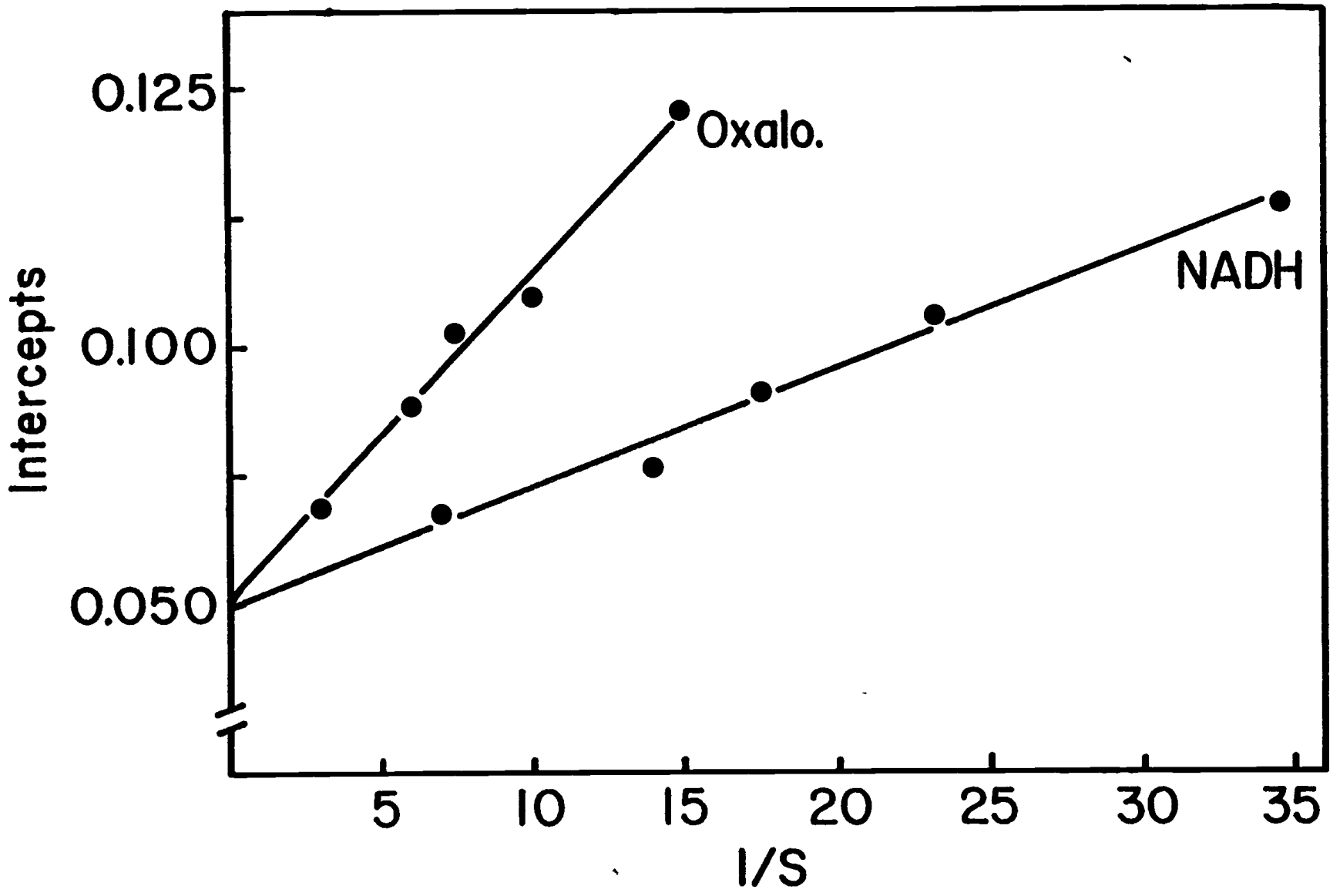
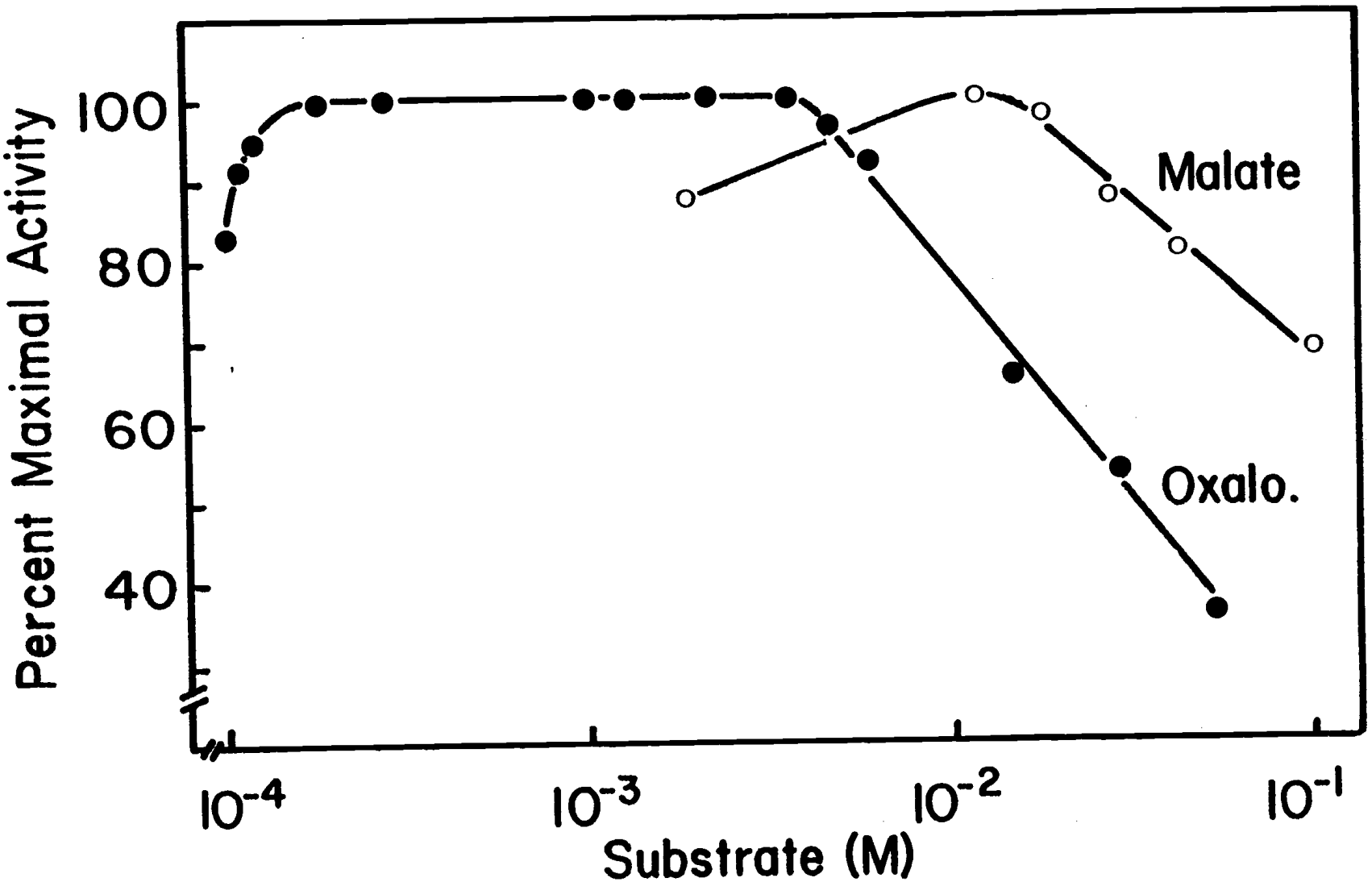

0.125
0.100
0.050
Intercepts
Oxalo.
NADH
5
10
15
20
25
30
35
1/S

Figure 9. Effect of substrate concentration on enzymatic activity expressed as percent of optimal activity.


Percent Maximal Activity
100
80
60
40
Malate
Oxalo.
10^{-4}
10^{-3}
10^{-2}
10^{-1}
Substrate (M)

CHAPTER II

Immunochemical Quantification and Characterization of Brine Shrimp Malate Dehydrogenase

Abstract

The cytosolic malate dehydrogenase (s-MDH) from brine shrimp nauplii has been immunochemically characterized with quantitative rocket immunoelectrophoresis (IEP) and antibody catalytic inhibition studies. Monospecific rabbit antiserum was produced against purified s-MDH and used for rocket IEP as a quantitative assay for the cytoplasmic isozyme. Since cross-reactivity is not observed against the mitochondrial MDH (m-MDH), the assay allows specific measurement of the s-MDH in crude naupliar supernatants in the presence of contaminating m-MDH. The assay has a sensitivity of approximately 100 nanograms s-MDH protein with a 4% standard deviation using either the purified enzyme or supernatant preparations. Catalytic inhibition studies using the monospecific antiserum gave 85% inhibition of s-MDH but no significant inhibition of the brine shrimp m-MDH. Lack of complete s-MDH inhibition by the antiserum suggests a difference between the enzyme active site and immunological binding site. Although the antiserum was produced against s-MDH from nauplii of the San Francisco Bay population, the antiserum inhibits equally well the s-MDH in supernatants of Great Salt Lake (Utah) nauplii, indicating little if any structural difference in the immunological sites of the s-MDHs from the two sources. Porcine s-and m-MDH, and beef and pigeon m-MDH were also tried as antigens against the brine shrimp s-MDH antiserum, and reactivity was not achieved with the enzymes during rocket IEP or catalytic inhibition experiments.

Introduction

In animal cells malate dehydrogenase exists primarily as two isozymes, differing in intracellular location and physiochemical properties (for review, see Banaszak and Bradshaw, 1975). The mitochondrial, Kreb's cycle isozyme (m-MDH)[1] and the soluble, cytoplasmic form (s-MDH) are both present in homogenates used for quantitative measurement of total MDH catalytic activity. Often studies require quantification of only one of the two isozymes, for example s-MDH. Since a variable amount of mitochondria are unavoidably ruptured during homogenation, it is not possible to rely solely on catalytic activity even in "post-mitochondrial" supernatants as a measure of s-MDH. Separation of the isozymes can be achieved by salt fractionation and column chromatography (Gerding and Wolfe, 1969; Hand, 1979), but the percent recovery of s-MDH is variable, making calculations of total s-MDH units in the original homogenate (or per animal) unreliable.

In hypersaline environments energy obtained from oxidative metabolism by Artemia salina nauplii is insufficient to maintain adenylate energy pools at a constant level (Conte et al., 1979), presumably due to large energy utilization by the sodium-potassium transport system for salt regulation (Conte, Droukas and Ewing, 1977;

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[1]Abbreviations used are: malate dehydrogenase (s-MDH, m-MDH), L-malate: NAD+ oxidoreductase (EC1.1.1.37); immunoelectrophoresis, IEP; homogenizing medium, HM.

Ewing, Peterson and Conte, 1974; Peterson et al., 1978). Brine shrimp cytoplasmic MDH is a component of an energy-yielding glycolytic pathway which may play a role in meeting the energy demands imposed on nauplii in high salt environments. To investigate this possibility, it is necessary to quantitatively measure s-MDH levels under varying developmental and environmental conditions.

This report describes the use of quantitative rocket immunoelectrophoresis (IEP), originally developed for serum proteins by Laurell (1966), for measurement of s-MDH from brine shrimp nauplii. The assay is shown to be specific for the cytoplasmic isozyme and has a sensitivity comparable to catalytic assays. Immunoprecipitation and catalytic inhibition of brine shrimp and vertebrate malate dehydrogenases by the antiserum against purified brine shrimp s-MDH is investigated.

Materials and Methods

Materials. Oxaloacetate, malate, NADH (Grade III), NAD^+ (Grade III), nitro blue tetrazolium, phenozine methosulfate, barbitol and sodium barbitol (barbitone; 5-5'-diethyl barbituric acid), crystalline bovine serum albumin and the purified vertebrate malate dehydrogenases were purchased from Sigma Chemical Company. Sodium azide was obtained from Eastman Kodak Company and ultra-pure ammonium sulfate from Schwartz-Mann Company. Complete and incomplete Freund's adjuvant were supplied by Difco Laboratories, and HBT Agarose was a product of Marine Colloids, Inc.

Brine shrimp encysted gastrulae were purchased from San Francisco Bay Brand Company, and the Great Salt Lake (Utah) cysts acquired from Longlife Products, Harrison, N.J.; both were stored *in vacuo* at -20°C. Nauplii were reared as previously described (Hand, 1979).

Purification and Activity Assay of s-MDH. Cytoplasmic malate dehydrogenase was purified from 27-hour San Francisco Bay nauplii following the protocol of Hand (1979). Routine assays were performed at 25°C in the following 3 ml assay mixture: 50 mM Tris-HCl buffer (pH 8.0), 0.33 mM oxaloacetic acid, 0.14 mM NADH and 0.1 ml of enzyme solution. Enzymatic oxidation of NADH was followed at 340 nm using a Varian-Cary 219 Dual Beam spectrophotometer. Crystalline bovine serum albumin (BSA) was present at 0.2 mg/ml in the enzyme diluent to prevent inactivation at low protein concentrations.

Antibody Preparation against Brine Shrimp s-MDH. Antigen samples were dialyzed extensively against phosphate buffered saline (0.01 M potassium phosphate buffer, pH 7.5, 0.1 M NaCl) and then emulsified 1:1 with Complete Freund's adjuvant (for the primary injection) using a 20 gauge microemulsifying needle. Sample emulsification was continued until the material was quite viscous and exhibited no phase separation when a droplet was placed in cold water.

New Zealand white rabbits received subcutaneous injections of the emulsified s-MDH protein (400 µg total) at 8-10 sites, paired on opposite sides of the lumbar spinal region. Maximum volume injected per site was 0.1 ml. Four weeks after the initial injection, a 400 µg booster in incomplete adjuvant was administered in the same manner. Whole blood was collected 10-14 days after the booster from the marginal ear vein or by heart puncture. For comparison of relative protein antigenicity, antiserum against crystalline bovine serum albumin (BSA) was prepared in a similar manner.

After collection, whole blood was allowed to clot in glass centrifuge tubes at room temperature and refrigerated overnight for clot contraction. Antiserum was separated by centrifugation (30 min, 1000 X g), and the straw-colored supernatant stored refrigerated under 0.1% sodium azide. For prolonged storage the antiserum was fractionated with 3 sequential 0-45% ammonium sulfate precipitations, and the isolated immunoglobulins kept as a 50% AS precipitate at 4°C with 0.1% sodium azide. Antibody titer was routinely checked using Ouchterlony double diffusion plates (1% agarose, 0.1 M KCl, 0.1% NaN_3 in 0.05 M borate buffer, pH 8.5).

Quantitative Rocket Immunoelectrophoresis. For preparation of thin slab gels (1.5 x 84 x 94 mm), s-MDH mono-specific antiserum was added to a solution of 1% agarose in 0.05 M barbitol buffer (pH 8.6) at 48°C immediately before pouring. Adherence of the antiserum-agarose gel to the glass plates was facilitated by pre-coating with 0.5% agarose, then blow drying with warm air. Approximately 13 ml of solution was required for the size plates above, and final antiserum concentrations of 0.5-5% were routinely employed. To minimize diffusion of the applied protein solutions (17 µl) from the sample wells, the application was completed within 3 minutes and electrophoresis started immediately. Electrophoresis was performed in the anodal direction on a water-cooled surface using a LKB Multiphor 2117 immunoelectrophoretic unit set at 2 V/cm across the gel for 18-20 hours (voltage regulation). The barbitol solution used in preparing the gel mixture served as the reservoir buffer.

After electrophoresis, excess buffer and serum protein were wicked from the gels by covering with Miracloth and pressing with absorbent tissue overlaid with a glass plate for 15 minutes. Gel plates were then soaked in several changes of PBS for at least 2-4 hours with agitation to remove all residual non-precipitated protein, including unreacted serum; the precipitation complex itself is too large to be washed from the gel matrix. After a 15 min distilled water rinse, the plates were again pressed and dried with a warm air blower with Miracloth in place to prevent gel cracking. Gels were stained for protein with Coomassie Brilliant Blue (0.5% stain in 45% ethanol, 45% water, 10% glacial acetic acid) for 10 min, and destained with several changes of the above solution minus the 0.5% stain.

Since antibody-precipitated s-MDH still exhibited significant catalytic activity, gels could be stained for MDH activity with the tetrazolium salt-formazan derivative technique as used by Chen (1968). Duplicate gels were stained with a reaction mixture lacking malate to insure the observed staining was substrate dependent. For this technique gels were washed in PBS as above at 4°C, but not dried before activity staining. To visualize the unprecipitated mitochondrial MDH present in electrophoresed samples, gels were not washed prior to staining.

Purified brine shrimp s-MDH (25 µg/ml) was used as the standard by diluting with reservoir buffer containing 0.2 mg/ml BSA. Protein was assayed using the method of Lowry, et al. (1951) as recently modified by Peterson (1977). The presence of BSA was essential during serial dilutions to achieve linear standard curves. Although either rocket height or area could be used for measurement, height was more convenient providing symmetrically-shaped rockets were obtained. Measuring error was generally less than 3%.

Sample Preparation for Immunoelectrophoresis. Brine shrimp nauplii were homogenized at a ratio of 1 g wet weight:2 ml homogenizing medium (HM), and the homogenates were centrifuged 30 min at 39,000 X g in a Sorvall SR-5B refrigerator centrifuge. The post-mitochondrial supernatant was used for IEP analysis of s-MDH.

To obtain preparations of m-MDH, homogenates prepared as above were centrifuged to remove cell debris at 1000 X g for 10 min. The pellet was resuspended in an equal volume of HM, recentrifuged, and the

combined low-speed supernatants centrifuged at 30,000 X g for 30 min. The pellet containing mitochondria overlayed with a lighter buff-colored sediment was gently resuspended in 35 ml of HM and the high-speed centrifugation repeated. This washing procedure was performed twice. The washed mitochondrial pellet, suspended in 5 ml of HM, was sonicated (Sonifier Cell Disruptor, Model W185, Heat-Systems-Ultra-Sonics, Inc.) for 2 minutes at 75 watts and then subjected to a freeze-thaw procedure using liquid nitrogen. The solution was centrifuged for 30 minutes at 30,000 X g and the supernatant used as the m-MDH preparation for IEP.

Antibody Inhibition of s-MDH Catalytic Activity. Brine shrimp s-MDH was diluted with buffer (1.0 mM dithiothreitol, 0.5 mM EDTA, 10% glycerol and 0.2 mg/ml BSA in 0.05 M tris-HCl buffer, pH 8.0) to 6-8 µg/ml and incubated for 15 minutes at 0°C with varying dilutions of s-MDH antiserum. Inhibition occurred rapidly, and no further inhibition was achieved by using longer incubation times. Control s-MDH samples were incubated with buffer under the same conditions with no loss of catalytic activity observed over the incubation period. Addition of BSA was of particular importance in preventing inactivation of the diluted MDH and reducing enzyme adherence to the glass test tubes (Frieden and Fernandez-Sousa, 1975; Hand, 1979). In the absence of BSA, significant loss of catalytic activity occurred. Additional inhibition studies on porcine s- and m-MDH, and pigeon breast and beef heart m-MDH were performed in a similar manner.

It should be noted that undiluted rabbit antiserum possessed measureable MDH activity (0.1 unit/ml), but at the dilutions used in

inhibition studies it was negligible and did not contribute significantly to the measured enzyme activities. There are numerous reports of MDH and other dehydrogenases being present in serum from various sources; the MDH is most likely released into the serum via erythrocyte degradation (Hess, 1959; Vesell and Bern, 1958).

Results

Antigenicity of Brine Shrimp s-MDH. The relative antigenicity of brine shrimp MDH and BSA are compared in Figure 1, which plots serum antibody titer versus time after initial and booster injections of the soluble proteins. At 4 weeks BSA elicited a slightly higher titer than did s-MDH, although both titers were very low. After the booster injection, antibody titers against both proteins displayed a rapid rise to maximum levels in 10 days, followed by a decline to low titers which remained for several weeks. A typical Ouchterlony plate for brine shrimp s-MDH is pictured in Figure 2 showing the single precipitin band observable after 8-10 hours of diffusion at room temperature.

Rocket Immunoelectrophoresis. As demonstrated in Figures 3 and 4, precipitin rocket height was directly proportional to s-MDH protein using serial dilutions of the purified enzyme. The lower limit of sensitivity was approximately 100 µg s-MDH protein per sample well (17 µl). Although below this level the response was still linear, measurement of the small rockets became difficult without photographic or optical enlargement. The reproducibility of the IEP assay with purified brine shrimp s-MDH is shown in Table I.

Crude post-mitochondrial supernatants containing s-MDH exhibited the same proportionality and linearity observed with the purified brine shrimp s-MDH standards over a comparable concentration range. Not unexpectedly however, antiserum cross-reactivity was evident using the crude PMS. In contrast to the single precipitin rocket observed when

Table 1. Reproducibility of quantitative rocket IEP assay with brine shrimp s-MDH. Replicates for purified s-MDH samples were collated from several different gel plates. SD = standard deviation.

Purified Brine Shrimp s-MDH Standards				Brine Shrimp Post-Mitochondrial Supernatant s-MDH Samples						
Sample	Rocket Length (mm)	Average ± SD	% SD	Sample	Dilution	Rocket Length (mm)	ng	µg/ml	Average ± SD	% SD
1	16.2			1	1/4	17.0	177	41.6		
	16.2				1/4	16.8	176	41.6		
	15.5								42.6 ± 2.0	4.7
	16.8	16.6 ± 0.8	4.0		1/8	9.6	98	46.1		
	17.5				1/8	8.6	88	41.4		
	16.0									
	16.8			2	1/4	22/4	222	52.2		
	17.5				1/4	23.9	238	56.0		
2	25.9	25.8 ± 0.1	0.5		1/8	12.4	122	57.4	57.0 ± 3.7	6.5
	25.9				1/8	13.5	133	62.6		
	25.6									
3	23.6	22.9 ± 0.5	2.3							
	22.6									
	22.4									

purified s-MDH was assayed with IEP, the PMS gave at least two rockets, one due to s-MDH and the other being a non-MDH soluble protein. Identification of the s-MDH rocket was based on MDH-specific enzymatic activity staining. The cross-reacting protein(s) did not show MDH catalytic activity (Fig. 5) and was probably of extra-mitochondrial origin, since washed mitochondrial preparations (see methods) showed no such precipitin reaction (Fig. 6). Additional characterization of the protein has not been pursued.

It was important to establish that the rocket IEP assay was specific for the cytoplasmic MDH isozyme, and does not show reactivity with the m-MDH. For this purpose, brine shrimp m-MDH extracted from washed mitochondrial preparations was compared to supernatant samples containing s-MDH using the IEP assay. Precipitin rockets were not seen with mitochondrial samples at any dilution assayed (Fig. 6). However, at the anodal end of the plate a heavily activity-stained region was observed which represented the unprecipitated m-MDH protein which electrophoresed across the plate. This protein could be washed from the gel, was very diffuse, and in no way resembled a precipitin rocket. In contrast, PMS samples prepared from the same naupliar material gave distinct s-MDH precipitin rockets which stained for enzyme activity, and prolonged washing did not remove the precipitated protein. Small amounts of unreacted m-MDH were visualized at the anodal end of the plates by activity staining. This expected observation was due to unavoidable rupture of some mitochondria during PMS preparation. From this data it is clear that this antiserum produced against brine shrimp s-MDH had no immunoprecipitation reaction against m-MDH judged by immunoelectrophoresis.

Antibody Inhibition of s-MDH Catalytic Activity. At a dilution of 1/1024, antiserum prepared against San Francisco Bay naupliar s-MDH inhibited the enzyme catalytic activity by 15%. Figure 7 depicts the reduction in brine shrimp s-MDH activity as a function of antiserum concentration. Maximum inhibition (85% reduction from control values) occurred in the presence 3% antiserum (1/32 dilution); complete inhibition was never achieved. Crude preparations of brine shrimp mitochondrial MDH showed no significant inhibition with the s-MDH specific antiserum at any concentration. The lack of m-MDH activity inhibition was in agreement with the rocket IEP analyses, which also demonstrated no cross-reactivity.

As a possible indication of populational differences, cysts from the Great Salt Lake (Utah) were cultured and naupliar PMS preparations assayed for s-MDH inhibition by the antiserum. The catalytic activity of s-MDH preparations from Great Salt Lake nauplii was inhibited to the same extent (85% inhibition with 3% antiserum) as the San Francisco Bay s-MDH, against which the antiserum was originally prepared.

Brine Shrimp s-MDH Antiserum Versus Vertebrate MDH. Porcine s- and m-MDH, and beef and pigeon m-MDH were assayed with rocket IEP to determine the presence or absence of precipitin reactions with brine shrimp s-MDH antiserum. Figure 8 demonstrates the lack of precipitin rocket formation using two concentrations of each vertebrate enzyme. In the case of beef m-MDH at 125/µg/ml, a faint, diffusely-staining streak was observable, but it had little resemblence to a true precipitin rocket and probably represented residual protein not completely washed from the

gel matrix at this concentration. Protein staining was not observed at the lower beef MDH concentration (25 µg/ml). Catalytic inhibition studies supported the above observations, since the brine shrimp s-MDH antiserum produced no inhibition of the vertebrate enzymes, including the beef m-MDH.

Discussion

The present investigation demonstrates the applicability of quantitative rocket immunoelectrophoresis for assaying specifically the cytoplasmic malate dehydrogenase isozyme from Artemia salina nauplii. The cytoplasmic isozyme can be quantitatively measured from post-mitochondrial supernatants in the presence of contaminating mitochondrial MDH with a sensitivity of 100 µg s-MDH protein. Using a specific activity of 550 µM NAD^+/min/mg protein for the purified enzyme, the 100 µg represents approximately 0.055 s-MDH units, which is approximately the number of units employed for routine catalytic activity assays (about 0.015 units). Therefore, the rocket IEP assay has sensitivity similar to catalytic assays and the additional advantage of being isozyme-specific, at least for the brine shrimp system. Although slightly less sensitive than the radioimmunoassay, rocket IEP is superior in certain respects. Simplicity, direct visual control of the reaction, and the possibility of comparing crude antigen preparations is particularly useful when changes in antigenic profiles cannot be predicted (Georgiades et al., 1977).

Regarding the occurrence of isozyme specificity by MDH antiserum, Kitto and Kaplan (1966) reported that antiserum to purified chicken s-MDH yielded a sharp precipitin band with the supernatant isozyme and inhibited catalytic activity, but no precipitin reaction or inhibition was observed with the mitochondrial form. Antiserum to chicken m-MDH showed exactly the opposite behavior. Similar absence of cross-reactivity was also noted for antiserum directed against crystalline

tuna and pig m-MDH, and crystalline ostrich s-MDH. In the cases of yeast and beef heart MDH's, patterns of immunological reactivity were somewhat different. Although antiserum against the cytoplasmic isozymes showed no cross-reactivity with mitochondrial forms, m-MDH antiserum exhibited precipitin reactions and catalytic inhibitions with the cytoplasmic isozymes (Hagele, Neeff and Mecke, 1978; Grimm and Doherty, 1961). From these examples it is evident that rocket IEP would probably possess isozyme specificity (at least for one of the isozyme forms) for many MDH sources.

Use of rocket IEP for enzyme protein analysis has been somewhat limited to date. Studies with erythrocyte carbonic anhydrase (Norgaard-Pedersen and Mondrup, 1971), serum lysozyme (Johansson and Malmquist, 1971) and erythrocyte acetylcholinesterase (Demus and Mehl, 1970) have utilized similar immunochemical methods, but examples employing quantitative IEP with enzymes of non-blood origin are even less numerous. Applying the method to a NAD^+/NADH oxidoreductase enzyme, as done in this report, permits utilization of specific enzymatic-activity staining of the precipitin rockets, providing all activity is not inhibited in the antigen-antibody complex. This staining is particularly advantageous for identification of the s-MDH rocket when antiserum cross-reactivity to unidentified proteins in crude homogenates results in multiple rocket formation. Additionally, the procedure results in virtually no background staining, giving the precipitin rocket high contrast for photographic records. After identification of the s-MDH rocket within the multiple pattern has been made, general protein staining (more economical) can be used for future plates.

The observation that complete inhibition of brine shrimp s-MDH cannot be achieved with mono-specific antiserum is similar to results from inhibition studies with beef s-MDH (Grimm and Doherty, 1961). These data suggest the s-MDH catalytic site may differ from the immunological binding site. The absence of immunological reactivity of vertebrate MDH's with the brine shrimp s-MDH antiserum observed with both inhibition and IEP assays implies the existence of different primary structures at the immunological sites compared to the brine shrimp enzyme.

The low-voltage (2 V/cm gel) or "slow rocket" technique employed with s-MDH gave greater reproducibility than the high voltage (8-10 V/cm gel) or "fast rocket" technique, presumably due to its lesser sensitivity to perturbing effects such as temperature, pH and voltage variations (for review of IEP methods, see Axelson, Kroll and Weeke, 1973). In this study sensitivity for s-MDH protein was maximized by using the lowest antiserum concentration still allowing adequate rocket visualization. However, this modification resulted in a relatively narrow s-MDH concentration range on any given plate. Since variation from plate to plate in rocket height was occasionally observed, at least one standard was included on all plates.

It has been recently suggested that the North American Artemia category (1 of 4) composed of 7 populations be reclassified as Artemia franciscana due to reproductive isolation observed among categories (Barigozzi, 1974; Bowen and Sterling, 1978; Clark and Bowen, 1976). With additional information on Artemia ecology and population genetics being rapidly generated, it is increasingly important to state the

locality from which experimental Artemia are collected; the number of Artemia sibling species is in a state of flux (Cole and Brown, 1967). Nauplii from two of the North American populations were used in the present study. The s-MDH antiserum, which was produced against the enzyme isolated from the San Francisco Bay brine shrimp population, inhibited naupliar s-MDH from both the Great Salt Lake (Utah) and San Francisco Bay populations to an equal extent. The result supports close structural similarity at the s-MDH immunological site, and therefore the enzymes from the 2 different populations are indistinguishable using this criterion.

Attempts have been made to use electrophoretic patterns of malate dehydrogenase "isozymes" for distinction of adult brine shrimp populations (e.g., Bowen and Sterling, 1978). However, brine shrimp MDH subforms observed on polyacrylamide gel electrophoresis (as many as 7 bands) have not yet been distinguished as true isozymes. It is probable that there are only 2 true isozymes (s- and m-MDH), and the other electrophoretic variants represent conformational, deaminational or proteolytic subforms (for review see Banaszak, 1975; Hand, 1979). The very small, or in some cases non-existent, variation in MDH polymorphism among Artemia adults supports this possibility (Bowen and Sterling, 1978), since genetic differences among individuals would be expected particularly if all 7 bands represented isozymes. Therefore, the seemingly common practice of applying the term "isozyme" to each and every multiple enzyme band displayed in electrophoresis, without detailed biochemical characterization of the enzyme system in question, may in some cases be misleading.

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Figure 1. Serum antibody titer (reciprocal of dilution as assayed with Ouchterlony double diffusion plates) versus days after injections of soluble proteins; antigens used were brine shrimp s-MDH (closed circles) and crystalline bovine serum albumin (open circles).

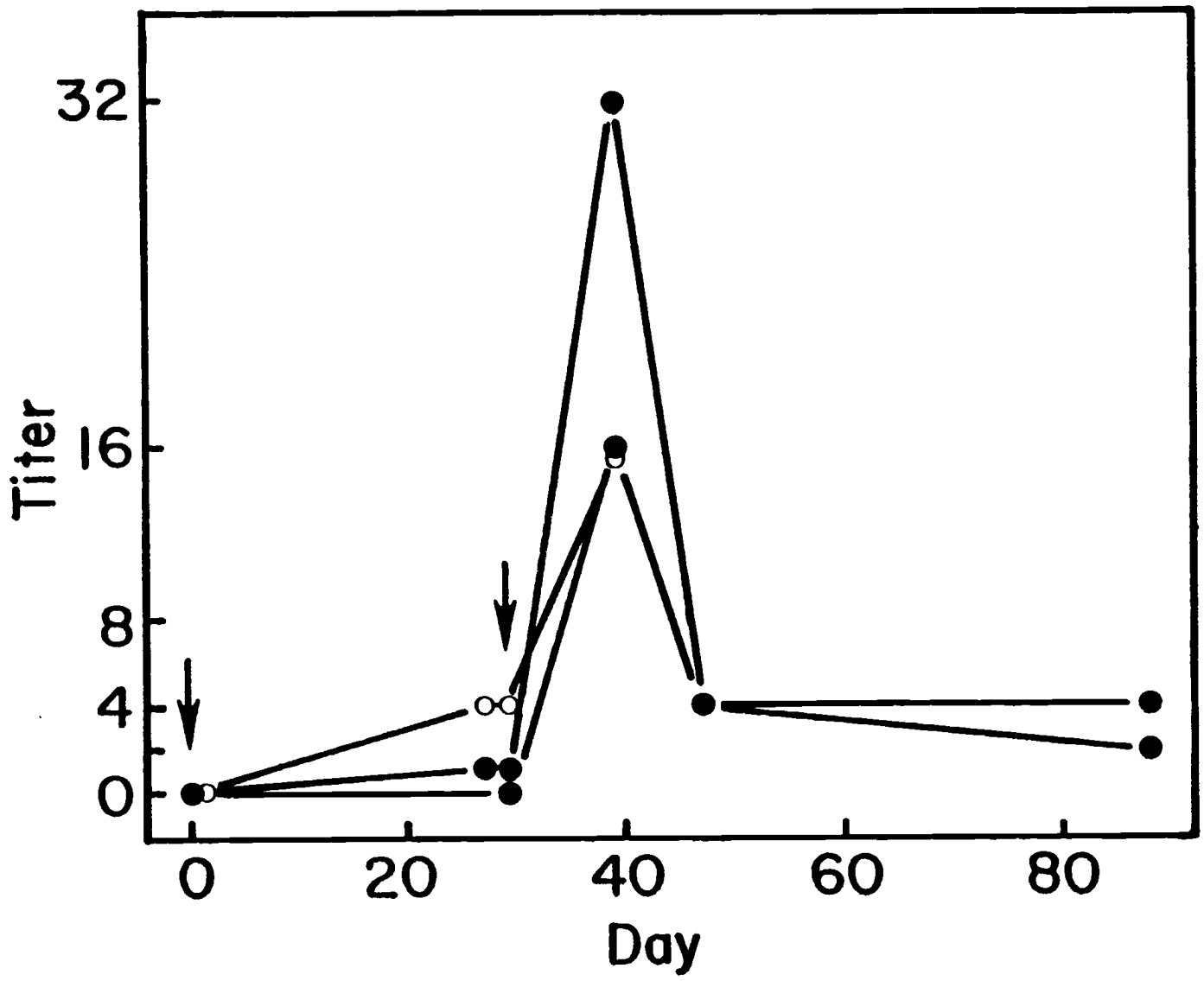

32
16
8
4
0
Titer
0
20
40
60
80
Day

Figure 2. Ouchterlony plate demonstrating the single precipitin band observed with 0.5 mg/ml brine shrimp s-MDH in center well and antiserum in outer wells at 1/4, 1/8, 1/16, 1/32, 1/64 dilutions (clockwise from top).

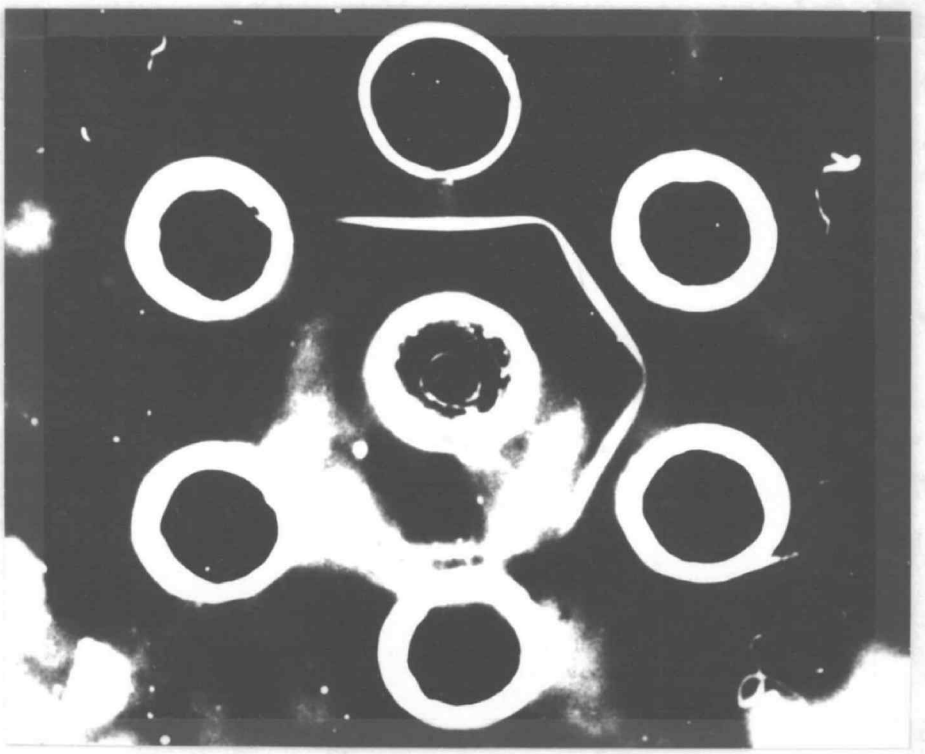


Figure 3. Rocket IEP plate showing the linear response of purified brine shrimp s-MDH. Dilutions of the 25 µl/ml stock solution are 5/5, 4/5, 3/5, 2/5 from left to right. Precipitin rockets are stained with Coomassie Brilliant Blue.

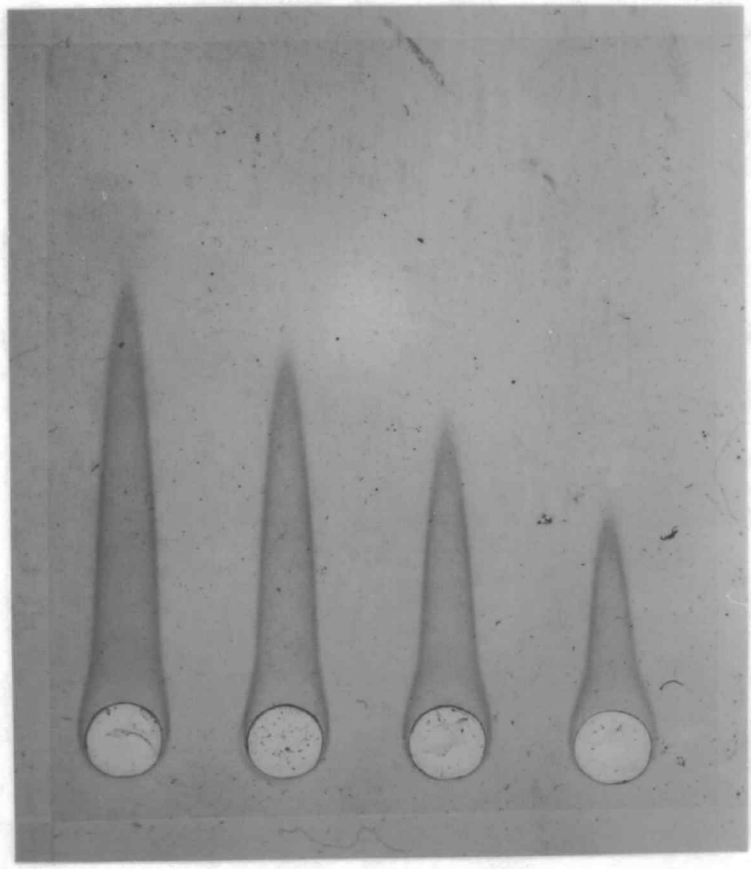


Figure. 4. Precipitin rocket height plotted against ng purified brine shrimp s-MDH for three concentrations of antiserum.

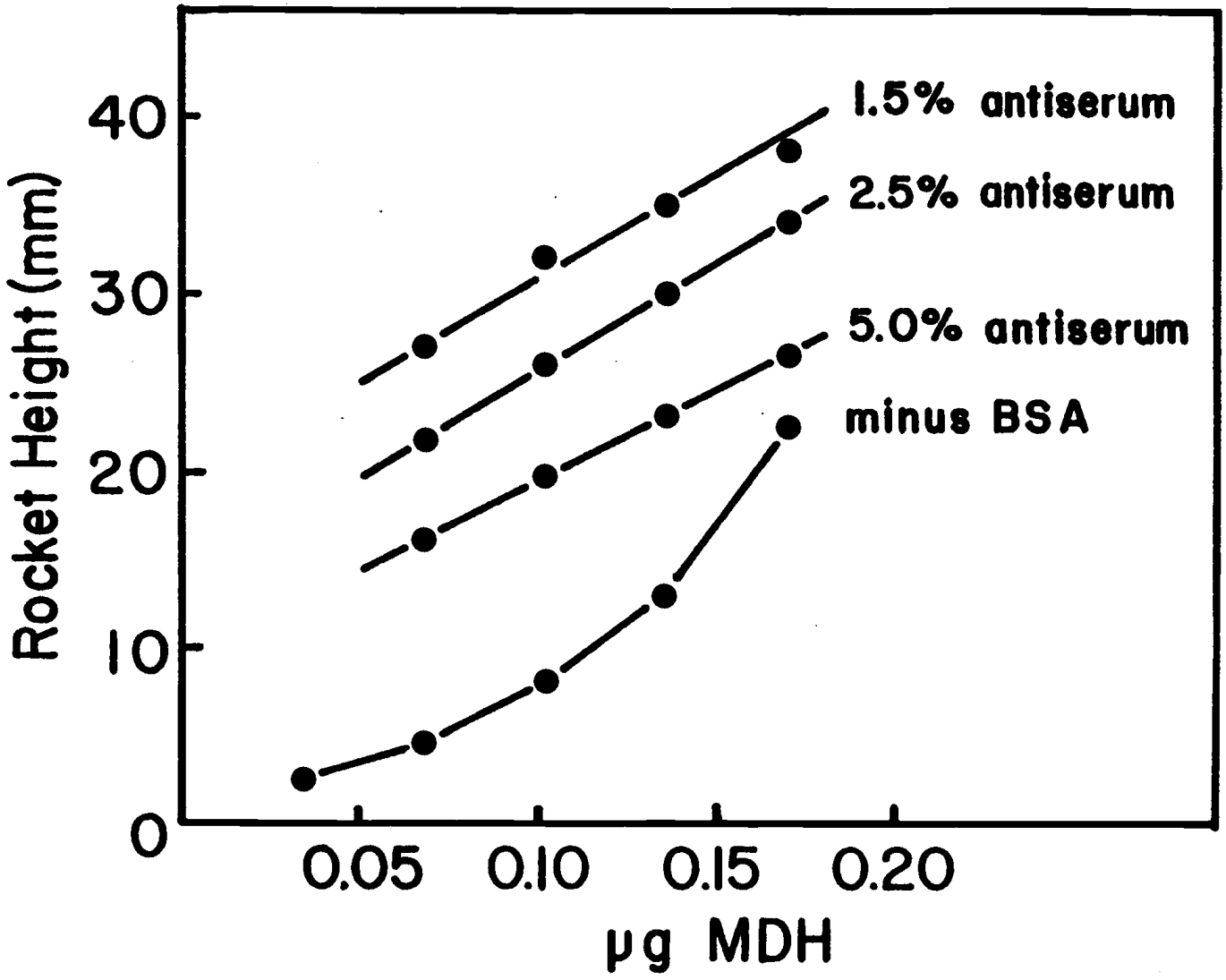
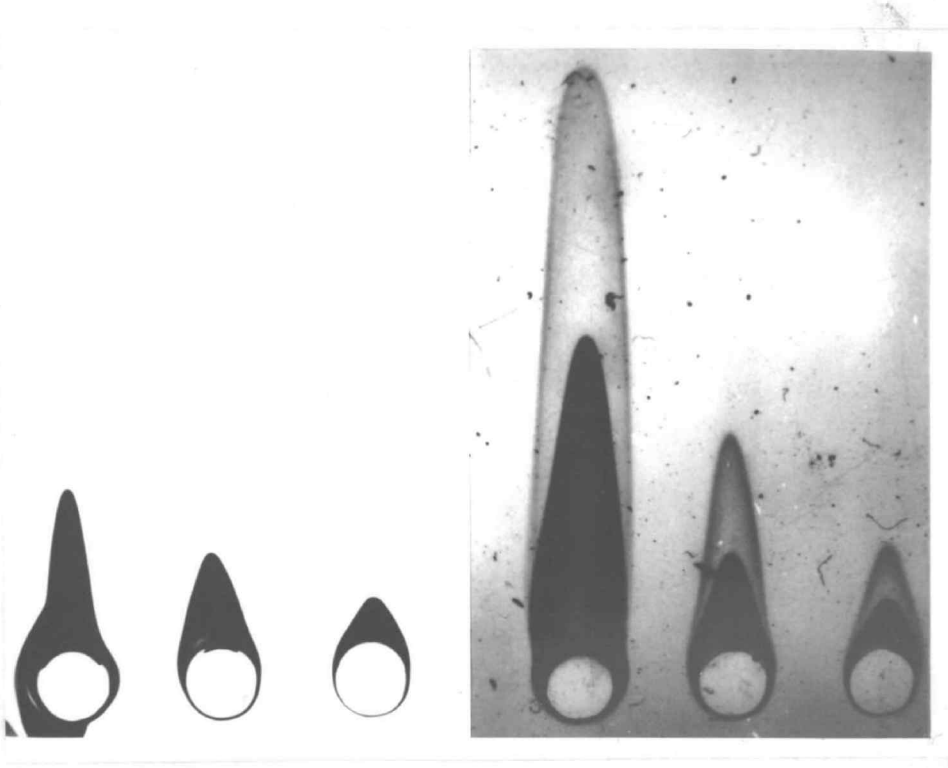

Rocket Height (mm)
40
30
20
10
0
0.05
0.10
0.15
0.20
µg MDH
1.5% antiserum
2.5% antiserum
5.0% antiserum
minus BSA

Figure 5. IEP plate of crude post-mitochondrial supernatant material demonstrating malate-dependent activity staining of the s-MDH precipitin rockets (A-C). A duplicate plate (wells D-F) stained with Coomassie Brilliant Blue protein stain demonstrates that in the crude preparation there is a cross-reacting protein which lacks MDH catalytic activity.



A B C D E F

Figure 6. Comparison of a naupliar mitochondrial preparation of m-MDH (A) to post-mitochondrial supernatant containing s-MDH (B,C). No antibody reactivity is observed against the m-MDH, which migrates to the upper (anodal) end of plate without precipitating (material seen at origin is carotenoid pigment).

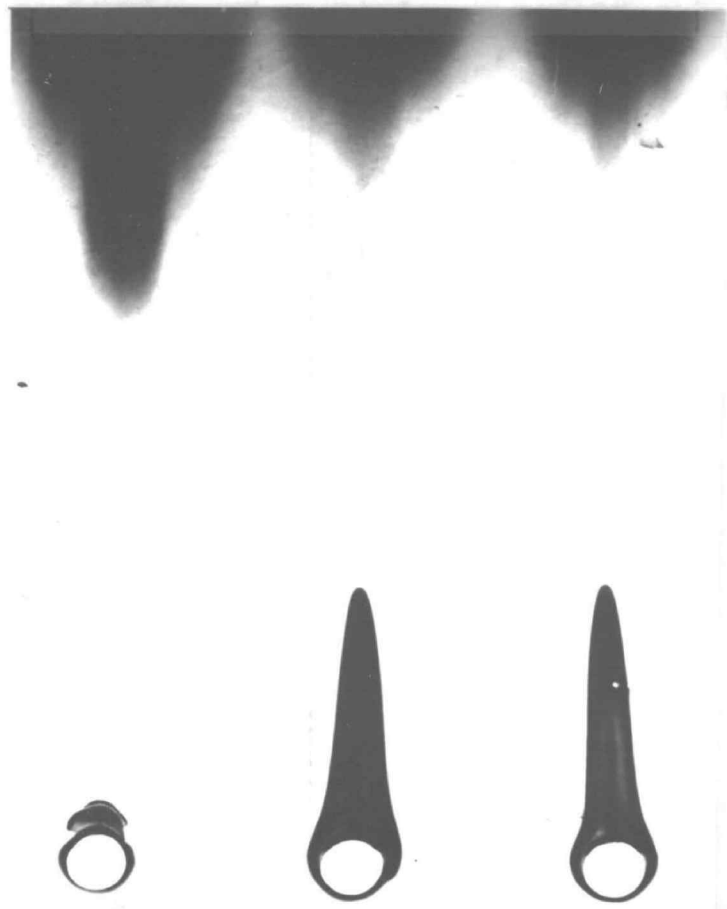

A
B
C

Figure 7. Inhibition of brine shrimp s-MDH catalytic activity as a function of antiserum concentration.

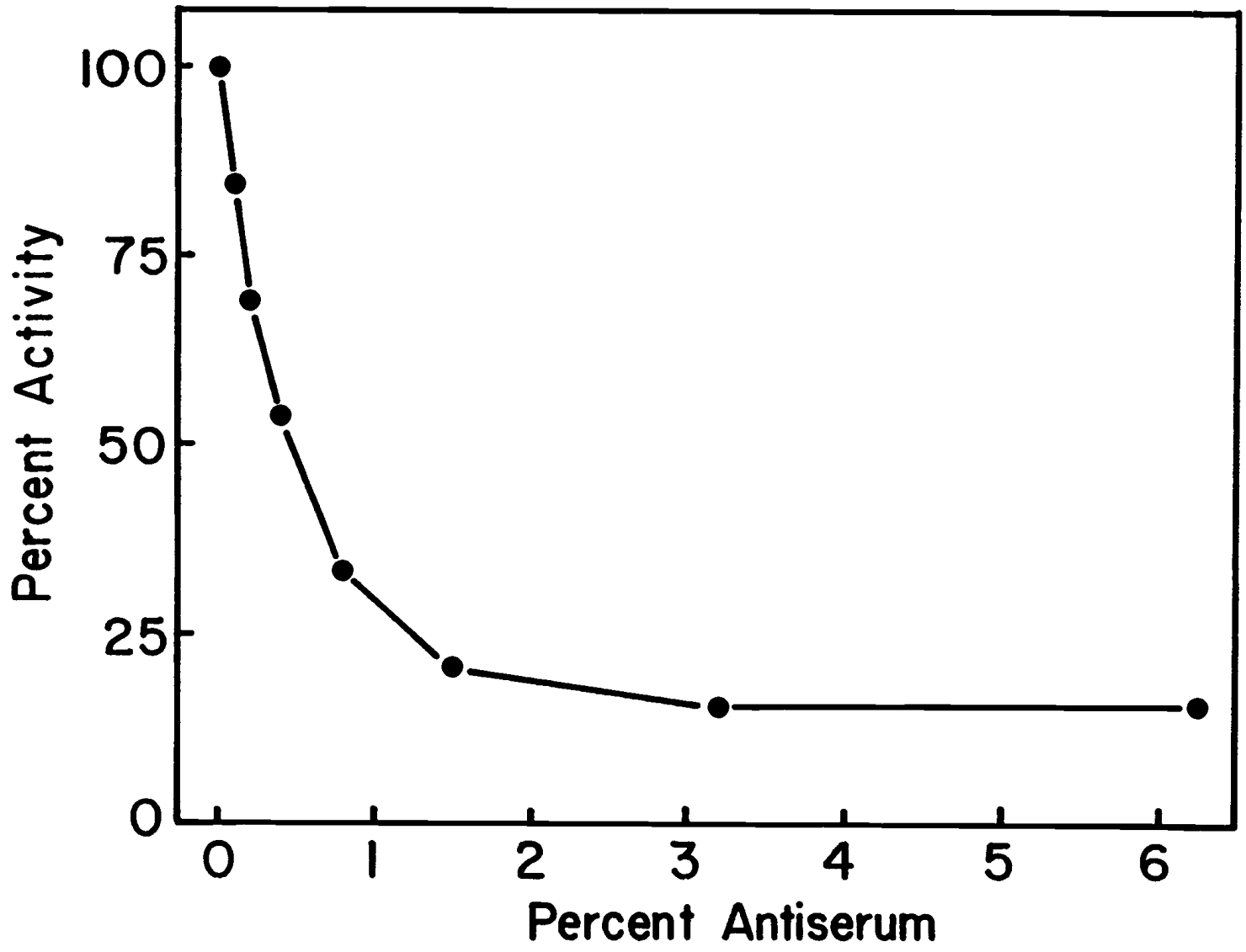

100
75
50
25
0
Percent Activity
0
1
2
3
4
5
6
Percent Antiserum

Figure 8. Rocket IEP plate using vertebrate MDHs as antigens against brine shrimp s-MDH antiserum. Antibody reactivity is not seen against any vertebrate enzyme. Pigeon breast m-MDH, 125 and 25 ug/ml (A,B); beef heart m-MDH, 125 and 25 ug/ml (C,D); pig heart m-MDH, 125 ug/ml (E); pig heart s-MDH, 125 and 25 ug/ml (F,G); brine shrimp s-MDH, 25 ug/ml (H).

A B C D E F G H

CHAPTER III

Effect of Environmental NaCl on the Biosynthesis of Cytoplasmic Malate Dehydrogenase during the Development of *Artemia salina* nauplii

Abstract

Brine shrimp nauplii challenged with fortified sea water (2.5 M NaCl) maintain significantly higher levels of cytoplasmic malate dehydrogenase (s-MDH) than larvae incubated in sea water. Eight to ten hours after emergence of free-swimming nauplii in sea water, s-MDH exhibits a steady decline for 20 to 40 hours; the decrease is smaller and stabilizes sooner in nauplii incubated in fortified sea water. Incorporation of $H^{14}CO_3$ into s-MDH protein was assayed using quantitative rocket immunoelectrophoresis (IEP) with monospecific antiserum prepared against purified brine shrimp s-MDH. A 40% faster rate of enzyme biosynthesis is observed in high salt, and together with the rapid s-MDH turnover (half-life of approximately one day), probably accounts for the difference in level between salt treatments. In contrast, $H^{14}CO_3$ incorporation into total TCA-precipitable protein in supernatant preparations decreases slightly in high salt, indicating a preferential synthesis of s-MDH. Temporal patterns of s-MDH during embryonic development were monitored using both catalytic activity and quantitative IEP assays. High s-MDH levels seen in encysted gastrulae (0.22 units or 0.57 μg s-MDH protein/100 embryos) remain relatively constant through the E_1 and E_2 emergent stages. The results are discussed in relation to the bioenergetics and temporal development of water and electrolyte regulation in hypersaline environments.

Introduction

Cell division is absent throughout pre-emergent development in encysted gastrulae of the brine shrimp Artemia salina but is resumed upon emergence of the embryo from the cyst (Nakanishi et al., 1962, 1963; Bellini, 1960). The E_1 and E_2 prenaupliar stages clearly represent periods of significant morphogenetic and physiological change. The de novo synthesis of the Na+K-activated ATPase and the development of the water and electrolyte regulation system used throughout naupliar development take place (Conte, Droukas and Ewing, 1977; Peterson, Ewing and Conte, 1978). Concurrently, structural development of the larval salt gland is observed (Conte, Droukas, Ewing, 1977; Hootman, Harris and Conte, 1972; Hootman and Conte, 1975), and it has now been definitively demonstrated that this gland possesses the salt-regulating machinery for the nauplius based on trans-epithelial measurements across the larval salt gland itself (Russler and Mangos, 1978; Conte, Dantzler and Beyenbach). Additionally, diguanosine tetraphosphate (diGDP) which is present at high, constant levels in encysted gastrulae is utilized extensively after hatching has occurred (Clegg, Warner and Finamore, 1967; Warner and Finamore, 1967; Warner and McClean, 1968) and may be linked in part to resumption of DNA synthesis (Bellini, 1960; Finamore and Clegg, 1969).

Differentiation during embryogenesis can often be followed by appearance, disappearance or change in level of enzyme proteins (Ozaki and Whiteley, 1970). For example, Ewing and Clegg (1969) reported a 35-40 fold increase in brine shrimp lactate dehydrogenase activity which began at hatching and marked a distinct change in carbohydrate

metabolism as the prenauplius differentiated into the free-swimming nauplius. Glycogen utilization and the ability to produce lactate in response to anaerobiosis both increased simultaneously with LDH activity.

Naupliar cytoplasmic malate dehydrogenase (s-MDH) is a component of an energy-yielding glycolytic shunt in which phosphoenolpyruvate (PEP) is carboxylated by PEP carboxykinase to form oxaloacetate, which is then one substrate for the s-MDH reaction:

$$\text{oxaloacetate} + \text{NADH} + \text{H}^+ \overset{\text{MDH}}{\rightleftharpoons} \text{malate} + \text{NAD}^+$$

In addition to aiding the maintenance of glycolytic redox balance, the pathway provides additional GTP which may be utilized to supplement energy requirements of active salt regulation. For the dicarboxylic acid pathway to serve in this capacity, it would appear necessary for its differentiation to have occurred by the time salt and water regulatory demands confront the organism (i.e., emergence from the cyst). Therefore, the temporal levels of s-MDH protein (assayed with quantitative rocket immunoelectrophoresis) and enzyme activity were measured during the larval development of Artemia salina and compared to the time of de novo appearance of the brine shrimp sodium-potassium regulation system. After establishment of this temporal pattern, the effect of environmental NaCl on s-MDH biosynthesis was then investigated by measurement of $H^{14}CO_3$ incorporation into the enzyme. Results suggest a rapid turnover of the enzyme pool occurs during development and that s-MDH synthesis is altered by NaCl.

Materials and Methods

Materials. Oxaloacetate, malate, NADH (Grade III), NAD^+ (Grade III), barbitol and sodium barbitol (barbitone; 5-5'-diethyl barbituric acid), crystalline bovine serum albumin and Tween 20 (polyoxyethylene sorbitan monolaurate) were purchased from Sigma Chemical Company. Sodium azide, D-19 Developer, Hypo II, and HBT-3 photographic emulsion were obtained from the Kodak Chemical Company. The HBT agarose was a product of Marine Colloids, Inc., and all other chemicals were reagent grade.

Rearing of Brine Shrimp Embryos. Brine shrimp encysted gastrulae were obtained from San Francisco Bay Brand Company and reared at 30°C in artificial sea water as previously described (Hand, 1979) with the following modifications. At hour 12 when free-swimming nauplii began to emerge in significant numbers, the larvae were "synchronized" to within three hours of hatch by using a double-cropping procedure (Ewing, Conte and Peterson, 1979; Peteson et al., 1978). These 15-hour synchronous nauplii were then collected and divided equally into flasks containing either fresh artificial sea water or sea water fortified with NaCl to produce a final concentration of 2.5 M. Due to the extended naupliar incubations in the present experiments (up to 72 hours), the marine unicellular alga, Pseudoisochrysis paradoxa, was used as a food source for the nauplii and introduced into the incubation medium at hour 25. Based on hemocytometer cell counts, algae were maintained at 30-100 cells/mm^3 by adding small aliquots of concentrated algal stock every 6-10 hours. This concentration was reported by Ivleva (1973) to be

optimal for maximum brine shrimp growth rate. Algae were definitely ingested by the nauplii based on the presence of partially digested and whole algal cells in fecal material. Nauplii were not fed during $H^{14}CO_3$ uptake experiments.

Sampling and Micro-homogenation of Embryos. Since the hatching of brine shrimp cysts is not synchronous, collection of triplicate samples composed of 100 embryos of the same stage required micromanipulation with pasteur pipets under a dissecting scope. Embryos were counted into 0.5 ml spot plate wells, filtered with miracloth and then quantitatively washed into 50 µl of homogenizing medium (1.0 mM dithiothreitol, 0.5 mM EDTA and 0.25 M sucrose in 50 mM tris-HCl buffer, pH 8.0). The homogenization was accomplished using a 100 µl capacity Uniform homogenizer (Jencons Scientific, Ltd.) with ground-glass body and pestle. Samples were transferred to 0.4 ml microfuge tubes and centrifuged 30 minutes at 39,000 X g in a Sorvall RC-5B refrigerated centrifuge. The high-speed supernatant was aspirated from the mitochondrial pellet and frozen under liquid nitrogen until analyses were performed. Recovery of malate dehydrogenase activity in the post-mitochondrial supernatant was 89 ± 1 percent (n=3) of that assayed in the crude homogenates. Upon thawing of stored samples, analyses were performed immediately due to enzyme instability in the crude PMS.

Activity Assays and Immunoelectrophoretic Analyses. Catalytic activity assays were performed at 25°C in the following 3 ml reaction mixture: 50 mM tris-HCl buffer pH 8.0, 0.33 mM oxaloacetate acid, 0.14 mM NADH

and 3 µl of enzyme solution. Enzymatic oxidation of NADH was followed at 340 nm using a Varian-Cary 219 dual beam spectrophotometer, and the rate was linear for 1-2 minutes.

Quantitative rocket immunoelectrophoresis of brine shrimp samples was carried out as previously described, using rabbit antiserum against purified brine shrimp s-MDH (Hand, 1979). The assay was specific for the cytoplasmic MDH isozyme. No cross-reactivity occurred with the mitochondrial isozyme which was unavoidably present in the PMS due to rupturing of some mitochondria during the homogenization and centrifugation. Therefore, the immunochemical assay possesses the distinct advantage of isozyme specificity, which cannot be achieved using activity assays alone. Slab gels were composed of 1% agarose dissolved in 0.05 M barbitol buffer (pH 8.6) and contained 0.5-5% s-MDH antiserum. Samples were electrophoresed for 18 hours at 2 V/cm gel using an LKB Multiphor 2117 immunoelectrophoresis unit. After electrophoresis plates were washed, dried and stained with 0.5% Coomassie Brilliant Blue.

Incorporation of $H^{14}CO_3$ into s-MDH Protein. Brine shrimp cysts are impermeable to amino acids (Clegg, 1966), but readily take up $H^{14}CO_3$, which is primarily fixed into amino acids and pyrimidines and later incorporated into proteins and nucleic acids (Clegg, 1966, 1976). To follow the labelling and subsequent turnover of the s-MDH pool, hydrated cysts (7.5 g) were placed in 250 ml artificial sea water and exposed to a 24-hour pulse of $NaH^{14}CO_3$ at 10 µCi/ml. Brine shrimp nauplii were then filtered out, rinsed and transferred to fresh sea water. At 1, 7

and 18 hours after this transfer equal aliquots of nauplii were filtered and homogenized (1 g nauplii:2 ml HM) using a Potter-Elvehjem glass-teflon tissue grinder. Post-mitochondrial supernatants (PMS) were prepared, and a portion of each sample was treated with perchloric acid (6% final concentration) and centrifuged for quantitative protein precipitation (Peterson, 1977). The resulting precipitated protein pellets (solubilized in 0.8 N NaOH), acid-soluble supernatants and untreated PMS samples were added to 10 mls of Handifluor scintillation mixture and counted to 1% error in a Model 3310 Packard Tri-Carb scintillation spectrometer. Protein determinations were performed following the method of Lowry et al. (1951) as modified by Peterson (1977).

The remainder of each PMS sample from above was assayed with quantitative rocket IEP for measurement of ^{14}C-incorporation into s-MDH protein (cpm/µg s-MDH). Each PMS sample was analyzed with three different immunoelectrophoresis treatments. (1) Small aliquots (17 µl) of each sample were applied to analytical IEP plates for accurate quantification of s-MDH protein (µg/ml PMS) as described earlier. (2) Preparative IEP gel plates were poured which contained 5% antiserum (10-fold the analytical antiserum level) and employed sample wells accommodating 0.05, 0.1 and 0.2 ml volumes. The triplicate samples applied to these scaled-up IEP plates resulted in sufficient cpm per precipitin rocket for measurement with liquid scintillation. (3) A duplicate set of preparative plates were prepared using 5% unchallenged rabbit serum in place of s-MDH antiserum. These plates served as controls to correct for non-specific PMS protein interaction with the serum proteins and/or gel matrix.

All plates were electrophoresed and processed following the methods described previously. However, after electrophoresis, the preparative gels were given a prolonged (24-hour) wash in several changes of phosphate buffered saline with continual agitation. Buffer salts were removed with a 15 minute distilled water rinse and gels were left in the hydrated state. Over a dark-field viewer rocket-shaped slices were cut from the experimental gel plates (s-MDH antiserum), and by positioning experimental slices over the corresponding sample tracts of control plates (unchallenged serum), equivalent slices were obtained lacking precipitin rockets. Each gel slice was placed in a counting vial and solubilized in 2 ml of distilled water by boiling for 1 minute. Ten ml of Handifluor scintillation mixture was added to the vial, shaken vigorously, and counted as described above. All samples were internally standardized with a spike of ^{14}C-toluene for quench correction. Using values from both the analytical and preparative gel plates, the cpm per µg s-MDH protein was calculated with the equation:

$$\frac{\text{cpm (experimental)} - \text{cpm (control)}}{\text{PMS sample volume (ml)}} \times \frac{1}{\text{µg s-MDH protein/ml PMS}} = \text{cpm/µg s-MDH protein}$$

To investigate the effect of environmental NaCl on $H^{14}CO_3$ incorporation into s-MDH protein, equal wet weights of 15-hour nauplii were transferred into either fresh artificial sea water or fortified sea water (2.5 M NaCl). At hour 23.5 both treatments received spikes of $NaH^{14}CO_3$ (10 µCi/ml medium) and the nauplii incubated for 17.5 hours.

At the end of the pulse, nauplii were filtered, rinsed and placed into their respective media (minus radiotracer) for one additional hour. The nauplii were then processed and analyzed as above.

Audioradiography of IEP Plates. To insure that the radioactivity measured by liquid scintillation in the antibody-containing gels was indeed located specifically in the precipitin rockets, additional plates were subjected to audioradiography. Kodak HBT-3 photographic emulsion was warmed to 45°C and diluted 1:1 with distilled water to which had been added two drops of Tween 20 per 25 ml of diluted emulsion to achieve a homogeneous consistency. The agarose on preparative IEP plates to be dipped in emulsion was dried to a thin film as described for analytical plates. Plates were warmed to 45°C and then dipped for 10 seconds and hung to dry in a special audioradiographic drying box (Hillemann and Ritschard, 1964). After transfer to a desiccated, light-tight box, the plates were stored at -20°C for 9 weeks.

The emulsion was developed in Kodak D-19 Developer (2 minutes), followed by a 10 second distilled water rinse, a 2 minute Hypo II treatment and a 10 minute distilled water wash. After photography the plates were stained through the emulsion with Coomassie Brilliant Blue (0.5% stain in 45% ethanol, 45% water, 10% glacial acetic acid).

Results

Developmental Profile of Brine Shrimp Embryos. Embryos from the San Francisco Bay population had a developmental profile similar to that reported for Great Salt Lake (Utah) animals (Conte, Droukas and Ewing, 1977; Peterson, Ewing and Conte, 1978; Warner, MacRae and Wahba, 1979), but developmental stages of San Francisco Bay embryos appeared to be slightly less synchronous. Figure 1 shows the profile routinely obtained by incubating the hydrated cysts in fernbach flasks at a concentration of 15 g (1.4×10^6 hydrated cysts) per 500 ml artificial sea water on a rotary shaker. After 35 hours of incubation, 45% of the cysts had developed into free-swimming nauplii, with combined E_1 and E_2 stages[2] representing less than 2% of the total. Figures 2 a-b demonstrate the importance of continuous agitation for normal development of San Francisco Bay embryos. When cysts were incubated in either spot plates (50-100 cysts/0.5 ml) or inverted hanging drops

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[2]The embryonic stages referred to in this paper follow the nomenclature of Nakanishi et al. (1962). The E_1 is recognized as a ruptured cyst from which a slight dome of embryonic tissue can be seen emerging. At the E_2 stage, the embryo (still within the hatching membrane) has completely emerged from the cyst, although remaining loosely attached to the cyst by a bit of membrane or connective material. Upon emergence from the hatching membrane the free-swimming embryo is termed a nauplius larvum.

(10-20 cysts/drop) without any agitation, embryos accumulated at the E_2 stage and did not emerge from the hatching membrane; the incubation medium in both cases had a high surface to volume ratio insuring sufficient oxygen supply. Sometimes the emerging E_2 became completely detached from the ruptured cyst, but still remained enclosed in the membrane. Development of an eye spot and appendages could be observed through the hatching membrane. The percentage of free-swimming nauplii at hour 35 was less than 6% in both cases. Therefore, s-MDH staging experiments were performed in fernbach flasks with continuous shaking (40 RPM) to avoid abnormal embryo development. It should be noted that Great Salt Lake embryos hatched equally well with all the above hatching procedures.

Brine Shrimp s-MDH Levels during Development. Activity assays and quantitative rocket immunoelectrophoretic analyses were performed on brine shrimp samples within 48 hours of their initial preparation and storage under liquid nitrogen. As demonstrated in Table 1, significant changes in both enzyme activity and s-MDH protein occurred during storage, with 10% increases observable by day 7.

Large quantities of s-MDH were present in hydrated encysted gastrulae, and levels were constant through the E_2 stage (Fig. 3). Each stage up to the nauplius was assayed at two or three different hours during development, and the results were comparable using either enzymatic activity assays or quantitative rocket IEP. Embryo protein in post-mitochondrial supernatants was not constant during development, and consequently s-MDH values in the histograms were expressed as units (or µg s-MDH protein) per 100 embryos, instead of per mg protein. Either

Table 1. Stability of brine shrimp s-MDH stored as a crude post-mitochondrial supernatant (PMS) in liquid nitrogen as a function of time.

Time (days)	Enzyme activity		s-MDH Protein*	
	units/ml PMS	Percent of Initial	µg/ml PMS	Percent of Initial
0	39	100	59.8	100
2	40	102	61.6	103
7	43	110	65.8	110
24	43	110	---	---

*Measured by quantitative rocket IEP.

two or three hundred synchronous embryos were manually isolated for each sample point.

Temporal Patterns of s-MDH in Different NaCl Concentrations. In Figure 4 brine shrimp s-MDH specific activity (units/mg protein) and total PMS protein (μg/100 embryos) were monitored through development of free-swimming 72-hour nauplii incubated in either sea water (0.5 M NaCl) or fortified sea water (2.5 M NaCl). The pattern of s-MDH specific activity was generally a reflection of protein fluctuations. For example, a 50% increase in specific activity over cyst values occurred in the newly-emerged nauplius (hour 13), but concurrently total protein exhibited a decrease through hour 13 to 56% of cyst levels. Protein returned to cyst values by hour 19 before beginning a slow decline which stabilized by hour 40 at 42% of hour 0 levels. Since embryos were fed unicellular algae beginning at hour 25 (development of a functional gut complete[3]), the overall drop in total protein may represent in part utilization of yolk protein. However, it is also possible that the feeding regime may not have been optimal.

Even presented as specific activity based on protein values, a difference was observed after 40 hours between the two salt treatments.

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[3]The time of differentiation of a functional gut was estimated by observing the ingestion of carmine red particles. When the dye was able to pass completely through and out of the gut, it was deemed functional.

The s-MDH activity from nauplii in the 2.5 M NaCl medium had average specific activities higher than their 0.5 M counterparts, although statistically the difference was not highly significant (95% confidence limits).

A more accurate representation of s-MDH profile during development was acheived by expressing quantities on a per 100 embryo basis (Fig. 5). When analyzed in this manner, both activity and s-MDH protein measurements showed a steady s-MDH decline in nauplii raised in artificial sea water beginning shortly after hour 19. The decline in enzyme activity stabilized by hour 40, while levels measured with quantitative IEP continue the downward trend until hour 60. The naupliar s-MDH remaining at hour 72 represented 30-40% of the maximum values reached earlier in development. A similar decline after hour 17-19 in s-MDH level was also seen in nauplii reared in fortified sea water (2.5 M NaCl). However, the decline stabilized much earlier (hour 72) and resulted in highly significant differences in level between the two treatments. The s-MDH was present at higher levels in the high salt treatment and remained so through hour 72. Although the trend was not as pronounced with IEP measurements, the difference was highly significant by hour 72.

Differences between enzyme profiles measured by catalytic activity as opposed to immunoelectrophoresis may be due to variation in the amount of contaminating m-MDH present in the PMS during development. This m-MDH variation would be reflected in the activity assay but not in the IEP analyses due to its immunochemical specificity for the cytoplasmic isozyme. Cytochrome oxidase, a mitochondrial enzyme, showed

two pronounced peaks in activity during brine shrimp development (Peterson, Ewing and Conte, 1978). One peak (12-16 hours) corresponded to the early mitochondrial biosynthesis period reported for brine shrimp by Schmidt et al. (1973). The second peak (40-50 hours) could also represent mitochondrial biogenesis, if increase in cytochrome oxidase activity is taken as a valid indicator of this process. Increased numbers of mitochondria could easily result in a larger m-MDH contribution to the measured malate dehydrogenase activity. It is also possible that an s-MDH subform with a higher catalytic activity increases in porportion to other subforms during development.

Turnover of s-MDH Protein during Naupliar Development. The $NaH^{14}CO_3$ pulse introduced into the artificial sea water medium from hour 0-23.5 resulted in extensive labelling of the s-MDH pool (147 cpm/10 µg s-MDH protein). Since the overall level of s-MDH did not significantly increase during this period, the significant $H^{14}CO_3$ incorporation implies the enzyme pool possesses a rapid turnover. This implication was strongly supported by the disappearance rate of radioactivity from the s-MDH pool after transfer to tracer-free sea water (Table 2). In 17 hours the cpm/100 µg s-MDH dropped approximately 50%, indicating an in vivo s-MDH half-life of less than one day. This half-life value was most probably an overestimate since labelled precursors were not immediately cleared from the intracellular pool upon transfer to tracer-free medium. The s-MDH protein had a higher turnover than the total cytoplasmic protein as a whole, based on the analysis of total TCA-precipitable protein during the same period (Table 2).

Table 2. (a) Incorporation of $H^{14}CO_3$ into brine shrimp s-MDH protein was determined using quantitative rocket immunoelectrophoresis. Embryos received a $H^{14}CO_3$ pulse from hour 0 to 24 of incubation, and nauplii were sampled at 1, 7, and 18 hours after transfer into tracer-free sea water. Rapid turnover of the s-MDH pool is demonstrated with approximately 50% degradation observed in 17 hours. The s-MDH precipitin rockets were cut from the gels and radioactivity counted by liquid scintillation. Radioactivity of control gels (prepared with unchallenged rabbit serum instead of s-MDH antiserum) was substrated from experimental values. (b) $H^{14}CO_3$ incorporated into total TCA-precipitable protein of the post-mitochondrial supernatant (PMS) was analyzed over the same time period.

(a) $H^{14}CO_3$ incorporation into s-MDH protein

Sample	Total cpm/gel tract			µg s-MDH/ ml PMS	cpm/µg s-MDH protein			
	0.05	0.1	0.2 (ml)		0.05	0.1	0.2 (ml)	ave.
Hour 1								
Experimental	142	234	458					
Control	106	175	320					
Difference	36	59	138	45.2	159	130	153	147
Hour 7								
Experimental	71	118	205					
Control	38	70	101					
Difference	33	48	104	40.5	163	118	128	136
Hour 18								
Experimental	37	57	115					
Control	25	39	69					
Difference	12	18	46	29.0	83	62	79	75

Table 2. (continued)

(b) $H^{14}CO_3$ incorporation into total TCA-precipitable PMS protein

Sample	Unfractionated PMS		TCA-soluble supernatant		TCA-precipitable protein	
	cpm/ml	cpm/mg protein	cpm/ml	cpm/mg precipitated protein	cpm/ml PMS	cpm/mg protein
Hour 1	375,800	50,510	166,640	22,400	196,000	26,340
Hour 2	295,400	47,040	157,300	25,050	134,900	21,480
Hour 18	214,650	47,700	108,900	24,200	102,600	22,800

Effect of NaCl on $H^{14}CO_3$ Incorporation into s-MDH. During the incubation period from hour 23.5 to 42, nauplii in 2.5 M NaCl exhibited a 45% greater incorporation of $H^{14}CO_3$ into s-MDH protein (cpm/µg s-MDH protein) than did to larvae in sea water (Table 3). This elevated s-MDH biosynthesis was consistent with the 30-75% higher s-MDH pool observed for nauplii reared in the high salt environment by hour 42. However, radioactivity in total PMS protein (TCA-precipitable) was slightly lower in the 2.5 M treatment than in the sea water treatment (Table 3). It is suggested therefore that while cytoplasmic protein biosynthesis in general is slightly reduced in high salt, s-MDH synthesis is preferentially increased.

Since in vivo incorporation of radioactive precursors into protein is dependent on the specific activity of the internal pool, the specific activities must be similar between treatments for comparisons to be made. Direct measurement of these values are complicated when the primary radiotracer ($H^{14}CO_3$) is not the species actually incorporated. However, TCA-soluble supernatants from the two salt treatments had very comparable counts per minute (normalized with protein levels) as seen in Table 3. In the TCA supernatant (precipitated protein removed) all residual $H^{14}CO_3$ had been released as $^{14}CO_2$, and the remaining counts represented radioactive precursors of which amino acids make up approximately 80% (Clegg, 1976). Since the ^{12}C-amino acid pools have been shown to moderately increase in vivo with increasing environmental NaCl (Emerson, 1967; Conte, Peterson, Ewing, 1973), the specific activity may be slightly lower if anything in the 2.5 M treatment. Yet, even in light of this possible decrease in specific activity, incorporation into s-MDH is still greater in 2.5 M NaCl.

Table 3. (a) Incorporation of $H^{14}CO_3$ into brine shrimp s-MDH protein was determined as a function of environmental NaCl using quantitative rocket immunoelectrophoresis. Nauplii of each salt treatment received an $H^{14}CO_3$ pulse from hour 23.5 to 41 of incubation and were sampled one hour after transfer into their respective tracer-free media. Radioactivity was measured as described in Table 2. (b) The $H^{14}CO_3$ incorporated into total TCA-precipitable protein of the post-mitochondrial supernatant (PMS) was analyzed for each salt treatment.

(a) $H^{14}CO_3$ incorporation into s-MDH Protein

Sample	Total cpm/gel tract			µg s-MDH/ ml PMS	cpm/µg s-MDH protein			
	0.05	0.1	0.2 (ml)		0.05	0.1	0.2 (ml)	ave.
Incubation in 0.5 M NaCl								
Experimental	93	175	288					
Control	58	119	234					
Difference	35	56	54	43.8	160	128	62	117
Incubation in 2.5 M NaCl								
Experimental	117	228	417					
Control	60	160	247					
Difference	57	88	170	56.2	203	156	151	170

Table 3. (continued)

(b) $H^{14}CO_3$ incorporation into total TCA-precipitable PMS protein

Sample	Unfractionated PMS		TCA-soluble supernatant		TCA-precipitable protein	
	cpm/ml	cpm/mg protein	cpm/ml	cpm/mg precipitated protein	cpm/ml PMS	cpm/mg protein
0.5 M NaCl Incubation	430,000	118,130	322,840	88,690	127,500	35,030
2.5 M NaCl Incubation	591,250	91,670	371,800	57,640	195,300	30,280

Audioradiography of IEP Plates. Audioradiography of duplicate IEP plates from $H^{14}CO_3$ incorporation studies indicated the radioactivity measured in gel slices was indeed localized specifically in precipitin rockets (Fig. 6). Grain development over the gel plate matched precisely the s-MDH precipitin rocket stained through the emulsion with Coomassie Brilliant Blue. The faster-migrating, cross-reacting rocket(s), shown previously to possess no MDH activity (Hand, 1979), did not produce any detectable grain development in the emulsion. Therefore, any minor amount of this precipitin rocket not completely eliminated in gel slices could not contribute any to radioactivity measured by liquid scintillation. Finally, grain counts of the audioradiographs revealed the same rapid turnover of s-MDH that was observed using liquid scintillation, therefore serving as a double check of the results.

Discussion

Levels of s-MDH during Development and Possible Metabolic Function. Enzyme activity measurements and quantitative rocket IEP have demonstrated high levels (7.5 units or 20 µg/mg protein) of cytoplasmic malate dehydrogenase are present in encysted gastrulae of Artemia salina. This activity is 500-fold higher than that observed by Ewing and Clegg (1969) for a similar oxidoreductase, lactate dehydrogenase, in hydrated brine shrimp cysts. Generally, in tissues possessing vastly higher MDH activities than LDH, one suggested function of the s-MDH isozyme is maintenance of redox balance during periods of anaerobiosis. However, as shown by the work of Ewing and Clegg (1969) and Dutrieu and Chrestia-Blanchine (1966), the rate of carbohydrate metabolism in hydrated cysts is reduced to almost undetectable levels by anaerobic conditions, which led these workers to conclude that the reduction of metabolic activity provided the basis for anaerobic tolerance by the gastrulae. Since there is an absence of anaerobic metabolism in cysts, the question is raised as to the function of the s-MDH under aerobic conditions in the cyst.

One aerobic metabolic process functioning in hydrated Artemia cysts and nauplii is the incorporation of CO_2 into a variety of amino and organic acids (Clegg, 1976; Conte, 1977). Using $^{14}CO_2$, Clegg determined that aspartic acid always contained the largest portion of incorporated radioactivity (40-50%), and among organic acids most radioactivity was associated with malate. A similar distribution of radioactivity was demonstrated in nauplii using $H^{14}CO_3$ (unpublished results). The most

direct way to fix CO_2 into aspartic and malic acids would be the carboxylation of phosphoenol-pyruvate (PEP) via PEP carboxykinase. The ^{14}C-oxaloacetate so formed could be converted in one step to aspartic acid by glutamate-oxaloacetate transaminase (present in cysts and nauplii; Emerson, 1967), or to malate by s-MDH. Therefore, one probable role of s-MDH in developing embryos is in the CO_2-fixation metabolic pathway.

The benefit of this CO_2-fixation pathway to the cyst is not yet clear, but in post-emergent stages, evidence exists which suggests a definitive role. Beginning at the E_2 stage when the embryo is confronted with an environmental salt gradient, the (Na+K)-ATPase salt transport enzyme increases dramatically from undetectable levels in the cyst to maximal levels in the nauplius after 40 hours of sea water incubation (Conte, Droukas and Ewing 1977). In hypersaline environments, active salt regulation in the nauplius (Conte, Dantzler and Beyenbach) would represent a major metabolic energy demand. Surprisingly, when ouabain (a specific inhibitor of the Na+K-ATPase) was administered to nauplii at levels completely inhibiting the enzyme, no decrease was observed in aerobic metabolism as judged by oxygen consumption (Conte et al., 1980). However, there was marked reduction in glycogen utilization by the nauplius, accompanied with little change in the already very low lactate level. The data strongly suggest a link between the metabolic energy demands of the (Na+K)-ATPase and an energy-producing glycolytic pathway which does not result in lactate formation. The CO_2-fixation pathway meets these criteria. The temporal patterns of s-MDH during brine shrimp embryonic development presented in

this paper together with the CO_2-fixation data (Clegg, 1976; Conte, 1977) demonstrate that components of the functional dicarboxylic acid pathway are present by the time energy demands of salt regulation are encountered at emergence.

The developmental pattern of s-MDH showed that the enzyme is not a reasonable marker or indicator of the structural-biochemical differentiation occurring during the cyst to nauplius transition. Although of possible value for the earlier egg to gastrula developmental period, the high s-MDH level in encysted gastrulae and the stable levels during the E_1, E_2 and early nauplius stages were not sufficiently dynamic to be a distinctive developmental character. In addition to LDH (Ewing and Clegg, 1969), other brine shrimp enzymes which do exhibit rapid increases during the emergence period are ribonuclease, amylase, a protease and a lipase (Urbani and Bellini, 1958; Bellini, 1957a,b; Bellini and Lavizzari, 1958).

In contrast to the brine shrimp, malate dehydrogenase levels during development of the sea urchin embryo, Strongylocentrotus purpuratus, are more dynamic. Total MDH activity in homogenates tripled as development proceeded from the unfertilized egg to the young pluteus stage (Ozaki and Whiteley, 1970), but the majority of the increase took place by the gastrula stage. Based on a DEAE-cellulose chromatographic separation of the two isozymes at several stages of development, Ozaki and Whiteley suggested that the ratio between isozymes was constant, and the three-fold increase noted for homogenates would hold for either isozyme individually.

Effect of NaCl on s-MDH Biosynthesis. In the present paper significant differences in s-MDH level were observed between nauplii incubated in 0.5 versus 2.5 M NaCl as early as hour 42 (via catalytic activity) and later with IEP analysis. The s-MDH level in nauplii reared in 2.5 M NaCl was maintained higher through development, while in the 0.5 M medium the level continually declined, eventually resulting in a difference as great as 85% between treatments. Few other larval brine shrimp enzymes have been studied in regard to environmental salt effects on activity or enzyme level during development. Emerson (1967) compared the activities of three enzymes from nauplii reared in distilled water, 0.5 M NaCl and 1.0 M NaCl. Cysts were incubated from hour 0 in one of the three solutions, and enzymes were assayed at the incubation hour in which 50% of the viable nauplii had emerged (distilled water, 20 hours; 0.5 M NaCl, 24 hours; 1.0 M NaCl, 44 hours). No significant differences in the activities of glutamate-oxaloacetate transaminase, glutamate-pyruvate transaminase or glutamate dehydrogenase were found among the three groups. Since cysts are not permeable to NaCl, nauplii had limited time exposure to salt in Emerson's studies. In contrast to the above method, rearing procedures in the present study allowed longer exposure of nauplii to the experimental NaCl media.

Because the difference in brine shrimp s-MDH level between salt treatments was not due to actual elevation in the high salt, the phenomenon should not be termed salt induction per se. Studies with $H^{14}CO_3$ incorporation into s-MDH were designed to provide more insight into the nature of this salt effect. The 40% lower incorporation (cpm/μg s-MDH) exhibited by nauplii in 0.5 M NaCl suggests a slower rate

of s-MDH biosynthesis compared to the high salt treatment. This rate of biosynthesis from hour 23.5 to 42 is insufficient to offset the rapid s-MDH degradation (approximate protein half-life of less than 1 day), resulting in a continual decline of the s-MDH pool in low salt. The half-life estimated here is somewhat shorter than the four days reported for the rat liver enzyme (Tarentino, Richert and Westfeld, 1966). This value was determined by following activity decay after induction by thyroid hormone, and therefore it is probably an overestimate due to enzyme synthesis still occurring during the decay period.

In 2.5 M NaCl the naupliar s-MDH biosynthesis is higher and apparently adequate to support a stable pool size. This pattern of s-MDH synthesis is in contrast to total naupliar protein synthesis. Conte, Peterson and Ewing (1973) showed that incorporation of ^{14}C-leucine into total TCA-precipitable protein dramatically decreased as the salt concentration was raised from 0.05 to 0.5 M NaCl. Increasing the environmental NaCl further to 2.5 M resulted in only a small additional reduction in protein synthesis. These results are supported in the present study, since $H^{14}CO_3$ incorporation into total TCA-precipitable protein found in post-mitochondrial supernatants was slightly diminished as NaCl was elevated from 0.5 to 2.5 M. Therefore, when compared to the reduced total protein synthesis, the biosynthesis of naupliar s-MDH is preferentially increased in the presence of high environmental NaCl.

If the brine shrimp s-MDH indeed functions as a component of an energy-yielding glycolytic shunt, then maintenance of high enzyme levels would appear advantageous in an environment where metabolic energy is at

a premium. As mentioned previously, active ion transport systems undoubtedly represent a sizable drain on ATP pools for continued salt secretion against a steep NaCl gradient. As suggested by Conte, Peterson and Ewing (1973), these ATP requirements could explain in part the reduction in total protein synthesis (a massive ATP-requiring process) in hypersaline environments, since at present little evidence exists supporting a direct regulatory role for Na^+ or Cl^- on brine shrimp protein synthesizing machinery in vitro (Bagshaw, 1969; Golub and Clegg, 1968).

In conclusion, the effect of environmental NaCl on s-MDH biosynthesis during naupliar development is consistent with the hypothesis that the enzyme (and associated dicarboxylic acid shunt) may be important in high salt adaptation via production of additional metabolic energy and metabolic water (Conte, 1977), and maintenance of glycolytic redox balance. However, how important the pathway's contribution is to total nauplius energy production remains to be determined. In this regard, it should be noted that without an additional energy source (such as this pathway) the aerobic scope for activity in the nauplius would appear to be very limited; naupliar oxygen consumption is maintained at a very high level (probable maximal) and does not increase in hypersaline conditions (Ewing, Conte and Peterson, 1979).

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Figure 1. Developmental profile of San Francisco Bay brine shrimp embryos reared at 30°C in sea water. Embryos were incubated in 2400-ml fernbach flasks on a rotary shaker at 40 rpm.

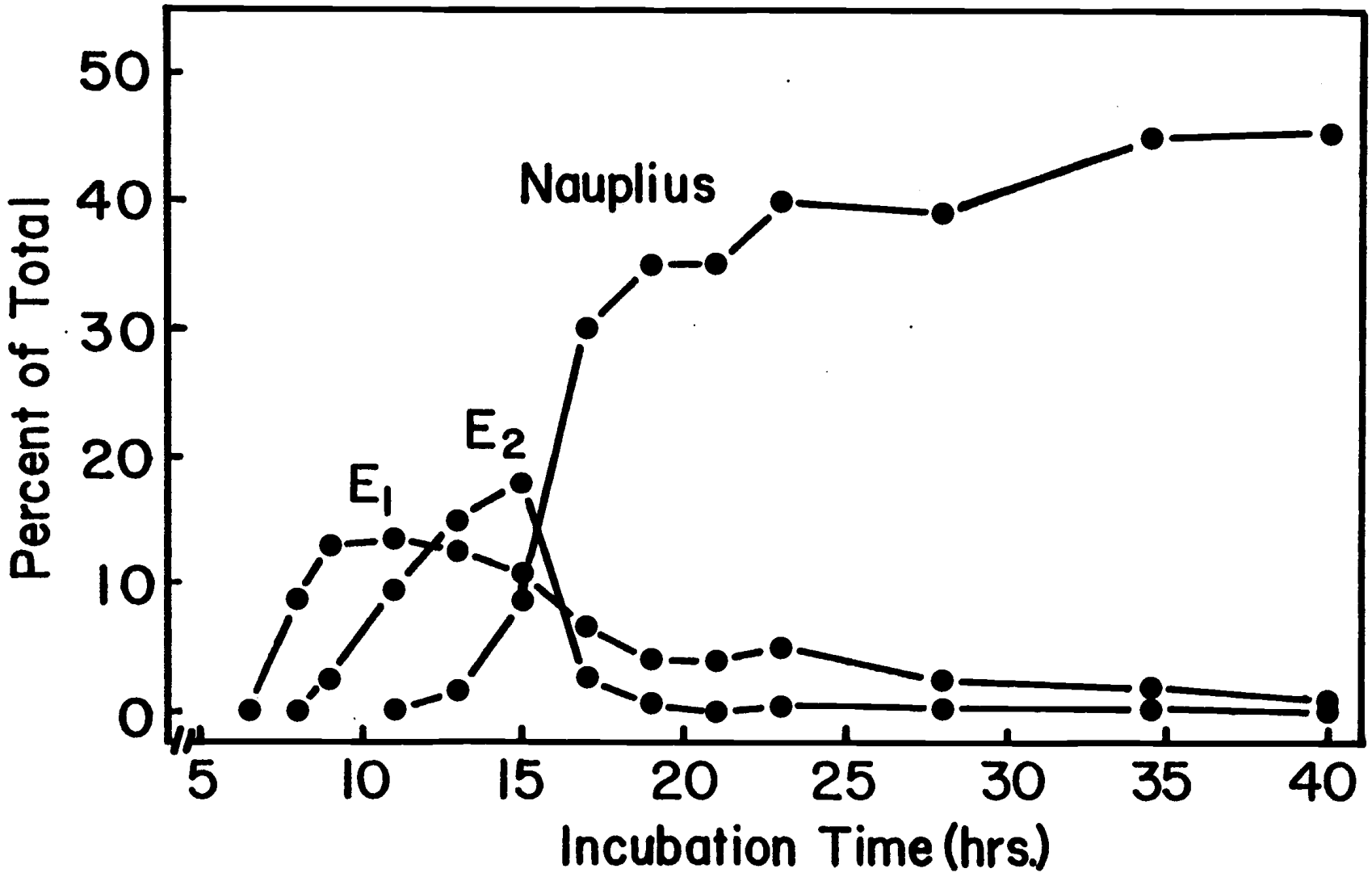

Percent of Total
50
40
30
20
10
0
Nauplius
E2
E1
5
10
15
20
25
30
35
40
Incubation Time (hrs.)

Figure 2. Developmental profile of San Francisco Bay brine shrimp embryos incubated in 0.5 ml spot plates (a) or hanging drops (b), both without agitation. Note the build up of embryos at the E_2 stage and the low percentage of free-swimming nauplii.

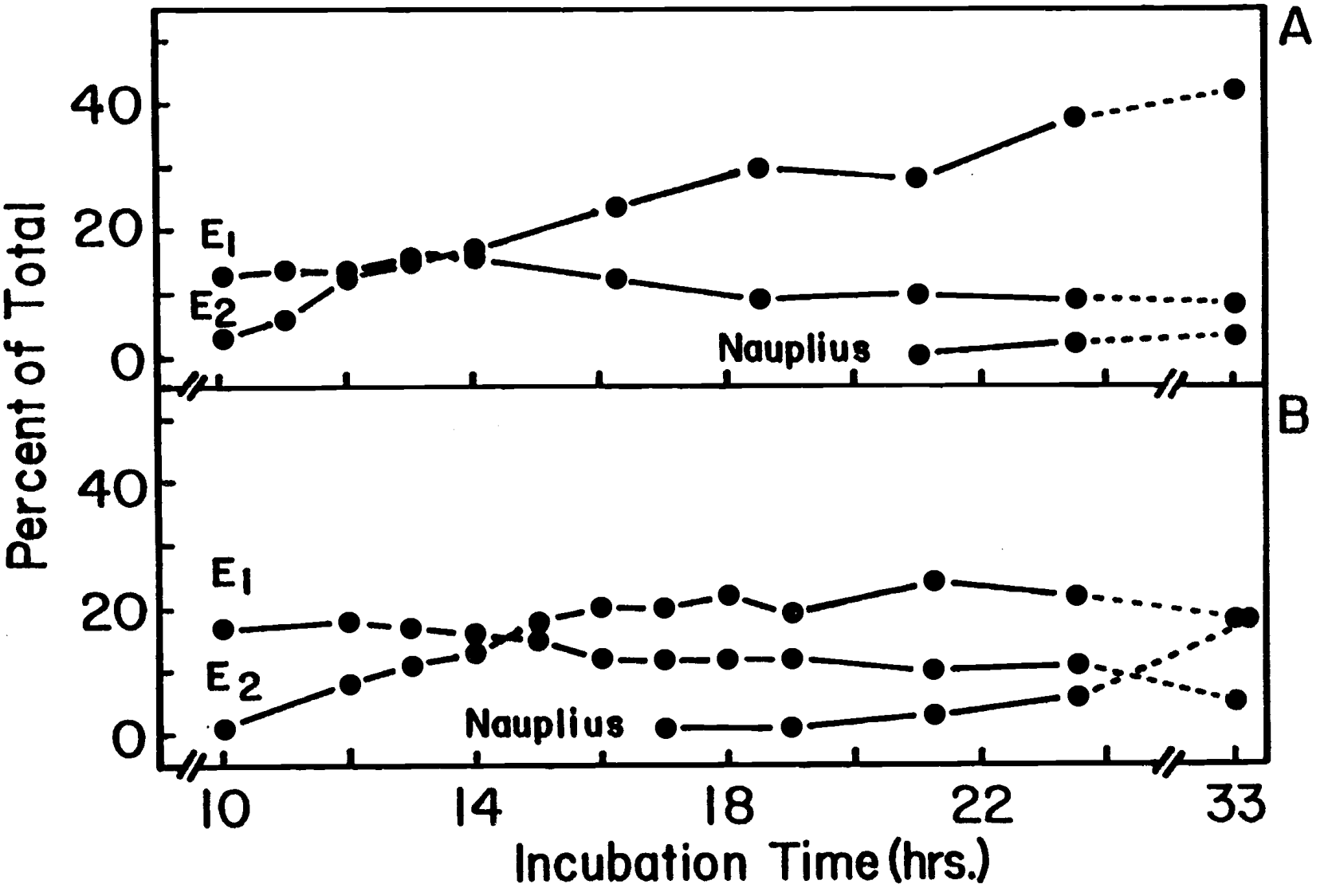

A
B
40
20
0
Percent of Total
E1
E2
Nauplius
10
14
18
22
33
Incubation Time (hrs.)

Figure 3. Histograms of brine shrimp s-MDH levels during early development measured by enzymatic activity (a) or quantitative rocket IEP (b). Developmental stages (described in text) were assayed at two or three different hours during incubation.

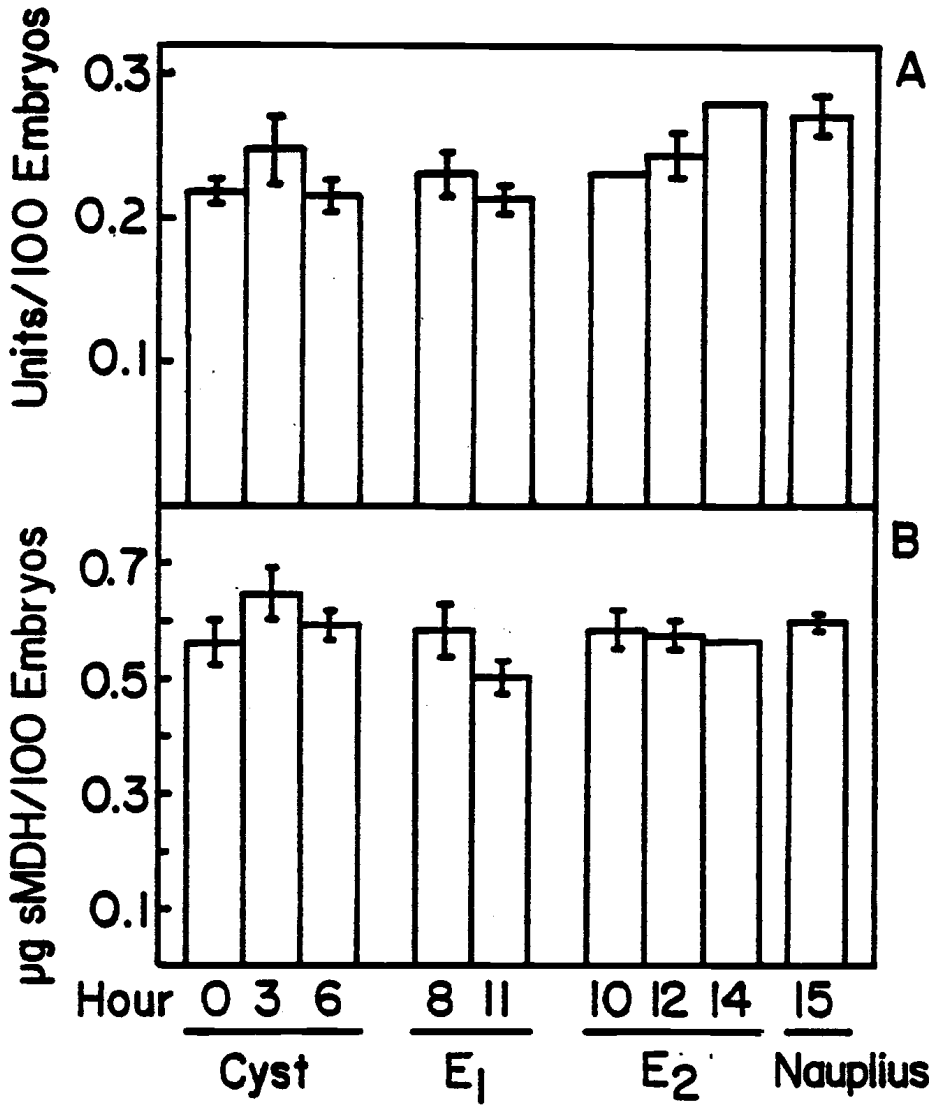

Units/100 Embryos
0.3
0.2
0.1
A
µg sMDH/100 Embryos
0.7
0.5
0.3
0.1
B
Hour 0 3 6 8 11 10 12 14 15
Cyst
E1
E2
Nauplius

Figure 4. (a) Specific activity of s-MDH (units/mg protein) during development of nauplii. (b) Total protein measured in naupliar post-mitochondrial supernatants and expressed per 100 embryos. Embryos were reared in either artificial sea water (○) or sea water fortified with 2.5 NaCl (●). Vertical bars at each sample point represent ± one standard deviation (n=3).

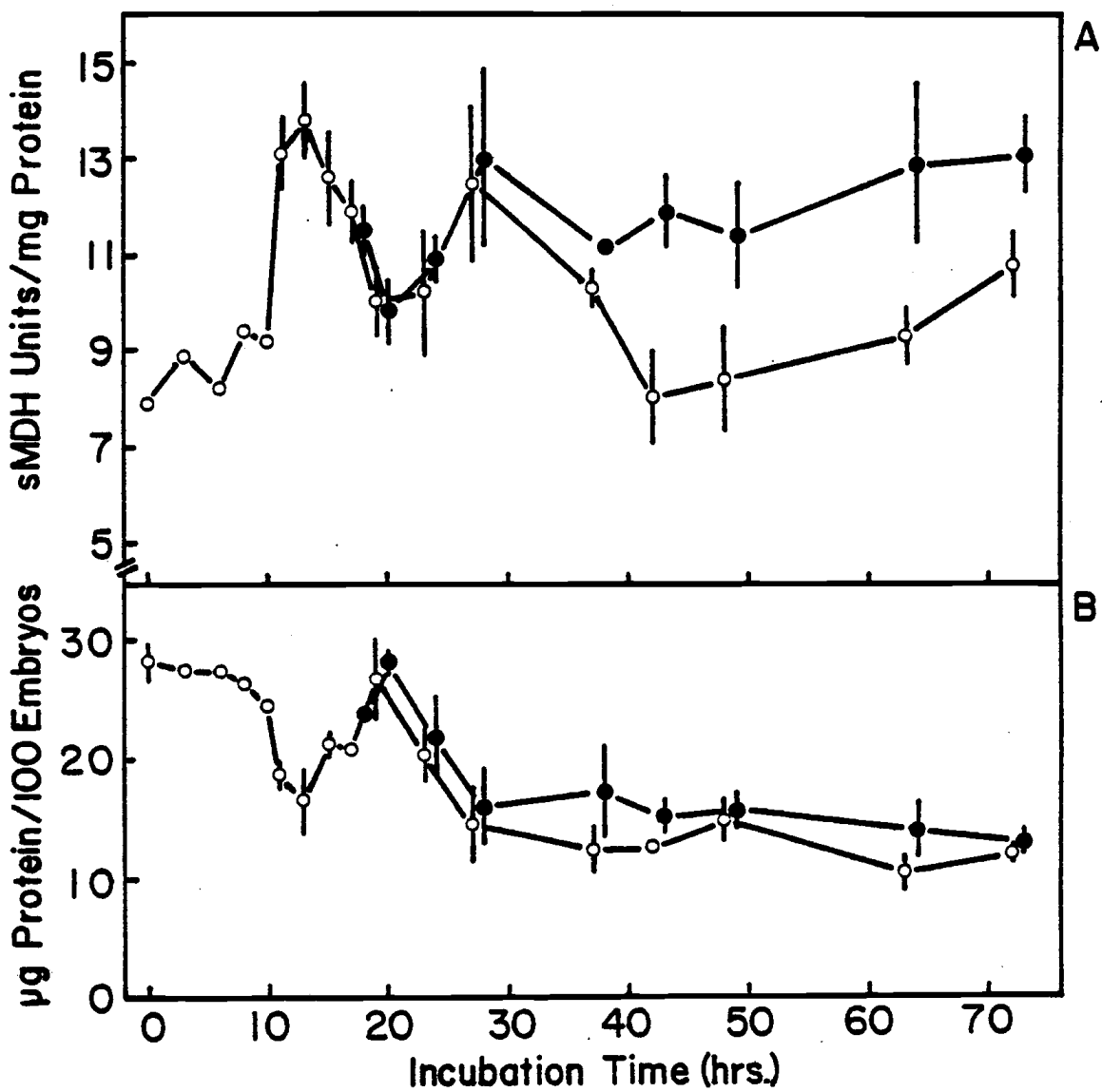

A
B
sMDH Units/mg Protein
µg Protein/100 Embryos
15
13
11
9
7
5
30
20
10
0
0
10
20
30
40
50
60
70
Incubation Time (hrs.)

Figure 5. Temporal patterns of s-MDH activity (a) and enzyme protein measured with the IEP assay (b) during brine shrimp development expressed per 100 embryos. Embryos were reared in either artificial sea water (○) or sea water fortified with 2.5 NaCl (●). Vertical bars at each sample point represent ± one standard deviation (n=3).

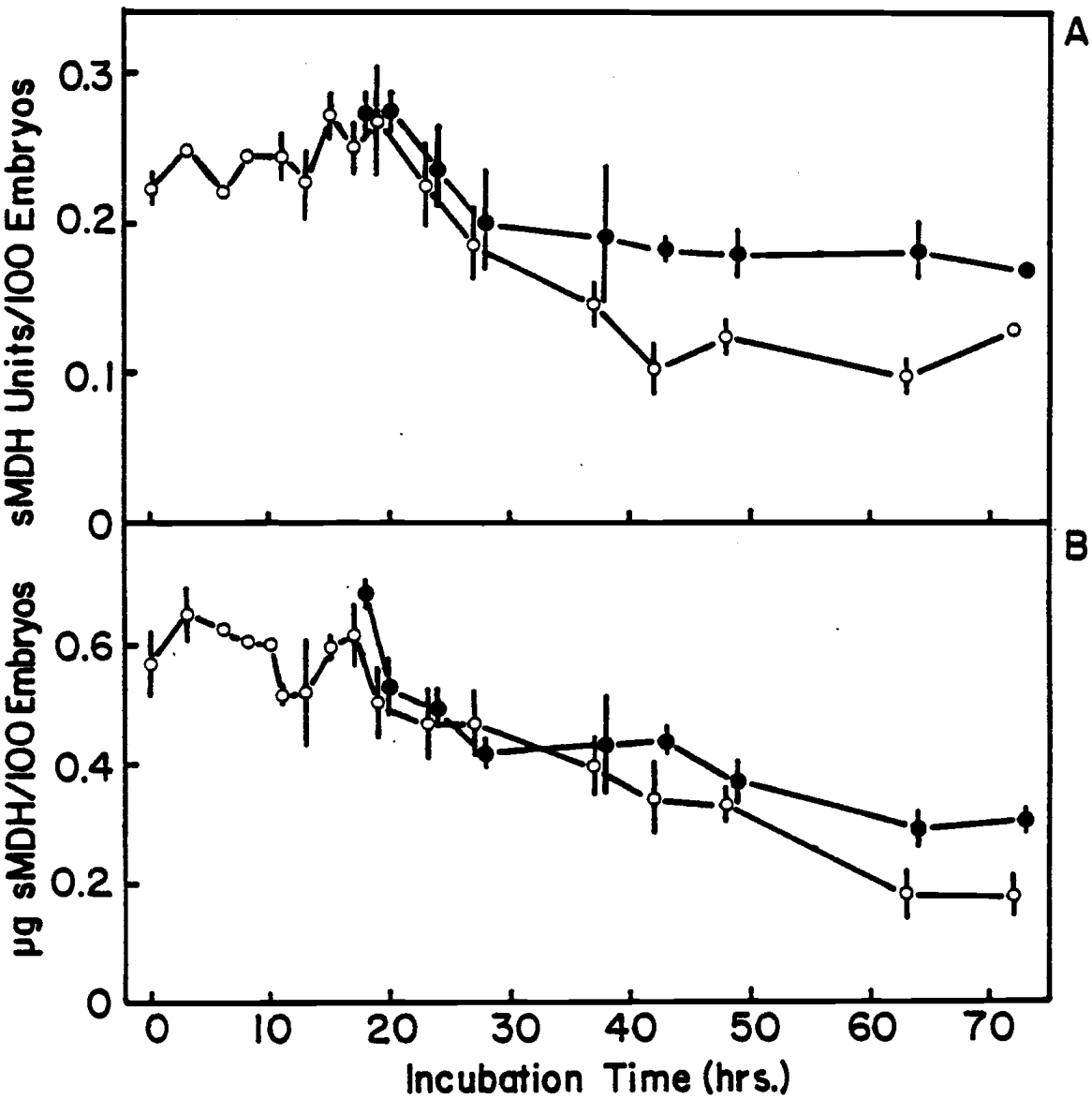
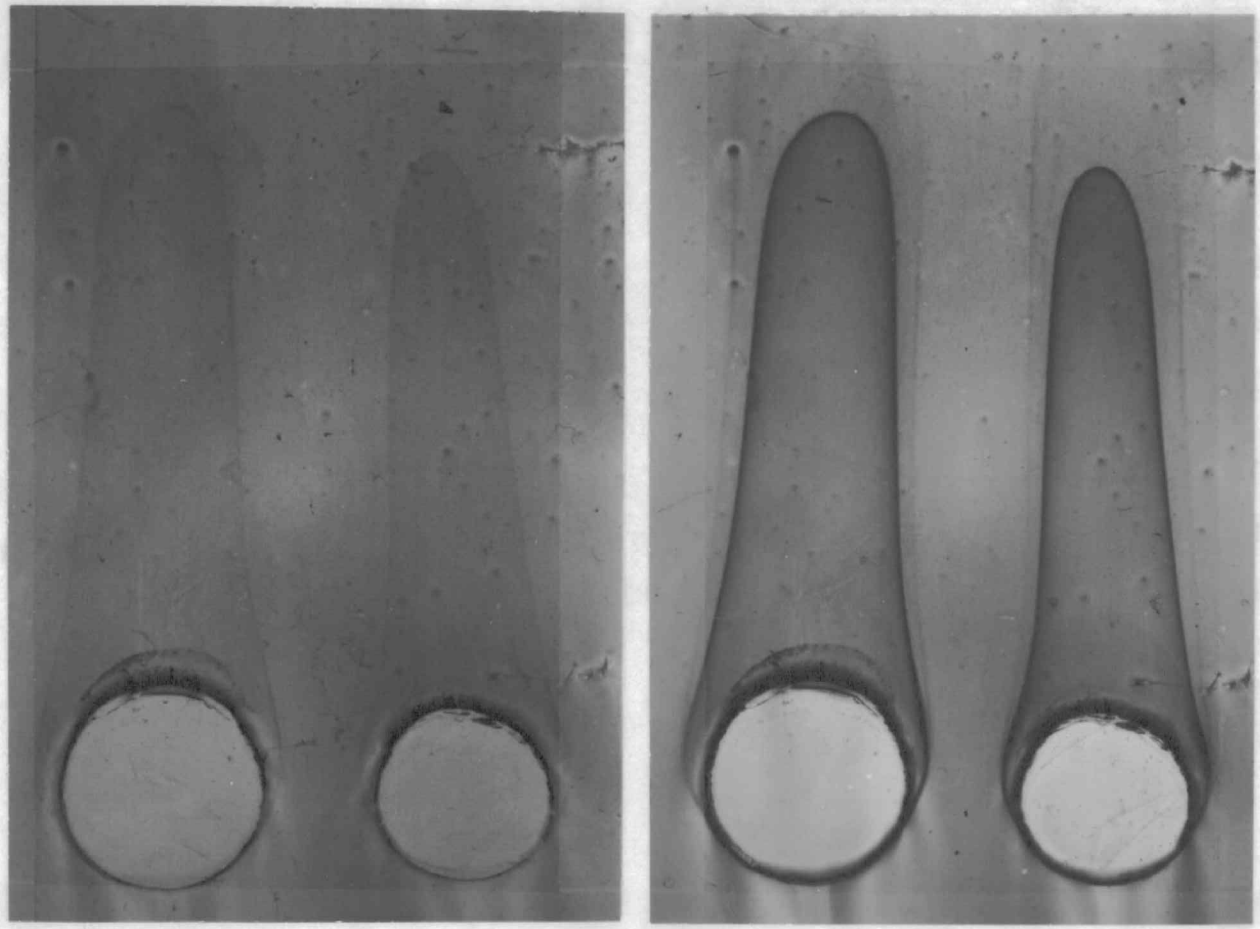

A
B
sMDH Units/100 Embryos
µg sMDH/100 Embryos
0.3
0.2
0.1
0
0.6
0.4
0.2
0
0
10
20
30
40
50
60
70
Incubation Time (hrs.)

Figure 6. Audioradiograph of preparative IEP plate demonstrating radioactivity in the s-MDH precipitin rocket (A,B). Same plate stained through the emulsion with Coomassie Brilliant Blue protein stain (C,D).



A B C D