MALATE DEHYDROGENASE FROM PHYSARUM POLYCEPHALUM AND PHYSARUM FLAVICOMUM: A PHYSICAL, CHEMICAL AND FUNCTIONAL STUDY OF THE MITOCHONDRIAL AND SUPERNATANT ISOZYMES IN GROWING AND DIFFERENTIATED STAGES

A Dissertation

Presented to

the Faculty of the Department of Biology

University of Houston

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

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by

William Martin Teague

May 1976

ACKNOWLEDGEMENTS

I would like to thank Dr. H. B. Gray and the Department of Biophysics, University of Houston for the use of the ultracentrifuge and related equipment, and Dr. D. S. Kirkpatrick, Department of Opthalmology, Baylor College of Medicine, Houston, for the use of the amino acid analyzer.

I would also like to thank my wife Katie for extensive help in the preparation of this dissertation.

I would also like to thank Dr. Henry R. Henney, Jr. for his patience and help in the development of this dissertation.

This research was supported in part by grant # E-247 from the Robert A. Welch Foundation awarded to Dr. Henry R. Henney, Jr., and by an award from the University of Houston New Research Opportunities Program.

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ABSTRACT

Two isozymes of malate dehydrogenase have been demonstrated for Physarum polycephalum and Physarum flavicomum plasmodia as well as P. flavicomum haploid cells by the techniques of polyacrylamide-gel disc electrophoresis and isoelectric focusing. Analyses of mitochondria from these sources by polyacrylamide-gel electrophoresis revealed that only the more cathodal protein was associated with these organelles (m-MDH). The other, more anodal form is found in the soluble cytoplasm (s-MDH).

The isozymes from P. polycephalum and P. flavicomum plasmodia have been purified to homogeneity as confirmed by gel filtration chromatography, polyacrylamide-gel disc electrophoresis and analytical ultracentrifugation. Final specific activities, as measured by oxaloacetate reduction for P. polycephalum were 1,020 for m-MDH and 810 for s-MDH. The specific activities for P. flavicomum were 1,080 for m-MDH and 839 for s-MDH.

The isozymes from both species were composed of dimeric, size equivalent subunits as demonstrated by sodium dodecyl sulfate gel electrophoresis. The sedimentation coefficients and molecular weights were determined. The molecular weights as established by sedimentation equilibrium were 69,500 for both P. polycephalum isozymes,

70,000 for P. flavicomum m-MDH, and 65,000 for P. flavicomum s-MDH.

Total amino acid analyses revealed the isozymes from each species were different. In P. polycephalum the s-MDH contained more isoleucine, serine, threonine and valine but less arginine, glutamate, glycine, half cystine, lysine and methionine than m-MDH. In P. flavicomum the m-MDH had higher values for arginine, aspartate, glutamate, glycine, isoleucine, lysine, methionine, serine and threonine but much lower values for phenylalanine.

The determination of optimum pH and substrate concentrations as well as Michaelis constants revealed the Physarum isozymes to be similar to each other as well as to a variety of other malate dehydrogenases from other sources. Analyses of the catalytic mechanism using substrate analogues revealed the mechanism was similar in all Physarum isozymes. Dicarboxylic acids with alpha carbon unsaturation were the best inhibitors of the reactions.

The m-MDH isozymes from Physarum were inhibited by adenosine phosphates with ATP giving the highest level of inhibition. The s-MDH isozymes were not inhibited. Other nucleoside phosphates had no effect.

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INTRODUCTION

Systems of proteins having equivalent catalytic activity, called isozymes, have been demonstrated only since 1959 (1). Now, many isozymes have been discovered throughout the biological system. The conceptualization and discovery of isozymes *per se* were perhaps not as interesting as the biological question their discovery raised: that of *raison d'être*.

Since 1959 the exploration of possible biological roles has indicated several functions, but all are directed toward the same goal of modulating metabolic activity. Certain modulation effects, at the simplest level, are under direct influence of the isozymes. This situation can be exemplified by considering the end product feedback inhibition of a pathway producing phenylalanine, tyrosine, and tryptophan demonstrated in *Escherichia coli* (2). The syntheses of these products are controlled by all three amino acids exerting their influence on a single, commonly required aldolase. However, each end product exerts its influence on but one of three isozymes of the aldolase, thus allowing for the finer modulation of precursor flow.

At more complex levels of modulation the role of isozymes is subjugated to a smaller theme in a grander scheme of metabolic activity control through increasingly more abstract compartmentation. This

compartmentation begins at the cellular level, where certain metabolic pathways are segregated in organelles, and advances to the multicellular organism level with the evolution of organs and organ systems (3). In these situations the isozymes have influence.

At the cellular level the compartmented space is a sealed and selectively permeable organelle. Such being the case the space must have entries and exits for the multitude of functions entertained by it. Indeed, metabolic connections between the extra- and intra-organelle system have to be deciphered before cellular metabolism can be explained. These entries and exits are, in part, under the control of isozymes, proteins having the same or equivalent catalytic activity but balanced on either side of the compartmented wall. An example of such a situation can be found in consideration of mitochondrial metabolic activity in relation to the whole cell. The reduced nicotinamide adenine dinucleotide (NADH) formed during glycolysis is reoxidized in the mitochondria (3), a structure refractory to nicotinamide adenine dinucleotide (NAD) penetration (4). Instead of direct NAD-mitochondrial interaction, the reducing equivalents from NAD are carried into the mitochondria on substrates which can penetrate the selective membrane. An example would be the malate-aspartate shuttle determined in rat tissue (5, 6), where the reducing equivalents

of cytoplasmic NAD are catalytically passed to malate, a substrate capable of penetrating mitochondria, via a cytoplasmic malate dehydrogenase. Once the malate enters the mitochondria, the reducing equivalents on malate are transferred to an intramitochondrially located NAD through the reverse reaction catalyzed by an isozyme.

The final sophistication of metabolic control is compartmentation through organ or organ system isolation of certain functions. Here again isozymes are contributory to this modulation concept. The now classic studies on lactate dehydrogenase biology have indicated the control of pyruvate metabolism in higher animals is strongly influenced by the type of isozyme present (7, 8). Here, the isozymes are not found in the same cell but in different tissues in the same species. In heart tissue from vertebrates where glycolysis is only an emergency measure, lactate dehydrogenase is structured to favor pyruvate as product. In muscle, where glycolysis is more common, the lactic acid formation is not as restrictive. The metabolic role in the compartmented modulation is further complicated by changes in the total cellular metabolism.

These changes studied in terms of various differentiational processes also indicate isozymes as metabolic modulators. The scope of the influence encompasses sea urchin development (9), Saccharomyces

(10), and Neurospora (11). These and other studies indicate that genetic control of differentiation is expressed in part by the time based, sequential appearance of certain isozymes.

These examples offered are not without their problems. Frequently the schemes, or interrelationships outlined, are based on one example or tissue. Also, the molecular biology of the enzymes involved is either severely incomplete or limited to one tissue.

The work offered here concerns the isozyme participation in cellular level compartmented metabolic modulation. More specifically it endeavors to explore the molecular biology of malate dehydrogenase (L-malate: NAD oxidoreductase, E. C. 1.1.1.37) for two reasons. The isozymes are implicated in the function of mitochondria and the knowledge gained would contribute to the whole of eucaryotic metabolism. Also, studies on these isozymes' biology would support or qualify the many functions assigned.

There are basically two major groups of malate dehydrogenase isozymes: a mitochondrial malate dehydrogenase (m-MDH) and one never found associated with any organelle, the cytoplasmic malate dehydrogenase (s-MDH) (3). There are other groups of isozymes found exclusively with plants. The roles attributed to these particular isozymes are numerous.

The role of m-MDH as a participant in the tricarboxylic acid cycle, where it catalyzes the conversion of malate to oxaloacetate with the production of one reduced NAD, is universally accepted (12). As mentioned before, the isozymes are participants in the malate-aspartate shuttle (5, 6). This is a complex shuttle involving not only the malate dehydrogenase isozymes but also glutamate-aspartate transaminase isozymes, and two membrane located, substrate carriers. The malate, formed in the cytosol from oxaloacetate and NADH by the catalytic activity of s-MDH is transported across a membrane barrier. This transport is done by an ∝ketoglutarate-malate carrier which functions by exchanging one malate molecule for an ∝ketoglutarate molecule. Thus, for every malate which enters the mitochondrion, an ∝ketoglutarate leaves. The malate inside the mitochondrion would be converted to oxaloacetate by m-MDH, thus regenerating the NADH. The oxaloacetate is then transaminated with glutamate by the mitochondrially localized isozyme of aspartate transaminase, forming aspartate and ∝ketoglutarate. The ∝ketoglutarate exits the mitochondria by the carrier as explained above. The aspartate, however, exits the mitochondria via an aspartate-glutamate carrier which exports the aspartate and, at the same time, imports a molecule of glutamate, thus supplying glutamate intramitochondrially for

the necessary transamination.

The extramitochondrially located aspartate and αketoglutarate are transaminated once again to oxaloacetate and glutamate. This completes the cycle with the net entry of one NADH.

Another metabolic pathway which has elements both inside and outside a mitochondrial barrier, and functions in part due to the role of s-MDH, is the export of acetyl-CoA made intramitochondrially to the outside where it is used for activities such as lipid synthesis. Although acetyl-CoA is required outside the mitochondria, the rate of direct diffusion or direct transport from within the mitochondria is 100 times too slow to meet the needs of the cell (13). A shuttle scheme has been proposed which involves citrate, formed intramitochondrially, being transported to the exterior (13). There it is cleaved to acetyl-CoA, furnishing the needed lipid precursor, and oxaloacetate, which re-enters the mitochondria after being converted to malate by s-MDH. Once malate is inside the mitochondria it is recycled by m-MDH. This shuttle scheme has been supported by evidence confirming the ability of citrate to cross the mitochondrial barrier (14).

At least two and possibly three other sources of membrane isolated malate dehydrogenase occur in plants in addition to the

s-MDH and m-MDH commonly found. These other sources are located in peroxisomes (15, 16), glyoxysomes (17) and, perhaps, in chloroplasts (12). There is definitely a participation of one malate dehydrogenase isozyme in photosynthesis of certain tropical plants, but this activity seems confined to the cytoplasm (18).

These studies on the gross function of malate dehydrogenase have been sparingly supported by competent biochemical analyses of the isozymes from a wide variety of tissues. These studies are more complete from mammalian systems but less so in other areas. It is the purpose of this dissertation to undertake a vigorous, cross modal, biochemical and biophysical study of the malate dehydrogenase isozymes in an attempt to assess the multiple roles indicated, and to discern the possible minimal function necessary for any malate dehydrogenase isozyme set. A brief survey of the literature in 1972 revealed that a serious gap existed in such an attempt.

The physicochemical structures have been investigated in complex animal tissue (19-24) and, somewhat, in complex plants (12, 15, 17, 25). The studies had also been undertaken in fungi (10, 11, 26) and invertebrates (9, 27-29). The protists, especially the simple animal type cells were poorly represented. A study of the malate dehydrogenase isozymes from the myxomycetes was in order.

The myxomycetes, or true slime molds, are free living, eucaryotic organisms which have characteristics in common with both plants and animals (30). These interesting organisms are characterized in part by demonstrating both a diploid and haploid stage in the life cycle. Also demonstrated in both stages are several interesting differentiational stages. The haploid organisms have an intraconvertible, bifold morphology. There is an amoeboid type cell and a biflagellated, morphologically static type cell; these forms are intraconvertible depending on nutrient and moisture availability. In starvation situations, the haploid cells will encyst forming dormant structures, microcysts, which are resistant to adverse environmental conditions.

Under favorable conditions the haploid cells will enter into syngamy which results in the formation of the diploid, vegetative plasmodia. The morphology of the vegetative stage is characterized as a unicellular, multinucleate, motile cell. The cell size is apparently genetically unlimited, a state supported by cytoplasmic streaming which acts to reduce diffusional limits. This diploid stage may, potentially, enter into either of two differentiational stages.

Under starvation conditions in the absence of light the vegetative cell will form a sclerotium, a berry-like dormant structure. If light is supplied under starvation conditions the vegetative cell will undergo

sporulation and form sporophores. These spores when, once again, favorable conditions are available, will germinate releasing haploid cells, a step which completes the life cycle.

The myxomycetes are considered to have both plant and animal characteristics. The plant similarity is based on the sporophore formation, a process more similar to the various fungi. The animal type characteristics are pronounced. The organisms have an unsaturated fatty acid pattern like Acanthamoeba while lacking the diunsaturated fatty acids which are characteristics of Dictyostelium discoideum (3, 32). The organisms lack chlorophyll, chloroplasts or any other photosynthetic apparatus (30). Their energy and carbon requirements can be met entirely through holozoic means. In the myxomycete genus Physarum, the most studied genus of all myxomycetes, the plasmodial stage utilizes glucose as well as other carbon sources (33). The organism demonstrates Krebs cycle as well as pentose phosphate cycle activity (34).

The organisms demonstrate a mobile, diploid stage. The mobile ability of the diploid organisms is made possible by a muscle-like protein system, a system normally associated with animals (35). Only the dormant stages have walls, but present indications are that these walls are not made of cellulose (36, Henney and Chu,

unpublished data).

The sensitive evolutionary position the organisms occupy plus these many interesting features make the organism ideal for comparative studies.

Preliminary studies were done on one species of myxomycete, Physarum polycephalum (37, 38). By electrophoretic analyses two malate dehydrogenase isozymes could be demonstrated. Electrophoretic analyses of soluble proteins from mitochondria isolated by techniques developed specifically for the tissue revealed the more cathodal band was isolated with this organelle; the malate dehydrogenase protein associated with mitochondria was designated m-MDH. The other band could not be found associated with any organelle and was designated s-MDH. A purification technique was developed which allowed the partial purification of both isozymes from crude extracts. In brief, the isozymes were separated by acetone fractionation of plasmodial homogenates acidified to pH 5.0. The m-MDH was further purified by cetylpyridinium chloride treatment and gradient elution from sulfoethyl cellulose at pH 6.0. The s-MDH was purified by ammonium sulfate fractionation, diethylaminoethyl cellulose chromatography, and gradient elution from sulfoethyl cellulose at pH 5.5. These two isozymes were examined for pH and substrate optima as

well as thermostability and response to certain cofactor analogues. These data have been published as a thesis (37) and as a journal article (38). The conclusions reached in those papers were that two separate proteins are responsible for the catalytic malate dehydrogenase activity in P. polycephalum. These two proteins are catalytically more similar to mammalian malate dehydrogenase isozymes than plant type malate dehydrogenase.

Based on these optimistic data the studies have been expanded. The purification has been altered to yield totally pure enzyme by the inclusion of isoelectric focusing and Sephadex gel filtration. The studies have been extended to Physarum flavicomum, variety 1 so that interspecic comparisons may also be made. The previous purification and analytic techniques performed on P. polycephalum have been repeated on isozymes from P. flavicomum. Further explorations on both species include determinations of isoelectric points, sedimentation coefficients, molecular weights, amino acid analyses and an extensive survey of compounds with intention of assessing their effect on catalytic activity. Certain limited active site studies have been performed. A journal article containing the physical and chemical analyses on isozymes from P. polycephalum has been accepted for publication (39).

Also included here are electrophoretic analyses of the haploid stage of P. flavicomum as well as of plasmodia grown on substrates other than glucose. These various data are compared extensively with malate dehydrogenase data from other tissues and conclusions are drawn with regard to the general function of malate dehydrogenase isozymes.

MATERIALS AND METHODS

Organism and growth conditions. The Physarum flavicomum, variety 1 plasmodial culture was obtained from H. R. Henney, Jr. and routinely grown in a soluble, semi-defined medium (33, 40). The culture of Physarum polycephalum plasmodia was obtained from C. J. Alexopoulos and has been maintained for over 8 years in the medium referred to above (33, 40). The identities of the cultures were confirmed after inducing the plasmodia to sporulate. Cultural conditions and minimal growth requirements for these strains have been described (33, 40). Physarum flavicomum myxamoeba, adapted to liquid media by Mary Henney (41) and maintained for over 2 years, were obtained from H. R. Henney, Jr.

With respect to enzyme isolation, cell growth in batch culture has been described previously (37, 38). The growth of myxamoeba in batch culture using the techniques developed for plasmodia (37, 38) was successful.

In certain experiments galactose and mannitol were substituted for glucose. These substitutions were based on published information. Substitutions of these compounds were made in media defined with respect to the vitamin source (33).

Mitochondrial isolation. The procedure has been developed and

reported previously (37, 38). These techniques and methods have been extended to both P. flavicomum, variety 1 diploid and haploid stages.

Protein purification. The basic purification scheme developed initially for P. polycephalum (37, 38) has been modified and applied to both species. The modifications consist of slight technical changes in procedures using cetylpyridinium chloride precipitation and gel filtration, and the addition of the isoelectric focusing technique.

Cetylpyridinium chloride (CPC) precipitation. All parameters previously described for CPC precipitation of the m-MDH acetone fraction were unchanged (37, 38), but the process was greatly facilitated by using plastic containers throughout the precipitation step. If a plastic vessel was used, only one addition of CPC to a 1% (w/v) final concentration was necessary for removal of all viscous contaminant (37).

Previous methods (37, 38) employed ammonium sulfate precipitation of m-MDH after CPC precipitation as a means of concentrating the enzyme prior to liquid chromatography. However, with the improved method for CPC precipitation, the m-MDH would not precipitate in salt but would float in high salt much like phospholipids. Therefore, the m-MDH protein was collected by substituting acetone

precipitation for ammonium sulfate precipitation. The solution temperature was lowered to 4 C, the container was placed in an ethanol-ice bath at -5 C, and 1 volume of acetone (-10 C) was added. Fractional additions of acetone did not prove satisfactory. The solution was allowed to stand 30 min and then centrifuged at 16,300 X g for 15 min at -5 C. The precipitate was dissolved in minimal amounts of 0.01 M potassium phosphate buffer (pH 6.0) containing 1 mM 2-mercaptoethanol (ME). The purification procedure as described (37, 38) was continued with the m-MDH being chromatographed on cationic exchange sulfoethyl (SE) cellulose. After chromatography the protein fraction was subjected to isoelectric focusing.

Isoelectric focusing. Isoelectric focusing was performed according to Vesterberg (42) using either the 440 ml LKB isoelectric focusing column for preparative work or the 110 ml LKB isoelectric focusing column for analytical work. The principle employed in the technique is that the proteins or other ampholytes have an iscelectric point, pI, in the pH spectrum where the ampholyte bears no net charge. In the isoelectric focusing technique it is possible to obtain a buffer with a continuously changing pH generated by the migration in an electric field and interaction of a mixture of compounds called ampholytes (LKB). Such a gradient has the lowest pH value at the anode and the

highest at the cathode.

When a mixture of proteins is introduced into this buffer and an electric field is applied across it, every protein will have a charge determined by the pI for that protein and the pH corresponding to the position of the protein. A heterogeneous population of proteins is thus separated and exactly focused at a point where the pH is equal to the pI. This technique has become popular only recently due to the commercial availability of ampholine (LKB) solutions necessary for the pH gradient. These ampholytes used in the gradient consist of mixtures of aliphatic polyamino-polycarboxylic acids weighing less than 10,000. They have the following characteristics: high electrical conduction, high buffering capacity, low ultraviolet absorption, low molecular weight, and aqueous solubility.

The particular format of isoelectric focusing used in our laboratory is density stabilized isoelectric focusing in liquid buffer. The technique employs a specially prepared piece of glassware, commercially available under the terms isoelectric focusing column, from LKB. The basic design of the apparatus is a tube within a tube. Given such an apparatus, both electrodes can be exposed to air while maintaining a vertical space for the focusing experiment. The gas produced by electrolysis does not bubble through the focusing chamber.

The apparatus is designed so that the lower electrolyte can be sealed from the focused proteins, and the vertical area containing the focused proteins can be emptied into a fraction collector. A prerequisite for this particular technique is the use of a density gradient formed with a non-ionic solute in order to retard thermal convection and to stabilize the protein zones during the collection phase.

Linear density gradients were formed and enzyme solutions distributed via the LKB gradient mixing apparatus, which is specifically constructed to compensate for the variable density encountered in making such a gradient. Because of the obvious buffering problem imparted by salt in the procedure, the sample solution is limited to 0.5 mmole of salt for the small column or 1.5 mmole for the large column. The volume allowed for the sample is quite liberal, the volume maximum being 55 ml for the small column and 210 ml for the large column.

The column temperature was maintained at a constant, low temperature to prevent proteolytic activity and arrest local thermal degradation. A Lauda K4/RD circulating cooling bath maintained the column at 5 C.

For a typical run, the column was mounted in a vertical position and connected to the constant temperature water bath. Water was

circulated through the cooling jackets. A dense electrode solution was prepared. With the anode at the bottom of the column, a dilute phosphoric acid electrolyte was used. This was prepared by mixing 0.2 ml of concentrated phosphoric acid with 14 ml of water and 12 g of sucrose. With the cathode at the bottom of the cell, a sodium hydroxide solution was prepared. This was made by mixing 0.2 g of solid sodium hydroxide with 14 ml of water and 12 g of sucrose. For the 440 ml column, 0.8 ml of phosphoric acid was mixed with 56 ml of water and 48 g of sucrose, or 0.8 g of sodium hydroxide was mixed with 56 ml of water and 48 g of sucrose depending on polarity. This mixture was passed into the central tube and allowed to rise 16 mm above the lower end of the central tube in the 110 ml column, and 20 mm in the 440 ml column. A dense focusing solution was prepared by diluting 3/4 of the carrier ampholytes plus 42 ml of water and 28 g of sucrose. For the larger column, 150 ml of water and 100 g of sucrose were used. All ampholyte concentrations used were at 1% (w/v), which corresponds to 3 ml of 40% (w/v) ampholytes in the small column or 10.75 ml in the larger column. A light solution was prepared by mixing the sample, 1/4 of the carrier ampholytes and water to a total volume of 60 ml for the small column or 215 ml for the larger column. The heavy solution and the light solution were

then put into a gradient mixing apparatus, and the gradient was introduced into the outer focusing tube at a flow rate not exceeding 4 ml/min.

After the gradient had been introduced into the column, a light electrode solution was added to the top. This light electrode solution was made from phosphoric acid or sodium hydroxide solutions depending upon the polarity of the experiment. The meniscus of the light electrode solution reached about 1 cm above the upper electrode. A power supply was then connected to the column. Maximum power at the start of the experiment did not exceed 2 to 3 watts for the small column or 4 to 6 watts for the large. During the experiment the voltage was increased as the amperage dropped, but the initial wattage settings were never exceeded. When the current throughout the column was constant, the power was terminated and the column emptied.

Termination of the isoelectric focusing experiment involved closing the valve separating the inner electrolyte tube from the outer focusing tube and removing the dense electrolyte. The sample outlet at the bottom of the focusing tube was opened and the liquid pumped from the column by forcing water into the column from the top at a flow rate not exceeding 1 to 2 ml/min. The liquid containing sample

was passed through a uvicord II (LKB) ultraviolet monitor and collected in an LKB fraction collector. The fractions were analyzed for enzymatic activity, and the pH of the fractions were measured at the temperature of the experiment using a Beckman model 3500 pH meter and Futura combination electrode. This basic experimental design was used in making isoelectric focusing experiments in pH ranges 3.0 to 11.0, 6.0 to 8.0, and 9.0 to 11.0 with certain modifications.

For crude extract isoelectric focusing the pH range 3.0 to 11.0 was used as described. For the m-MDH, ampholyte (LKB) solution in the pH range 9.0 to 11.0 was used at a concentration of 0.5% (w/v) along with a 0 to 55% (w/v) sorbitol gradient. Sodium hydroxide solution (0.1 M) was used at the cathode (bottom) and a 0.01 M acetic acid solution was used at the anode.

For s-MDH the ampholyte solution in the pH range 6.0 to 8.0 was used at a concentration of 1% (w/v). The anode solution, oriented to the bottom of the column, was composed of 0.01 M phosphoric acid; the cathode solution was 0.1 M sodium hydroxide. Sucrose was used to form the stabilizing gradient (42).

The ampholytes were difficult to remove by dialysis from the protein solutions; four and five changes of 100 volume buffer were

required to remove the ampholytes. The presence of ampholytes in the protein solutions interfered with protein determinations, thus affecting specific activity values. Therefore, the ampholytes were removed by gel filtration on Sephadex G-200.

Gel filtration chromatography. The original purification scheme (37, 38) called for a final step of gel filtration over Sephadex G-100. But, because the molecular size of malate dehydrogenase approached the separation limits of Sephadex G-100 chromatography, Sephadex G-200 was substituted for G-100. Column size and methods were unchanged (37, 38).

Electrophoresis. Polyacrylamide-gel disc electrophoresis was performed in either of two formulations, one designed for migration of compounds toward the anode and one for migration toward the cathode. The anodic migration system was performed in accordance with Ornstein (43) and Davis (44) in an apparatus similar to the one described by Davis (44). The system has been described (37, 38). The acrylamide concentration was 7% (w/v) in the gel.

Cathodic migration was done according to Taber and Sherman (45). In this system the separating gel consisted of a 3.5% (w/v) acrylamide matrix polymerized in a 0.06 M potassium glycinate buffer (pH 7.3) with a N,N,N',N'-tetramethylethylenediamide (TEMED)

and ammonium persulfate catalytic polymerization system. The ammonium persulfate was 0.007% (w/v) in the polymerization mixture.

The stacking gel consisted of a 2.5% (w/v) polyacrylamide-gel in a 0.06 M potassium glycinate buffer (pH 10.3). The stacking gel was polymerized with the same TEMED, ammonium persulfate system. The reservoir buffer was composed of 1.37 g of glycine and 3.82 ml of 2,6 lutidine in 1 liter of water (pH 8.3). The general techniques involved in casting the gel were equivalent to those used for the Davis (44) system. The tracking dye used for the cathodic system was basic fuchsin, but the dye must be used in tubes not containing protein. The system is described as concentrating proteins (stacking) at pH 8.3 and causing migration at pH 6.6 toward the cathode.

Sodium dodecyl sulfate (SDS) polyacrylamide-gel disc electrophoresis was done according to Weber and Osborn (46). The principle involved is that proteins first exposed to SDS under reducing conditions will migrate through a polyacrylamide matrix with a mobility directly related to the molecular weight of the subunit. The gels as used here were composed of a 10% (w/v) acrylamide matrix polymerized in a 0.1 M sodium phosphate buffer (pH 7.2) containing 0.1% (w/v) SDS. The polymerization was catalyzed by a TEMED, ammonium persulfate system. The reservoir buffer was composed of the same buffer

system found in the gel.

The samples were first incubated in a 0.01 M sodium phosphate buffer (pH 7.2) containing 1% (w/v) SDS and 1% (v/v) ME (final concentration) for 2 min at 100 C. A portion of this mixture containing 25 to 150 μg of protein was mixed with sucrose and 5 microliters of 0.05% (w/v) bromphenol blue so that the total volume did not exceed 150 microliters nor the sucrose concentration drop below 20% (w/v). The samples were subjected to 3 milliamps per gel. The electrophoretic process was discontinued when the blue bromphenol tracking band approached the bottom of the tube. The gel was cut at the center of the tracking band to mark the electrophoretic bottom of the gel.

All gels were stained for 2 hr in 0.5% (w/v) aniline blue black and eluted with a solution of acetic acid, methanol, and water (7:5:88, v/v). Proteins were characterized according to their migratory ability determined by the method of Weber and Osborn (46).

Analytical ultracentrifugation. Ultracentrifugation studies were performed using a Beckman model E analytical ultracentrifuge equipped with an electronic speed control, a monochromator (47), photoelectric scanner system (47, 48) and multiplexer system (49).

The monochromator light source (47) allows analysis at the maximum absorption wavelength. The photoelectric scanner system

(47, 48) and multiplexer accessory (49) allow direct optical density versus distance recordings of multiple centrifuge cells in one experiment.

The instrument has been modified by the installation of the Hurst and Gray (50) temperature control system which overcomes thermal convection in the sample cell, a problem at low protein concentrations.

Protein samples for ultracentrifugation were dialyzed extensively against a solution of 0.01 M potassium phosphate buffer (pH 7.5) containing 1 mM ME and 0.15 M sodium chloride.

High speed sedimentation velocity experiments were performed using an An-D type rotor. These experiments were recorded using the photoelectric scanner and multiplexer system with standard 12 mm, double sector, charcoal-filled Epon center pieces fitted with quartz windows and scanner window holders.

Centrifugation parameters for boundary sedimentation velocity experiments were 60,000 rev/min at 20 C with the monochromator adjusted to 265.4 nm. The sedimenting boundary positions were determined from the photoelectric scanner tracings of optical density versus radial distance using the half height method. Photoelectric scanner tracings were taken at 4 min intervals. No less than 18 data

points were collected per sample.

The observed sedimentation coefficients, $\underline{s_{obs}}$, were calculated using the equation:

$$\underline{s_{obs}} = \frac{d\mathrm{Ln}\bar{r}}{dt} \cdot \frac{1}{w^2}$$

where $\bar{r}$ is the distance in centimeters of the boundary half height from the axis of rotation, t is the time in seconds, and w is the angular velocity of a rotor in radians per second.

This equation is developed from consideration that the force field responsible for sedimentation in the ultracentrifuge is defined by the square of the angular velocity times the distance ($\bar{r}$) from the axis of rotation. The units of force are in dynes per gram. The sedimentation of a solute in the ultracentrifuge is then described by the velocity of sedimentation per unit force field or

$$\underline{s_{obs}} = \frac{d\bar{r}/dt}{w^2 r}$$

Only slight rearrangement is needed to generate the previous equation, which was calculated by the least squares method, using an Olivetti programma desk top computer programmer. Deviant values on $\mathrm{Ln}\bar{r}$ versus T plots were discarded as described by Ostrander and Gray (51).

The coefficient $\underline{s}$ varies with density as well as viscosity of the solvent. The observed sedimentation coefficients were corrected to standard $\underline{s_{20,w}}$ values which are defined as sedimentation in a solvent having the viscosity and density of water at 20 C. The particular equation used is based on a two component system:

$$\underline{s_{20,w}} = \underline{s_{obs}} \frac{(1 - \bar{v}\rho)_{20,w}}{(1 - \bar{v}\rho)} \cdot \frac{N_s}{N_{20,w}}$$

where $\bar{v}$ is the partial specific volume of the protein, here assumed to be 0.74 ml/g (20), ρ is solvent density, and N is solvent viscosity. Subscripts 20 and w refer to the conditions of water.

Because the $\underline{s_{20,w}}$ value varies with concentration, multiple determinations are usually made at various macromolecule concentrations. The various determinations are then used to extrapolate the $\underline{s_{20,w}}$ values to infinite dilution. However with the photoelectric scanner attachment, the $\underline{s_{20,w}}$ can be determined on such low solute concentrations as to be effectively an "infinite dilution" value. The values determined here approximate an $\underline{s^o}$ value because of the low protein concentrations employed (0.7 mg/ml).

Low speed sedimentation equilibrium studies were done with the same data collection system and instrumentation described for high speed sedimentation. At low speeds the solute will redistribute

relative to the sedimentation and diffusional characteristics of the macromolecule under a given set of force field conditions. The molecular weight can be obtained from the redistribution profile according to the following relationship:

$$M = \frac{dLnC}{d\bar{r}^2} \cdot \frac{2RT}{w^2(1 - \bar{v}\rho)}$$

where M is the molecular weight, C is the absolute concentration of the redistributed solute, $\bar{r}$ is the distance from the axis of rotation, R is the gas constant, T is the absolute temperature and other symbols are as defined for sedimentation. The data needed to complete the calculations are obtained by allowing solute to redistribute to equilibrium in a particular force field. Then data are collected on the relative concentration versus radial distance.

Sample chambers of double sector cells were first loaded with 0.02 ml of fluorocarbon (FC-43) oil to define the bottom of the liquid column. Then 0.1 ml of protein solution was introduced. In the absorbance blank chamber of the double sector cell, 0.01 ml of FC-43 was introduced followed by 0.12 ml of dialysate buffer.

The ultraviolet absorption system was set as described for velocity experiments, and the equilibrium was established at 13,000 rev/min at 20 C for both isozymes from each species in an

An-F type rotor. The data were collected 38 hr after reaching proper centrifugal conditions. The data collected are equilibrium distribution profiles generated by the sedimentation of the protein being in equilibrium with diffusion. This profile is in the form of a graph generated on pressure sensitive paper by a fixed point pen.

Because the pen is fixed at one point, the pen deflection is not equal for increasing units of absorption. Therefore, absorption versus pen deflection plots were constructed from absorbance calibration stair steps generated electronically. These plots were used to obtain the absorbance value corresponding to the concentration of the protein at selected distances from the axis of rotation along the equilibrium redistribution profile.

After the relative concentration profile was recorded, the rotor was accelerated to 44,000 rev/min and the protein allowed to sediment. The concentration of protein at the meniscus of the previous data could then be determined, and the relative concentration recorded previously could be corrected to absolute macromolecular concentrations versus radial distance. Plots of natural log absolute concentration versus (radial distance)^2 could then be made and the slope determined. The molecular weight was then easily calculated.

Amino acid analysis. Samples for amino acid analysis were

hydrolyzed for 24, 48 or 72 hr in vacuo at 105 C with constant boiling HCl. Flash evaporated samples were dissolved in buffer containing 0.1 mM taurine as an internal standard. All samples prepared for amino acid analysis were chromatographed on a single column automatic amino acid analyzer using Durrum DC-1A resin in a system described by Hamilton (52). Buffer systems were described by Kirkpatrick and Bishop (53) with the exception that the first buffer was pH 2.9.

Serine and threonine concentrations were corrected by extrapolation to 0 hr of hydrolysis; isoleucine, leucine and valine were corrected by extrapolation to 96 hr of hydrolysis. Half cystine content was determined as cysteic acid using the performic acid method (54). Comparisons were made between the amino acid values for m-MDH and s-MDH, and among several literature values, by assuming reported values were accurate to plus or minus 5%.

Total cysteine determinations. Protein total sulfhydryl groups were determined using Ellman's reagent, 5,5' dithiobis (2-nitrobenzoic acid) (DTNB) (55) on proteins denatured with SDS (56). The reaction involves a quantitative yield of a nitrylbenzene thiol anion per sulfhydryl group. The anion released is yellow, having a molar extinction coefficient of 14,600 at 412 nm.

The protein, 3 to 15 nanomoles, was dissolved in 1.94 ml of solution containing 2% SDS, 0.5 mg/ml ethylenediaminetetraacetate (EDTA) and 0.08 M sodium phosphate buffer (pH 8.0). This mixture was divided equally between two tubes giving a final total volume of 0.97 ml in each. To the reaction tube was added 0.1 ml of 1.33 mg/ml DTNB. To the tube designated as a spectrophotometric blank was added 0.1 ml of water. The color was allowed to develop for 15 min. At the end of that time the absorbance of the reaction tube was determined against the blank tube at 412 nm.

Tryptophan analyses. The procedure for tryptophan determination was essentially that of Spies and Chambers (57) with modifications by Harrison and Hoffman (58). Standard curves were prepared using tryptophan incubated with para-dimethylaminobenzaldehyde (DAB). The procedure is done in the dark until total color development is complete.

The DAB (82.5 mg) was dissolved in 2.75 ml of 2 N sulfuric acid. This solution was mixed with 22 ml of 23.7 N sulfuric acid, and 2.25 ml of the resulting mixture was added to each of 10 test tubes. Tryptophan stock solutions of 120 and 60 μg/ml were prepared in water. To each of nine test tubes containing DAB an aliquot of tryptophan was added so that the range of 1 to 30 μg was covered, but the total volume added did not exceed 0.25 ml. Water was added to all

10 test tubes to bring the final volume to 2.5 ml. These were cooled and incubated for 24 hr at 25 C. Then 0.025 ml of 0.04% (w/v) sodium nitrite was added to each. The color was allowed to develop for 1 hr at room temperature. A plot of 100 times log transmittance (at 590, 600 and 610 nm) versus μg tryptophan was made.

The preparation of whole proteins for analyses required enzymatic digestion of the proteins to expose the tryptophan residues (57). The proteases used to digest the test proteins must be treated separately to negate any color the proteases would contribute to the color developed. Also the test proteins might contribute nonspecific color to the reaction mixture which must be negated by performing the test on proteins minus the DAB.

The directions used will be given for the complete reaction mixture. The suitable controls consisted of (a) a companion set of experimental tubes minus the DAB and (b) a repeat of the whole experiment including the control described in (a) but lacking the unknown protein. To each of five test tubes were added aliquots of enzyme solution to span a 5 to 25 μg range in a total volume not exceeding 0.25 ml. These test tubes were boiled for 15 min, then cooled, and 0.01 ml of 0.2 M Tris buffer (pH 9.2) was added to each. In each of the test tubes 0.2 ml of 1 mg/ml chymotrypsin (Worthington) and

0.02 ml of 1 mg/ml trypsin (Worthington) was added. The samples were incubated for 6 hr at 37 C. At the end of this time the volume was corrected to 0.25 ml.

The tubes were then treated as in the standard curve determination, but the protein samples were incubated with DAB only 12 hr, not 24 hr. At the end of the incubation period 0.025 ml of 0.045% (w/v) sodium nitrite was added to each.

After color development the transmittance was determined against the companion tubes minus DAB at 590, 600 and 610 nm. The lowest transmission was converted to μg tryptophan with the proper standard curve. The tryptophan contained in the proteases was negated by treating the control tubes (minus unknown protein) with the same procedure.

Malate dehydrogenase assays. All L-malate: NAD oxidoreductase E. C. 1.1.1.37 assays were done as described previously (37, 38). All procedures and equipment used to determine pH and substrate optima have been described in detail (37) as have all features related to Michaelis constant (K_m) determinations.

Functional inhibitor studies. Potential inhibitors of the malate dehydrogenase catalytic activity were substituted for buffers in the assay mixture described under Malate dehydrogenase assays.

The inhibitors, where possible, were purchased as the free ion. All were titrated to the proper pH with assay buffer ions. The molecular weight of the inhibitor used was adjusted by data available from the manufacturer on the purity, water content, and extraneous ions present. The inhibitors were made as stock solutions at 10 times the final concentration used in the assay. They were then added to the assay at 0.1 ml/ml of reaction mixture.

These inhibitors were used in assay mixtures containing various substrate and cofactor concentrations. Control sets of assays (minus inhibitor) were done every 8 hr of assay time. All data were treated as described in a previous publication (37, 38) under Michaelis constant (K_m). All data intercepting control data at the reciprocal of maximum velocity (1/Vm) but with less negative reciprocal K_m values than control data were treated as competitive inhibitors. The inhibitor constant (K_i) was calculated according to the following assumed relationship:

$$K_i = \frac{K_m [I]}{K_{app} - K_m}$$

where I is the molar concentration of the inhibitor in solution, K_{app} is the apparent Michaelis constant with the inhibitor in the assay mixture, and all other units are as defined. The units of K_i are

moles/liter.

All data intercepting control data at $-1/K_m$ but with more positive 1/Vm were treated as noncompetitive inhibitors. The inhibitor constants for these compounds were calculated from the following assumed relationship:

$$K_i = [I] \ (Vm/V_{app} - 1)^{-1}$$

where V_{app} is used for the apparent maximal velocity obtained in the presence of the inhibitor, and other terms are as described.

Thermostability determination. The time dependent thermostability of P. flavicomum malate dehydrogenase isozymes was determined at 50 C as explained before (37, 38).

Active site sulfhydryl determination: para-hydroxymercuribenzoate. The potential for an active site sulfhydryl group was investigated using para-hydroxymercuribenzoate (PMB).

Organic mercurial compounds such as PMB are sensitive and specific reagents which will react with sulfhydryl groups. The affinity of the compounds for sulfhydryl groups increases as the pH decreases, and the reaction is stable to a number of ions and ionic strengths (59).

The PMB solutions were prepared fresh daily by suspending the

solid material in water and titrating the PMB into solution with 0.1 M sodium hydroxide. A sample of this stock solution was then diluted with 0.1 M potassium phosphate buffer (pH 7.0) and the spectrophotometric absorbance obtained at 232 nm. The molar extinction coefficient, ε M = 1.69 X 10^4 (59) was used to calculate the final concentration.

The protein was prepared by extensive dialysis against the buffer of choice. The general procedure used involved mixing the stock solution of PMB with protein and buffer so that a finite ratio of PMB to protein was achieved. Variations within the specific experimental designs included variations of PMB:protein molar ratios, variation of incubation time with PMB (from 15 min to 20 hr) and variation of incubation temperature (from 4 to 25 C). The effects of PMB were assessed on the basis of activity alteration. The PMB and protein mixture was diluted with 0.1 M potassium phosphate buffer (pH 7.5) and assayed in the direction of oxaloacetate reduction.

Active site sulfhydryl determination: N-ethylmaleimide. The possibility of an active site sulfhydryl group was also investigated using N-ethylmaleimide (NEM). This compound undergoes first order reaction with sulfhydryl groups by addition at activated double bonds (60). In the pH range 5.0 to 7.0 the compound reacts with no other

amino acid. Above pH 7.0 the NEM hydrolyzes (61). NEM may be used as a probe of the active site by incubating it with enzyme and following the decrease in catalytic activity (62, 63).

The NEM was prepared in 50 mM phosphate buffer (pH 6.0); the absolute concentration was determined spectrophotometrically at 305 nm using the extinction coefficient $\varepsilon'M = 620$. The protein was prepared by dialysis against the same buffer. The protein (200 μg) was mixed with NEM solution so that the final concentration of NEM was in excess (500:1 molar ratio). The volume did not exceed 1 ml. A 0.01 ml sample of the incubation mixture was extracted every 5 min for the first 30 min, followed by extraction every 10 min for the next 90 min, and assayed for malate dehydrogenase activity in the standard oxaloacetate reduction assay. Control incubation tubes lacking the NEM were used.

Active site arginyl residue: 2,3-butanedione. The possibility of an active site arginine residue was investigated by inhibition kinetics using 2,3-butanedione as an arginyl residue inhibitor.

The initial reaction between 2,3-butanedione and the guanidinium group of arginine involves the addition of two N-H bonds across the carbonyls of 2,3-butanedione. This adduct is then stabilized by the borate ion (64).

The 2,3-butanedione was prepared as a 10% (v/v) solution in 50 mM sodium borate buffer (pH 7.5) (64). The protein in borate buffer (pH 7.5) was introduced into the 10% (v/v) 2,3-butanedione solution so that the 2,3-butanedione was in 500 molar excess with respect to the protein. This mixture was allowed to incubate at 25 C. At intervals an aliquot was removed, diluted in 0.1 M potassium phosphate buffer (pH 7.5), and assayed immediately in the direction of oxaloacetate reduction.

Active site nitration: tetranitromethane. Tetranitromethane (TNM) is an effective nitration agent, especially of tyrosine residues. The compound has been used to investigate the possibility of an active site tyrosine group in m-MDH from bovine heart muscle (65). The reagent is used here to indicate the possibility of an active site group sensitive to nitration.

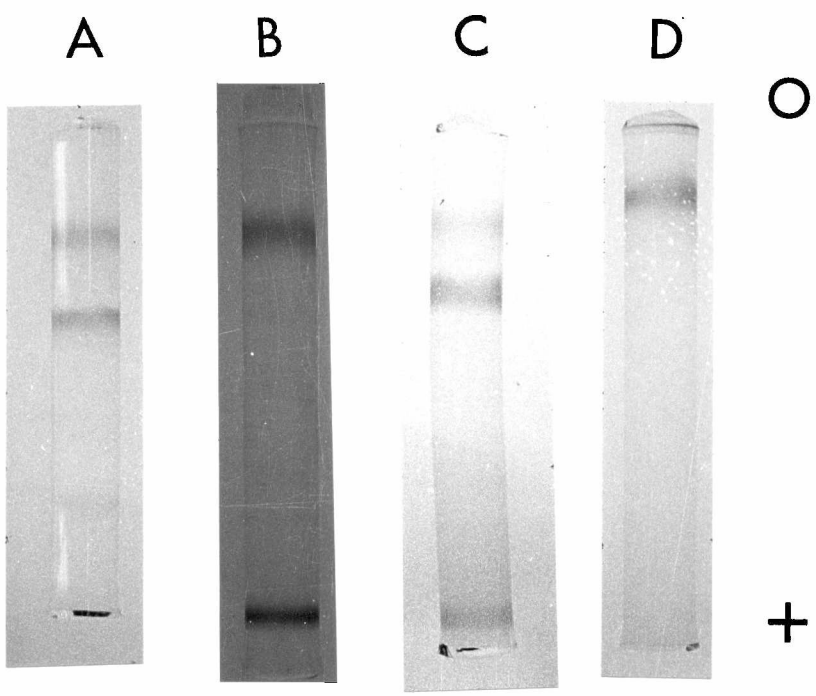
The reaction mixture consisted of 6 μmoles of TNM, 0.005 ml of ethanol (used for dilution of TNM), 0.1 mg of enzyme, and 1 μmole of potassium phosphate (pH 7.5) in a total volume of 1 ml. The incubation was allowed to proceed for 1 hr at 35 C. At the end of this time an aliquot was removed, diluted in 0.1 M potassium phosphate buffer (pH 7.5), and assayed for activity in the standard oxaloacetate reduction assay. Control tubes containing everything but TNM were used.

RESULTS

Malate dehydrogenase isozyme localization in Physarum. Previous data (37, 38) obtained from P. polycephalum plasmodia revealed that two electrophoretically distinct proteins were responsible for malate oxidation. Polyacrylamide-gel electrophoresis of soluble proteins from P. flavicomum plasmodia revealed similar results: a more cathodal band of rf 0.28 and a more anodal band of rf 0.38 (0.36 to 0.40) (Fig. 1A). The soluble protein extracts from P. flavicomum mitochondria, when subjected to polyacrylamide-gel electrophoresis, have indicated that only the cathodal band (Fig. 1B) was associated with mitochondria.

This basic pattern of two electrophoretically distinct proteins demonstrating malate dehydrogenase activity was also revealed in the electrophoresis of soluble proteins from P. flavicomum haploid cells: a more cathodal band of rf 0.25 and a more anodal band of rf 0.35 (Fig. 1C). Only the more cathodal band was seen when soluble proteins extracted from mitochondria were subjected to electrophoresis (Fig. 1D). This slower band was, therefore, designated mitochondrial malate dehydrogenase (m-MDH) as was the cathodal band from P. polycephalum (37, 38). An electron micrograph of the mitochondria isolated from the P. flavicomum plasmodia is shown

Figure 1. Polyacrylamide-gel disc electrophoresis (43, 44) of soluble proteins from P. flavicomum stained for malate dehydrogenase activity. All gels were 7% (w/v) polyacrylamide run at pH 9.5 at 4 C. Each contained 0.5 enzyme unit as measured by oxaloacetate reduction. The bottom band is the tracking dye. The O indicates the origin; the + indicates the anode. The identities of the stained isozymes are as follows: A, crude extract of plasmodia; B, plasmodial mitochondrial isolation; C, crude extract of haploid cells; D, haploid mitochondrial isolation.


A
B
C
D
O
+

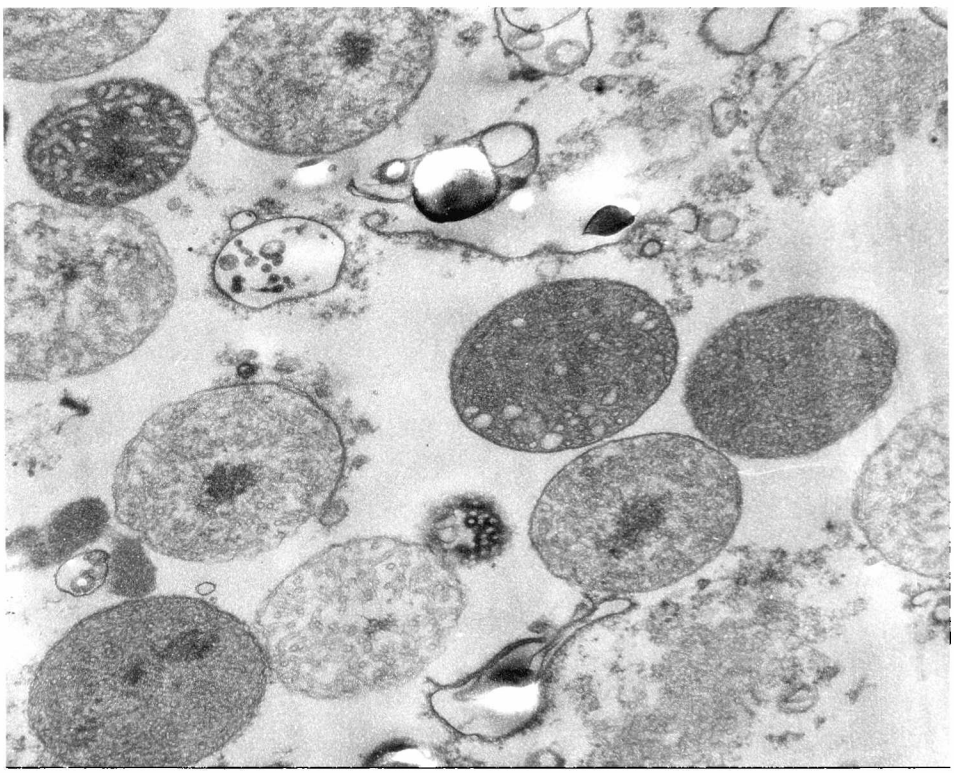
(Fig. 2). The malate dehydrogenase with the more anodal migration could never be localized with any particulate fraction. It was designated supernatant malate dehydrogenase (s-MDH).

Isolation and total purification of malate dehydrogenase isozymes from Physarum plasmodia. The basic purification procedure described here was developed and used to generate highly purified isozymes from P. polycephalum plasmodia (37, 38). A major alteration in the purification scheme was made with the addition of isoelectric focusing. As before, the P. polycephalum isozymes were separated by acetone fractionation of soluble plasmodial homogenates acidified to pH 5.0 (37, 38). The m-MDH was then freed of a viscous contaminant with CPC.

Certain technical changes were made in this CPC procedure. The present CPC technique was more rapid; it yielded a 42% recovery of enzyme in relation to crude extracts as compared to a previous 32% recovery (37, 38). The 10% difference was responsible for conserving some 12,000 enzyme units of P. polycephalum m-MDH per 1,000 g of plasmodia. However, the protein had to be collected from the CPC precipitated supernatant with acetone mediated precipitation, as the previous ammonium sulfate mediated precipitation was useless.

The purification scheme was continued for m-MDH with salt

Figure 2. Electron microscopic analysis of mitochondrial isolates from P. flavicomum. The mitochondria were first fixed in 2.5% glutaraldehyde and 0.1 M sodium phosphate buffer, pH 7.4. The mitochondria were then fixed in 1% osmium sulfate with 0.1 M sodium phosphate, pH 7.4. For full details of the preparation of these mitochondria for electron microscopic analysis, refer to Lena W. Cheung's Master's Thesis (Ultrastructural studies of the life cycle of Physarum flavicomum grown in pure culture. 1972. University of Houston). All electron microscopic analyses were performed by Ms. Cheung.



gradient elution from SE-cellulose at pH 6.0 (37, 38), isoelectric focusing and Sephadex G-200 chromatography.

The isolated P. polycephalum s-MDH was purified by ammonium sulfate fractionation, diethylaminoethyl (DEAE) cellulose chromatography, gradient elution from SE-cellulose at pH 5.5 (37, 38), isoelectric focusing and Sephadex G-200 chromatography. The purification procedures and results, based on an initial 1,000 g (wet weight) are summarized in Table 1 for P. polycephalum isozymes.

The purification procedures generated pure malate dehydrogenase proteins from P. flavicomum as well as P. polycephalum, but differences between homologous isozymes from those two species (Table 2) were evident.

Both P. flavicomum isozymes were more sensitive to acetone fractionation, as judged by enzyme yields, than the isozymes from P. polycephalum. However, the loss incurred was balanced by certain enzyme yield gains for both isozymes from P. flavicomum when compared to yields from P. polycephalum. Perhaps the most dramatic gain with respect to the m-MDH isozyme was in the response to SE-cellulose chromatography. Only 50% of the P. polycephalum m-MDH isozyme could be recovered after chromatography while 81% of the P. flavicomum isozyme was recoverable after the same

TABLE 1. Summary of the purification of P. polycephalum malate dehydrogenase isozymes[a]

Fraction	Total enzyme units (x 10^{-3})[b]	Total protein (mg)	Sp act[c]	Purification factor[d]
Crude extract	185.0	37,000.0	5	1.0
m-MDH				
0.3 to 0.6 vol acetone	120.0	2,000.0	60	12.0
CPC-acetone[e]	77.0	409.0	188	37.6
SE-cellulose	39.0	42.8	910	182.0
Isoelectric focusing-G-200 Sephadex	16.0	15.7	1,020	204.0
s-MDH				
0.6 to 1.0 vol acetone	46.3	1,715.0	27	5.4
$(NH_4)_2SO_4$	33.3	476.0	70	14.0
DEAE-cellulose	22.5	122.0	184	36.8
SE-cellulose	16.8	27.0	622	124.4
Isoelectric focusing-G-200 Sephadex	6.8	8.4	810	162.0

[a] Based on an initial 1,000 g (wet weight) of microplasmodia.
[b] As measured by oxaloacetate reduction at 25 C in 0.1 M potassium phosphate, pH 7.5.
[c] Units per milligram of protein.
[d] Specific activity of each fraction/specific activity of crude extract.
[e] Cetylpyridinium chloride.

TABLE 2. Summary of the purification of P. flavicomum malate dehydrogenase isozymes[a]

Fraction	Total enzyme units ($\times 10^{-3}$)[b]	Total protein (mg)	Sp act[c]	Purification factor[d]
Crude extract	170.0	18,900.0	9	1.0
m-MDH				
0.3 to 0.6 vol acetone	80.0	2,105.0	38	4.2
CPC-acetone[e]	45.5	392.0	116	12.9
SE-cellulose	38.5	56.6	680	75.5
Isoelectric focusing-G-200 Sephadex	15.5	14.4	1,080	120.0
s-MDH				
0.6 to 1.0 vol acetone	31.0	886.0	35	3.9
$(NH_4)_2SO_4$	24.5	408.0	60	6.7
DEAE-cellulose	19.6	93.0	210	23.3
SE-cellulose	14.7	19.0	775	86.0
Isoelectric focusing-G-200 Sephadex	8.1	9.7	839	93.2

[a] Based on an initial 1,000 g (wet weight) of microplasmodia.
[b] As measured by oxaloacetate reduction at 25 C in 0.1 M potassium phosphate, pH 7.5.
[c] Units per milligram of protein.
[d] Specific activity of each fraction/specific activity of crude extract.
[e] Cetylpyridinium chloride.

procedure.

With respect to s-MDH enzyme sensitivity, no procedure indicated as dramatic a difference between the two species as that for the m-MDH chromatographic response difference, but ammonium sulfate fractionation and isoelectric focusing both yielded P. flavicomum s-MDH enzyme at levels about 15% higher than those techniques did with the homologous isozyme from P. polycephalum.

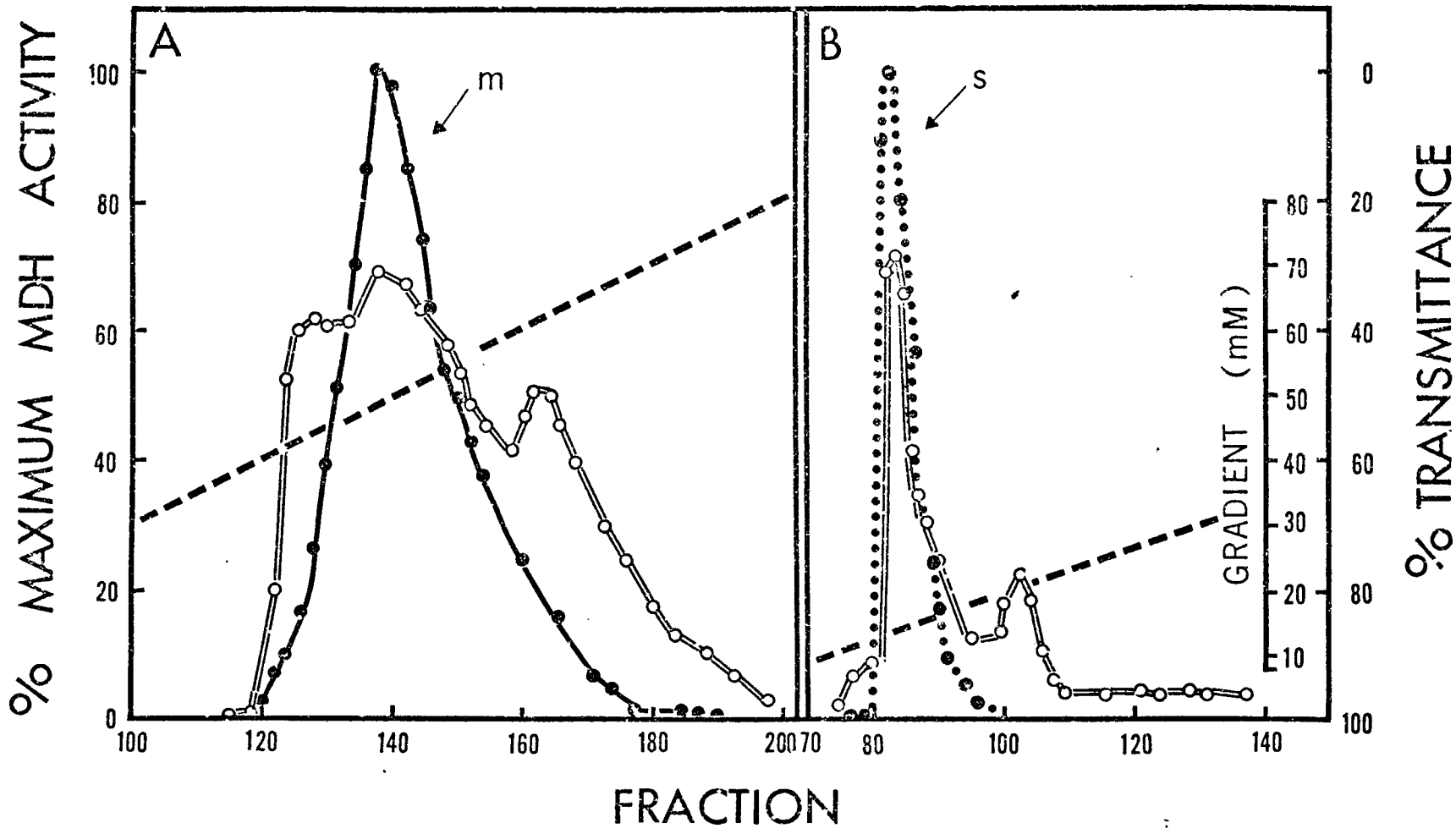
Response differences to the purification techniques were not limited to the isozymes alone. A brief comparison of the specific activities (Tables 1 and 2) between results from P. polycephalum and P. flavicomum revealed that the behavior of the soluble proteins derived from these two species in response to purification techniques were different. This lack of identical behavior indicated that the differences between these species extend throughout the molecular organization of these cells. These response differences were reflected in the gradient elution profiles of the isozymes when subjected to SE-cellulose chromatography.

SE-cellulose chromatography of P. flavicomum m-MDH. The CPC treated, acetone precipitated m-MDH was chromatographed on SE-cellulose (Table 2) under similar conditions as P. polycephalum m-MDH (37, 38). The gradient elution profile is given (Fig. 3A).

Figure 3. Gradient elution profile of malate dehydrogenase isozymes on SE-cellulose. Only portions of the gradients containing malate dehydrogenase activity are shown. (A) The elution gradient profile of CPC-acetone precipitated m-MDH from P. flavicomum. The figure represents 400 mg of protein contained in 132 ml of 30 mM potassium phosphate buffer (pH 6.0) with 1 mM ME loaded on a 2 by 30 cm column containing 25 g of SE-cellulose (0.17 meq/g) equilibrated with the same buffer. Then a linear gradient (525 ml) extending from 30 to 80 mM potassium phosphate (pH 6.0) was used to elute the column. This gradient was followed by 60 ml of 80 mM potassium phosphate buffer (pH 6.0) with 1 mM ME. Each fraction contained 5 ml per tube. (B) The profile of the DEAE-cellulose enzyme active peak of s-MDH loaded on SE-cellulose. The elution gradient profile

Figure 3 (continued)

represents 209 mg of protein contained in 49 ml of 8 mM citrate-phosphate buffer (pH 5.5) with 1 mM ME loaded on a 2 by 30 cm column containing 15 g of SE-cellulose equilibrated with the same buffer. After the sample was applied, the column was eluted with 135 ml of 8 mM citrate-phosphate buffer (pH 5.5) followed by a linear gradient (230 ml) from 8 to 50 mM citrate-phosphate buffer (pH 5.5). Each fraction contained 2 ml per tube. Symbols: closed circles and solid line (● ——), percent maximum m-MDH activity; closed circles and dotted line (● ••••), percent maximum s-MDH activity; double line (═══), percent transmittance at 280 nm; dashed line (– –), salt gradient.


A
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The bulk of the P. flavicomum m-MDH eluted at 50 mM buffer, and the elution range was between 45 and 70 mM buffer. This was judged equivalent to the behavior of P. polycephalum m-MDH. However, the central protein peak containing the enzyme was fused on either side with two other peaks of protein. These two accessory peaks were not present in the P. polycephalum elution (37, 38).

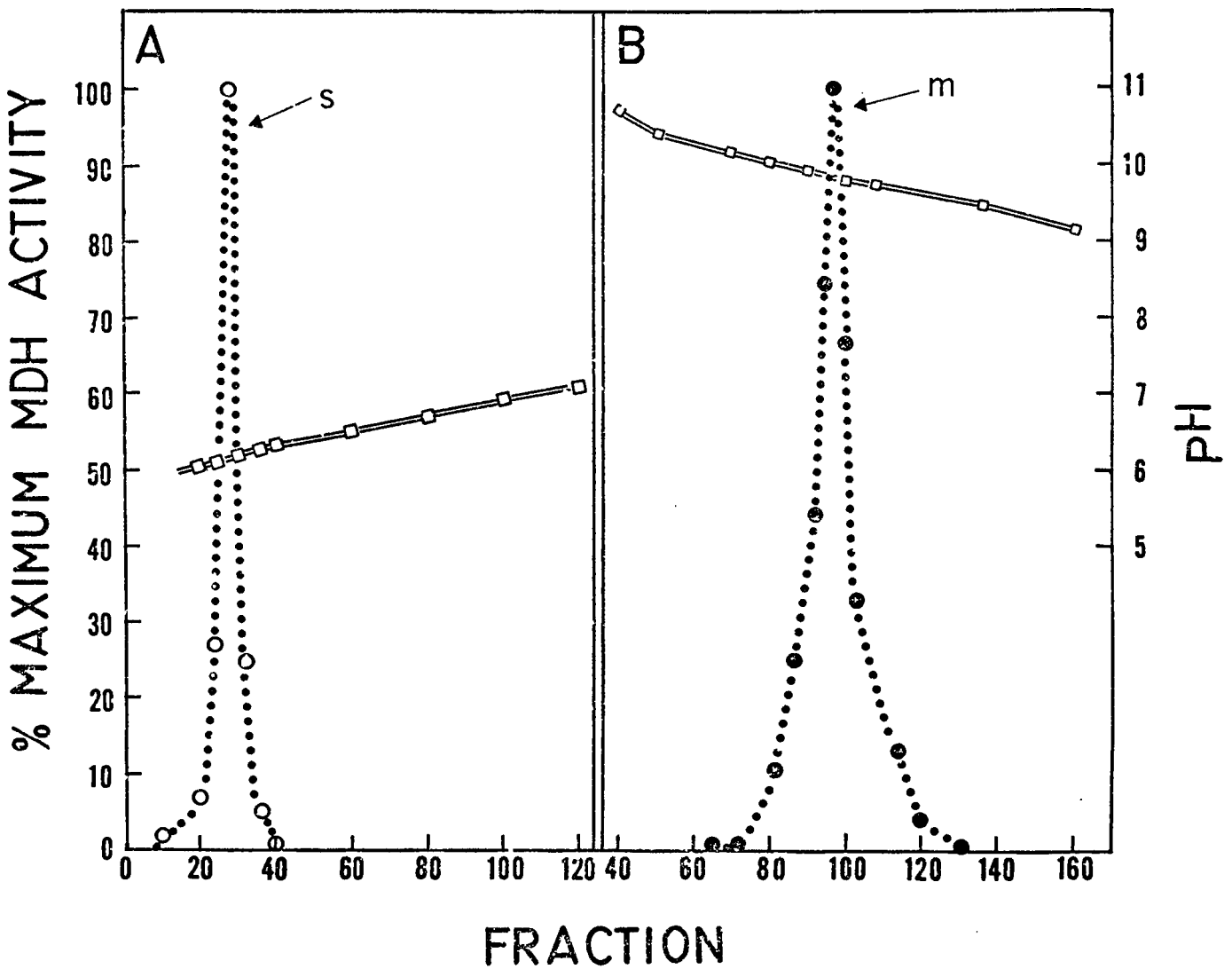
SE-cellulose chromatography of P. flavicomum s-MDH. The s-MDH from P. flavicomum, partially purified by acetone fractionation, was first fractionated with ammonium sulfate and then chromatographed on a DEAE-cellulose column (Table 2) under identical conditions used for the s-MDH isozyme from P. polycephalum. The unstructured, nonretained peak of protein contained the active enzyme, a situation identical to that for the s-MDH from P. polycephalum (37, 38). This nonretained peak was then chromatographed on SE-cellulose. The gradient elution profile is given (Fig. 3B). The s-MDH began eluting at about 14 mM buffer, and 90% of the enzyme eluted by the time the gradient reached 16 mM buffer. The maximum elution occurred at 13.5 mM buffer. This response was not entirely identical to P. polycephalum s-MDH (37, 38). The P. polycephalum enzyme had its maximum elution at 17 mM. These differences indicated the subtle inequality between the s-MDH isozymes from the two species.

Not only did the homologous isozymes behave differently but also the nonspecific protein elution pattern was different. The trailing side of the SE-cellulose gradient elution of P. polycephalum derived proteins (37, 38) was characterized by a broad band of contaminating protein. The trailing peak in the system derived from P. flavicomum was reduced and more structured (Fig. 3A).

Isoelectric focusing of Physarum plasmodial malate dehydrogenases eluted from SE-cellulose chromatography. Both s-MDH and m-MDH preparations from P. polycephalum demonstrated only one band of enzymatically active protein with a pI of 6.14 for s-MDH (Fig. 4A) and a pI of 9.83 for m-MDH (Fig. 4B) (Table 3). Similar results were obtained for the isozymes from P. flavicomum. The s-MDH had a pI identical with that of the P. polycephalum s-MDH (Fig. 5A) while the pI for P. flavicomum m-MDH was 9.95 (Fig. 5B) (Table 3). All isozymes were stable during focusing experiments.

Gel filtration chromatography of Physarum plasmodial malate dehydrogenases collected from isoelectric focusing. Chromatography of each isozyme from both species on Sephadex G-200 produced only a single protein fraction per isozyme. This method served as the first criterion of purity. Both isozymes from P. polycephalum eluted at 112 ml of effluent which would correspond to a molecular weight of

Figure 4. Isoelectric point determination of P. polycephalum s-MDH and m-MDH. Fractions of 2 ml were collected and assayed. Only portions of gradients containing malate dehydrogenase activity are shown. (A) Isoelectric point determination of s-MDH. Focusing of s-MDH was carried out in 1% (w/v) ampholyte (pH 6.0 to 8.0) for 36 hr. Anode: 0.01 M phosphoric acid at column bottom; cathode: 0.1 M NaOH. A stabilizing gradient was formed using sucrose. (B) Isoelectric point determination of m-MDH. Focusing of m-MDH was carried out in 0.5% (w/v) ampholyte (pH 9.0 to 11.0) for 72 hr. Anode: 0.01 M acetic acid at column top; cathode: 0.1 M NaOH. Stabilizing gradient was generated with sorbitol. Symbols: open squares (▫), pH; open circles (○), percent maximum s-MDH activity; closed circles (●), percent maximum m-MDH activity.


A
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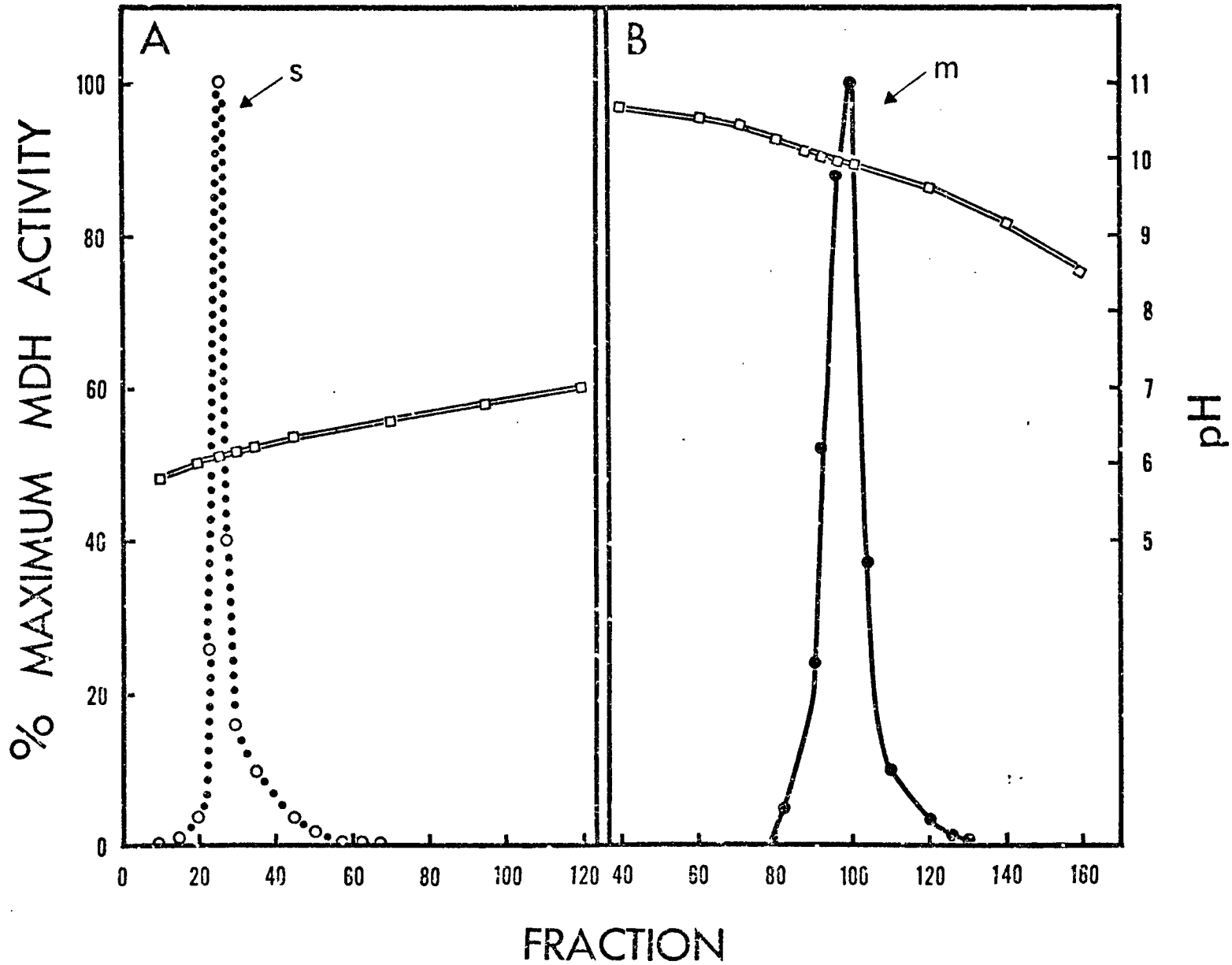
120
140
160

TABLE 3. Summary of the physical properties for the malate dehydrogenase isozymes from Physarum

Method	P. polycephalum		P. flavicomum	
	m-MDH	s-MDH	m-MDH	s-MDH
pI[a]	9.83	6.14	9.95	6.14
$s_{20,w}$	4.64 S	4.43 S	4.52 S	4.12 S
mol wt[b]	69,500	69,500	69,000	65,000

[a] Isoelectric point as determined by isoelectric focusing in a density gradient stabilized liquid system.
[b] Determined by sedimentation equilibrium.

Figure 5. Isoelectric point determination of P. flavicomum s-MDH and m-MDH. All details are identical to the legend for Figure 4. Symbols: open squares (□), pH; open circles (o), percent maximum s-MDH activity; closed circles (●), percent maximum m-MDH activity.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B
s
m
% MAXIMUM MDH ACTIVITY
pH
FRACTION
0
20
40
60
80
100
120
140
160
5
6
7
8
9
10
11

about 70,000 at the calculated partition coefficient of 0.43 for Sephadex G-200. The m-MDH and s-MDH activity peaks are shown for comparative purposes (Fig. 6A). The isozymes from P. flavicomum showed unequal elution (Fig. 6B) with the s-MDH eluting slightly faster indicating a slightly higher molecular weight.

The combined methods of isoelectric focusing and Sephadex G-200 chromatography yielded enzyme recoveries for isozymes from P. polycephalum equivalent to previously reported values for Sephadex G-100 chromatography. The final specific activities (37, 38) as measured by the oxidation of NADH at 340 nm were 1,020 for m-MDH and 810 for s-MDH from P. polycephalum (Table 1) and 1,080 for m-MDH and 839 for s-MDH from P. flavicomum (Table 2).

Gel electrophoresis of isozymes purified from Physarum plasmodia. Samples of the purified isozymes from P. polycephalum plasmodia were subjected to electrophoresis (43, 44) and stained for protein. The m-MDH preparation (Fig. 7A) consisted of one band of protein with an average rf of 0.17 (0.15 to 0.19). The s-MDH activity (Fig. 7C) also resided in one band of protein with an average rf of 0.38 (0.36 to 0.40). This method served as the second criterion of purity.

Comparative migrations can best be observed in Figure 7B where

Figure 6. Sephadex gel filtration chromatography of the malate dehydrogenase isozymes. The Sephadex G-200 was rehydrated and equilibrated in 50 mM potassium phosphate buffer (pH 7.5) with 1 mM ME. In each case the Sephadex bed was constructed in a 1.5 by 60 cm Pharmacia column. The total volume was 180 ml while the void volume was 68 ml. The column was eluted at 7.5 ml/hr using reverse flow techniques. The fraction volume was 1 ml. (A) The elution profile of P. polycephalum m-MDH and s-MDH. The m-MDH sample contained 32 mg of protein dissolved in 7 ml buffer. The s-MDH load contained 17.5 mg of protein dissolved in 4.7 ml of buffer. (B) The elution profile of P. flavicomum m-MDH and s-MDH. The s-MDH was loaded at 15.5 mg of protein contained in 5.3 ml of buffer while the m-MDH sample contained 28 mg of protein dissolved in 6.9 ml of buffer. Symbols: closed circles (•), percent maximum m-MDH activity; open circles (o), percent maximum s-MDH activity.

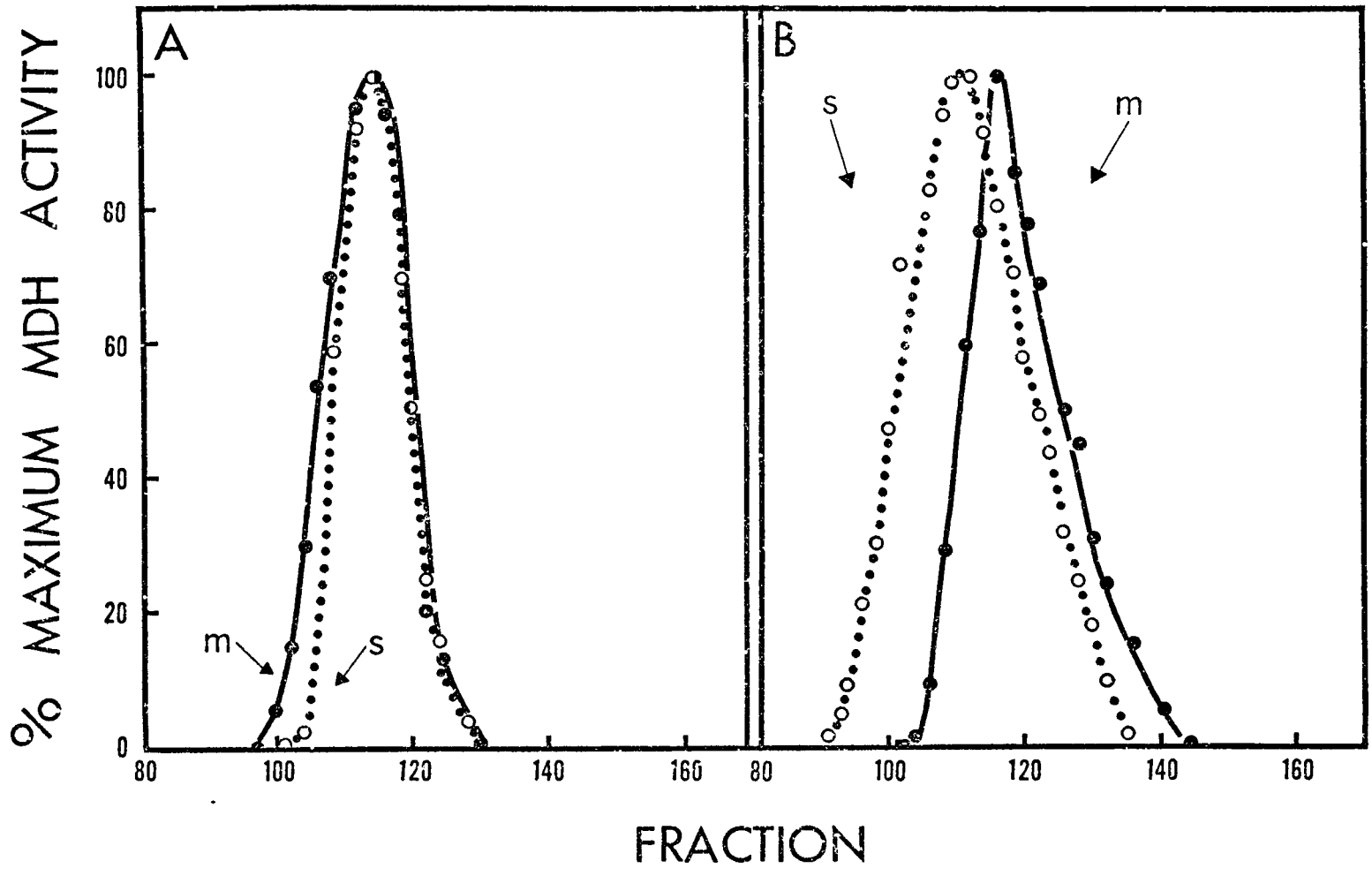
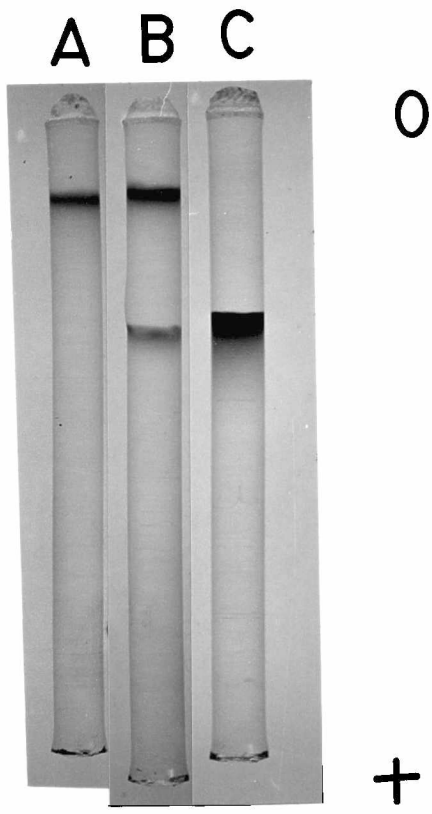

A
B
m
s
s
m
% MAXIMUM MDH ACTIVITY
0
20
40
60
80
100
80
100
120
140
160
80
100
120
140
160
FRACTION

Figure 7. Native P. polycephalum malate dehydrogenase analyzed by 7% (w/v) polyacrylamide-gel electrophoresis in a discontinuous pH system (43, 44). The proteins were stained with 0.5% (w/v) aniline blue black. The O indicates the origin; the + indicates the anode. The identities of the stained proteins are as follows: A, 50 μg purified m-MDH; B, mixed 50 μg pure m-MDH and 25 μg pure s-MDH; C, 75 μg purified s-MDH.


A
B
C
O
+

m-MDH and s-MDH were mixed and subjected to identical electrophoretic conditions as were the separate, pure samples.

Samples of the purified isozymes from P. flavicomum were subjected to similar electrophoretic conditions (43, 44). The results were equivalent to the data on electrophoresis of isozymes from P. polycephalum. The m-MDH preparation (Fig. 8A) and the s-MDH preparation (Fig. 8B) demonstrated single bands of protein with rf values indistinguishable from values obtained for isozymes isolated from P. polycephalum. A sample of the P. flavicomum m-MDH isozyme was subjected to gel electrophoresis at pH 6.6 with migration toward the cathode (45). This experiment indicated that the protein preparation was indeed electrophoretically homogeneous. Only one band of rf 0.55 was again revealed on gels stained for protein (Fig. 8C).

Sedimentation velocity ultracentrifugation: Physarum plasmodial malate dehydrogenase isozymes. Single, symmetrical boundaries were observed for isozyme preparations from P. polycephalum (Fig. 9) and P. flavicomum (Fig. 10) when they were subjected to velocity sedimentation. With respect to the P. polycephalum isozymes the $s_{20,w}$ for m-MDH was 4.64 S while the s-MDH value was 4.43 S (Table 3). The P. flavicomum isozymes had $s_{20,w}$ values of

Figure 8. Native P. flavicomum malate dehydrogenase analyzed by polyacrylamide-gel electrophoresis in a discontinuous pH system. The proteins were analyzed by anodic migration (43, 44); the m-MDH was analyzed by cathodic migration (45) also. The proteins were stained with 0.5% (w/v) aniline blue black. The O indicates the origin; the + indicates the anode while the - indicates the cathode. The identities of the stained proteins are as follows: A, 50 μg purified m-MDH; B, 50 μg purified s-MDH; C, 50 μg purified m-MDH (analyzed by cathodic migration).

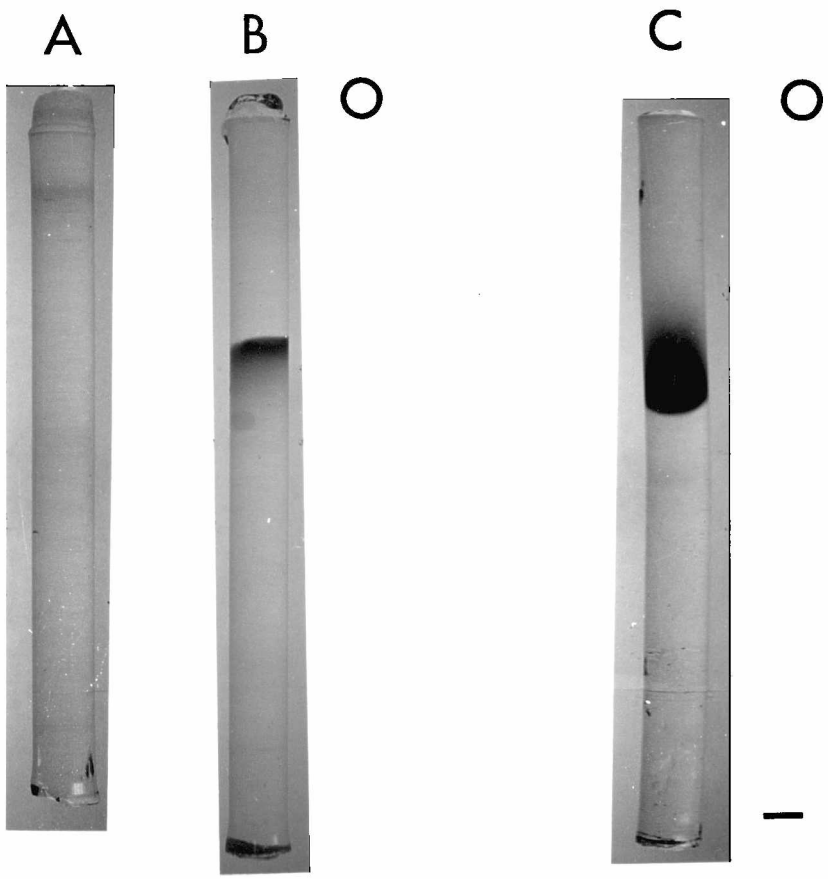

A
B
O
C
O
—

Figure 9. Sedimentation velocity of malate dehydrogenase isozymes from P. polycephalum. Typical photoelectric scanner tracings of absorbance (265.4 nm) versus distance from the axis of rotation (R) are represented. Centrifugation parameters for boundary sedimentation velocity experiments were 60,000 rev/min at 20 C in a Beckman model E ultracentrifuge. Protein samples were dissolved in 10 mM potassium phosphate buffer (pH 7.5) containing 1 mM ME and 150 mM sodium chloride. (A) P. polycephalum m-MDH (732 μg protein/ml) at 32 min after initiation of the experiment. (B) P. polycephalum s-MDH (650 μg protein/ml) at 32 min after initiation of experiment.

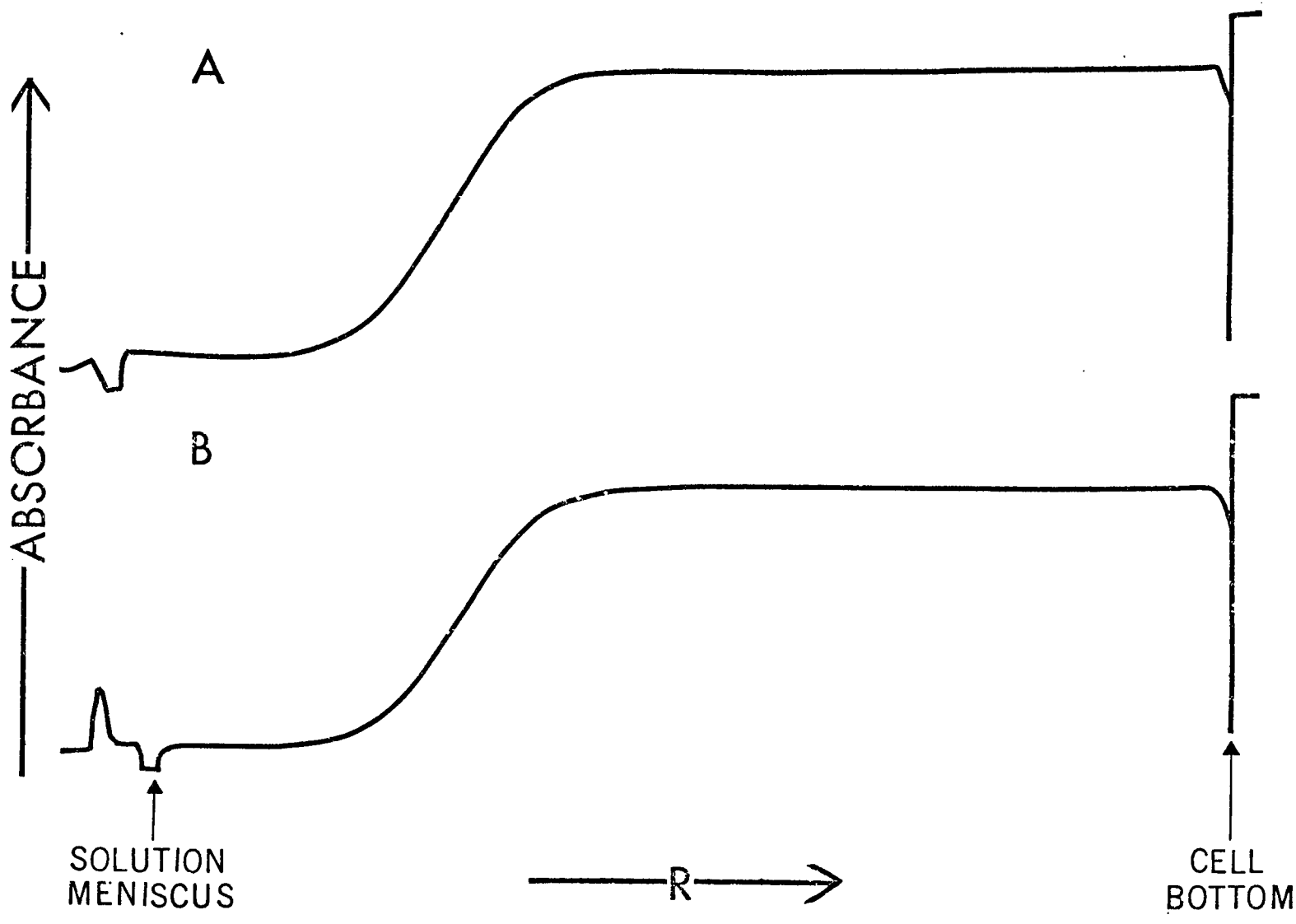
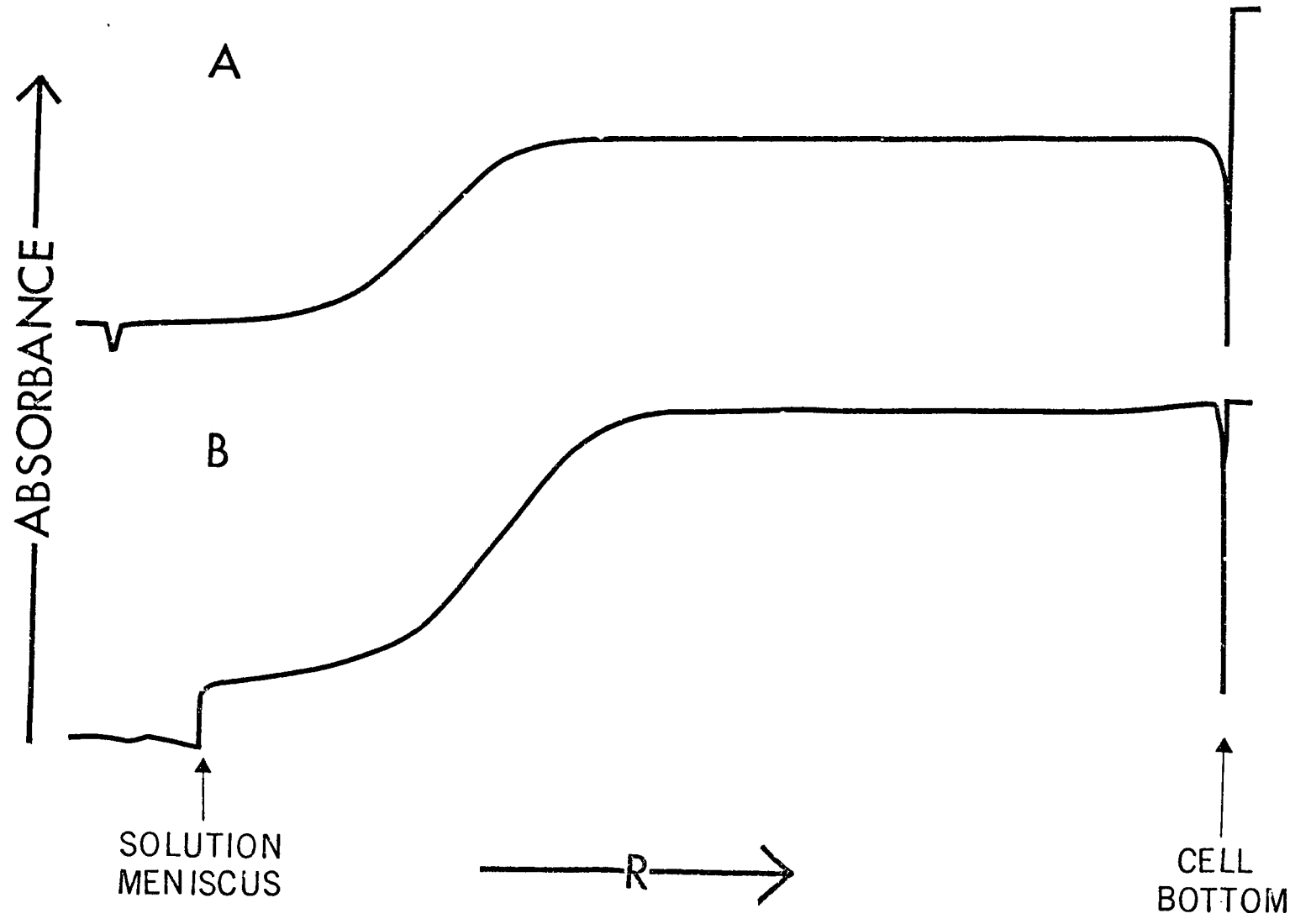

A
B
ABSORBANCE
SOLUTION
MENISCUS
R
CELL
BOTTOM

Figure 10. Sedimentation velocity of malate dehydrogenase isozymes from P. flavicomum. All qualifying parameters are identical to the legend for Figure 9. (A) P. flavicomum m-MDH (580 µg protein/ml) at 30 min after initiation of the experiment. (B) P. flavicomum s-MDH (500 µg protein/ml) at 32 min after initiation of the experiment.


A
B
ABSORBANCE
SOLUTION
MENISCUS
R
CELL
BOTTOM

4.52 S for the m-MDH and 4.12 S for the s-MDH (Table 3).

Sedimentation equilibrium ultracentrifugation: Physarum plasmodial malate dehydrogenase isozymes. From sedimentation equilibrium experiments, the molecular weights of 69,500 for both P. polycephalum isozymes were found (Table 3). Typical scanner tracings of the concentration distribution profile versus radial distance are shown (Fig. 11). A typical calibration curve used to translate pen deflection distance into absorbance units is shown (Fig. 12). Curvature was not indicated for any of the natural log concentration versus $(\text{radius})^2$ plots for the isozymes from this species (Fig. 13).

The molecular weights of P. flavicomum malate dehydrogenase isozymes were 69,000 for the m-MDH and 65,000 for the s-MDH (Table 3). The equilibrated protein distribution profiles along with the calibration stair steps are shown (Fig. 14). Once again no curvature was indicated for any of the natural log concentration versus $(\text{radius})^2$ plots (Fig. 15).

These combined sedimentation velocity and sedimentation equilibrium methods served as a third criterion of purity.

Total amino acid analyses. The amino acid compositions of the two purified isozymes from P. polycephalum are summarized in Table 4. Results are expressed as residues per mole (70,000 g/mole).

Figure 11. Sedimentation equilibrium centrifugation of P. polycephalum malate dehydrogenase isozymes. The data represent photoelectric scanner tracings of proteins redistributed after 38 hr at 13,000 rev/min in a Beckman model E ultracentrifuge. Each tracing is accompanied by its calibration block which allows interpretation of distance in terms of absorbance (265.4 nm). The parameters of the redistribution are absorbance (265.4 nm) versus distance (R) from the axis of rotation. (A) P. polycephalum m-MDH at 732 μg protein/ml. (B) P. polycephalum s-MDH at 650 μg protein/ml.

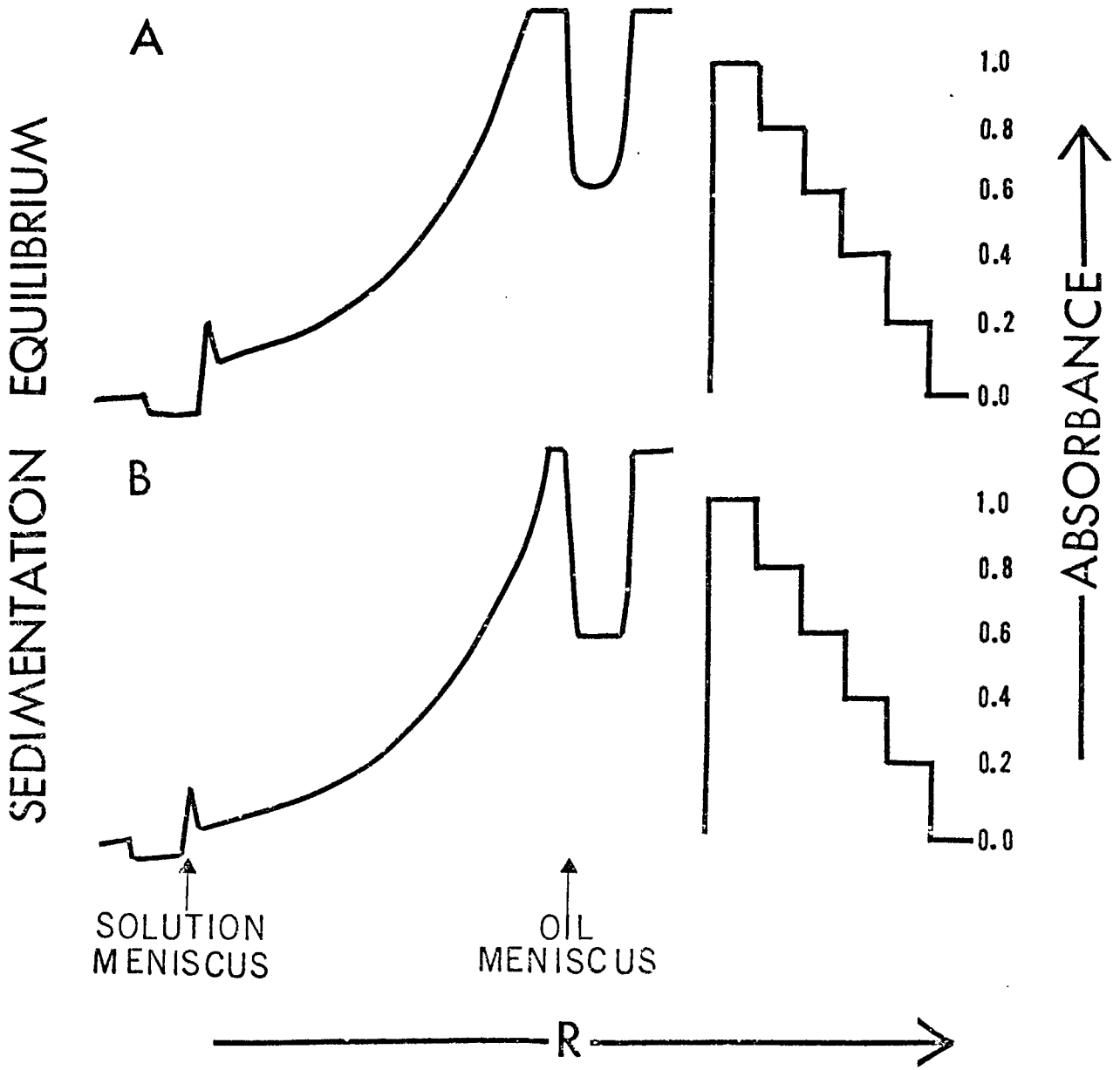

SEDIMENTATION EQUILIBRIUM
A
B
1.0
0.8
0.6
0.4
0.2
0.0
ABSORBANCE
SOLUTION MENISCUS
OIL MENISCUS
R

Figure 12. Sedimentation equilibrium centrifugation absorbance correction curve. What is shown here is typical of the correction curves that need to be used in order to convert photoelectric scanner pen deflection distance (cm) into absorbance.

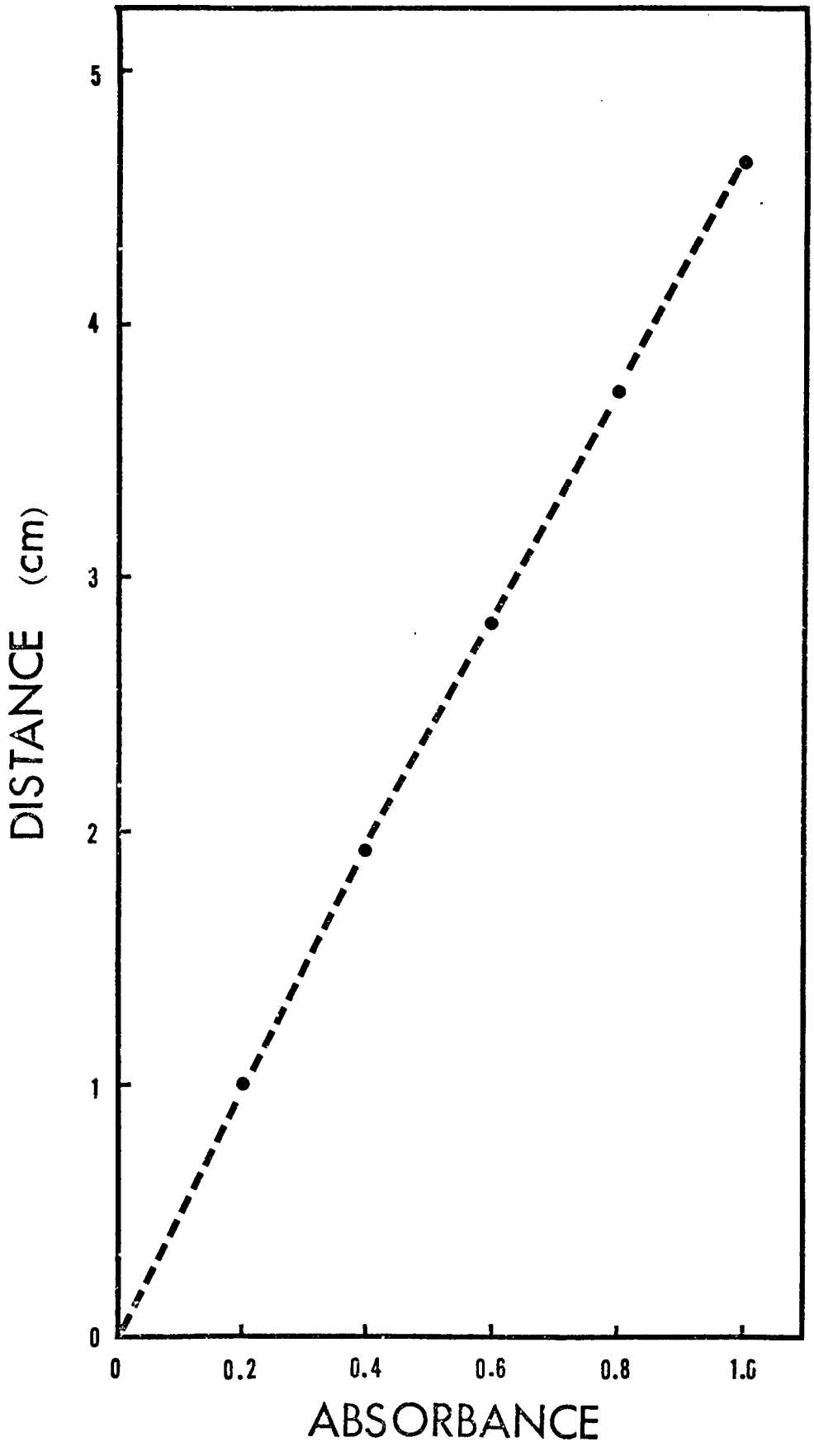

DISTANCE (cm)
5
4
3
2
1
0
0
0.2
0.4
0.6
0.8
1.0
ABSORBANCE

Figure 13. Sedimentation equilibrium centrifugation slope plots for P. polycephalum. The line slope was generated by relating the natural logarithm of the absolute protein concentration (as measured in terms of absorbance at 265.4 nm) versus the square of the absolute distance from the axis of rotation (r^2). The data were derived from photoelectric scanner tracings. The line (slope) giving the best fit of the data was determined by linear regression. Symbols: closed circles (•), m-MDH; open circles (o), s-MDH.

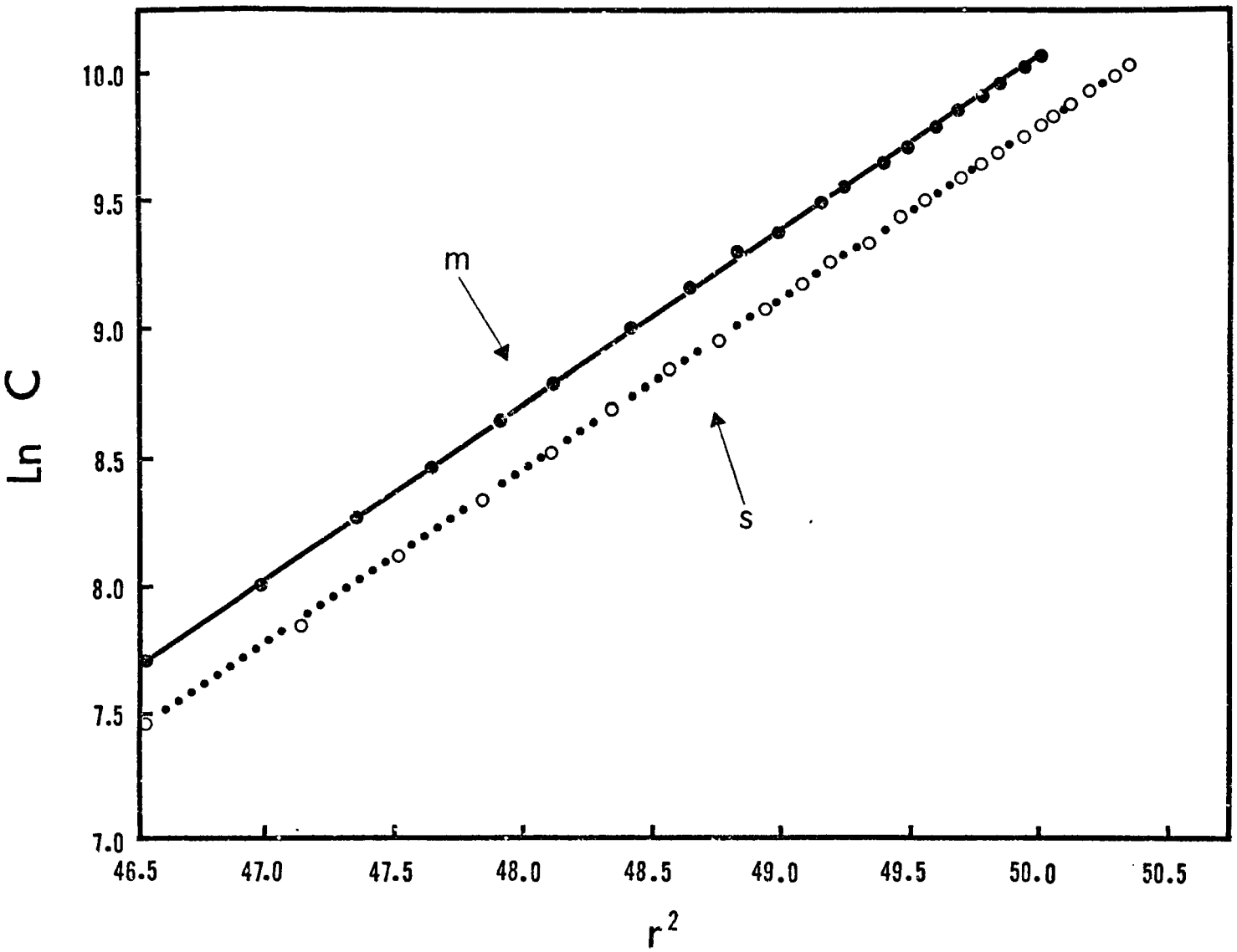

Ln C
10.0
9.5
9.0
8.5
8.0
7.5
7.0
46.5
47.0
47.5
48.0
48.5
49.0
49.5
50.0
50.5
r 2
m
s

Figure 14. Sedimentation equilibrium centrifugation of P. flavicomum malate dehydrogenase isozymes. All details are identical to the legend for Figure 11. (A) P. flavicomum m-MDH at 580 µg protein/ml. (B) P. flavicomum s-MDH at 500 µg protein/ml.

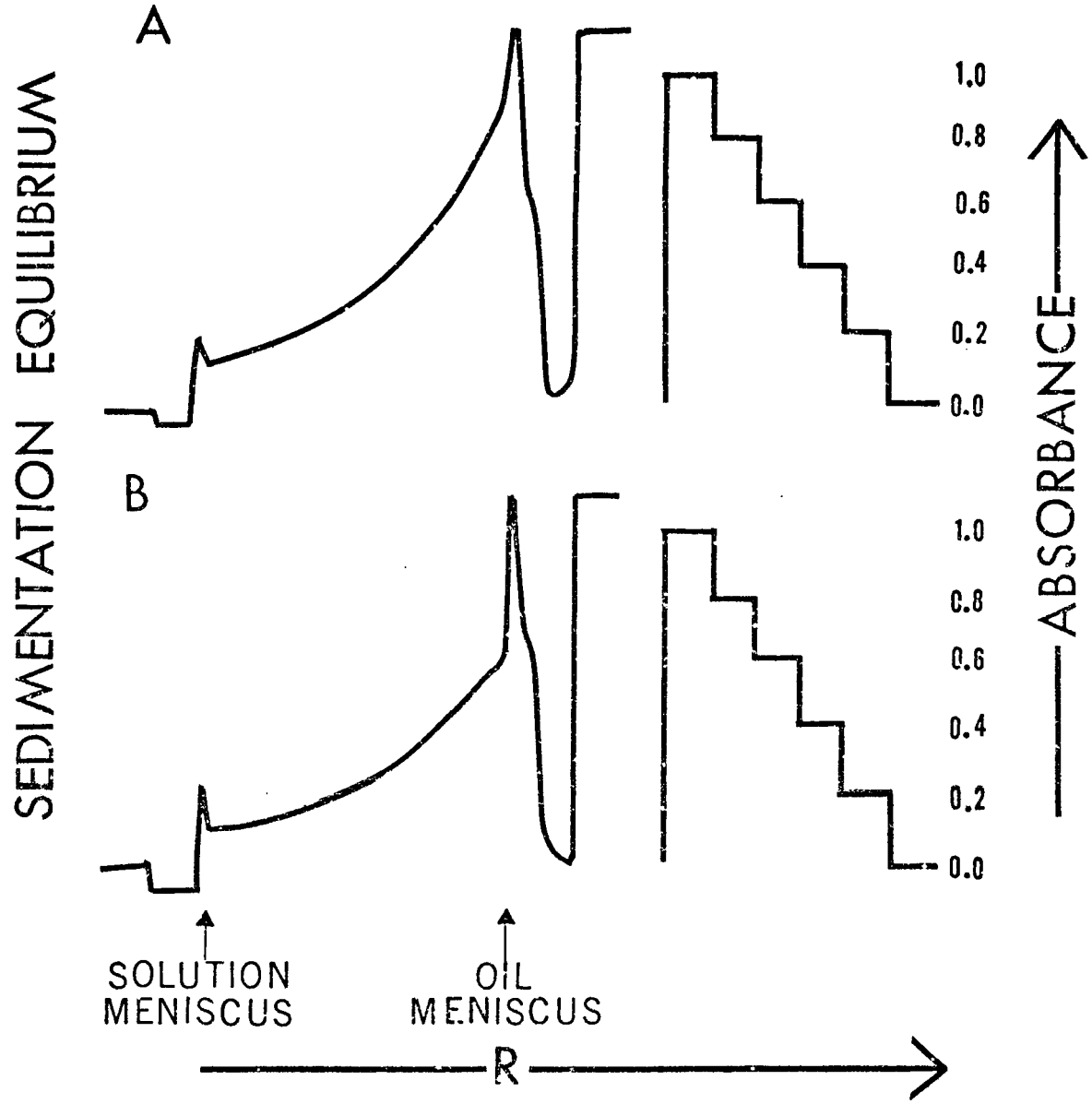

A
B
SEDIMENTATION EQUILIBRIUM
1.0
0.8
0.6
0.4
0.2
0.0
ABSORBANCE
SOLUTION MENISCUS
OIL MENISCUS
R

Figure 15. Sedimentation equilibrium centrifugation slope plots for P. flavicomum. All details are as explained in Figure 13. Symbols: closed circles (•), m-MDH; open circles (o), s-MDH.

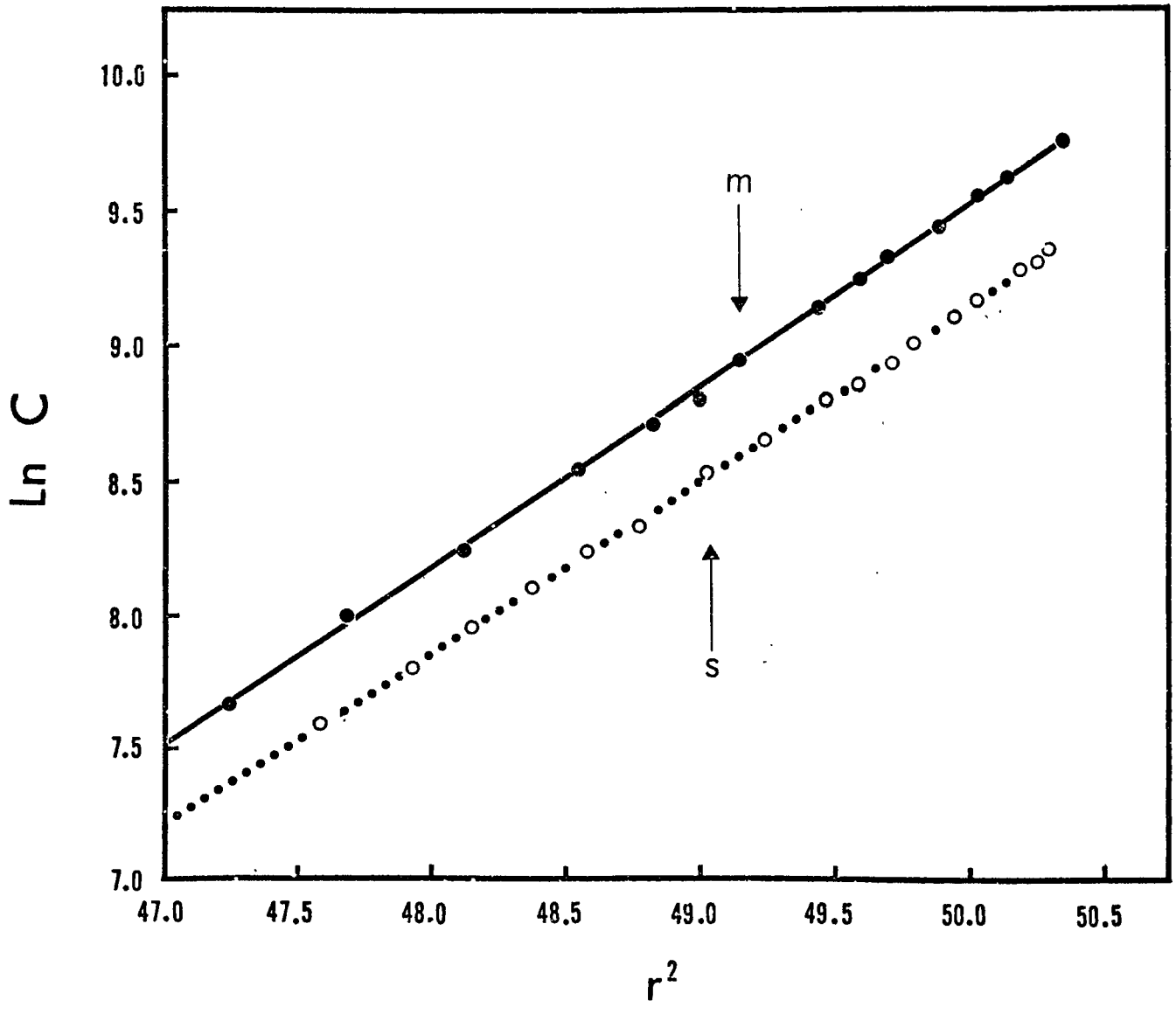

10.0
9.5
9.0
8.5
8.0
7.5
7.0
Ln C
47.0
47.5
48.0
48.5
49.0
49.5
50.0
50.5
r 2
m
s

TABLE 4. Amino acid composition of m-MDH and s-MDH from Physarum polycephalum

Amino acids	m-MDH[a]	s-MDH[a]
Lys	47	40
His	12	12
Arg	27	21
Asp	68	67
Thr[b]	33	41
Ser[b]	34	43
Glu	63	46
Pro	26	28
Gly	54	42
Ala	63	64
1/2 Cys[c]	6	3
Val[d]	39	67
Met	15	8
Ile[d]	39	48
Leu[d]	63	63
Tyr	16	14
Phe	17	19
Trp[e]	3	3

[a] Results reported as residues per mole (70,000 mol wt).
[b] Extrapolated to 0 hr hydrolysis.
[c] Determined as cysteic acid.
[d] Extrapolated to 96 hr of hydrolysis.
[e] Determined by the method of Harrison and Hoffman (58).

Analyses with Ellman's reagent (55, 56) indicated that the cysteine content was 5 residues per mole of m-MDH and 2 residues per mole of s-MDH. This same method gave 0.5 residues per mole of bovine serum albumin which was comparable to literature values (56). Both forms had equivalent amounts of tryptophan.

The amino acid compositions of the purified malate dehydrogenase from P. flavicomum are summarized in Table 5. Results are expressed as residues per mole (70,000 g/mole for m-MDH; 65,000 g/mole for s-MDH). Analyses with Ellman's reagent indicated that the cysteine content was identical to that found for P. polycephalum.

Gel electrophoresis of SDS denatured enzyme. The subunit number and subunit molecular weight of the purified isozymes derived from Physarum plasmodia were estimated by SDS gel electrophoresis (46). Each isozyme exhibited only one band of protein when subjected to the electrophoretic procedure. The results of electrophoresis on P. polycephalum m-MDH (Fig. 16B) and s-MDH (Fig. 16C) are shown along with results for commercially available catalase (Fig. 16A) and pig heart m-MDH (Fig. 16D). The results of analyses on P. flavicomum m-MDH (Fig. 17A) and s-MDH (Fig. 17B) are given, also.

The subunit molecular weights were estimated from plots of log molecular weight versus mobility of proteins with known molecular

TABLE 5. Amino acid composition of m-MDH and s-MDH from Physarum flavicomum

Amino acids	m-MDH[a]	s-MDH[b]
Lys	55	43
His	15	14
Arg	29	24
Asp	69	60
Thr[c]	42	34
Ser[c]	50	42
Glu	61	53
Pro	31	30
Gly	56	46
Ala	71	69
1/2 Cys	-	-
Val[d]	45	48
Met	11	7
Ile[d]	35	39
Leu[d]	60	54
Tyr	22	20
Phe	3	22

[a] Results reported as residues per mole (70,000 mol wt).
[b] Results reported as residues per mole (65,000 mol wt).
[c] Corrected to 0 hr of hydrolysis.
[d] Corrected to 96 hr of hydrolysis.

Figure 16. SDS denatured malate dehydrogenase from P. polycephalum analyzed by 10% (w/v) polyacrylamide-gel electrophoresis at 25 C in 0.1% (w/v) SDS and 10 mM sodium phosphate buffer (pH 6.8). Each gel contained 50 μg of protein stained with 0.5% (w/v) aniline blue black. The O indicates the origin; the + indicates the anode. The identities of the stained proteins are as follows: A, catalase; B, purified m-MDH; C, purified s-MDH; D, pig heart m-MDH.

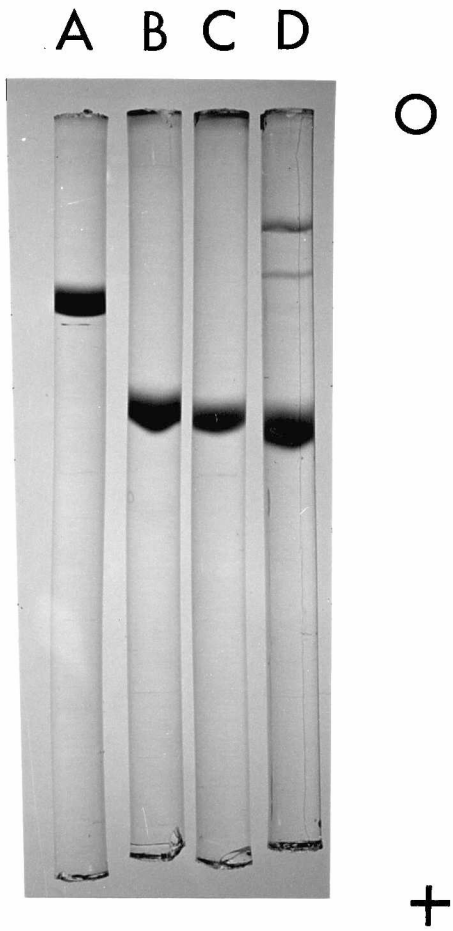
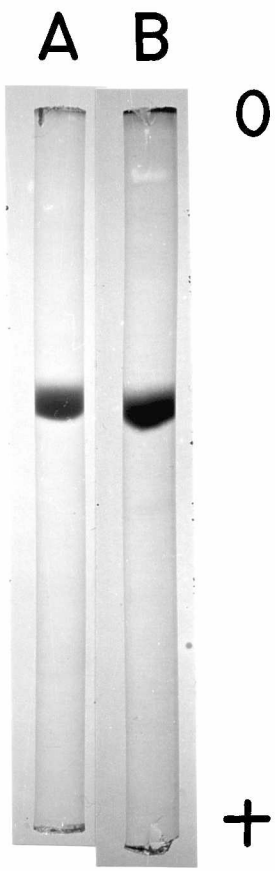

A B C D
O
+

Figure 17. SDS denatured malate dehydrogenase from P. flavicomum analyzed by 10% (w/v) polyacrylamide-gel electrophoresis at 25 C in 0.1% (w/v) SDS and 10 mM sodium phosphate (pH 6.8). Each gel contained 50 µg of protein stained with 0.5% (w/v) aniline blue black. The O indicates the origin; the + indicates the anode. The identities of the stained proteins are as follows: A, purified m-MDH; B, purified s-MDH.


A
B
0
+

weight. The molecular weight of the subunit of m-MDH from both P. polycephalum and P. flavicomum was estimated to be 37,000 (Fig. 18). The molecular weight of the subunit of s-MDH from P. polycephalum and P. flavicomum was estimated to be 35,600 (Fig. 18).

Kinetic analyses of Physarum malate dehydrogenase. All kinetic analyses were performed on pure isozyme, as defined with respect to electrophoretic, hydrodynamic and gel filtration properties, derived from Physarum plasmodia. The pure isozymes were stored at 4 C at concentrations of 1 mg/ml or higher. The enzymes were quite stable over a period of months under these conditions.

Optimum pH: oxaloacetate reduction. The pH of 0.1 M potassium phosphate buffer was varied between 6.0 and 8.5 in the standard oxaloacetate reduction assay. The optimum for both isozymes from P. flavicomum was pH 7.5. An average of two separate determinations from two isolates for each isozyme is shown in Figure 19A. The m-MDH had a lower activity than s-MDH at suboptimum pH values.

These results were quite compatible with the pH optima for the m-MDH (7.5) and s-MDH (7.6) isozymes from P. polycephalum (37, 38).

Figure 18. Subunit molecular weight determination of the polypeptide chain of malate dehydrogenase isozymes by SDS gel electrophoresis. All criteria concerning the electrophoretic technique are found in the legend of Figure 16. Mobility was calculated according to Weber and Osborn (46). The marker proteins (and their subunit molecular weights) are identified as follows: A, bovine serum albumin (68,000); B, catalase (58,000); C, ovalbumin (45,000); D, lactate dehydrogenase (35,000); E, pig heart m-MDH (33,500); F, cytochrome c (11,700). Symbols: closed circles (•), m-MDH; open circles (o), s-MDH; closed squares (■), marker proteins.

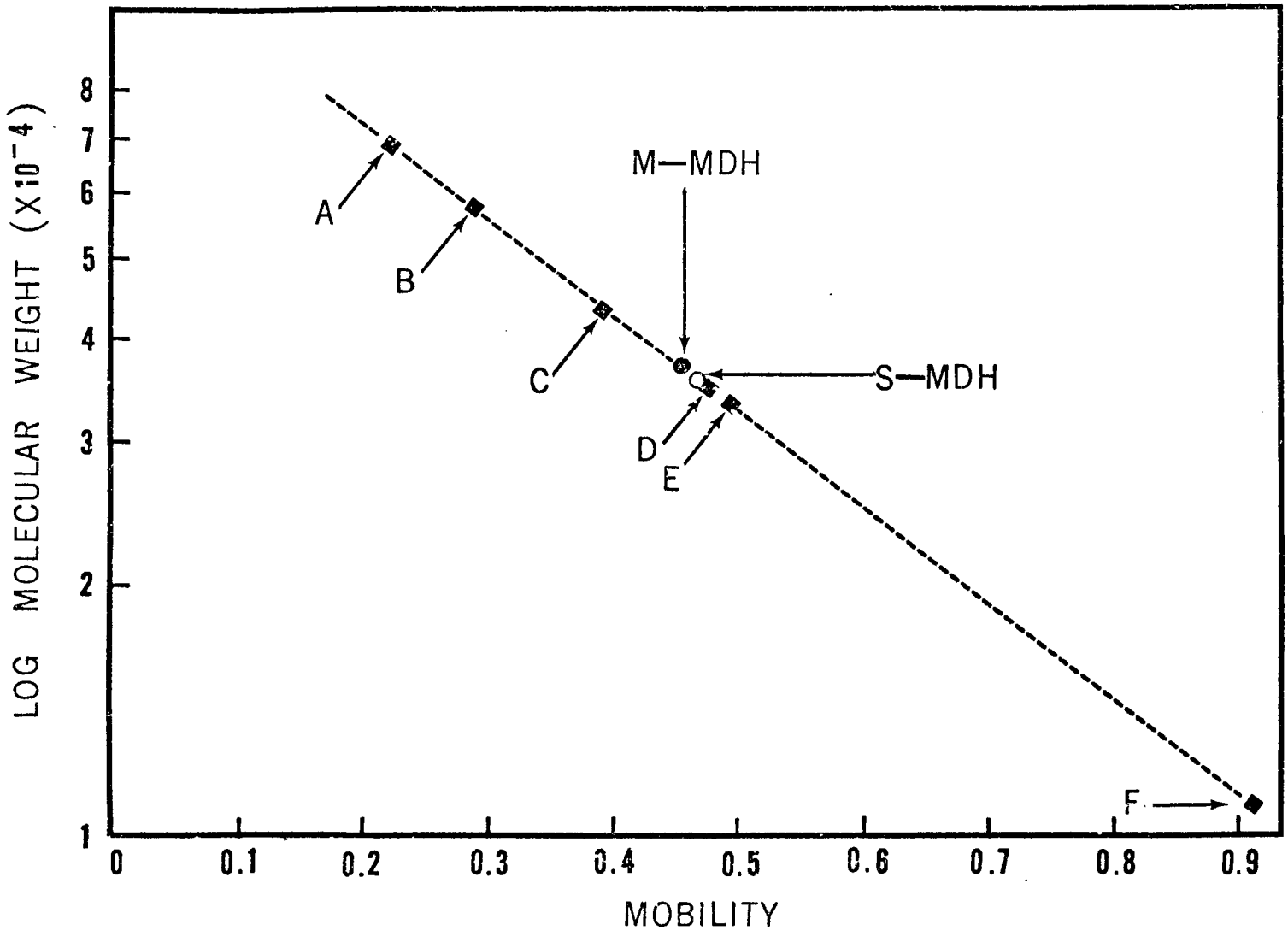
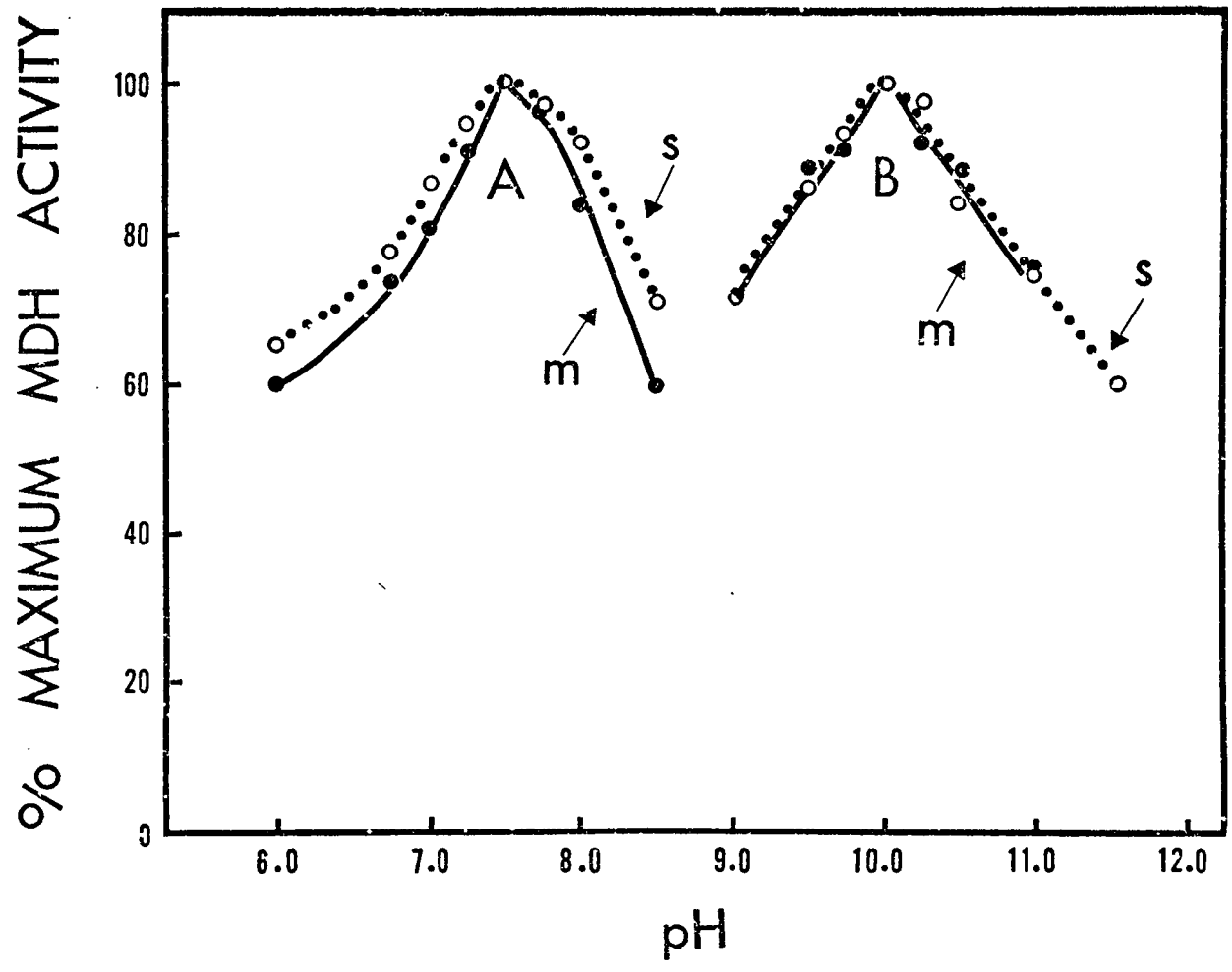

LOG MOLECULAR WEIGHT (X10⁻⁴)
8
7
6
5
4
3
2
1
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
MOBILITY
A
B
C
D
E
F
M—MDH
S—MDH

Figure 19. Optimum pH determination for the malate dehydrogenase isozymes of P. flavicomum. (A) Oxaloacetate reduction. The reaction mixture consisted of 0.39 μmole of NADH, 0.99 μmole of oxaloacetate, sufficient enzyme in 0.01 ml to cause an absorbance change between 0.15 and 0.05 units per minute, and sufficient 0.1 M potassium phosphate buffer to equal a total volume of 3 ml. The spectrophotometer was adjusted to 0.300 A at 340 nm with a blank consisting of everything but enzyme. The temperature of reaction was 25 C. (B) Malate oxidation. The reaction mixture consisted of 1.23 μmoles of NAD, 60 μmoles of malate, sufficient enzyme in 0.01 ml to cause an absorbance change between 0.15 and 0.05 units per minute, and sufficient 0.075 M sodium glycinate buffer to equal a total volume of 3 ml. The spectrophotometer was adjusted to 0.000 A at 340 nm with a blank consisting of everything but enzyme. Symbols: closed circles (•), percent maximum m-MDH activity; open circles (o), percent maximum s-MDH activity.

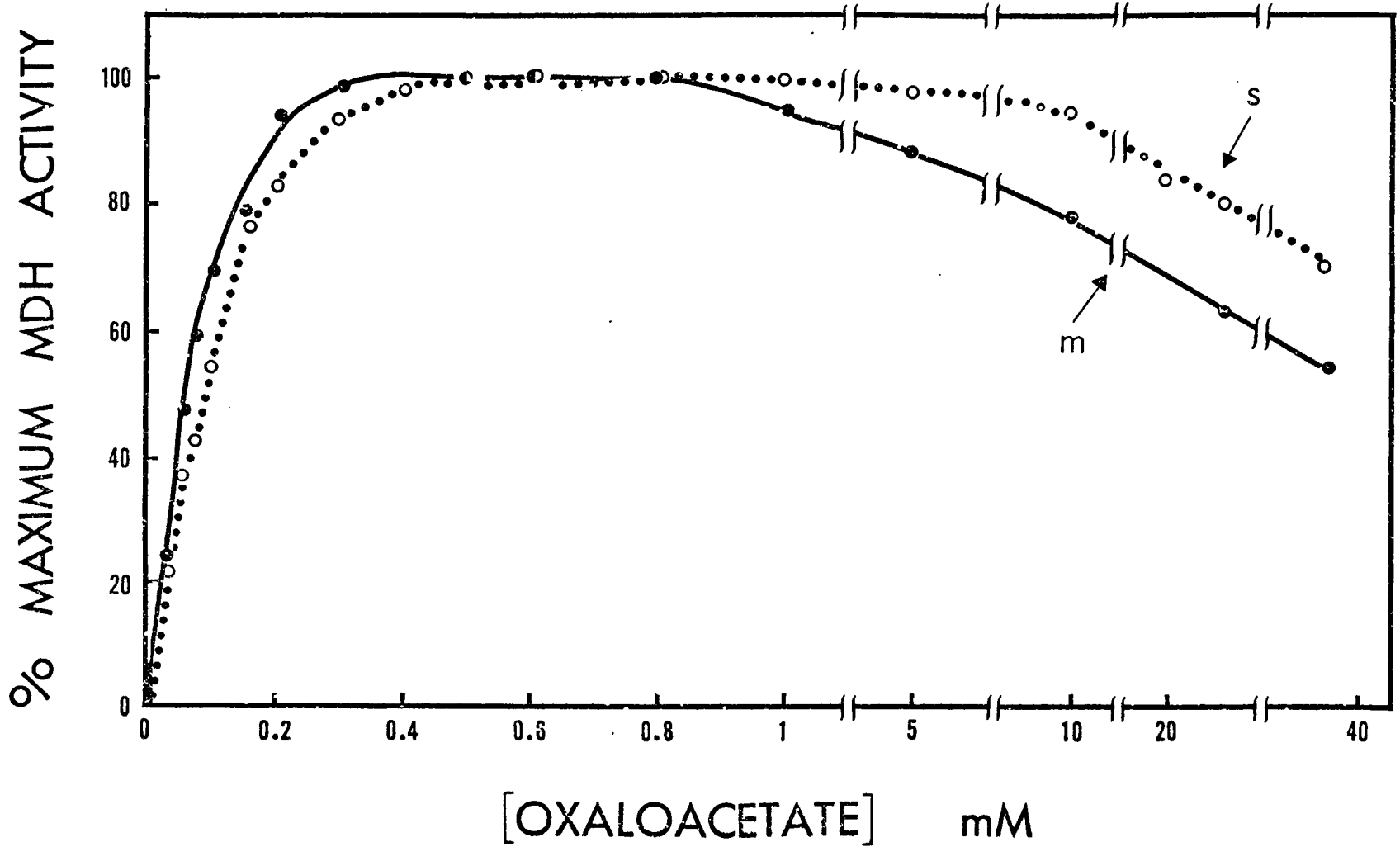

% MAXIMUM MDH ACTIVITY
100
80
60
40
20
0
A
B
s
m
s
m
6.0
7.0
8.0
9.0
10.0
11.0
12.0
pH

Optimum pH: malate oxidation. The pH of 0.075 M sodium glycinate buffer was varied between 9.0 and 11.5 in the standard malate oxidation assay. The optimum for both m-MDH and s-MDH from P. flavicomum was pH 10.0. There was essentially no difference in the response to pH changes between the two forms (Fig. 19B). These results were identical with data on P. polycephalum (37, 38).

Substrate optimum: oxaloacetate reduction. The optimum oxaloacetate concentration for malate dehydrogenase isozymes from P. flavicomum was measured in 0.1 M potassium phosphate buffer (pH 7.5) at 25 C. The optimum oxaloacetate concentration for the m-MDH was 0.35 mM (0.30 to 0.40 mM) oxaloacetate while that for s-MDH was 0.45 mM (0.40 to 0.50 mM) oxaloacetate (Fig. 20). Beyond 0.80 mM oxaloacetate, both isozymes were increasingly inhibited. At 40 mM substrate the s-MDH was inhibited 30% while the m-MDH was inhibited 50%. These values were obtained from three separate determinations.

The data on isozymes from P. polycephalum have been reported previously (37, 38). The maximal rates were obtained at about 0.35 to 0.70 mM oxaloacetate for m-MDH and 0.25 to 0.35 mM oxaloacetate for s-MDH. Beyond 0.70 mM oxaloacetate both

Figure 20. Determination of optimum oxaloacetate concentrations for the malate dehydrogenase isozymes of P. flavicomum. All reactions were performed in 3 ml cuvettes at 25 C. Each assay mixture contained 0.39 μmole NADH, enough enzyme in 0.01 ml to cause an absorbance drop between 0.15 and 0.05 units per minute, 0.3 ml of 10-fold oxaloacetate neutralized to pH 7.5, and sufficient 0.1 M potassium phosphate buffer (pH 7.5) to equal a total volume of 3 ml. The spectrophotometer was previously set at 0.300 A at 340 nm, with a mixture containing everything but enzyme, its volume being replaced by buffer. The reaction was initiated with enzyme. Only initial rates were used for the calculations. Enzyme velocity changes with concentration were expressed as percent maximum malate dehydrogenase activity using the maximum rate for each isozyme at 100%. Symbols: closed circles (●), m-MDH activity; open circles (o), s-MDH activity.


% MAXIMUM MDH ACTIVITY
100
80
60
40
20
0
0
0.2
0.4
0.6
0.8
1
5
10
20
40
S
m
[OXALOACETATE] mM

P. polycephalum isolated isozymes were increasingly inhibited (37, 38).

Kinetic behavior with malate: optimum L-malate concentration and behavior with D-malate. The optimum L-malate concentration, as measured in 0.075 M sodium glycinate buffer (pH 10.0) at 25 C, for both isozymes from P. flavicomum was 6.0 mM (5.5 to 6.5 mM) (Fig. 21). Both isozymes were inhibited by the substrate, but the effect was asymmetric. At 200 mM, s-MDH was inhibited 30% while m-MDH was inhibited only 5%. These values were obtained from four separate determinations. Substitution of D-malate for L-malate under the conditions for optimal L-malate oxidation gave no reduction of NAD at 100-fold increases in enzyme concentration. These results compared well with results from P. polycephalum where 5.0 to 7.0 mM malate gave maximum activity for s-MDH, and 6.0 to 9.0 mM malate gave maximum activity for m-MDH (37, 38).

Michaelis constant determinations of oxaloacetate for the isozymes of Physarum plasmodia. The apparent K_m for m-MDH was 9.1×10^{-5} M oxaloacetate, and the K_m for s-MDH was 1.1×10^{-4} M oxaloacetate (Table 6). Plots of reciprocal velocity versus reciprocal oxaloacetate were linear for both substrates (Fig. 22A). Similar results were obtained and reported for isozymes from P. polycephalum

Figure 21. Determination of optimum malate concentrations for the malate dehydrogenase isozymes from P. flavicomum. All reactions were performed in 3 ml cuvettes at 25 C. Each assay mixture contained 1.23 μmoles NAD, enough enzyme in 0.01 ml to cause an absorbance rise between 0.05 and 0.15 units per minute, 0.3 ml of a 10-fold concentration of malate neutralized to pH 10.0, and sufficient 0.075 M sodium glycinate buffer (pH 10.0) to equal a total volume of 3 ml. The spectrophotometer was previously set at 0.000 A at 340 nm with a mixture containing everything but enzyme, its volume being replaced by buffer. The reaction was initiated with enzyme. Only initial rates were used for the calculations. Rate changes with concentration were expressed as percent maximum malate dehydrogenase activity using the maximum rate for each isozyme as 100%. Symbols: closed circles (•), m-MDH activity; open circles (o), s-MDH activity.

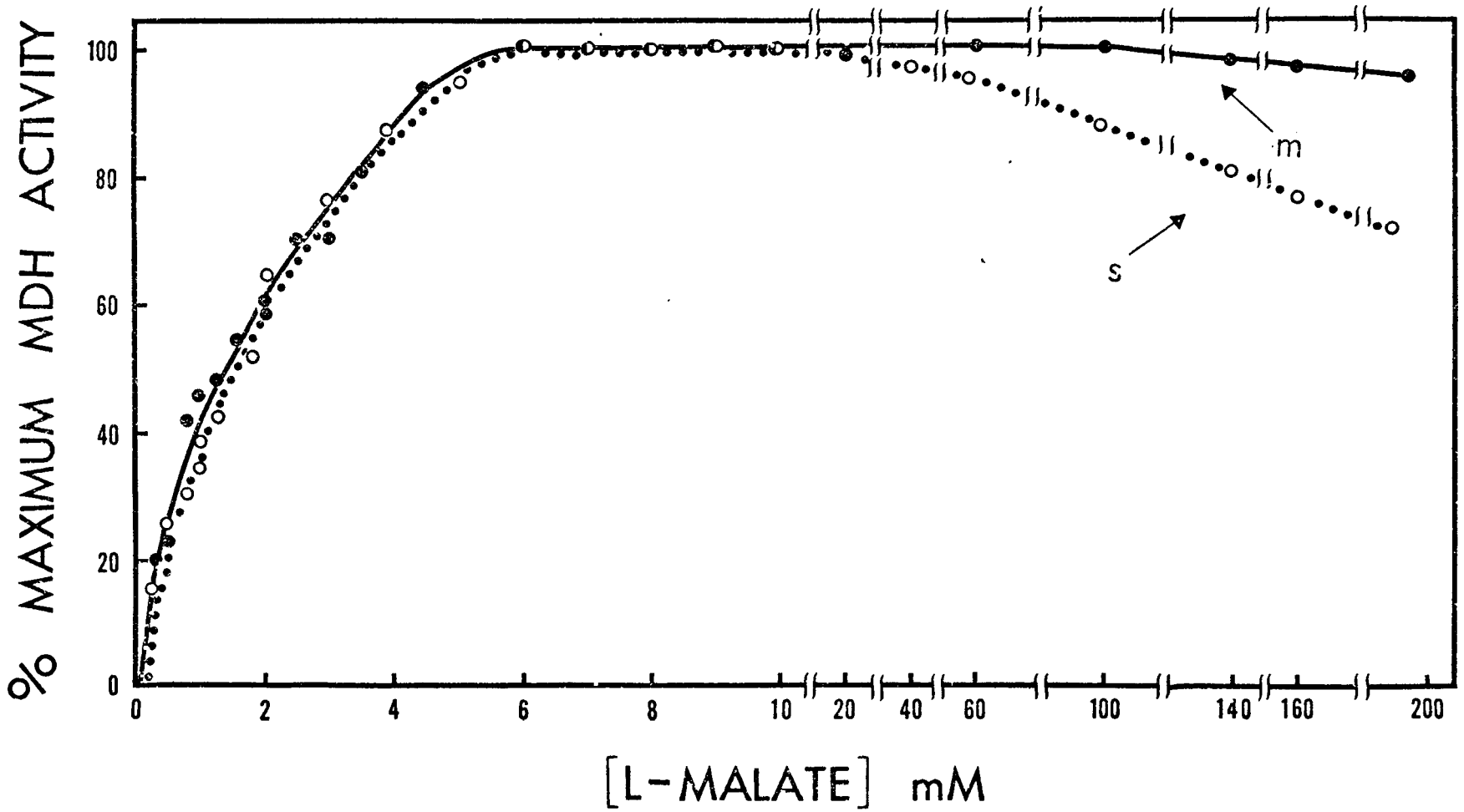
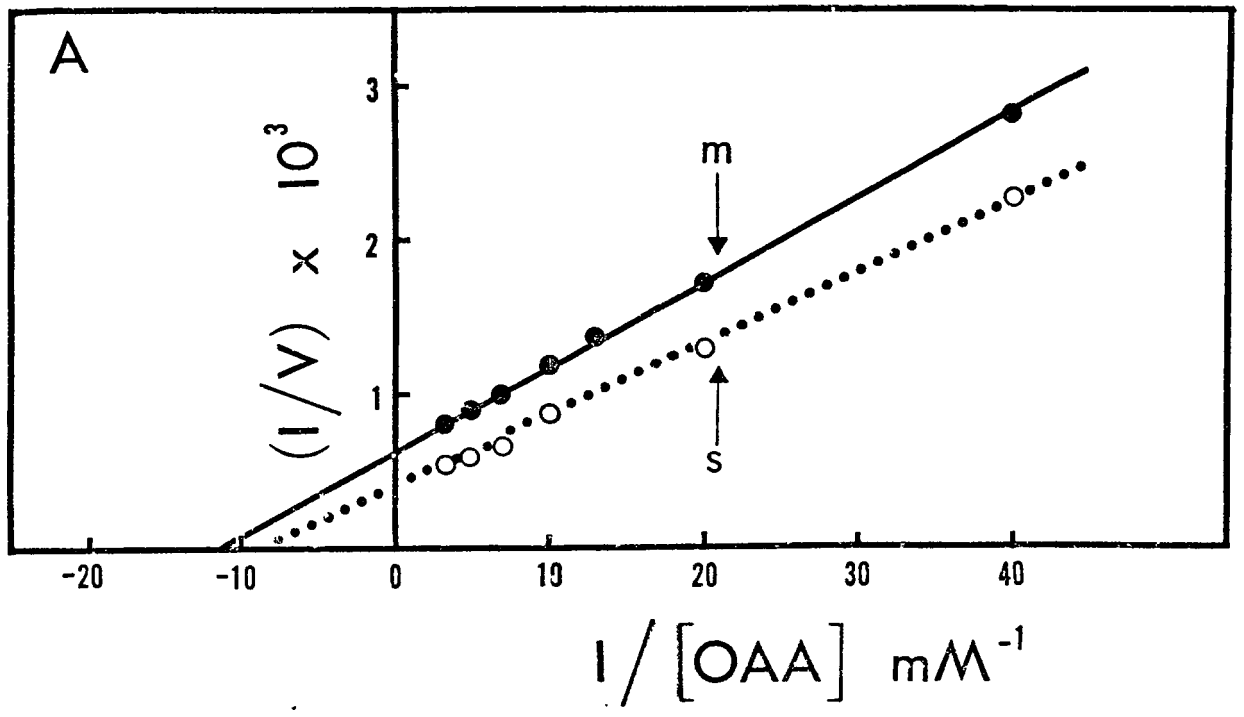
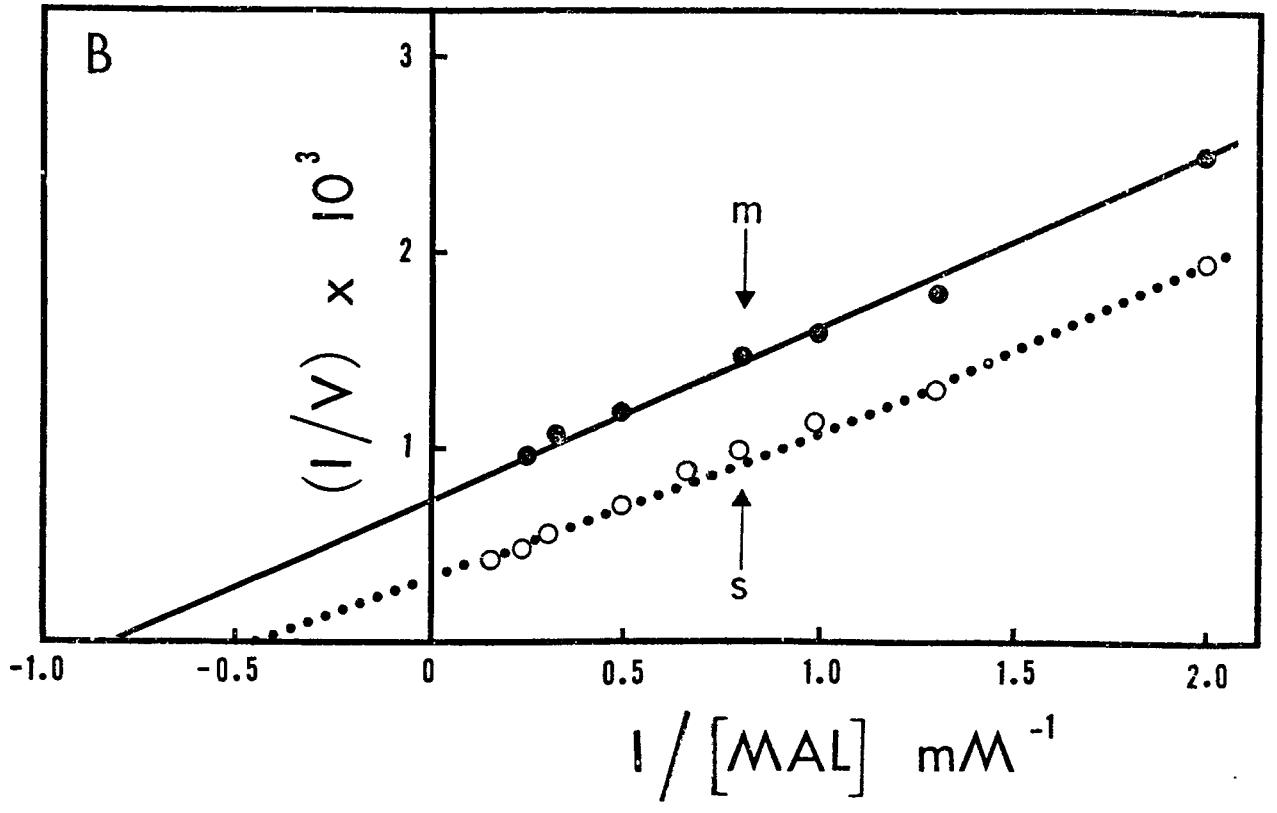

% MAXIMUM MDH ACTIVITY
100
80
60
40
20
0
0
2
4
6
8
10
20
40
60
100
140
160
200
m
s
[L-MALATE] mM

TABLE 6. Michaelis constants for the Physarum malate dehydrogenase isozymes

Substrate	Michaelis constants: K_m (μM)			
	P. flavicomum		P. polycephalum	
	m-MDH	s-MDH	m-MDH	s-MDH
Oxaloacetate	91	110	74	60
Malate	1,220	1,860	980	1,070
NADH	72	75	200	48
NAD	230	450	190	750

Figure 22. Double reciprocal plots of the initial velocity (expressed as enzyme units/ml) at 25 C versus substrate concentration for the malate dehydrogenase isozymes of P. flavicomum. (A) The relationship of reciprocal velocity to reciprocal oxaloacetate (OAA) concentration. In addition to the oxaloacetate the reaction mixture contained 0.13 mM NADH, enzyme in 0.01 ml, and sufficient 0.1 M potassium phosphate buffer (pH 7.5) to equal a 1 ml total volume. The photic absorbance decrease at 340 nm accompanying the oxidation of NADH was followed. (B) The relationship of reciprocal velocity to reciprocal malate (MAL) concentration. In addition to the malate the reaction mixture consisted of 0.41 mM NAD, enzyme in 0.01 ml, and sufficient 0.075 M sodium glycinate buffer (pH 10.0) to equal a 1 ml total volume. The photic absorbance increase at 340 nm accompanying the reduction of NAD was followed. Symbols: closed circles (•), m-MDH activity; open circles (o), s-MDH activity.


A
(1/V) x 10^3
3
2
1
m
s
-20
-10
0
10
20
30
40
1/[OAA] mM^-1


B
(1/V) x 10^3
3
2
1
m
s
-1.0
-0.5
0
0.5
1.0
1.5
2.0
1/[MAL] mM^-1

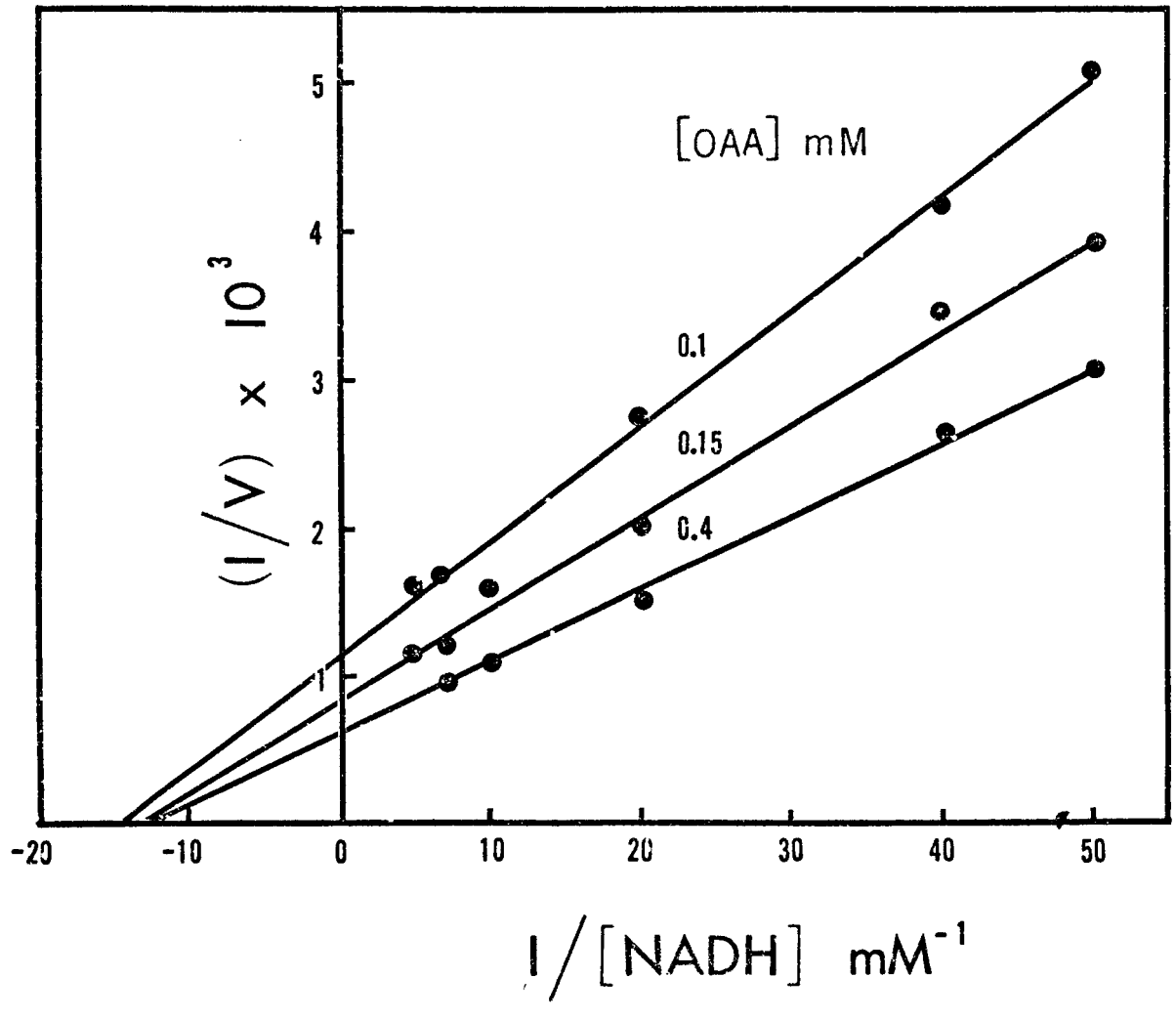
(Table 6) (37, 38). These results have been repeated over 10 times from multiple isolates.

Michaelis constant determinations of malate for the isozymes of Physarum plasmodia. The apparent K_m for m-MDH was 1.22×10^{-3} M malate, and the K_m for s-MDH was 1.86×10^{-3} M malate (Table 6). Typical plots of reciprocal velocity versus reciprocal substrate concentration are shown (Fig. 22B). These results were compatible with data for the isozymes of P. polycephalum. The apparent K_m for m-MDH from P. polycephalum was 9.8×10^{-4} M malate while the K_m for s-MDH was 1.07×10^{-3} M malate (Table 6).

Michaelis constant determinations of the coenzymes NADH and NAD for the m-MDH isozyme of P. flavicomum. The apparent K_m value of NADH for m-MDH from P. flavicomum was 7.2×10^{-5} M NADH (Table 6). This value was derived from analyses at various oxaloacetate concentrations (Fig. 23). The concentration of 0.13 mM NADH used in previous assays was not inhibitory to the isozyme. Reduced nicotinamide adenine dinucleotide phosphate (NADPH) at a similar concentration in the oxaloacetate reduction assay would not substitute for NADH.

The apparent K_m value of NAD for m-MDH from P. flavicomum was 2.3×10^{-4} M NAD (Table 6). This value was derived from

Figure 23. The relationship of reciprocal velocity (expressed as enzyme units/ml) at 25 C versus reciprocal NADH concentration for m-MDH from P. flavicomum. In addition to NADH the reaction mixture contained either 0.1, 0.15 or 0.4 mM oxaloacetate (OAA) as indicated, enzyme in 0.01 ml, and sufficient 0.1 M potassium phosphate buffer (pH 7.5) to equal a 1 ml total volume. The photic absorbance decrease at 340 nm accompanying the oxidation of NADH was followed.


[OAA] mM
0.1
0.15
0.4
(1/V) x 10^3
1/[NADH] mM^-1
5
4
3
2
1
-20
-10
0
10
20
30
40
50

analyses at various malate concentrations (Fig. 24). The concentration 0.41 mM NAD was not inhibitory to the isozyme. NADP would not replace NAD in the malate oxidation assay.

These various results were similar to those found for m-MDH from P. polycephalum. The K_m of NAD was 1.9×10^{-4} M which was in close agreement to the above results but the K_m of NADH was 2.0×10^{-4} M for the m-MDH isozyme from P. polycephalum. This was considered a bit high to be equivalent to the K_m for m-MDH from P. flavicomum (Table 6) (37, 38).

Michaelis constant determinations of the coenzymes NADH and NAD for the s-MDH isozyme of P. flavicomum. The apparent K_m value of NADH for s-MDH from P. flavicomum was 7.5×10^{-5} M (Table 6). This value was derived from analyses at various oxaloacetate concentrations (Fig. 25). The concentration of 0.41 mM NAD was not inhibitory to the s-MDH isozyme. NADPH would not replace NADH in the oxaloacetate reduction assay.

The apparent K_m value of NAD for s-MDH from P. flavicomum was 4.5×10^{-4} M (Table 6). This value was derived from analyses at various malate concentrations (Fig. 26). The concentration 0.41 mM NAD was not inhibitory to the isozyme. NADP would not replace NAD in the malate oxidation assay.

Figure 24. The relationship of reciprocal velocity (expressed as enzyme units/ml) at 25 C versus reciprocal NAD concentration for m-MDH from P. flavicomum. In addition to NAD the reaction mixture contained either 2, 4 or 6 mM malate (MAL) as indicated, enzyme in 0.01 ml, and sufficient 0.075 M sodium glycinate buffer (pH 10.0) to equal a 1 ml total volume. The photic absorbance increase at 340 nm accompanying the reduction of NAD was followed.

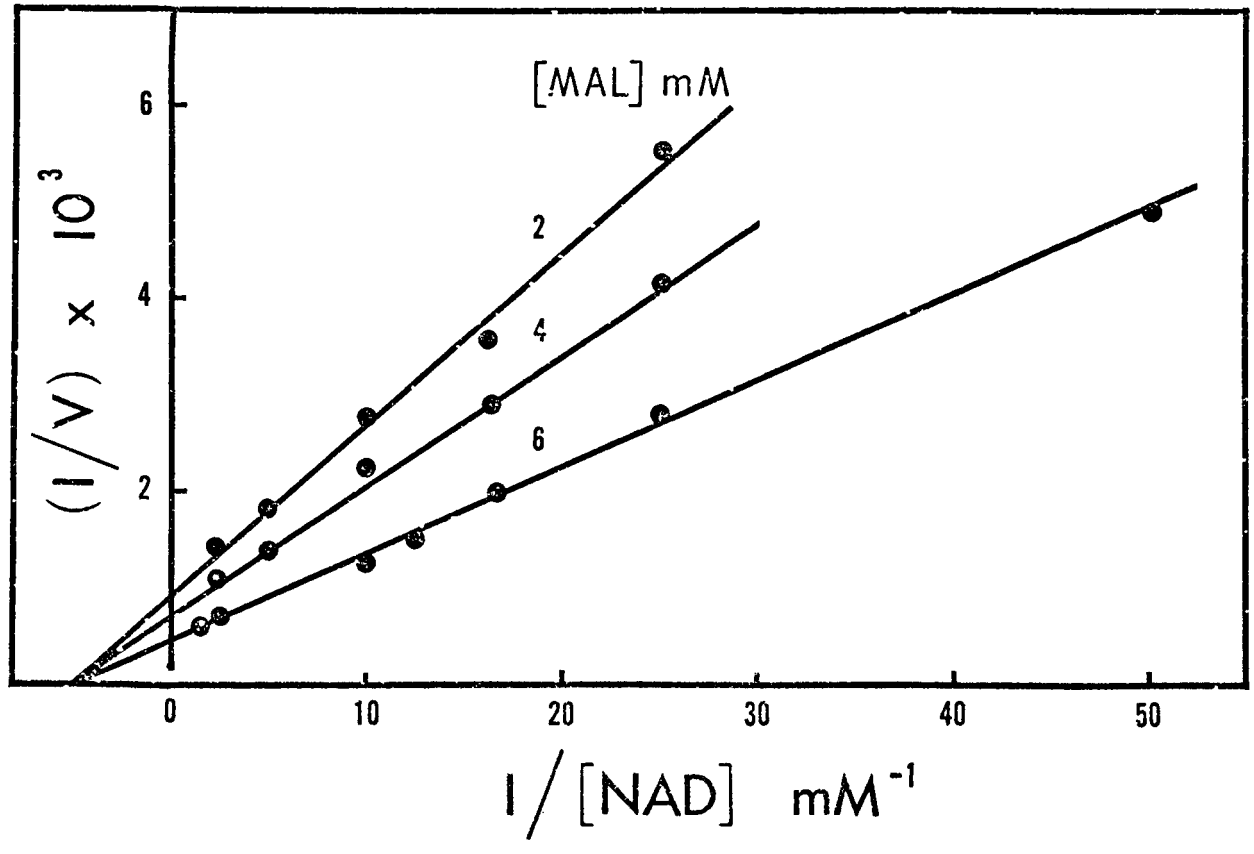

[MAL] mM
2
4
6
(1/V) x 10^3
6
4
2
0
10
20
30
40
50
1/[NAD] mM^-1

Figure 25. The relationship of reciprocal velocity (expressed as enzyme units/ml) at 25 C versus reciprocal NADH concentration for s-MDH from P. flavicomum. In addition to NADH the reaction mixture contained either 0.1, 0.15 or 0.4 mM oxaloacetate (OAA) as indicated, enzyme in 0.01 ml, and sufficient 0.1 M potassium phosphate buffer (pH 7.5) to equal a 1 ml total volume. The photic absorbance decrease at 340 nm accompanying the oxidation of NADH was followed.

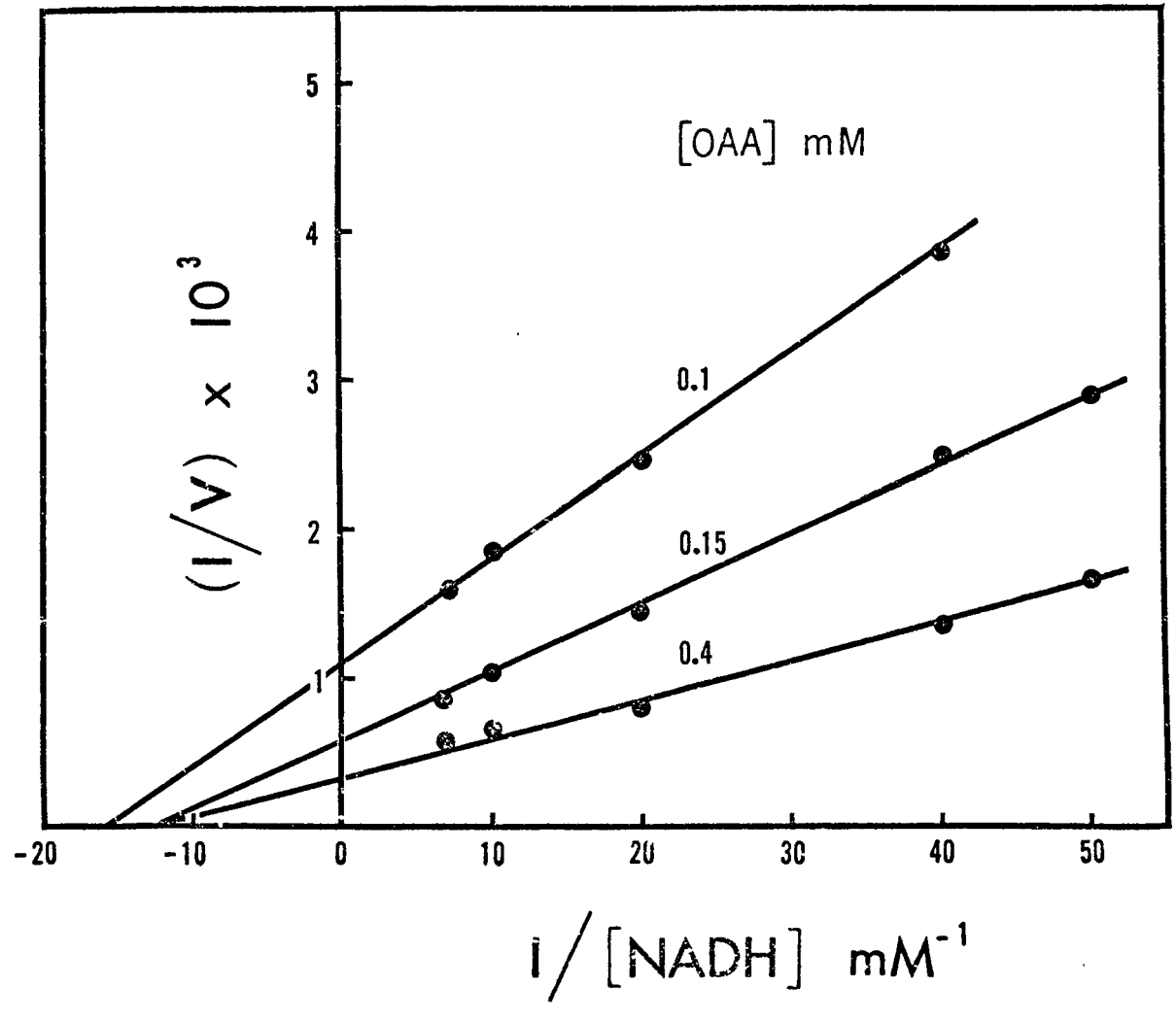
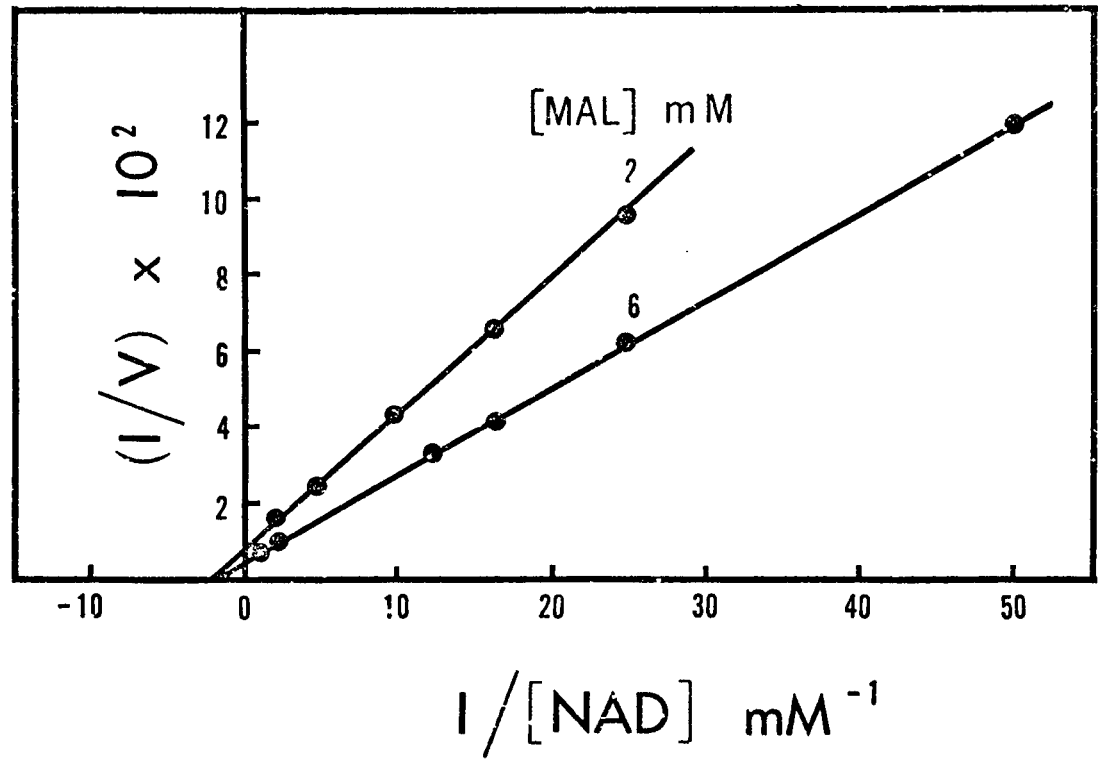

[OAA] mM
0.1
0.15
0.4
(1/V) x 10^3
1/[NADH] mM^-1
5
4
3
2
1
-20
-10
0
10
20
30
40
50

Figure 26. The relationship of reciprocal velocity (expressed as enzyme units/ml) at 25 C versus reciprocal NAD concentration for s-MDH from P. flavicomum. In addition to NAD the reaction mixture contained either 2 or 6 mM malate (MAL) as indicated, enzyme in 0.01 ml, and sufficient 0.075 M sodium glycinate buffer (pH 10.0) to equal a 1 ml total volume. The photic absorbance increase at 340 nm accompanying the reduction of NAD was followed.


[MAL] mM
?
6
(1/V) x 10²
12
10
8
6
4
2
-10
0
10
20
30
40
50
1/[NAD] mM⁻¹

These various results were similar to those found for s-MDH from P. polycephalum (Table 6) (37, 38).

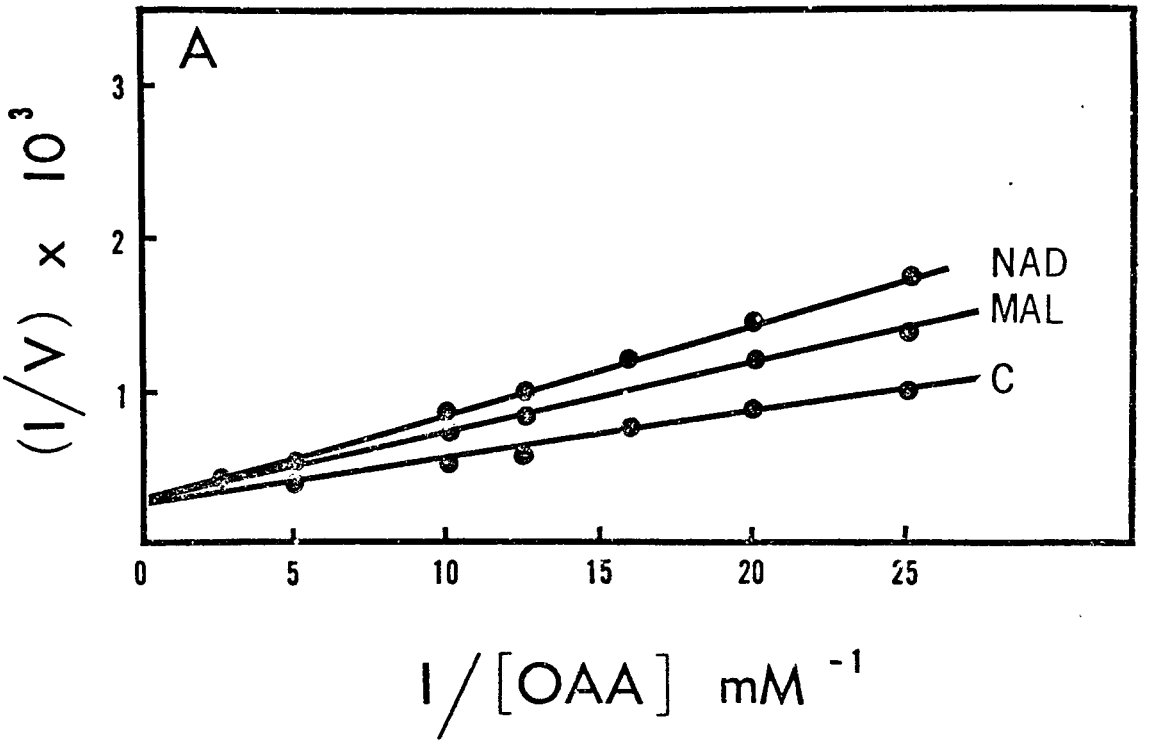
Product effects on the oxaloacetate reduction ability of m-MDH from P. flavicomum: malate and NAD. The effects of the products malate and NAD were tested against the activity of m-MDH from P. flavicomum. Both products were competitive with oxaloacetate for the active site of m-MDH (Fig. 27A). The apparent K_i of malate for m-MDH was 14 mM, and the K_i of NAD for m-MDH was 4.5 mM when oxaloacetate was used (Table 7).

The effects of these products were also tested against variable NADH concentrations. They were both competitive (Fig. 27B); malate had a K_i of 23.3 mM, and NAD had a K_i of 3.92 mM.

Product effects on the oxaloacetate reduction ability of s-MDH from P. flavicomum: malate and NAD. The effects of the products malate and NAD were tested against the activity of s-MDH from P. flavicomum. The effects of these products against variable oxaloacetate concentrations revealed both products to be competitive inhibitors of the enzyme for oxaloacetate (Fig. 28A). The apparent K_i of malate for s-MDH was 7.24 mM, and the K_i of NAD for s-MDH was 7.20 mM (Table 7).

The effects of these products were also tested against variable

Figure 27. The effects of reaction products on the P. flavicomum m-MDH catalytic activity. Plots of reciprocal velocity (expressed as enzyme units/ml) at 25 C versus reciprocal substrate concentration in the presence of NAD (5 mM) or malate (MAL) (8 mM) were made. The control reaction containing no product (C) is shown. The photic absorbance decrease at 340 nm accompanying the oxidation of NADH was followed. (A) The effects of products on the relationship of reciprocal velocity versus reciprocal oxaloacetate (OAA) concentration. In addition to oxaloacetate and product the reaction mixture contained 0.1 mM NADH, enzyme in 0.01 ml, and sufficient 0.1 M potassium phosphate buffer (pH 7.5) to equal a 1 ml total volume. (B) The effects of products on the relationship of reciprocal velocity versus reciprocal NADH concentration. In addition to NADH and product the reaction mixture contained 4 mM oxaloacetate, enzyme in 0.01 ml, and sufficient 0.1 M potassium phosphate buffer (pH 7.5) to equal a 1 ml total volume.


A
(1/V) x 10³
3
2
1
0
5
10
15
20
25
NAD
MAL
C
1/[OAA] mM⁻¹

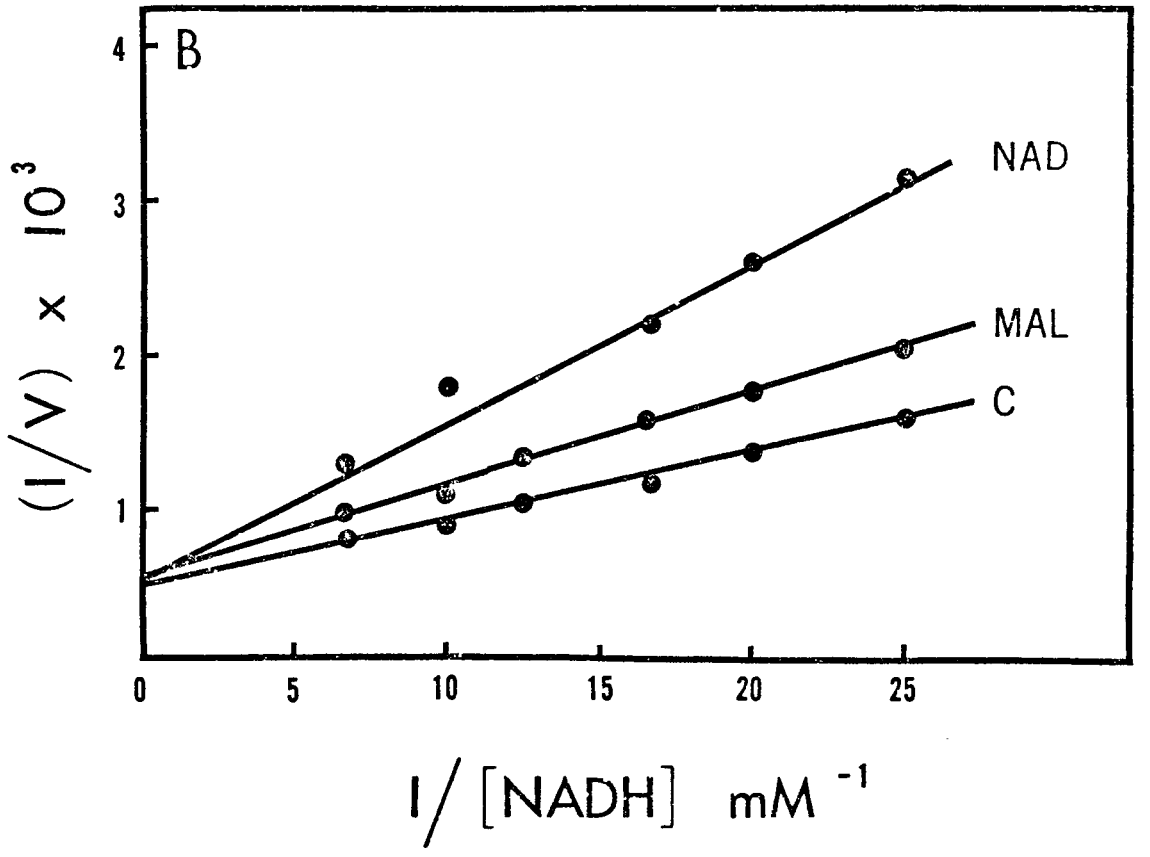
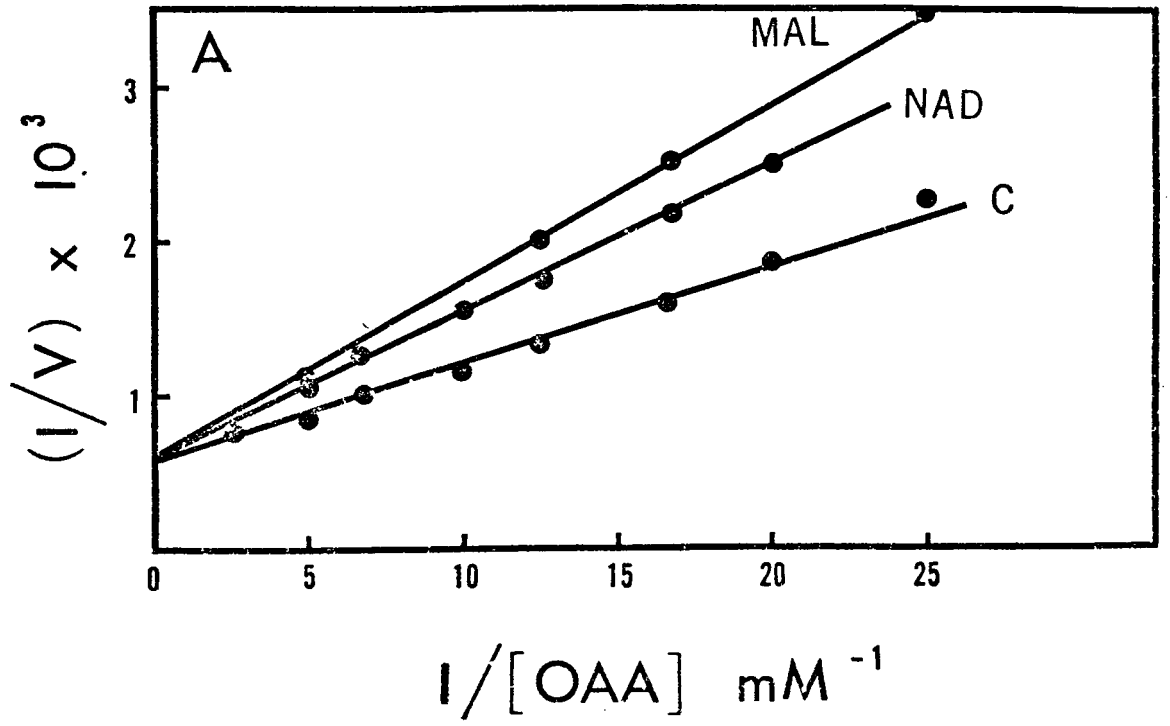
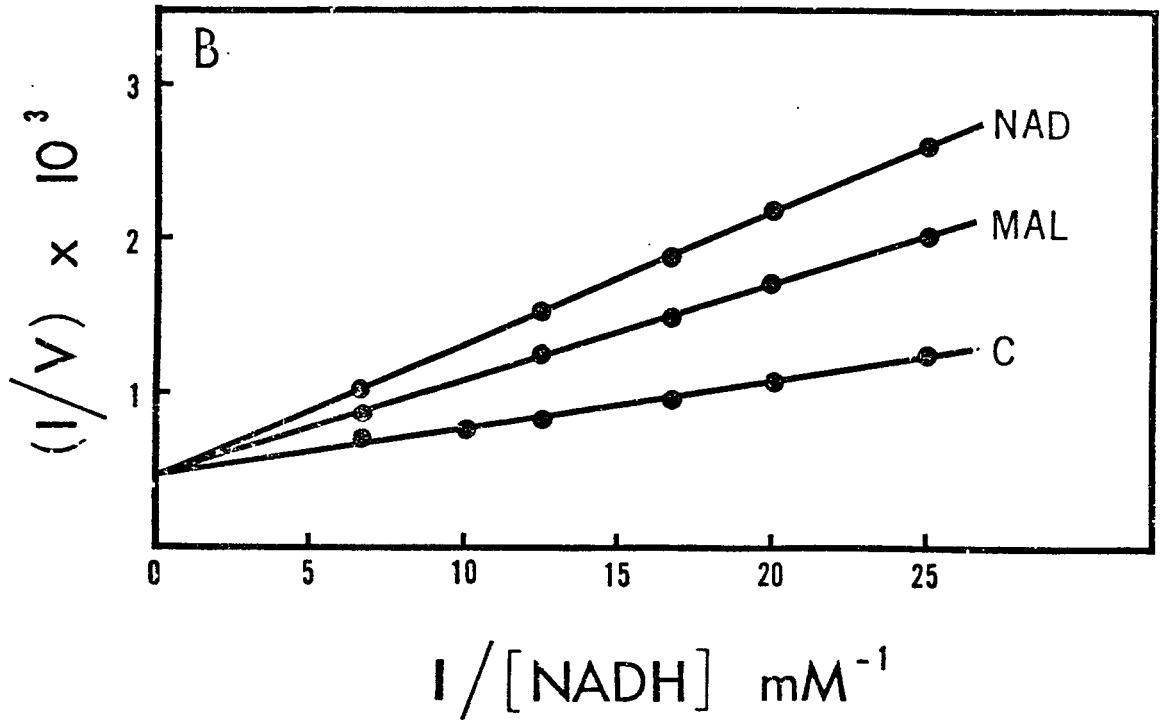

B
(1/V) x 10³
4
3
2
1
0
5
10
15
20
25
NAD
MAL
C
1/[NADH] mM⁻¹

TABLE 7. Product inhibitor constants for the P. flavicomum malate dehydrogenase isozymes

Substrate	Product inhibitor constants (mM)			
	Oxaloacetate	Malate	NADH	NAD
m-MDH				
Oxaloacetate	-	14.00	-	4.50
Malate	1.59	-	0.012	-
NADH	-	23.30	-	3.92
NAD	1.11	-	0.046	-
s-MDH				
Oxaloacetate	-	7.24	-	7.20
Malate	1.63	-	0.017	-
NADH	-	9.11	-	3.03
NAD	0.58	-	0.013	-

Figure 28. The effects of reaction products on the P. flavicomum s-MDH catalytic activity. Plots of reciprocal velocity (expressed as enzyme units/ml) at 25 C versus reciprocal substrate concentration in the presence of NAD (5 mM) or malate (MAL) (8 mM) were made. The control reaction containing no product (C) is shown. The photic absorbance decrease at 340 nm accompanying the oxidation of NADH was followed. (A) The effects of products on the relationship of reciprocal velocity versus reciprocal oxaloacetate (OAA) concentration. In addition to oxaloacetate and product the reaction mixture contained 0.1 mM NADH, enzyme in 0.01 ml, and sufficient 0.1 M potassium phosphate buffer (pH 7.5) to equal a 1 ml total volume. (B) The effects of products on the relationship of reciprocal velocity versus reciprocal NADH concentration. In addition to NADH and product the reaction mixture contained 4 mM oxaloacetate, enzyme in 0.01 ml, and sufficient 0.1 M potassium phosphate buffer (pH 7.5) to equal a 1 ml total volume.


A
MAL
NAD
C
(1/V) x 10^3
1
2
3
0
5
10
15
20
25
1/[OAA] mM^-1


B
NAD
MAL
C
(1/V) x 10^3
1
2
3
0
5
10
15
20
25
1/[NADH] mM^-1

NADH concentrations. They were both competitive (Fig. 28B) with NADH for the enzyme; malate had a K_i of 9.11 mM, and NAD had a K_i of 3.03 mM (Table 7).

Product competition with malate on the malate oxidation ability of m-MDH and s-MDH: oxaloacetate and NADH. The effects of products oxaloacetate and NADH were tested against variable malate concentrations in the malate oxidation assay. Both products demonstrated competitive inhibition patterns when m-MDH was used (Fig. 29) or when s-MDH was used (Fig. 30).

For m-MDH, the K_i of oxaloacetate was 1.59 mM, and the K_i of NADH was 0.012 mM (Table 7). For s-MDH the K_i of oxaloacetate was 1.63 mM while the K_i of NADH was 0.017 mM (Table 7).

Product competition with NAD on the malate oxidation ability of m-MDH and s-MDH: oxaloacetate and NADH. The effects of products oxaloacetate and NADH were tested against variable NAD concentrations in the malate oxidation assay. Both products demonstrated competitive inhibition patterns for the NAD active site of m-MDH (Fig. 31A) and s-MDH (Fig. 31B).

The oxaloacetate had a K_i of 1.11 mM versus NAD when used with m-MDH while it had a K_i of 0.58 mM when used with s-MDH (Table 7). The NADH had a K_i of 0.046 mM versus NAD when used

Figure 29. The effects of reaction products on the P. flavicomum m-MDH catalytic activity. Plots of reciprocal velocity (expressed as enzyme units/ml) at 25 C versus reciprocal malate (MAL) concentration in the presence of NADH (0.05 mM) or oxaloacetate (OAA) (2 mM) were made. The control reaction containing no product (C) is shown. In addition to malate and product the reaction mixture contained 0.43 mM NAD, enzyme in 0.01 ml, and sufficient 0.075 M sodium glycinate buffer (pH 10.0) to equal a 1 ml total volume. The photic absorbance increase at 340 nm accompanying the reduction of NAD was followed.

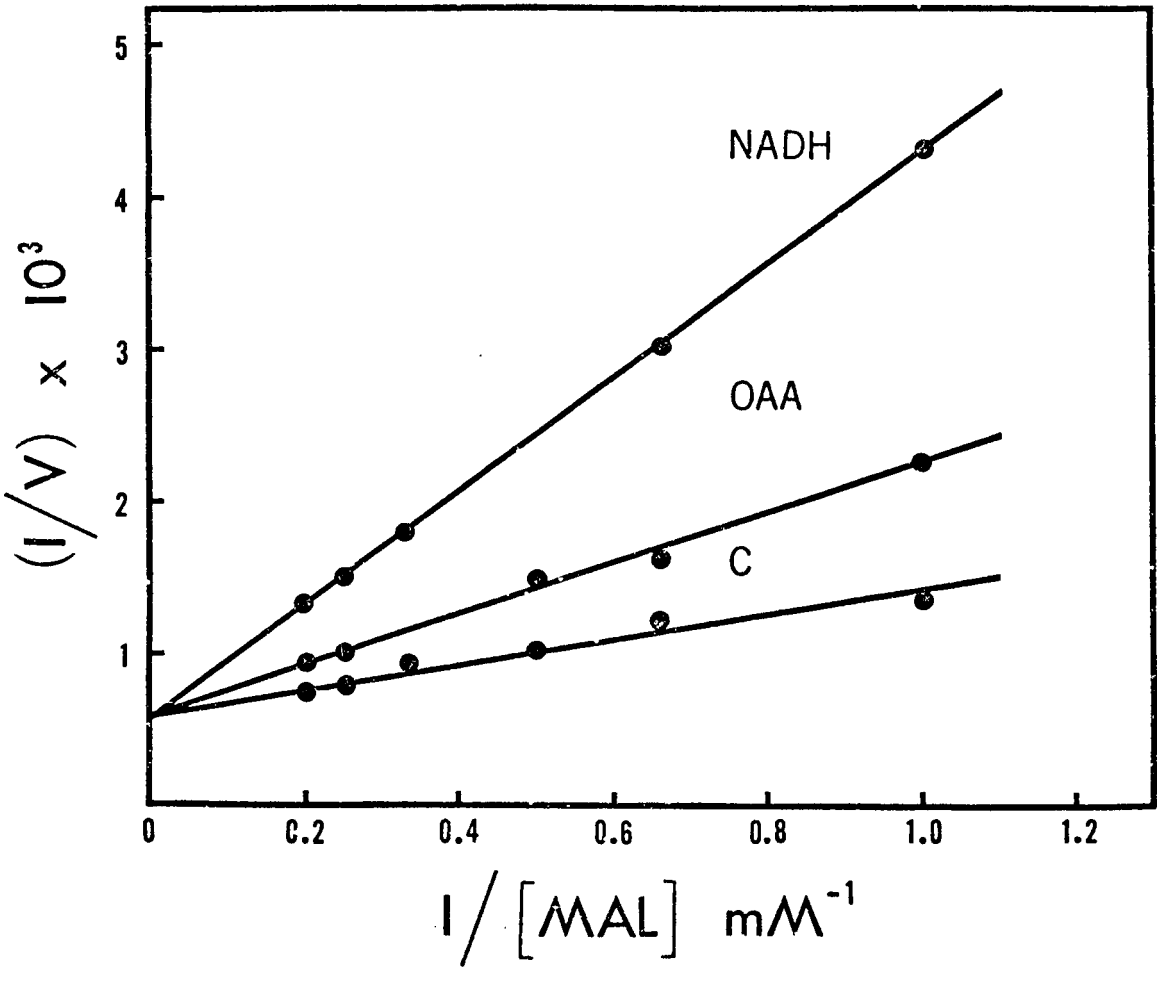

5
4
3
2
1
0
0.2
0.4
0.6
0.8
1.0
1.2
(1/V) x 10³
1/[MAL] mM⁻¹
NADH
OAA
C

Figure 30. The effects of reaction products on the P. flavicomum s-MDH catalytic activity. Plots of reciprocal velocity (expressed as enzyme units/ml) at 25 C versus reciprocal malate (MAL) concentration in the presence of NADH at 0.05 and 0.1 mM, and oxaloacetate (OAA) at 1 and 2 mM were made. The control reaction in the absence of product is shown (C). In addition to malate and product the reaction mixture contained 0.43 mM NAD, enzyme in 0.01 ml, and sufficient 0.075 M sodium glycinate buffer (pH 10.0) to equal a 1 ml total volume. The photic absorbance increase at 340 nm accompanying the reduction of NAD was followed.

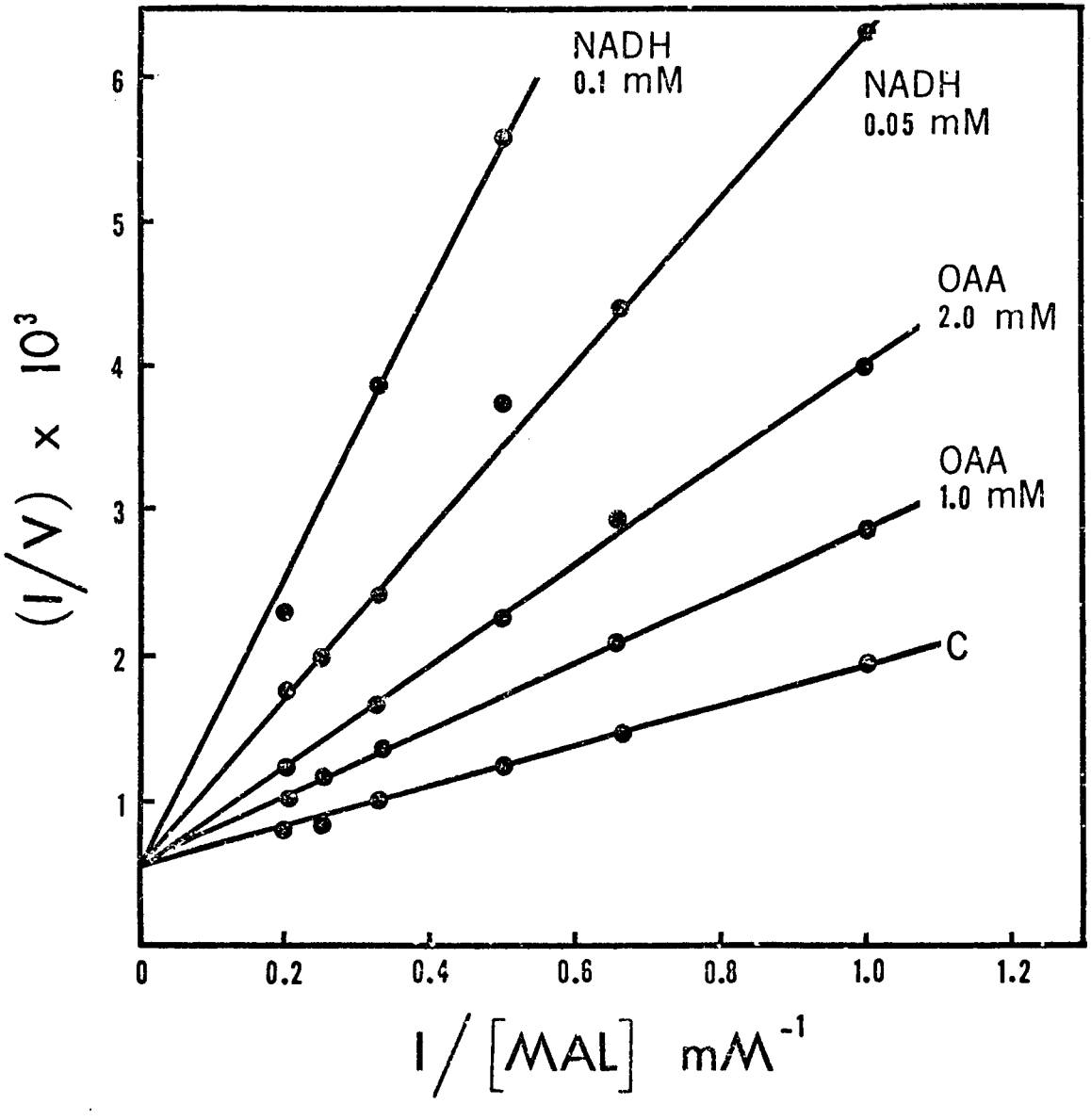
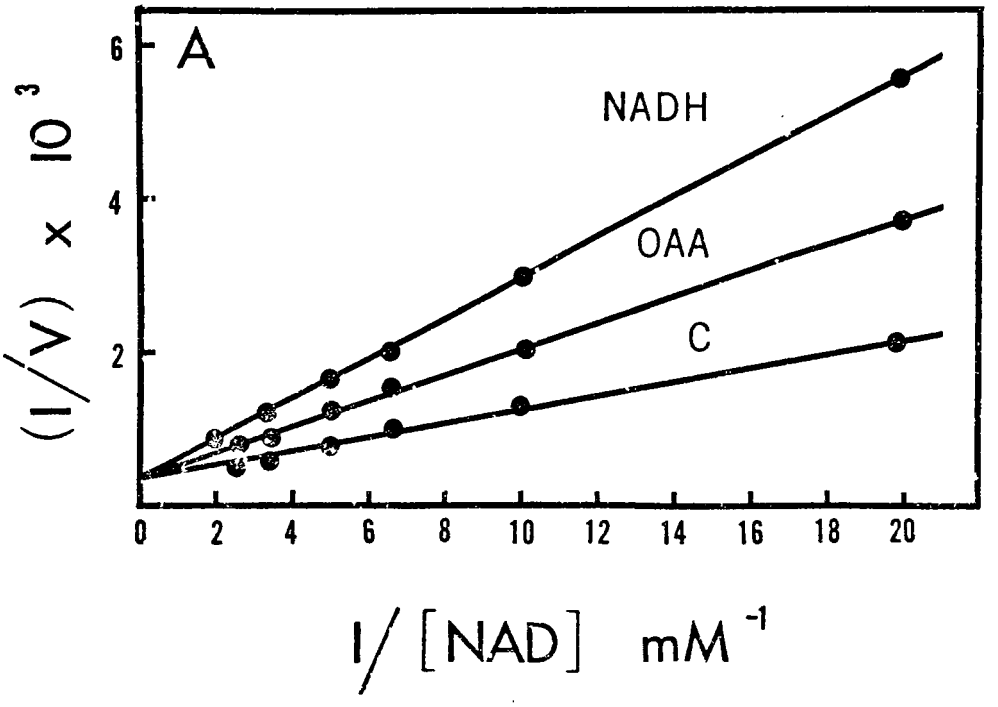
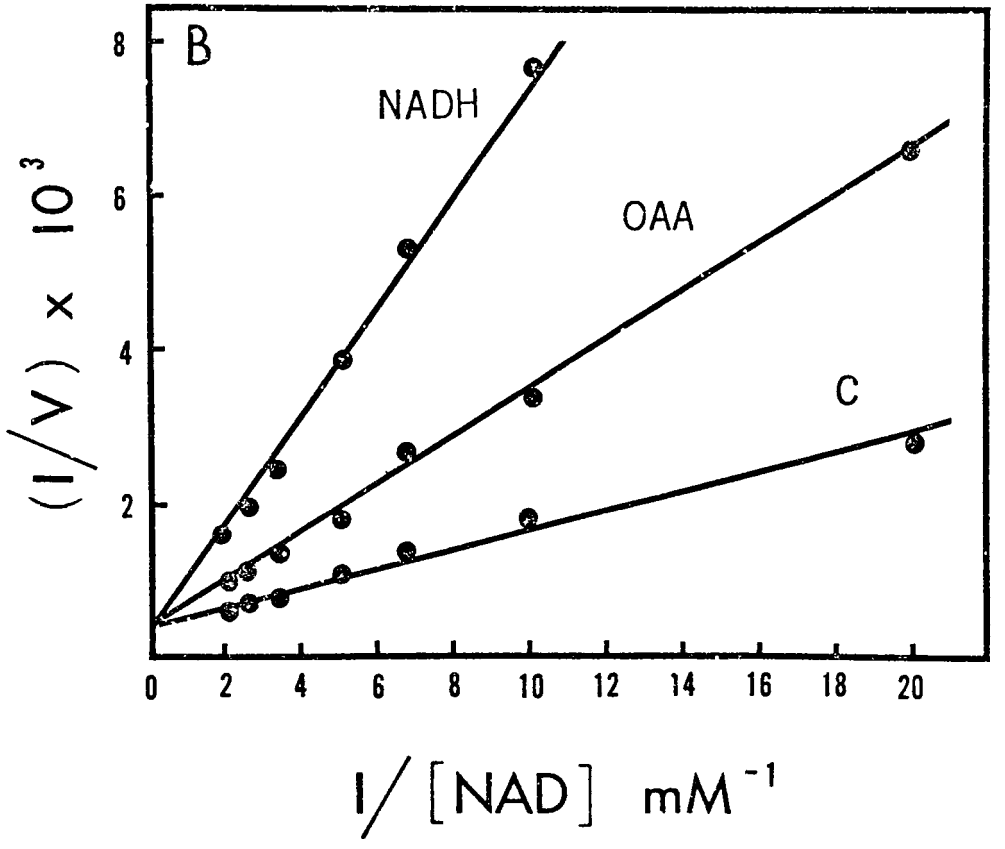

NADH
0.1 mM
NADH
0.05 mM
OAA
2.0 mM
OAA
1.0 mM
C
(1/V) x 10³
1/[MAL] mM⁻¹
0
0.2
0.4
0.6
0.8
1.0
1.2
1
2
3
4
5
6

Figure 31. The effects of reaction products on the relationship of reciprocal velocity (expressed as enzyme units/ml) and reciprocal NAD concentration at 25 C for the isozymes of malate dehydrogenase from P. flavicomum. The effects of NADH at 0.1 mM and oxaloacetate (OAA) at 1 mM were tested. The control reaction in the absence of product is shown (C). In addition to NAD and product the reaction mixture contained 6 mM malate, enzyme in 0.01 ml, and sufficient 0.075 M sodium glycinate buffer (pH 10.0) to equal a 1 ml total volume. The photic absorbance increase at 340 nm accompanying the reduction of NAD was followed. (A) The effects of reaction products on the m-MDH activity. (B) The effects of reaction products on the s-MDH activity.


A
NADH
OAA
C
(1/V) x 10^3
1/[NAD] mM^-1
0
2
4
6
8
10
12
14
16
18
20


B
NADH
OAA
C
(1/V) x 10^3
1/[NAD] mM^-1
0
2
4
6
8
10
12
14
16
18
20

with m-MDH while it had a K_i of 0.013 when used with s-MDH.

Inhibition of the activity of P. polycephalum malate dehydrogenase isozymes by substrate analogues. Various compounds were tested as inhibitors of the P. polycephalum malate dehydrogenase activity (oxidative as well as reductive). Certain compounds had no effect (K_i greater than 60) on activity of either isozyme (Table 8). A list of these compounds included glycolytic cycle intermediates as well as monocarboxylic acids and nonsubstituted, saturated dicarboxylic acids having a carbon chain length longer than three.

Other compounds demonstrated competitive inhibition with the substrates for the isozymes. The most effective inhibitors (Table 9) were dicarboxylic acids with alpha carbon keto or hydroxyl groups. The keto or hydroxyl group was not a prerequisite as maleate and fumarate were fairly strong inhibitors as well. It may be that the chemistry of the alpha carbon itself, rather than the group attached, was the true deciding factor as to whether a dicarboxylic acid would be an inhibitor or not. There is no single term that describes the various chemical interactions of the alpha carbon of effective inhibitors. For lack of a better term the alpha carbon of effective inhibitors shall be described as "unsaturated".

Groups attached to the beta carbon could modify the inhibition as

TABLE 8. Compounds demonstrating no interference with malate dehydrogenase activity[a, b]

cis-aconitate
adipate
asparagine
fructose 1, 6-diphosphate
glucose 6-phosphate
glutamate
glutamine
ketomalonate
lactate
phosphoenol pyruvate
3-phosphogluconate
pyruvate
succinate

[a] As determined in both oxaloacetate reduction and malate oxidation.
[b] The reactivity refers to m-MDH and s-MDH from both species.

TABLE 9. The inhibitor constants of compounds competitive with substrate for malate dehydrogenase from P. polycephalum

Inhibitor	Inhibitor constants: K_i (mM)			
	Oxaloacetate reduction		Malate oxidation	
	s-MDH	m-MDH	s-MDH	m-MDH
∝ ketoglutarate	2.1	1.3	6.5	4.9
D, L-isocitrate	2.2	5.1	3.8	8.6
Maleate	8.2	18.0	20.2	43.0
Aspartate	14.6	>60.0	5.5	21.3
Malonate	15.6	20.0	17.8	10.6
Fumarate	17.3	24.7	3.9	3.7
Citrate	21.5	25.0	7.0	5.7
L-tartrate	>60.0	28.5	>60.0	>60.0

indicated by the lack of inhibition demonstrated by cis-aconitate while maleate or fumarate were effective as inhibitors. The data for maleate and fumarate were particularly interesting. The cis oriented maleate was a better inhibitor of oxaloacetate reduction than the trans oriented fumarate while the reverse was true of the malate oxidation. This would indicate some substrate orientational effects were important during catalysis.

Aspartate was diagnostic in differentiating between the two isozymes. It had moderate inhibitory effects against s-MDH but only weak effects against m-MDH. Maleate, also, inhibited the s-MDH more than m-MDH although the difference was not as dramatic.

Inhibition of the activity of P. flavicomum malate dehydrogenase isozymes by substrate analogues. A few of the substrate analogues inhibiting malate dehydrogenase isozymes derived from P. polycephalum were tested against P. flavicomum isozymes for comparative purposes (Table 10).

The data indicated the isozymes from P. flavicomum have an equivalent response to substrate analogues as the homologous isozymes of P. polycephalum. This similarity of responses to substrate analogues, along with similarity of pH and substrate optima, allowed the use of product inhibition data determined on P. flavicomum as a

TABLE 10. The inhibitor constants of compounds competitive with substrate for malate dehydrogenase from P. flavicomum

Inhibitor	Inhibitor constants: K_i (mM)			
	Oxaloacetate reduction		Malate oxidation	
	s-MDH	m-MDH	s-MDH	m-MDH
∝ketoglutarate	2.0	1.0	4.6	5.3
D, L-isocitrate	2.4	4.9	3.5	8.6
Maleate	8.0	16.7	18.5	47.0
Aspartate	18.5	> 60.0	5.1	23.2
Citrate	25.0	25.0	8.4	10.3

means of evaluating the effects of substrate analogues of Physarum isozymes. An inhibitor which demonstrated inhibition levels approximating (or better than) substrate inhibition levels was judged a physiologically important inhibitor.

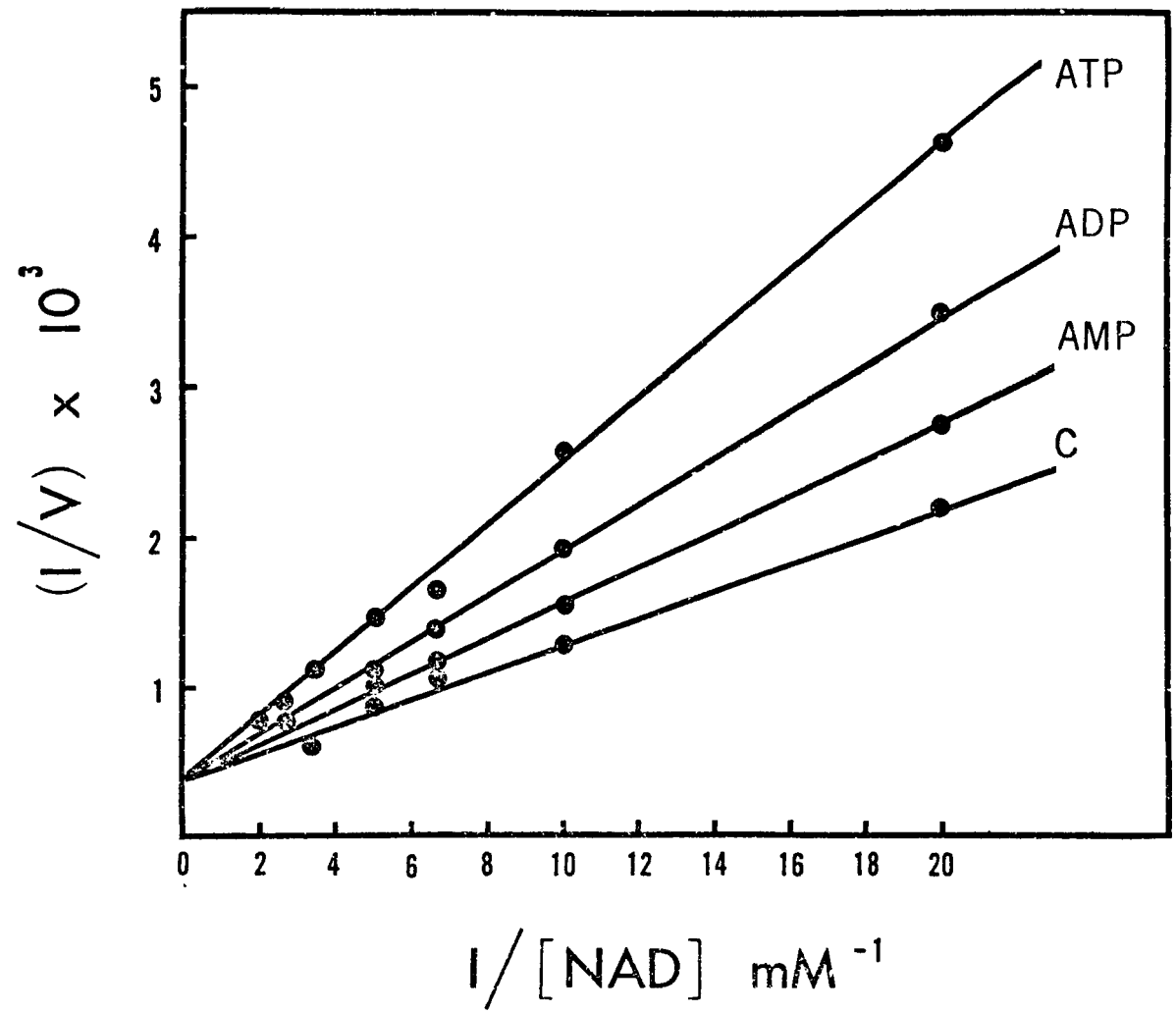
Inhibition of the activity of the Physarum malate dehydrogenase isozymes by nucleoside phosphates. The effects of various nucleoside triphosphates were tested as inhibitors of the P. polycephalum malate dehydrogenase catalyzed reaction. The nucleotides cytosine triphosphate (CTP), guanosine triphosphate (GTP), thymidine triphosphate (TTP), and uridine triphosphate (UTP) had no effect. The adenosine phosphates, however, did demonstrate competitive inhibition with both substrate and cofactor for the m-MDH. The s-MDH was not affected. The inhibition of m-MDH was greatest using adenosine triphosphate (ATP). The adenosine diphosphate (ADP) had less inhibitive effect while adenosine monophosphate (AMP) had the least (Table 11). Adenine had no inhibitive effects at all. This selective inhibition of m-MDH was observed for the isozyme from P. flavicomum, also (Table 11). A typical pattern of the inhibition is shown (Fig. 32). Inhibition tests using adenosine 3', 5'-cyclic phosphate and guanosine 3, 5'-cyclic phosphate demonstrated no discernible effects.

Thermal characteristics of the malate dehydrogenase isozymes

TABLE 11. Inhibitor constants of adenosine phosphates for m-MDH from Physarum during malate oxidation

Substrate	Inhibitor constants: K_i (mM)		
	ATP	ADP	AMP
P. polycephalum			
Malate	0.47	2.33	3.72
NAD	0.44	1.39	2.53
P. flavicomum			
Malate	0.49	2.51	3.53
NAD	0.45	1.36	2.61

Figure 32. The effects of adenosine 5'-triphosphate (ATP), adenosine 5'-diphosphate (ADP) and adenosine 5'-monophosphate (AMP) on the relationship of reciprocal velocity (expressed as enzyme units/ml) at 25 C versus reciprocal NAD concentration for the m-MDH isozyme of P. flavicomum. The reaction mixture contained in addition to the NAD concentration, 1 mM adenine nucleotide, 6 mM malate, enzyme in 0.01 ml, and sufficient 0.075 M sodium glycinate buffer (pH 10.0) to equal a 1 ml total volume. The response of the enzyme in the absence of inhibitor is shown (C). The photic absorbance increase at 340 nm accompanying the reduction of NAD was followed.

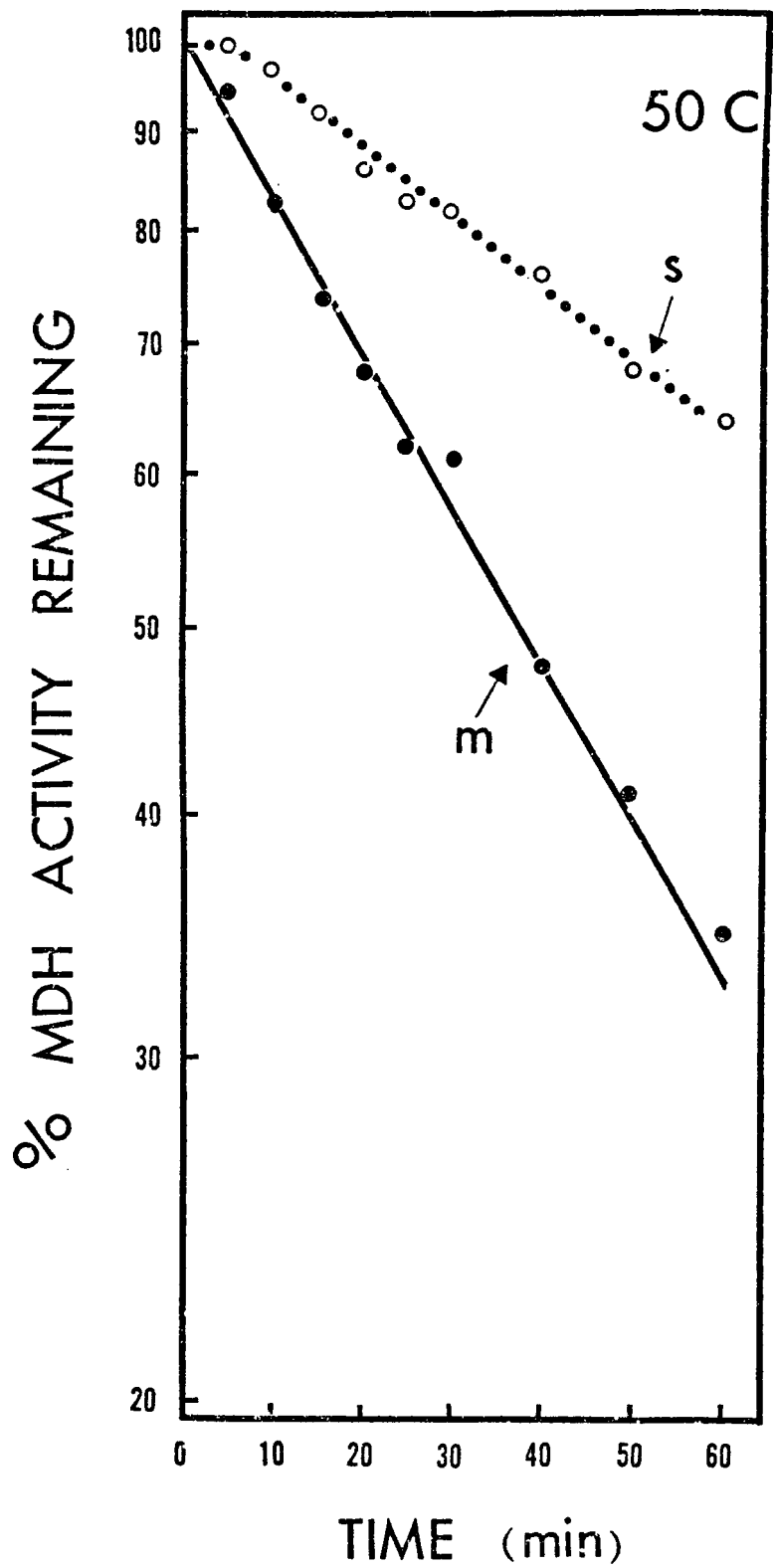

ATP
ADP
AMP
C
(1/V) x 10^3
1/[NAD] mM^-1
0
2
4
6
8
10
12
14
16
18
20
1
2
3
4
5

from P. flavicomum. The heat stability of the isozymes was analyzed by exposing them to 50 C for a period of 60 min (Fig. 33). The activity remaining after 60 min, as compared with unheated samples of each isozyme, was 33% for the m-MDH and 64% for the s-MDH. These results were similar, but not identical, to thermal characteristics of the isozymes from P. polycephalum (37, 38). After 60 min exposure to 50 C, the P. polycephalum m-MDH activity was reduced 80% while the s-MDH was reduced 34%.

Active site analyses: sulfhydryl group modification. The possibility of sulfhydryl groups being important in the catalysis of oxaloacetate reduction was tested by several methods. The isozymes were incubated with 0 to 300-fold molar excesses of PMB over enzyme for periods ranging from a few minutes to days. The temperature of incubation was varied from 4 to 20 C. The buffer and pH were altered. In no instance was the activity of either isozyme from either species of Physarum affected. These methods would inactivate pig heart m-MDH. The inactivation was also attempted with NEM at 500-fold excess of reagent over protein. Full activity was retained even after 120 min of incubation.

Active site analyses: arginine group modification. The possibility of an active site arginine group in malate dehydrogenase from

Figure 33. A comparison of the thermal stability of m-MDH and s-MDH from P. flavicomum. The stability was compared by exposure to 50 C for 60 min. Ten samples of each isozyme in test tubes containing 1 ml of 1 enzyme unit (as measured by oxaloacetate reduction) were previously warmed to 25 C in a water bath for 15 min. At the end of this period all tubes but one of each isozyme were immediately plunged into a preset 50 C water bath. The two tubes not placed in the bath were placed in ice and served as 100% maximum activity. One tube of each isozyme was removed and cooled immediately every 5 min for 30 min and then every 10 min for an additional 30 min. Each tube was analyzed for enzyme activity (oxaloacetate reduction) using previously determined optimal conditions. Controls of unheated samples of each isozyme were checked to insure that only an insignificant loss, less than 3%, had occurred by the warming process. Symbols: closed circles (•), percent maximum m-MDH activity; open circles (o), percent maximum s-MDH activity.


50 C
s
m
% MDH ACTIVITY REMAINING
100
90
80
70
60
50
40
30
20
0
10
20
30
40
50
60
TIME (min)

P. polycephalum was assessed by incubating protein with a 500 molar excess of butanedione in borate buffer. After 1 hr of incubation the m-MDH retained only 25% of the activity of the control; s-MDH retained only 32%. If the incubation mixture was made 75 mM in NADH the activity of m-MDH was reduced only 30%. The s-MDH activity in the same incubation mixture was reduced only 15%. Addition of malate or oxaloacetate to the reaction mixture was uneventful. These results indicated a butanedione sensitivity which was relieved in part by cofactor.

Active site analyses: tyrosine modification. The possibility of a tyrosine group at the active site of P. polycephalum malate dehydrogenase was investigated using the reagent tetranitromethane. The tetranitromethane and enzyme were incubated at 37 C for 1 hr either in the absence or presence of 1 μmole of substrate. Both m-MDH and s-MDH were quite sensitive to nitration. Each form retained only 20% of its activity after the incubation process.

No substrate by itself would protect m-MDH from nitration; but, when 0.5 μmole of NADH and 0.5 μmole of malate or 0.5 μmole of NAD and 0.5 μmole of oxaloacetate were substituted, the enzyme retained 60% of its activity after the hour incubation. When s-MDH was examined, all substrates caused retention of an additional 30% of

activity over control. When mixed substrates were used, the s-MDH retained 70% of its activity after the hour incubation. These results indicated a sensitivity of both isozymes to nitration. This sensitivity was relieved when both substrate and cofactor were present.

Electrophoretic investigation of crude extracts from Physarum. The possibility that malate dehydrogenase isozymes might exist at levels below the detection level of polyacrylamide-gel disc electrophoresis was investigated using isoelectric focusing. The isoelectric focusing technique permits low level activity to be detected as long as the isoelectric point of the masked enzyme differs from the major protein. Investigation by this type of analyses of crude extracts from P. polycephalum and P. flavicomum plasmodia indicated no other malate dehydrogenase isozymes were present. The isoelectric points of the isozymes were not identical to the purified forms. The s-MDH had a pI of 6.6 (6.5 to 6.7) while the m-MDH had a pI of 10.0 (9.9 to 10.1). These fluctuations may have been due to protein-protein interactions because the ionic strengths of the crude extracts were drastically reduced, a requirement of the technique.

Isoelectric focusing investigation of malate dehydrogenase from plasmodia grown on galactose rather than glucose revealed the same two isozymes with pI values identical to glucose grown plasmodia.

Investigation of haploid cells grown both on glucose as well as galactose showed identical patterns.

Polyacrylamide-gel disc electrophoresis of crude extracts of P. flavicomum sclerotia revealed two bands of malate dehydrogenase with identical migrations as found in crude extracts. The conclusions are that the malate dehydrogenase isozymes purified from P. polycephalum and P. flavicomum plasmodia were representative of the malate dehydrogenase isozymes in any portion of the life cycle of Physarum.

DISCUSSION

Proteins responsible for malate dehydrogenase activity have been studied in a number of tissues including complex plant (12, 15-17), vertebrate (19-24), invertebrate (9, 27-29), and fungal systems (10, 11, 26). The physical, chemical and kinetic study of malate dehydrogenase from Physarum offered here permits a cross modal comparison of characteristics of malate dehydrogenase from all tissues, and an assessment of the minimal function of malate dehydrogenase.

From a number of sources it has been determined that there are several basic groups of malate dehydrogenase enzymes as distinguished by compartmentation and electrophoretic analyses. These groups include malate dehydrogenases associated with mitochondria, malate dehydrogenases not associated with any organelle, and malate dehydrogenases associated with peroxisomes or glyoxysomes of plant tissue (15, 66).

In certain organisms there are more complicated isozyme relationships found within each malate dehydrogenase group. This situation is found more often in plants such as maize (67) and wheat (68). The intragroup m-MDH and s-MDH isozymes from maize have been shown to be truly isozymes, rather than conformational isomers of the same enzyme, by a number of experiments including ones

showing their inheritance follows Mendelian rules (69). The situation of intragroup isozyme multiplicity was also found in teleosts (24) but this intragroup multiplicity does not seem to be a common feature of vertebral systems (70, 71).

In Physarum species the malate dehydrogenase activity is defined in only two isozymes. One isozyme is found in the cytoplasm and the other is found in the mitochondria. This has been demonstrated by comparison of electrophoretic analyses of both soluble proteins derived from crude extracts and soluble proteins derived from mitochondria. This comparison has been done in P. polycephalum and P. flavicomum plasmodia as well as P. flavicomum haploid cells. The proteins extracted from mitochondria contained only the more cathodal malate dehydrogenase activity. There was no evidence of any intragroup isomers of the enzymes in either the polyacrylamide-gel disc electrophoretic analyses or isoelectric focusing analyses of crude extracts. The m-MDH being more cathodal and s-MDH more anodal in polyacrylamide-gel disc electrophoresis was consistent with Saccharomyces (10), Drosophila (27), cactus (12) and vertebral tissues (1). However, the electrophoretic mobility per se was not a definitive method of identifying the isozymes as can be seen from the many examples where the relative mobility was reversed, such as in

snail (28), sea urchin (29) and certain fish (24, 72).

The malate dehydrogenase isozymes from the plasmodia of the Physarum species were completely purified, as confirmed by electrophoretic, hydrodynamic, and conformational properties before proceeding with further analyses. The previous claim of high purity for the P. polycephalum isozymes (37, 38) was confirmed since these preparations exhibitied only slight increases in specific activity values over those reported earlier: 774 as compared with 810 for s-MDH and 1,007 as compared with 1,020 for m-MDH. These specific activities for P. polycephalum were equivalent to those for the isozymes isolated from P. flavicomum: 839 for s-MDH and 1,080 for m-MDH.

The purification procedures, besides furnishing the needed pure proteins, allowed discrimination between isozymes within the same species as well as discrimination between homologous isozymes from the two Physarum species. The response of the P. polycephalum tissue to the acetone extraction and column chromatographic procedures has been discussed extensively (37). The conclusion that the P. polycephalum derived isozymes were quite similar, but not identical, to animal and plant isozymes can only be strengthened by the results of response of P. flavicomum derived tissue to these various procedures. The differential responses to acetone and the subtle shift in

SE-cellulose elution of P. flavicomum s-MDH to a lower ionic strength release than that found for P. polycephalum s-MDH showed the subtle differences between the homologous isozymes from the Physarum tissues.

It is interesting to note that the m-MDH isozymes from both Physarum species were quite stable to the presence of CPC used for purification purposes. The initial losses observed in the CPC fractionation may be explained in terms of nonspecific entrapment of the m-MDH in precipitated protein matrices. Once the unwanted protein was discarded the m-MDH isozymes were quite stable in a solution of 1% (w/v) CPC. No loss in activity occurred in samples stored at 4 C for over 4 months. This observation is interesting in light of data reported showing m-MDH was stabilized by cardiolipin and lysophosphatidylcholine (73). The stabilization by CPC, a positively charged detergent, and the negatively charged cardiolipin hints that the m-MDH in vivo may be localized in mitochondria at hydrophobic subcompartments. This interpretation is clouded however by data showing inhibition by palmitoyl CoA of m-MDH activity (74) which contraindicate these previous results. It may be that the shape of the hydrophobic group is the key to understanding these contradictory phenomena.

The purification of these four isozymes was realized with the aid of isoelectric focusing. This technique was a significant contribution to the generation of totally pure proteins and served to characterize the various enzymes. One of the most distinguishing features of the Physarum isozymes was that the isoelectric points of the isozymes were widely divergent. The value for the m-MDH isozyme (9.83 for P. polycephalum and 9.95 for P. flavicomum) was in good agreement with that of pig heart m-MDH (9.30) (75). Also, for ox kidney m-MDH there was an indication that the pI might be above pH 7.5 (76). However, isoelectric points reported for malate dehydrogenase from maize (67), spinach microbody (77), Schizosaccharomyces (78) and beef heart (79) were all below 6.5. The trend of s-MDH having a pI below that of the m-MDH form in Physarum was consistent with all the above tissues (67, 77-79) but s-MDH from Physarum demonstrated a higher pI than these various tissues. The isoelectric focusing method furnished more information and was more discriminant than gel electrophoresis, the technique most commonly used to characterize isozymes.

The availability of completely purified Physarum malate dehydrogenases enabled the determination of additional physical and chemical properties. Along with compartmentation, electrophoretic

mobility and behavior in purification, thermostability could differentiate quite easily between the two isozymes. The P. flavicomum s-MDH being more stable than m-MDH was in agreement with most tissues (20, 21, 25) including P. polycephalum (37, 38). As yet, only cactus (12) and Drosophila (27) were exceptional.

The sedimentation values for P. polycephalum m-MDH (4.64 S) and s-MDH (4.43 S) as well as those for P. flavicomum m-MDH (4.52 S) and s-MDH (4.12 S) allowed discrimination among the Physarum isozymes, and agreed with values reported for other organisms. All sedimentation values for malate dehydrogenase from other tissues, except for two, fell between 4.0 and 5.4 S with the majority falling between 4.1 and 4.7 S (20, 27, 49, 67, 76, 77, 79, 80-85). The two exceptions were malate dehydrogenases with sedimentation values of 9.0 S (86, 87).

The sedimentation equilibrium data, in contrast to boundary velocity sedimentation, did not differentiate between the isozymes of P. polycephalum. While it did allow discrimination between the P. flavicomum isozymes (Table 3) the sedimentation equilibrium parameter was not discriminant among the various malate dehydrogenases from a number of sources. For the most part, the weight range was from 65,000 to 75,000 (20, 29, 49, 67, 76, 77, 79, 80-85)

with the majority of values falling between 67,000 and 71,000 (20, 29, 49, 67, 77, 79, 80-84). Plant tissue malate dehydrogenase apparantly can exist in a number of molecular sizes, formed through the aggregation of subunits, depending on the type of small ion associated (88). The most stable form, however, had a molecular weight of 64,000 (88). Schizosaccharomyces pombe malate dehydrogenase (78) was the only one with a lower value (60,000); Bacillus species malate dehydrogenase (117,000) (86) and an unusual form from pig heart (138,000) (87) have consistently higher molecular weights. The latter two proteins apparently have a tetrameric subunit structure.

Subunit analyses via SDS polyacrylamide-gel electrophoresis also demonstrated the similarity of Physarum malate dehydrogenase isozymes to other systems in that each isozyme was composed of two subunits of identical size. The occurrence of dimeric, equivalent size subunits agrees with reports on malate dehydrogenase from other tissues (85, 87). The exceptions were reports of unequal subunits from salmonid fish and brown shrimp (49, 72) and the two examples of tetrameric subunits previously mentioned (86, 87).

The amino acid analyses served to further define the Physarum isozymes. These analyses also provided a basis for comparison of Physarum isozymes with their counterparts in more complex

eucaryotic tissue. Inspection of the amino acid analyses showed conclusively the isozymes were distinct proteins. The m-MDH from P. polycephalum, in contrast to s-MDH from P. polycephalum contained more arginine, glutamate, glycine, half cystine, lysine, and methionine but less isoleucine, serine, threonine, and valine. The two forms were quite similar with regard to the content of alanine, aspartic acid, histidine, leucine, proline, and aromatic amino acids. The isozymes from P. flavicomum differed in the content of 10 amino acids also, with m-MDH having higher values for arginine, aspartate, glutamate, glycine, isoleucine, lysine, methionine, serine, and threonine but much lower values for phenylalanine. The alanine, histidine, leucine, proline, valine and tyrosine were equivalent between the two isozymes.

A cross modal comparison between species of Physarum revealed that the homologous m-MDH isozymes differed in the content of eight amino acids. The m-MDH from P. polycephalum had a higher content of lysine and methionine while having a lower alanine, glutamate, serine, threonine, tyrosine, and valine content. The s-MDH from P. polycephalum also differed in the content of eight amino acids from its homologue in P. flavicomum by having a higher aspartate, isoleucine, leucine, threonine, and valine content, but a lower glutamate,

phenylalanine, and tyrosine content.

The half cystine content of the P. polycephalum m-MDH being approximately twice that of s-MDH was harmonious with reports on isozymes from other tissues (23, 63). This was somewhat surprising since the total half cystine content of P. polycephalum malate dehydrogenase was lower than that of other tissues (20, 63, 89). The total sulfhydryl content being similar to the cysteic acid content suggested that the cysteine amino acids were present as free sulfhydryls. The sulfhydryl content of P. flavicomum malate dehydrogenases was equivalent to that found in the isozymes from P. polycephalum.

The further comparison of P. polycephalum s-MDH amino acid content data to that from various vertebral sources demonstrated that P. polycephalum s-MDH was similar to both chicken heart s-MDH (eight amino acids) (20) and pig heart s-MDH (seven amino acids) (23). This trend held true for data on s-MDH from P. flavicomum where comparison of amino acid content data demonstrated similarity to chicken heart s-MDH (five amino acids) (20) and pig heart s-MDH (eight amino acids) (23).

The P. polycephalum m-MDH was more similar to chicken heart m-MDH (seven amino acids) (20) than it was to pig heart m-MDH (four amino acids) (63), ox kidney m-MDH (four amino acids) (76) or

beef heart m-MDH (two amino acids) (90). A brief comparison of the data from P. flavicomum m-MDH revealed it to be similar to pig heart m-MDH (seven amino acids) (63) and chicken heart m-MDH (six amino acids) (20).

However, when all the amino acid content data were considered, each set compared to the other, it was interesting to note that no amino acid content was common among all m-MDH isozymes (20, 63, 76). This lack of common composition among the several m-MDH isozymes was in contrast to s-MDH amino acid content data where all the analyses revealed a similar content of at least five amino acids. Furthermore, the comparisons of amino acid content data among all the above sources of m-MDH isozymes showed that the content for chicken heart m-MDH (20) was more similar to another heart tissue (pig heart m-MDH, 13 amino acids) (63) than to another vertebral organism (ox kidney m-MDH, five amino acids) (76). Indeed, chicken heart m-MDH was more similar to the simple eucaryote tissue P. polycephalum m-MDH than it was to ox kidney m-MDH.

This chemical dissimilarity with regard to amino acid content is contrasted with kinetic parameters where the similarity among all the isozymes was striking. In P. polycephalum the optimum pH for

oxaloacetate reduction was 7.6 for both isozymes; the pH optimum for P. flavicomum isozymes was 7.5 for the same reaction. This is contrasted with 7.8 for m-MDH and 7.6 for s-MDH in vertebrates (20, 91). The optimum oxaloacetate concentration for the isozymes from P. polycephalum (37, 38) and P. flavicomum being between 0.25 and 0.35 mM showed the close similarity to the optimum of 0.33 mM for both s-MDH and m-MDH for vertebral systems (20). This is not to say that the differences among the s-MDH and m-MDH optima from both species of Physarum are not demonstrable. However, the physiological significance of the minor differences is minimal.

The Michaelis constants of the m-MDH from both Physarum species for oxaloacetate were similar among all the isozymes (Table 6). These values for m-MDH were slightly high for vertebral tissue (20, 21, 79, 91), more directly comparable to Drosophila (27) and Spinacia (25), but exceeded by Saccharomyces (10). The s-MDH Michaelis constants for oxaloacetate were directly comparable to vertebrates; examination of the literature showed that the s-MDH isozymes of all tissues were much more consistent with respect to the Michaelis constants for oxaloacetate (12, 21, 25, 27).

The optimum pH of 10.0 for both isozymes for malate oxidation was equivalent to that for vertebrates, as was the optimum malate

concentration (21). Michaelis constants of the isozymes for malate were also similar to other systems (20, 21, 25, 26, 79), and served to separate these isozymes from sea urchin (29), cactus (12), and Drosophila (27).

Comparison of Michaelis constants of the isozymes for the cofactors was difficult because of the wide range of values reported. As an example, the Michaelis constant for beef heart s-MDH was reported as 5.4×10^{-4} M (21, 39) and also 9.9×10^{-5} M (91). Indeed even within the same genus (Physarum) widely divergent values were found (Table 6). Perhaps of more significance, the Michaelis constants can be used to separate the isozymes on the basis of differences of affinity, especially with respect to cofactor. The differences of affinity between the two isozymes for the cofactor were additional indicators of the differences in structure between m-MDH and s-MDH in Physarum.

The substrate inhibition pattern for oxaloacetate from Physarum was unusual. From a number of sources the effect of excess oxaloacetate on the s-MDH was negligible, while the m-MDH was inhibited (10, 20, 21, 28, 72). In P. polycephalum, both isozymes were inhibited, with the s-MDH being inhibited more strongly than m-MDH (37, 38). In P. flavicomum once again the isozymes were

inhibited by high substrate, but the m-MDH was inhibited more, as is seen in other systems (10, 20, 21, 28, 72). The inhibition in either Physarum species did not begin until some 10-fold concentration over the Michaelis constant for oxaloacetate was met. The significance of the reversal for P. polycephalum has been discussed (37, 38). These additional findings on P. flavicomum only support the conclusion drawn previously that, although the variable response was species specific, the physiological significance of the oxaloacetate inhibition is minimal. It is interesting to note that the variable response was due to variability between the s-MDH homologues in the two species. The m-MDH from the two systems was affected equivalently.

The substrate inhibition patterns demonstrated by high concentrations of malate for the isozymes of Physarum were equivalent to those observed with other systems (20, 21); the s-MDH isozymes of both Physarum species were inhibited more by high malate concentration than the m-MDH isozymes. Differences were noted between the species in that the malate inhibition effects were not as pronounced with the P. flavicomum isozymes as they were with the P. polycephalum isozymes.

Product inhibition patterns obtained on Physarum derived malate dehydrogenase isozymes tentatively indicated a rapid equilibrium

random order mechanism. These results were qualified by the high substrate inhibition experienced by both isozymes. The possibility of mixed type inhibition being obscured in this type of analyses was extremely high. The presence of even one mixed type inhibition in the results could alter the interpretation of the mechanism to an ordered system.

The general mechanism indicated by various studies with pig heart (92-95) and beef heart (96, 97) malate dehydrogenase isozymes was an ordered, bi bi mechanism. However this mechanism has been elucidated only by extensive analyses.

One of the first kinetic analyses done with malate dehydrogenase derived from a vertebral source (97) indicated an unusual, Theorell-Chance type mechanism. The proposed mechanism allowed for either NADH or oxaloacetate to bind to enzyme in random fashion; the second substrate would then bind. However, the product malate was always released first, followed by NAD. This mechanism deviated from a Theorell-Chance mechanism in having an asymmetric number of binary complexes formed.

This unusual mechanism has not been confirmed by alternate methods of analyses. Analogue inhibition analyses (92, 93) and equilibrium kinetic analyses (96) have indicated an ordered, bi bi

mechanism. Because the enzymes studied were dimeric, these various analyses were dependent on data showing that the dual active sites per functional protein were equivalent for NADH binding in both beef heart (98) and pig heart (99); there was no indication of cooperativity, either positive or negative, between the active sites.

The kinetic analyses for plant derived malate dehydrogenase isozymes have not been done in a rigorous manner, but product inhibition patterns in mung bean (100) and analogue inhibition patterns in Opuntia (12) suggested some sort of ordered system as well. These various data were equivalent in the respect that the s-MDH and m-MDH isozymes both demonstrated an equivalent mechanism in each system studied. It may be that Physarum derived malate dehydrogenase isozymes demonstrate an ordered mechanism also, but confirmation would require an extensive amount of time and equipment not available at present. The more important aspect, the equivalency of s-MDH and m-MDH kinetic systems in the same tissue was shown here.

The data on product inhibition were used to evaluate substrate and cofactor analogue inhibition data. These inhibition data were more beneficial in discerning the functional equivalency of the reaction mechanism between the s-MDH and m-MDH in Physarum and among malate dehydrogenases of other tissues. Tests of various compounds

as inhibitors have indicated that dicarboxylic acids with unsaturated alpha carbon atoms produced the best inhibition in both s-MDH and m-MDH derived from Physarum. This interpretation was based on the lack of inhibition demonstrated by monocarboxylic acids, such as pyruvate, phosphoenol pyruvate, and lactate, and by dicarboxylic acids such as adipate, glutamate and succinate. The dicarboxylic acids with alpha carbon unsaturation such as ∝ketoglutarate, D, L-isocitrate, or maleate were effective inhibitors of the oxaloacetate reduction reaction. Their approximate inhibitor constants were equivalent to or better than the inhibitor constants for malate. In addition, the analogue maleate was replaced by fumarate as an effective inhibitor of the malate oxidation reaction, indicating that beta carbon substitutions may influence the activity as well. The lack of inhibition demonstrated by cis-aconitate reinforces the interpretation of beta carbon influence.

These results concerning the active site were equivalent to those obtained on malate dehydrogenase isozymes from animal and plant systems. Much of the earlier work on malate dehydrogenase specificity depended on assessment of a compound as an actual substrate rather than as an inhibitor. Such tests showed that ox heart and pig heart m-MDH could use D-tartrate, oxaloglycollate, tartronate, or ∝hydroxyglutarate as substrate, although the activity was far reduced

over the natural substrates: a maximum velocity of 1,350 for malate as compared to a maximum velocity of 50 for D-tartrate (19). The substrate structure data were expanded by inhibitor studies for beef heart m-MDH, where mesoxalate and oxalate had detectable activity but ∝ketobutyrate and pyruvate were untouched (101), and beef heart s-MDH, where ∝ketoglutarate and D-tartrate were active inhibitors but L-tartrate, ∝ketobutyrate and pyruvate were inactive (91). Hydroxymalonate (92), citrate, and fumarate (102) were only weakly effective against s-MDH. However, the data concerning citrate and fumarate inhibition were severely incomplete.

The general inhibition pattern described above has been supported by studies on malate dehydrogenases in plant tissue (12, 67, 77). In maize (67) the various isozymes were inhibited by ∝ketoglutarate, citrate and D, L-isocitrate while succinate, glutamate and aspartate were not effective. The spinach microbody malate dehydrogenase was also inhibited by glyoxylate and pyruvate, indicating that the alpha position might be more important in this malate dehydrogenase form (77).

Several exceptions were noted in the survey of analogue inhibition. For Physarum derived malate dehydrogenase isozymes, aspartate was a weak inhibitor of the s-MDH activity but ineffectual in regard to

m-MDH. This unequal inhibition level demonstrated that minor differences existed between the s-MDH and m-MDH isozymes. Because the inhibition levels were so low, when compared to the level produced by products, the selective inhibition produced by aspartate could have little physiological importance.

The beef heart s-MDH isozyme was unusual in that fructose 1, 6-diphosphate was an allosteric inhibitor (80, 103). Neither this compound nor other similar compounds (Table 8) have any inhibitory effect in Physarum, pig heart, or plant derived malate dehydrogenase isozymes. This difference reflects a change at a regulator site rather than at the catalytic site.

In both Opuntia (12) and maize (67) the isozymes were inhibited by cis-aconitate, a compound which has no effect in either Physarum or vertebral derived malate dehydrogenase isozymes. However, the inhibition demonstrated by this compound does not concern the substrate binding; it was demonstrated that cis-aconitate was binding at a site separate from the active site (12, 67).

Tests of nucleoside phosphates as inhibitors of NAD have shown that the Physarum m-MDH isozymes were inhibited by ATP, ADP and AMP. The level of inhibition was reduced as the number of phosphates attached was reduced. The lack of inhibition by adenine, GTP, CTP,

TTP or UTP highlighted the specificity for the adenosine phosphate. A more interesting aspect was that s-MDH was totally unaffected by these inhibitors, even at 10 times the concentration of ATP giving high inhibition with m-MDH. This would indicate that the regulation of interaction between m-MDH and s-MDH could be effected by ATP, ADP and AMP levels in the cell. These adenosine phosphate inhibition results were similar to those for animal derived malate dehydrogenases. Pig heart m-MDH, in one report, was inhibited by adenosine phosphates in NAD reduction catalysis but activated in NADH oxidation catalysis (104). The data concerning activation were not supported by later studies (105). The plant malate dehydrogenases were also consistent with respect to adenosine phosphate inhibition. Pea (106) and mung bean (100) derived m-MDH isozymes were inhibited but the s-MDH isozymes were not affected. The degree of inhibition by adenosine phosphates decreased with decreasing phosphorylation. Thus ATP was most inhibitory, ADP less and AMP least. With regard to pea m-MDH, inosine triphosphate and CTP were not effective; GTP had about equivalent inhibition activity as did ATP.

Tests of NAD analogues as inhibitors of NAD binding were done for P. polycephalum isozymes previously (37, 38). The results again indicated that the basic catalytic hydrogen transfer mechanism of

s-MDH was similar to that for the m-MDH isozyme. Examination of the literature revealed that the basic mechanism was shared among all malate dehydrogenase isozymes (37, 38). The tests were not repeated for the isozymes of P. flavicomum.

To summarize, these combined results indicate conservation of the kinetic mechanism for malate dehydrogenase isozymes from all sources. The question arises as to whether the isozymes demonstrate a similar catalytic activity using two different chemical reactions or whether the catalytic activity of malate dehydrogenase is performed by the same mechanism. This question can not be resolved as yet. The answer to this question requires extensive active site analyses in a number of tissues; this work has only begun.

The sulfhydryl group participation in the catalytic activity of m-MDH (but not s-MDH) derived from animal sources has been demonstrated using the sulfhydryl specific reagents NEM (63), PMB (65, 90, 108, 109) and 4,4'-bis dimethylaminodiphenylcarbinol (110). The accumulated evidence indicated that the sulfhydryl group was not a part of the active site, however. The titration of three to four sulfhydryls with PMB led to activation (108, 109) rather than inhibition of pig heart m-MDH. The titration of six to 10 sulfhydryls led to inactivation, a number which was quite high for active site specificity.

In Euglena the asymmetric PMB inhibition of malate dehydrogenase isozymes was reversed. The s-MDH was inactivated by PMB while m-MDH was insensitive (82). In Physarum neither m-MDH nor s-MDH were inhibited by PMB or NEM. These data confirm the hypothesis that the sulfhydryl group could not be a part of any general catalytic mechanism. Sulfhydryl groups were probably important only in maintaining ternary structure. The fluctuation of sulfhydryl group importance among s-MDH and m-MDH isozymes demonstrated that these isozymes were different both in respect to isozymes within the same cell as well as in respect to homologous isozymes in different tissues.

In contrast to the sulfhydryl variability several amino acids have been implicated as important in both s-MDH and m-MDH activity. The amino acid arginine has been suggested as important to the catalytic activity by using the arginine specific reagent butanedione (111, 112) which gives an acid stable hydroxymethylimidazoline compound. The analyses of derivatives, assayed by column chromatographic amino acid analyses, have indicated two arginines for s-MDH (111) and one arginine for m-MDH (112) were at the active site. The results for Physarum were not quantitated but they did reveal a butanedione sensitivity in both s-MDH and m-MDH as is found with

these vertebral systems. In an analogous situation it could be demonstrated that Physarum malate dehydrogenases were sensitive to tetranitromethane, a compound which has been used to nitrate tyrosine. The amino acid tyrosine has been demonstrated as important to the catalytic activity of malate dehydrogenase from beef heart by using the tyrosine specific reagents acetylimidazole and tetranitromethane (65). The possibility of these amino acids having specific catalytic interaction has not been explored in other tissues.

These two amino acids were not the only ones which have been found important to the catalytic activity of malate dehydrogenase. Histidine acetylation with iodoacetamide of a beef heart m-MDH (107) and pig heart m-MDH (113-115) has indicated the possible role of histidine in the catalytic mechanism. By using radioactive iodo[^{14}C]-acetamide, trypsin, and sequence analysis it has been possible to map the group around the possible active site histidine in pig heart m-MDH (115). The sequence of amino acids was (amino terminal) valine, serine, valine, proline, isoleucine, histidine, glycine, glycine (valine, alanine, glycine) and lysine. From a number of tests the histidine was considered near the NADH binding site (115).

Another amino acid, lysine, has also been tentatively identified as being important in NADH binding to malate dehydrogenase. The

data implicating lysine were obtained using pyridoxal 5'-phosphate (116). As yet this area has not been supported in other systems or isozymes (116).

The similarity of the kinetic parameters for malate dehydrogenase isozymes from Physarum as well as the malate dehydrogenase isozymes from a number of distantly related tissues indicates a universal catalytic mechanism may apply. The evidence regarding specific active site amino acids was slim, but it indicated that the catalytic mechanism in both isozymes may very well be carried out by an identical chemical reaction mechanism.

The variability of several physical and chemical parameters between m-MDH and s-MDH from any tissue, or between any two homologous isozymes in even closely related tissues, such as P. polycephalum m-MDH and P. flavicomum m-MDH or beef heart m-MDH and pig heart m-MDH, was as striking as the similarity of the kinetic parameters.

One phenomenon which could explain some of the structural disparity between isozymes is that mitochondrial malate dehydrogenase is not made by the mitochondria, but is produced at the cytoplasmic endoplasmic reticulum (117). This structural specificity of compartmentation has been suggested before with the hypothesis that soluble

mitochondrial proteins migrate as a complex with mitochondrial phospholipids (118). It would seem that, between groups of isozymes, structure most probably dictates designation within the cell.

The data showing variation in regulatory parameters indicated that the variation in physical and chemical parameters may also reflect differences in regulatory control. This regulatory control does not follow a recognizable, classic evolutionary trend such as could be classified as animal type or plant type control. An analogous condition exists for lactate dehydrogenase where the type of isozyme isolated from a particular vertebral tissue reflects the oxygen tension of that tissue (8) rather than whole tissue complexity.

The role served by malate dehydrogenase isozymes in vivo can not be explained specifically by the particular types of analyses offered here. However, the role proposed for vertebral malate dehydrogenase isozymes as a reducing equivalent "gate" through the selectively permeable mitochondrial wall is not denied. Indeed, this cross modal study of the biochemistry of malate dehydrogenase endorses the universal nature of the enzyme mechanism; the role of the isozymes is extended to all eucaryotic tissue. The malate-aspartate shuttle (5, 6) demonstrated in rat liver may well function in all eucaryotes involved with oxidative phosphorylation.

The enzymes would be responsive to the energy level of the cell, a useful attribute in light of the role proposed. These malate dehydrogenase isozymes, by definition, are directly responsive to the NAD/NADH ratio, a variable which is a likely major control of glycolysis (119). Also, the m-MDH isozyme is apparently under specific control of the adenosine phosphate level within the mitochondria. This selective inhibition would allow suppression of m-MDH activity within mitochondria thus allowing cytosolic s-MDH to balance the extracellular energy level without cyclization through mitochondria.

Both isozymes would also be responsive to the Krebs cycle intermediates citrate and D, L-isocitrate. Inhibition by these intermediates would not only modulate m-MDH activity in terms of Krebs cycle control but also would modulate s-MDH activity, an aspect important when considering lipogenesis may proceed via extramitochondrial citrate cleavage to oxaloacetate and acetyl-CoA (13).

This study supports the hypothesis that the role of malate dehydrogenase isozymes is to function as a gate between mitochondria and cytosol for the reducing equivalents contained in NADH. Future biochemical work will be directed at active site elucidation to determine if the isozymes do indeed utilize an equivalent chemical reaction.

SUMMARY

Malate dehydrogenase has been isolated and totally purified from the myxomycetes Physarum polycephalum and Physarum flavicomum. Extensive analyses of the chemical, physical and kinetic features of the isozymes reveal that the kinetic parameters of all malate dehydrogenase isozymes are quite similar, though distinguishable. The physical and chemical parameters, such as isoelectric points, amino acid analyses, and thermal denaturation, were quite dissimilar. The function of the malate dehydrogenase isozymes has been investigated in vertebral tissue. The investigation offered here indicates that the malate dehydrogenase isozymes are kinetically similar in all eucaryotic tissues. The function assigned to vertebral malate dehydrogenase isozymes as a reducing equivalent shuttle between cytosol and mitochondria can now be extended to all malate dehydrogenase isozymes in eucaryotic organisms.

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