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# Purification and Properties of Malate Dehydrogenase from *Chlorella pyrenoidosa*. Catalytic Mechanism of the Particulate Form*

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ABSTRACT: By means of ammonium sulfate fractionation and column chromatography, particulate and supernatant forms of malate dehydrogenase (MDH) from *Chlorella pyrenoidosa* have been purified to specific activities of 33 and 84, respectively. The two forms have been shown to be chromatographically, electrophoretically, and immunologically different, each showing a characteristic MDH activity when chromatographed on DEAE-cellulose, when electrophoresed on cellulose-acetate, and when developed by Ouchterlony double diffusion. Both forms have a molecular weight of approximately 61,000 as determined by Sephadex gel filtration. The kinetic properties of the particulate MDH were studied by: (1) analysis of initial velocity patterns in the direction of DPNH oxidation at pH 7.4 in 0.1 M potassium phosphate buffer; (2) determination of product inhibition patterns with $DPN^+$ and malic acid as product inhibitors; and (3) analysis of inhibition patterns of monoethyl oxaloacetic acid with respect to DPNH and oxaloacetic acid. All three studies yielded results supportive of a compulsory ordered addition mechanism for the particulate MDH. The $K_m$ of DPNH of $4 \times 10^{-5}$ M and $K_m$ of oxaloacetic acid of $5 \times 10^{-5}$ M as well as the substrate inhibition by oxaloacetic acid at levels above $8.48 \times 10^{-4}$ M for the particulate MDH are data similar to those obtained with animal mitochondrial malate dehydrogenases.

The malate dehydrogenase system (L-malate:DPN oxidoreductase, EC 1.1.1.37) of animal, plant, and microbial origin has been shown to generate a complex electrophoretic pattern. Two MDH[1] proteins, one associated with the supernatant fraction of the cell and the other with the particulate fraction, can generally be demonstrated in animal and microbial species; several plant systems have been suggested to consist of three MDH proteins (Mukerji and Ting, 1969; Ting, 1968; Yamazaki and Tolbert, 1969).

Physical properties such as subunit composition (Wolfenstein *et al.*, 1969), fluorescence spectra (Cassman and Englard, 1966a), amino acid composition (Kitto and Kaplan, 1966), and immunological reactivities (Henderson, 1968) have been examined for a number of highly purified animal MDH enzymes. In a few animal species, kinetic studies have led to the elucidation of catalytic mechanisms: either a basic compulsory ordered addition ("ordered bi–bi") mechanism or some variation thereof has been suggested in each case. But in plant species, though multiple forms of MDH from several systems (Ting, 1968; Mukerji and Ting, 1969) have been studied in terms of physical and kinetic properties, including thermal inactivation and molecular weight determinations and electrophoretic, chromatographic, and pH profiles, no catalytic mechanism has been defined. Examining the MDH system of *Chlorella pyrenoidosa*, Cole and his associates (1968) have demonstrated the existence of multiple electrophoretic forms. This communication reports the partial purification of MDH activities from this algal species and includes molecular weight determinations as well as immunological and electrophoretic studies. A detailed kinetic analysis of that MDH activity associated with the particulate fraction of the cell is presented.

## Experimental Procedures

*Organism and Culture Conditions.* Steady-state cultures of the high-temperature strain (7-11-05) (Sorokin and Myers, 1953) of *C. pyrenoidosa* were cultured as described previously (Cole *et al.*, 1968).

*Purification.* Cells were harvested by centrifugation (12,100-*g*) at 4°. The cell pellet, washed twice with 0.1 M Tris-HCl buffer, pH 8.6, and twice with distilled water, was recovered in 50 ml of 0.2 M potassium phosphate buffer with 0.3 M sucrose, pH 6.5. The sample was mixed with a one-third volume aliquot of glass powder in a 125-ml beaker and sonicated with the blunt tip probe of a Branson Sonic Power Sonifier, Model M52, operated on high power for a total of 3–5 min, in 15-sec periods. The beaker was immersed in an acetone–ice bath and the sample maintained at 5°. After sonic disruption, the sample was centrifuged at 480*g* for 5 min to sediment only

* From the Departments of Biochemistry and Medicine, Louisiana State University Medical Center, New Orleans, Louisiana 70112. *Received June 17, 1970.* This work was supported in part by NSF Grant GB-8317.

† This paper represents partial fulfillment of the requirements for the Master of Science Degree.

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[1] Abbreviation used is: MDH, malate dehydrogenase.

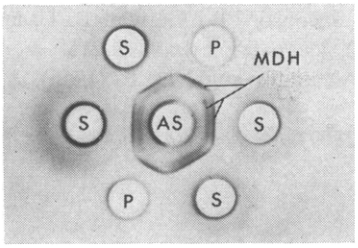


FIGURE 1: Ouchterlony double-diffusion study. Particulate (P) and soluble (S) fraction MDH developed with rabbit anti-*C. pyrenoidosa* serum (AS). Precipitin patterns have been stained for MDH activity and counterstained for protein.

whole cells and cellular debris. After two additional sonic treatments of the pellet and subsequent centrifugations, the three supernatant fractions were pooled and the pellet was discarded. The pooled supernatants, collectively termed "crude MDH," were centrifuged at 27,000*g*, yielding the soluble fraction and the particulate pellet; the latter was washed twice with 0.1 M sodium borate buffer, pH 8.6, and the washings were combined with the soluble fraction.

*Supernatant MDH Preparation.* The soluble fraction was dialyzed overnight against three 1-l. changes of 0.2 M potassium phosphate buffer, pH 8.0. Solid ammonium sulfate (176 g/l.) was added with constant stirring at 4° to give 30% saturation (Green and Hughes, 1955), and the pH was maintained at 8.0 by dropwise addition of concentrated ammonium hydroxide. After 30 min of mixing, the preparation was centrifuged at 12,100*g* for 15 min and the precipitate discarded. The supernatant was taken to 50% ammonium sulfate saturation (an additional 127 g/l.) and centrifuged; the precipitate was discarded. The remaining supernatant was taken to 85% ammonium sulfate saturation (an additional 258 g/l.). After centrifugation, the precipitate was recovered in 0.002 M potassium phosphate buffer with 0.001 M EDTA–0.001 M $\beta$-mercaptoethanol, pH 8.0, and applied to a DEAE-cellulose column (1.5 × 19 cm), prepared according to Peterson and Sober (1962) and equilibrated with the above buffer. All of the MDH activity was eluted with the 0.002 M buffer. The contents of tubes with MDH activity were pooled and dialyzed for 2 days at 4° against a 100% saturated solution of ammonium sulfate (729 g/l.) in 0.1 M potassium phosphate buffer with 0.001 M EDTA–0.01 M $\beta$-mercaptoethanol, pH 7.5. The precipitate was recovered by centrifugation, redissolved in 0.005 M potassium phosphate buffer with 0.001 M EDTA–0.001 M $\beta$-mercaptoethanol, pH 6.5, and dialyzed for 2 hr against this same buffer. The dialyzed sample was diluted approximately fourfold with dialysis buffer and applied to a CM-cellulose column (1.5 × 19 cm), prepared according to Kitto and Kaplan's modification (1966) of Pesce's method (Pesce *et al.*, 1964), but equilibrated in the final step with 0.005 M potassium phosphate buffer with 0.001 M EDTA–0.001 M $\beta$-mercaptoethanol, pH 6.5. Two overlapping peaks of MDH activity were eluted with this buffer. These activities were subsequently resolved by rechromatography at pH 8.0 on a larger DEAE-cellulose column (3 × 17 cm). Contents of the tubes representing the second peak of MDH activity eluted off the column were pooled and designated "supernatant MDH," specific activity 84.

*Particulate MDH Preparation.* After the 27,000*g* centrifugation and subsequent washings, the particulate pellet was recovered in 0.002 M potassium phosphate buffer, pH 8.0 (Yue, 1966), and the MDH activity repeatedly extracted by freezing and thawing at −60° in this same buffer. The sample was centrifuged at 27,000*g* after each thawing, and the supernatants were recovered and pooled. Ammonium sulfate fractionation (identical with that described for the supernatant fraction) of the pooled extracts yielded a 50–85% saturated fraction containing most of the MDH activity. This pellet was recovered in 0.002 M potassium phosphate buffer with 0.001 M EDTA–0.001 M $\beta$-mercaptoethanol, pH 8.0, and applied to a DEAE-cellulose column (1.5 × 19 cm, as described above). A single peak of MDH activity was eluted with this buffer. Contents of tubes with MDH activity were pooled and designated "particulate MDH," specific activity 33.

*Molecular Weight Determinations.* The molecular weight of various samples was determined by gel filtration with a Sephadex G-150 (Superfine) column (2.5 × 46 cm), prepared according to Murphey and coworkers (1967). For preparation of the selectivity curve, three standard proteins of known molecular weight, RNase, ovalbumin, and aldolase (Calibration Kit Proteins, Pharmacia Fine Chemicals, Inc., Piscataway, N. J.), and a commercial preparation of pig heart MDH (Boehringer Mannheim Corp., New York, N. Y.) were used. Protein concentration and MDH activity were assayed spectrophotometrically (see spectrophotometric section).

*Cellulose-Acetate Electrophoresis.* A Gelman electrophoresis unit was employed for electrophoresis of samples on cellulose-polyacetate paper strips, Sepraphore III (Gelman Instrument Co., Ann Arbor, Mich.), in barbital buffer (1.8 g of diethyl barbituric acid plus 10.3 g of sodium barbiturate), pH 8.6. Samples were subjected to electrophoresis for 55 min at a constant current of 2 mA/strip, immediately after which MDH activity was localized (Cole *et al.*, 1968) and strips were fixed in a methanol–water–acetic acid (1:1:0.2) solution and mounted in mineral oil between glass slides.

*Agar Gel Double Diffusion.* Double diffusion was performed according to Ouchterlony (1962). First antiserum and then antigen wells were filled with 25–50 $\mu$l of antiserum and antigen, respectively. Plates were prepared and incubated, and immunodiffusion was carried out for 2–5 days at room temperature using techniques similar to those described by Arquembourg *et al.* (1970). The gels were subsequently washed and then dried to a thin film; after MDH activity was localized and a counterstain (light green SF or Amido-Schwarz stain B) for protein applied, the gels were fixed and varnished.

*Preparation of Immunizing Antigen.* Steady-state cells of *C. pyrenoidosa* were harvested by centrifugation and the pellet was washed twice with 0.1 M Tris-HCl buffer, $9 \times 10^{-3}$ M $CaCl_2$, pH 7.0, and once with distilled water prior to lyophilization. The lyophilized (whole cell) immunizing antigen was stored at −7° for later use.

Three common-bred laboratory rabbits were immunized by subcutaneous injections (5 mg of immunizing antigen/kg of body weight) over a 15-month period. For preliminary injections, the immunizing antigen was emulsified (by sonication) in 1 ml of Freund's incomplete adjuvant (BBL, Division of Bioquest, Cockeysville, Md.); for "booster" injections, antigen was mixed with 1 ml of 0.85% saline (no emulsification required).

*Collection and Storage of Antiserum.* Whole blood was drawn from the animal's ear (central artery) and allowed to clot for several hours at room temperature; retraction of the clot was enhanced by overnight refrigeration. Samples of potent anti-*C. pyrenoidosa* serum were pooled and stored at −7° for later use.

*Spectrophotometric Assay of MDH and Protein.* For spec-

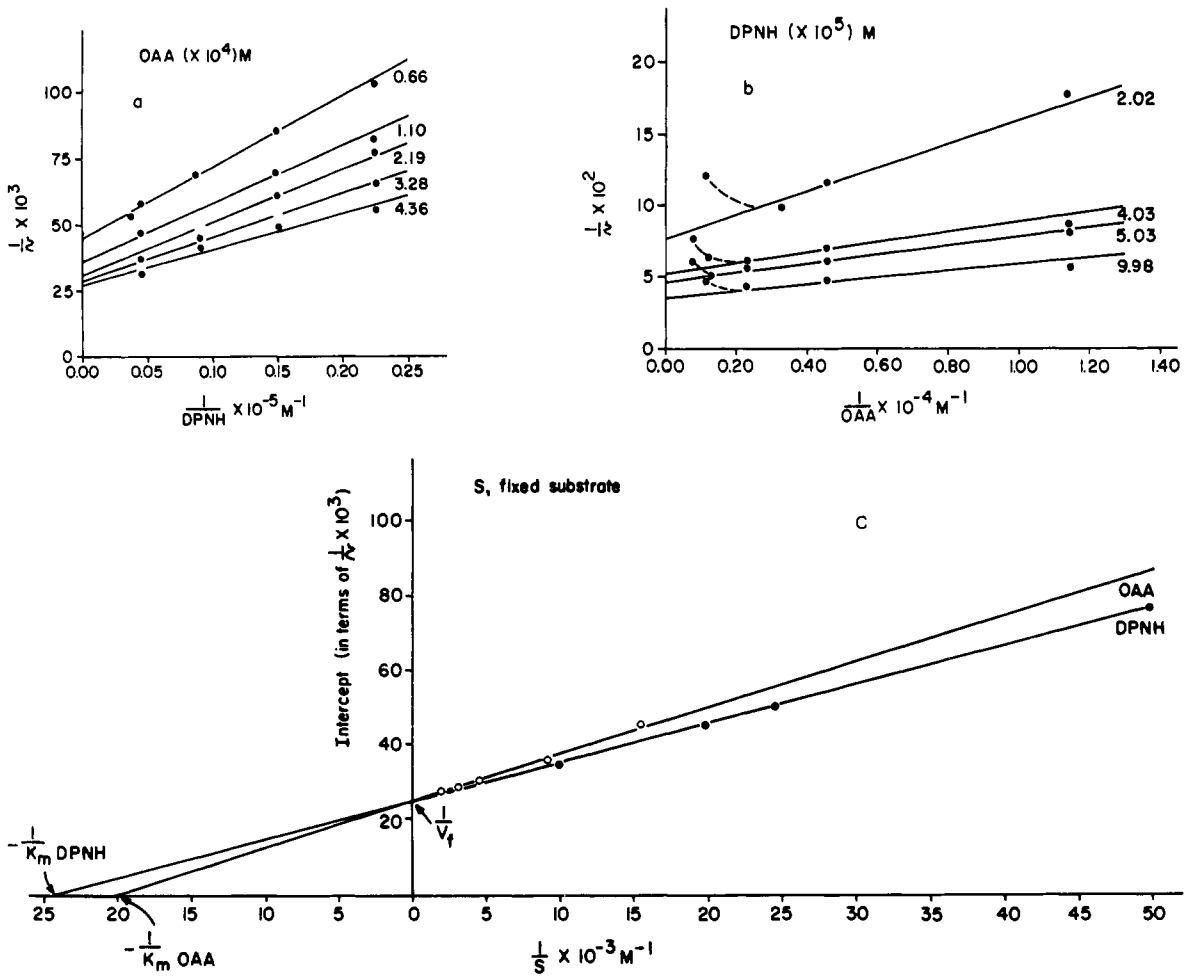


FIGURE 2: Initial velocity studies: primary and secondary plots. (a) Primary plot of reciprocal initial velocity with respect to reciprocal DPNH concentration. Various concentrations of oxaloacetic acid, the fixed substrate, are indicated. Assay mixtures included 2.8 ml of 0.1 M potassium phosphate buffer, pH 7.4, and approximately $1.1 \times 10^{-3}$ mg of enzyme (5 $\mu$l), representing $1.3 \times 10^{-2}$ unit of activity. Velocity is expressed as micromoles of DPNH oxidized per minute per milligram of protein; measurements were taken through a 1-cm light path. (b) Primary plot of reciprocal initial velocity with respect to reciprocal oxaloacetic acid concentration. Various concentrations of DPNH, the fixed substrate, are indicated. Other conditions as in Figure 2a. (c) Secondary plots of ordinate intercepts with respect to reciprocal fixed substrate concentrations. Values for the oxaloacetic acid and DPNH curves obtained from data in Figures 2a and 2b, respectively.

trophotometric determinations of MDH activity, the Beckman Model DB-G spectrophotometer and the Sargent automatic recorder (Model SRLG) were used. The initial rate of DPNH oxidation at room temperature was followed by monitoring the decrease in absorbancy at a wavelength of 340 m$\mu$, where the molar extinction coefficient of DPNH is $6.22 \times 10^3$ $M^{-1}$ $cm^{-1}$ (Horecker and Kornberg, 1948). A typical assay mixture consisted of 2.8 ml of 0.1 M potassium phosphate buffer, pH 7.4, 0.1 mM DPNH, 1.0 mM oxaloacetic acid, and a sufficient amount of enzyme to give a reproducible rate. One unit of MDH activity is that amount of enzyme required to convert 1 $\mu$mole of DPNH into $DPN^+$ per min. Specific activity is defined as units per milligram of protein. Multiple rate measurements were made for each point and protein concentrations were determined by optical density measurements at 280 m$\mu$ (Warburg and Christian, 1941).

*Chemicals.* The following chemicals were purchased from Sigma Chemicals (St. Louis, Mo.): DPNH, $DPN^+$, oxaloacetic acid, and malic acid. Monoethyl oxaloacetic acid was obtained from Nutritional Biochemicals Corp. (Cleveland, Ohio). The DEAE-cellulose and CM-cellulose used for the enzyme purification were products of Sigma Chemicals.

## Results

*Initial Purification of Supernatant MDH and Particulate MDH.* Ammonium sulfate fractionation of a sonicated sample of *C. pyrenoidosa*, combined with DEAE-cellulose column chromatography of the MDH-enriched fraction yielded a particulate MDH sample, specific activity 33, free of supernatant MDH activity; this same procedure (but with a subsequent CM-cellulose chromatographic step) yielded a soluble fraction containing two MDH activities (Figure 1). The single precipitin band associated with the particulate MDH sample suggests a single MDH immunodiffusing system. The soluble fraction possesses two MDH immunodiffusing systems, one of which is immunologically identical with that of the particulate MDH sample.

*Kinetic Analysis of Particulate MDH.* Kinetic studies of the approximately 100-fold-purified particulate MDH were performed. No endogenous DPNH oxidase activity was detected and there was no reaction rate with TPNH. Though aminotransferases, particularly aspartate aminotransferase (Munkres, 1968), have been recognized as contaminants of some MDH preparations, no such activity was detected in the

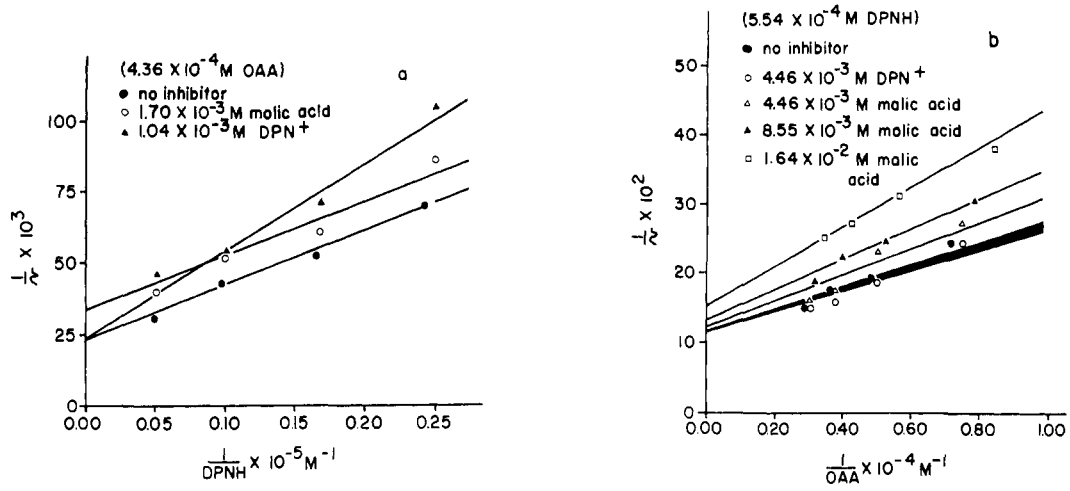


FIGURE 3: Product inhibition by $DPN^+$ and malic acid. (a) Reciprocal initial velocity with respect to reciprocal DPNH concentration; oxaloacetic acid is at an approximately saturating level. All assay conditions as in Figure 2a. (b) Reciprocal initial velocity with respect to reciprocal oxaloacetic acid concentration; DPNH is at an approximately saturating level. Assay mixtures included 0.5 ml of 0.1 M potassium phosphate buffer, pH 7.4, 0.025 ml of distilled water, and approximately $1.1 \times 10^{-3}$ mg of enzyme; measurements were taken through a 0.2-cm light path.

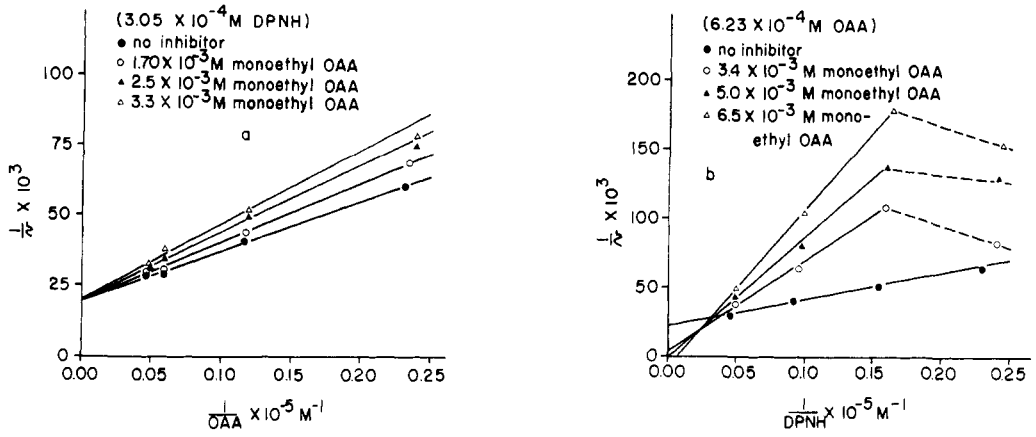


FIGURE 4: Inhibition by an analog of oxaloacetic acid, monoethyl oxaloacetic acid. (a) Reciprocal initial velocity with respect to reciprocal oxaloacetic acid concentration; DPNH is at an approximately saturating level. All assay conditions as in Figure 2a, except that $6.8 \times 10^{-4}$ mg of enzyme (3 $\mu$l), representing $7.8 \times 10^{-3}$ unit of activity, was used in this study. (b) Reciprocal initial velocity with respect to reciprocal DPNH concentration; oxaloacetic acid is at an approximately saturating level. All assay conditions as in Figure 2a.

*C. pyrenoidosa* particulate MDH. Neither D- nor L-lactate dehydrogenase was present in *C. pyrenoidosa* (Cole *et al.*, 1968). Visual evaluation of most probable lines and calculation of most probable lines by the least-squares method were equally valid since estimated slope and intercept errors were of the same magnitude, as in Dupourque and Kun (1969). Slope–intercept analyses were performed, and the theoretically more consistent visually estimated lines were plotted.

Initial velocity patterns for the particulate MDH preparation were determined, with DPNH and oxaloacetic acid, in turn, the variable substrate (Figures 2a and 2b, respectively). In these "primary" plots of reciprocal initial velocity with respect to reciprocal variable substrate concentration, the concentration range of DPNH was $4.4 \times 10^{-5}$ M to $2.6 \times 10^{-4}$ M (Figure 2a) and that of oxaloacetic acid was $0.87 \times 10^{-4}$ M to $1.25 \times 10^{-3}$ M (Figure 2b). Substrate inhibition by oxaloacetic acid was manifested in Figure 2b by a marked decrease in velocity at oxaloacetic acid concentrations of $8.48 \times 10^{-4}$ M and above. The "secondary" plots (Figure 2c) of ordinate intercepts with respect to reciprocal fixed substrate concentrations of oxaloacetic acid and DPNH represent the replots of data from Figures 2a and 2b, respectively. This plot (Florini and Vestling, 1957) enables the direct extrapolation of $K_m$ values for DPNH ($4 \times 10^{-5}$ M) and oxaloacetic acid ($5 \times 10^{-5}$ M) to be made.

Product inhibition studies with $DPN^+$ and malic acid were performed at "saturating" levels of fixed substrate and the patterns analyzed according to Cleland (1963). Both in the absence and presence of product inhibitors, the variable concentration range of DPNH was $4.05 \times 10^{-5}$ M to $2.00 \times 10^{-4}$ M (Figure 3a). Whereas $DPN^+$ exhibits competitive inhibition with respect to DPNH, malic acid exhibits uncompetitive inhibition with respect to this substrate. In one case—that of DPNH at a concentration of $2.00 \times 10^{-4}$ M and in the presence of $1.04 \times 10^{-3}$ M $DPN^+$—the data were not in agreement with the theoretically predicted pattern. It is possible that the total nucleotide concentration in this instance attained a level which initiated substrate inhibition by DPNH. Substrate inhibition by DPNH has been reported by Raval

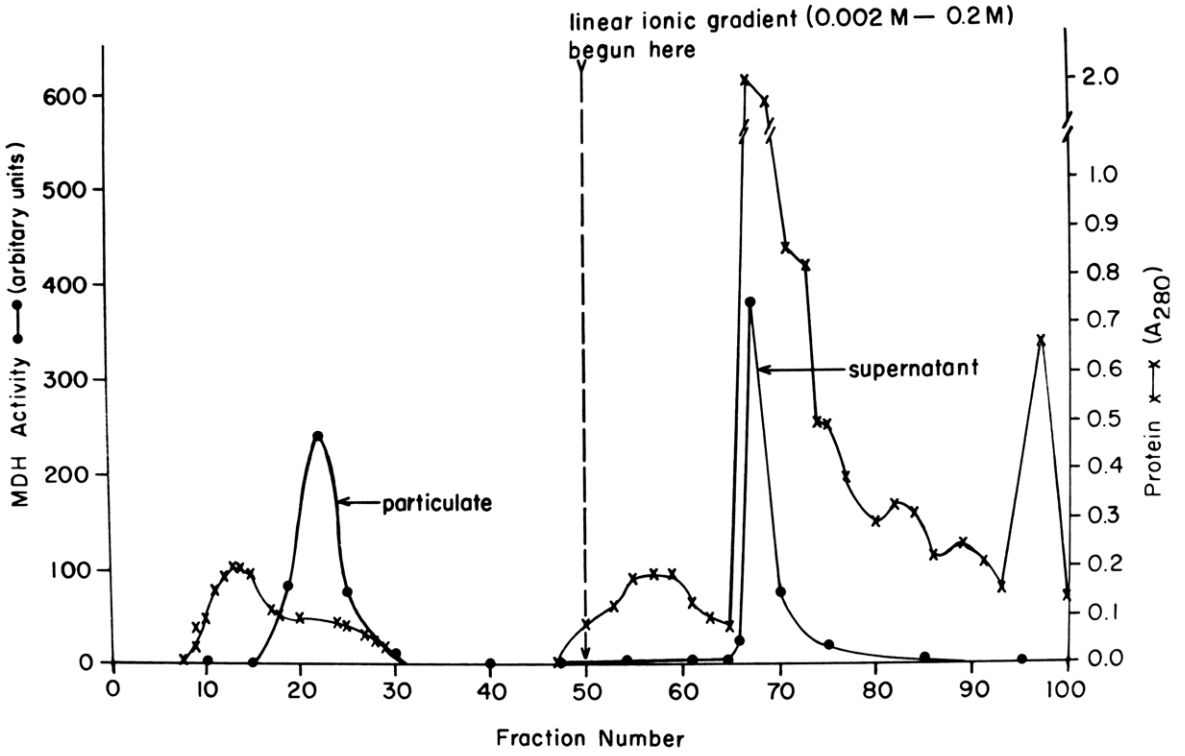


FIGURE 5: Rechromatography of the soluble fraction of MDH activity on DEAE-cellulose. A linear gradient (0.002 M → 0.2 M) of potassium phosphate buffer, pH 8.0, was begun with fraction number 50. Each fraction contained approximately 5.0 ml.

and Wolfe (1962). In the case of oxaloacetic acid as the variable substrate (Figure 3b) in the concentration range of 1.40–3.48 $\times$ $10^{-4}$ M, $DPN^+$ exerts virtually no inhibition with respect to oxaloacetic acid, while three levels of malic acid confirm the noncompetitive nature of malic acid product inhibition with respect to oxaloacetic acid.

Additionally, particulate MDH was assayed in the presence of a structural analog of oxaloacetic acid, monoethyl oxaloacetic acid. This analog proved to be a substrate with a rate about 2.5% that for the normal substrate. As shown in Figure 4a, kinetic analysis at a saturating level of fixed substrate revealed competitive inhibition by monoethyl oxaloacetic acid with respect to oxaloacetic acid, within the concentration range of 0.43–2.14 $\times$ $10^{-4}$ M oxaloacetic acid. The points of *lowest* oxaloacetic acid concentration for the top two curves have dropped off of their respective lines, suggesting that the significance of the analog as a substrate increases at low levels of the natural substrate. A "mixed-type" or noncompetitive inhibition with respect to DPNH was evident when the level of this the variable substrate was within the range of 4.37 $\times$ $10^{-5}$ M to 2.15 $\times$ $10^{-4}$ M and the level of oxaloacetic acid, the fixed substrate, was saturating (Figure 4b). At concentrations of DPNH below 6.54 $\times$ $10^{-5}$ M or in excess of 2.15 $\times$ $10^{-4}$ M, the reaction rate in the presence of this inhibitor exceeds the theoretically predicted value for a strictly noncompetitive inhibition pattern, again suggesting the involvement of the inhibitor as a substrate.

*Rechromatography of the Soluble Fraction.* Rechromatography of the soluble fraction on a larger DEAE-cellulose column resolved its two MDH activities (Figure 5). The chromatographic, electrophoretic, and immunological properties of the first peak of MDH, specific activity 27, are identical with those of the initial particulate MDH preparation; therefore, this peak represents contamination of the soluble fraction with particulate MDH. The second peak of MDH, specific activity 84, representing an approximately 250-fold purification of this component, was termed supernatant MDH.

*Immunological Studies.* As seen in Figure 6, the MDH peaks described above correspond to the two MDH immunodiffusing systems of the soluble fraction (refer to Figure 1). The precipitin arc between antiserum–particulate MDH and the precipitin arc between antiserum–supernatant MDH cross each other (not visible in the photographic reproduction), suggesting a certain degree of antigenic difference (Figure 6a). Adsorption of antiserum with either particulate or supernatant

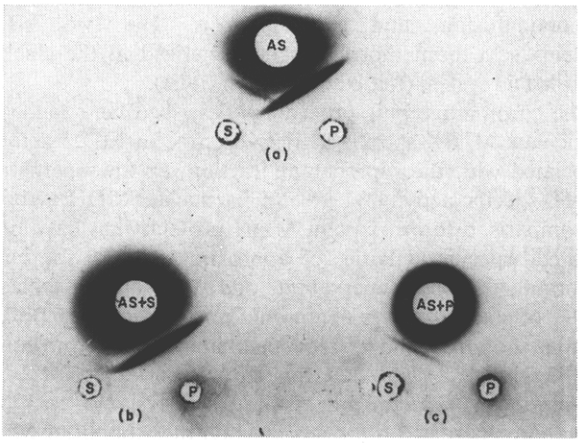


FIGURE 6: Double diffusion using supernatant MDH (S) and particulate MDH (P) *vs.* anti-*C. pyrenoidosa* serum (AS): (a) antiserum, nonadsorbed (AS); (b) antiserum adsorbed against supernatant MDH (AS + S); (c) antiserum adsorbed against particulate MDH (AS + P). Adsorption was carried out by adding to the antiserum well the same quantity of antigen as was placed in its respective antigen well.

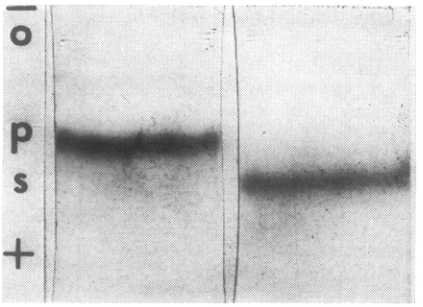


FIGURE 7: Cellulose-acetate electrophoresis (with subsequent MDH localization) of the supernatant (s) and particulate (p) MDH derived from the soluble fraction of *C. pyrenoidosa:* origin (O), anode (+), cathode (−).

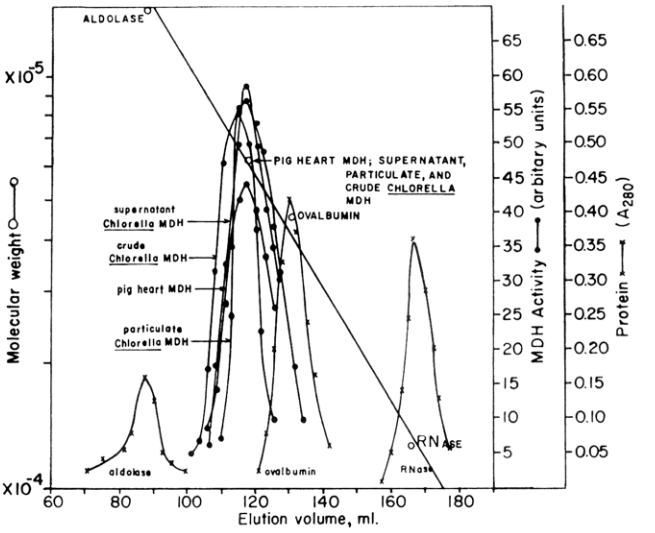


FIGURE 8: Molecular weight determinations of various MDH preparations by Sephadex G-150 gel fltration.

MDH completely removes reactivity against the homologous antigen, while reactivity against the heterologous antigen is not affected (Figures 6b and 6c). The two MDH enzymes are, therefore, immunologically different and have ellicited production of two different antibodies.

*Cellulose-Acetate Electrophoretic Study.* As seen in Figure 7, the two MDH peaks described above have different electrophoretic mobilities, the supernatant MDH having the greater anodal mobility. (Starch gel electrophoresis in borate, barbital, and phosphate–citrate buffer systems suggests that the supernatant and particulate MDHs are composed of conformers; these results will appear in a separate manuscript.)

*Molecular Weight Determinations.* Molecular weight determinations of MDH protein(s) in samples of crude, supernatant, and particulate fractions of *C. pyrenoidosa* and of commercial pig heart MDH were performed by Sephadex gel filtration (Figure 8). All samples contained MDH protein(s) with a molecular weight of approximately 61,000.

## Discussion

Similar to most animal, plant, and microbial MDH systems studied previously (Dupourque and Kun, 1969; Davies, 1969; Sulebele and Silverstein, 1969), *C. pyrenoidosa* appears to possess at least two MDH proteins—one associated with the supernatant fraction of the cell and one associated with the particulate fraction—as demonstrated by chromatography, immunodiffusion, and electrophoresis. The two MDH proteins have been thoroughly characterized in the chicken heart MDH system (Kitto and Kaplan, 1966).

The chromatographic procedures described here yielded a particulate MDH, specific activity 33, free of MDH activity associated with the supernatant fraction, and a supernatant MDH, specific activity 84, free of particulate MDH activity. In comparison to other plant MDH proteins that have been isolated—specific activities of approximately 0.08 for mitochondrial, 0.09 for supernatant, and 0.05 for chloroplastic MDH of spinach leaves (Yamazaki and Tolbert, 1969), 0.02 for mitochondrial, and 0.03 for supernatant MDH from barley seedlings (Yue, 1966), 2.0 for mitochondrial and 1.2 for cytoplasmic MDH from grapefruit juice vesicles (Vines, 1968), and approximately 3.6 for pea epicotyl mitochondrial MDH (Davies, 1969), have been reported—*C. pyrenoidosa* particulate MDH and supernatant MDH are highly purified, about 100- and 250-fold, respectively.

Kinetic data obtained for the particulate MDH are consistent with a compulsory ordered addition (ordered bi-bi) mechanism, with the environmental parameters described. Preliminary kinetic investigations of MDH enzymes from plants have been published (Yamazaki and Tolbert, 1969; Vines, 1968), but no catalytic mechanism has been defined. Reports in the literature on the catalytic mechanism of animal MDH enzymes include the following: two independent studies (Raval and Wolfe, 1962; Silverstein and Sulebele, 1969) of pig heart mitochondrial MDH enzyme suggesting a compulsory ordered addition mechanism, and a third study (Harada and Wolfe, 1968a,b) proposing a more complex reciprocating compulsory order variation of this basic mechanism; a partially random order mechanism (random order in the direction of DPNH oxidation, but compulsory order in the direction of $DPN^+$ reduction) reported by Cassman and Englard (1966b) for the beef heart supernatant MDH enzyme; the report of a compulsory ordered addition mechanism for both mitochondrial and supernatant MDH of ox kidney (Dupourque and Kun, 1969).

An extensive gel filtration survey of MDH from animal, plant, and microbial sources, published by Murphey *et al.* (1967), documented a single molecular weight species of approximately 60,000 from all species studied, except certain Gram-positive bacteria. Samples of MDH, both crude and crystalline, known to exhibit multiple electrophoretic bands likewise exhibited homogeneity by gel filtration. Supernatant MDH and particulate MDH of *C. pyrenoidosa* were estimated to have a molecular weight of 61,000 by Sephadex gel filtration.

Further purification and additional characterizations of physical properties of *C. pyrenoidosa* MDH are in progress in this laboratory.

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# Elastase. I. A New Inhibitor, 1-Bromo-4-(2,4-dinitrophenyl)butan-2-one*

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ABSTRACT: The hydrolytic action of elastase on both its natural substrate, elastin, and a synthetic ester, *p*-nitrophenyl *tert*-Boc-L-alaninate is inhibited irreversibly and stoichiometrically by a new inhibitor, 1-bromo-4-(2,4-dinitrophenyl)-butan-2-one.

The site of reaction of the inhibitor with the enzyme is the $\gamma$-carboxyl group of a glutamic acid—tentatively identified as Glu-6. Evidence for the esterification of this glutamic acid residue is obtained from amino acid analysis of the modified enzyme after Lossen rearrangement and from leucine aminopeptidase digestion of its cyanogen bromide peptides.

Elastase possesses remarkable similarities in its chemical, physical, and biological properties to two other pancreatic proteolytic enzymes, trypsin and chymotrypsin. All three enzymes possess a highly reactive serine which can be phosphorylated by diisopropyl fluorophosphate, four homologous disulfide bridges, and two histidine residues in homologous sequences (Brown *et al.*, 1967). Elastase is unique, however, in its ability to degrade elastin, a connective tissue protein with a very high content of glycine, alanine, and proline (Partridge and Davis, 1955).

Considerable work with site-specific irreversible inhibitors has been carried out with trypsin and chymotrypsin and has demonstrated one of the two homologous histidines in both chymotrypsin and trypsin to be essential for catalytic activity (Schoellmann and Shaw, 1963; Shaw *et al.*, 1965). Few examples of specific chemical modifications of elastase have been reported, due perhaps in part to the lack of a convenient assay system for this enzyme. The recent development of a spectrophotometric assay with *p*-nitrophenyl *tert*-Boc-L-alaninate (NBA)[1] as substrate (Visser and Blout, 1969) has made such studies more practicable.

The apparent specificity of elastase for alanine derivatives suggested that either the chloromethyl ketone or diazo ketone derivatives of alanine would be useful chemical modification reagents to demonstrate that elastase, like trypsin and chymotrypsin, possesses a histidine residue essential for enzymic activity. As will be shown below, none of the several alanine derivatives synthesized inhibited elastase activity. However, 1-bromo-4-(2,4-dinitrophenyl)butan-2-one [DPBB (XIII)] was found to react irreversibly and stoichiometrically with elastase, abolishing its esterolytic (with NBA as sub-

* From the Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts 02115. *Received July 13, 1970.* We are pleased to acknowledge the support in part of this work by U. S. Public Health Service Grant AM-07300.

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[1] The abbreviations used are: TPCK, L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone; NBA, *p*-nitrophenyl *tert*-Boc-L-alaninate; DPBB, 1-bromo-4-(2,4-dinitrophenyl)butan-2-one; PMSF, phenylmethanesulfonyl fluoride; HFIP, hexafluoro-2-propanol; DPB-elastase, dinitrophenylbutanonyl-elastase; LAP, leucine aminopeptidase; DEPP, diethyl *p*-nitrophenyl phosphate; NMT, *N*-methyl-*N*-nitroso-*p*-tosylamide.