LACTATE DEHYDROGENASE OF *HYMENOLEPIS DIMINUTA*:

ISOLATION AND CHARACTERIZATION

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Lactate dehydrogenase was isolated in pure form from crude extract of the cestode Hymenolepis diminuta by heat treatment and column chromatography. The purified enzyme has a specific activity of 106 units per mg protein. The molecular weight of the purified protein was 75,000 as determined by Sephadex gel filtration and analytical ultracentrifugation. An equilibrium ultracentrifugation study suggests a subunit molecular weight of 39,000. From these data, a dimer form of the native enzyme is proposed.

Only one molecular form of lactate dehydrogenase was demonstrated by cellulose acetate and polyacrylamide gel electrophoresis performed on crude extract.

Kinetic studies suggest that this enzyme from the tapeworm is similar to the heart type of vertebrate lactate dehydrogenase. The K_m for pyruvate was determined to be 0.17 mM, and the K_m for lactate was 6.3 mM. The enzyme was shown to be specific for L-lactate and DPN. Inhibition studies using N-ethyl maleimide and p-chloromercuribenzoate indicate the possibility of an essential histidine and/or cysteine residue(s) in the active site of the enzyme.

LACTATE DEHYDROGENASE OF *HYMENOLEPIS DIMINUTA*: ISOLATION AND CHARACTERIZATION

THESIS

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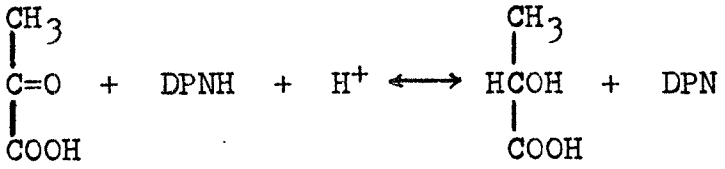
LACTATE DEHYDROGENASE OF HYMENOLEPIS DIMINUTA: ISOLATION AND CHARACTERIZATION

Lactate dehydrogenase (L-lactate:DPN oxidoreductase EC 1.1.1.27) is a DPN-dependent enzyme catalyzing the interconversion of lactate and pyruvate in the glycolytic pathway. It catalyzes the reaction:



In 1920, Thunberg recognized an enzyme in tissues which oxidized lactate and which depended on a water-soluble cofactor for its activity. The enzyme was first studied in cell-free extracts by Banga et al. in 1932. In 1940, using bovine heart as the enzyme source, Straub became the first researcher to crystallize lactate dehydrogenase. Since that time, preparations of crystalline enzyme have been described from rat skeletal muscle (Kubowitz and Ott, 1943), rabbit skeletal muscle (Racker, 1951), rat liver (Gibson et al., 1953; Hsieh and Vestling, 1965), rat heart (Wieland et al., 1959), and human heart (Nisselbaum and Bodansky, 1961; Pesce et al., 1967). Procedures for preparation of lactate dehydrogenases specifically from heart and striated muscle have been described by Pesce et al. for leopard frog, bullfrog, halibut, dogfish, pheasant,

ostrich, duck, rhea, turkey, and rabbit. These various lactate dehydrogenases have been characterized with respect to molecular weight, amino acid composition, and catalytic properties (Pesce et al., 1967).

An obligatory binding order of coenzyme followed by substrate has been shown for various lactate dehydrogenases (Novoa et al., 1961; Gutfreund et al., 1968). McPherson (1970) has shown in dogfish muscle lactate dehydrogenase that the AMP end of the coenzyme must first bind and generate, through an induced conformational change, a binding site for the nicotinamide mononucleotide end. Di Sabato (1971) has demonstrated that a covalent bond is formed between pyruvate and DPNH at the active site.

Lactate dehydrogenase is a cytoplasmic enzyme with reported molecular weights from 135,000 to 150,000 (Cahn et al., 1962; Kaloustian et al., 1969; Jaenicke and Knof, 1968). At present, lactate dehydrogenase is regarded as a tetrameric protein with four subunits, each having a molecular weight of 35,000 (Appella and Markert, 1961; Cahn et al., 1962). Isoenzyme forms of this enzyme have been demonstrated in many animals since the first report of multiple forms of the same enzyme by Wieland and Pfleiderer in 1957.

In mammals the tetrameric molecule is made up of four subunits of two parent molecular types. Dawson et al. (1964) have termed the parent forms "H" (heart) and "M"

(muscle), for the organs from which they are readily obtained. Five tetrameric isoenzymes are formed by the combination of subunits of the two parent types. Studies by Dawson et al. (1964), Cahn et al. (1962), and Kaplan and Cahn (1962) indicate that these M and H types of lactate dehydrogenase subunits have significantly different physiological roles. This concept is based on the demonstration that the H form of the enzyme in mammals is inhibited by excess pyruvate, whereas the M form maintains activity at high pyruvate concentrations. In aerobic tissue, such as heart muscle, where a steady supply of energy is required, this energy is supplied by the complete oxidation of pyruvate in the mitochondria, and the inhibition of the H form by pyruvate favors this oxidative pathway. In tissues such as striated muscle there is a requirement for sporadic, sudden releases of energy under relatively anoxic conditions. This energy is supplied by anaerobic glycolysis, which produces large amounts of pyruvate and requires its reduction to lactate to complete the oxidoreduction cycle of glycolysis. In tissues such as these, the M form of the enzyme predominates. Everse et al. (1970) term the H type a lactate dehydrogenase, whereas the M type is a pyruvate reductase.

The presence of lactate dehydrogenase in Hymenolepis diminuta was first reported by Read in 1951. Lactate has been reported as a fermentation product of anaerobic

metabolism in this tapeworm by Read (1951), Laurie (1957), and Scheibel and Saz (1966). The relative amounts of lactate (as percent millequivalents of total acid) produced by this cestode range from 14% or less (Fairbairn et al., 1961) to 37-98% (Laurie, 1957) to above 70% (Read, 1956).

Hymenolepis diminuta is a cestode which, as an adult, inhabits the relatively anaerobic small intestine of the rat. It possesses an unusual carbohydrate metabolism which resembles the Embden-Meyerhof pathway to the point of the production of phosphoenolpyruvate (PEP). At this point, two enzymes compete for this substrate. PEP can be converted to pyruvate by the enzyme pyruvate kinase (Bueding and Saz, 1968) as occurs in the Embden-Meyerhof pathway. The pyruvate then becomes the substrate for lactate dehydrogenase with the production of lactate and the regeneration of DPN necessary for glycolysis. Alternatively, the PEP can be converted to oxalacetate (OAA) by the enzyme phosphoenolpyruvate carboxykinase (Prescott and Campbell, 1965). This latter reaction appears to predominate the metabolic pathway at this point (Bueding and Saz, 1968).

The formation of OAA from PEP by PEP carboxykinase involves the production of ITP. The conversion of ITP to ATP by a nucleoside diphosphokinase in the worm has not yet been demonstrated, although it is probable that this reaction is present. The OAA is then acted on by malate dehydrogenase producing malate and regenerating DPN for

glycolysis. The malate thus formed then enters the mitochondrion. Since direct oxidative systems are absent in the H. diminuta mitochondrion (Ward and Fairbairn, 1970), malate must be utilized further by a dismutation system. Intramitochondrial reducing power, in the form of D(T)PNH, is obtained by the oxidative decarboxylation of malate to pyruvate and CO_2, thereby giving rise to pyruvate in the absence of pyruvate kinase (Saz, 1970). This reaction is catalyzed by the mitochondrial malic enzyme. D(T)PNH formed from this reaction then serves to reduce a corresponding amount of malate to succinate via fumarate and the fumarate reductase reaction with the concomitant formation of ATP (Scheibel et al., 1968).

The concentration of CO_2 in the small intestine varies from 2.16-79.89% (Read, 1950). When CO_2 is available in sufficient quantities, PEP carboxykinase will convert most of the PEP to OAA before pyruvate kinase can catalyze the production of pyruvate. However, when CO_2 is in lower concentrations, the PEP carboxykinase reaction may not be able to function to any great extent, allowing the pyruvate kinase reaction to produce the substrate for lactate dehydrogenase.

It has been pointed out that in the anaerobic habitat of intestinal helminths, succinate formation has a distinct advantage for the parasite over the lactate dehydrogenase reaction (Saz and Bueding, 1966). Both provide a means for reoxidizing DPNH, making DPN available for glycolysis,

but in contrast to the lactate dehydrogenase reaction, reduction of fumarate to succinate by DPNH, catalyzed by a mitochondrial electron transport system, generates energy in the form of ATP. However, the H. diminuta lactate dehydrogenase activity is 2.5 times higher than the activity of lactate dehydrogenase in A. suum muscle which possesses the same basic carbohydrate metabolism (Bueding and Saz, 1968). The presence of this enzyme in such quantity in H. diminuta may indicate a significant physiological difference between the two intestinal worms. Lactate dehydrogenase in this tapeworm may serve a unique role in the energy production of this animal. The importance of this enzyme in H. diminuta has never been discussed.

The purpose of this study was to isolate and study the kinetic properties of this metabolic enzyme in Hymenolepis diminuta. In this manner, the significance of this ubiquitous enzyme may be elucidated.

MATERIALS AND METHODS

Materials

Hymenolepis diminuta was kept in the laboratory in male Sprague-Dawley rats as described by Read et al. (1963). The worms were harvested 15-30 days after infection.

Cellex D DEAE cellulose with an exchange capacity of 0.74 meq per g was obtained from Calbiochem, cellulose phosphate with an exchange capacity of 0.89 meq per g was from Sigma, and Sephadex G-25 and Sephadex G-200 were purchased from Pharmacia.

DPNH, DPN, sodium pyruvate, L-lithium lactate, phenazine methosulfate, MTT tetrazolium, N-ethyl maleimide, and p-chloromercuribenzoic acid were obtained from Sigma. 2,6-dichlorophenolindophenol-sodium salt (DCIP) was from Fisher Scientific.

Enzyme Assays

Hymenolepis diminuta lactate dehydrogenase was assayed at 25° in a Beckman Model DB-GT Spectrophotometer. During purification of the enzyme, lactate dehydrogenase acitivity was determined routinely by assaying for the reverse (reduction of pyruvate to lactate) reaction. This reaction was assayed by measuring the initial rates of DPNH oxidation spectrophotometrically at 340 mμ. Quartz cuvettes of 1-cm

light path contained 300 µmoles of sodium phosphate buffer (pH 7.5), 3.0 µmoles of sodium pyruvate, 0.39 µmoles of DPNH, and enzyme in a total volume of 3.0 ml. The reaction was initiated by the addition of enzyme sufficient to cause a decrease in optical density between 0.01 and 0.10 in 60 sec. For the determination of Michaelis constant (K_m) for pyruvate, the pyruvate concentration was varied from 0.15 µmoles to 150 µmoles. One unit of activity is defined as the amount of enzyme catalyzing the reduction of 1 µmole of pyruvate per min at 25°.

The enzyme was assayed in the forward direction (oxidation of lactate to pyruvate) by measuring the reduction of dichlorophenolindophenol at 600 mµ. The cuvette contained 300 µmoles of sodium phosphate buffer (pH 7.5), lithium lactate varied from 3 µmoles to 450 µmoles, 0.66 µmoles of DPN, 0.06 µmoles of DCIP, 0.2 µmoles of phenazine methosulfate, and enzyme in a total volume of 3.0 ml. The reaction was initiated by the addition of enzyme sufficient to cause a decrease in optical density between 0.01 and 0.10 in 60 sec. This method of dye-linked enzyme assay is an adaption of the method of Singer and Kearney (1957) as modified by Arrigoni and Singer (1962) for determination of succinic dehydrogenase activity.

Protein Determination

Protein was estimated by a modification of the method of Lowry *et al.* (1951) using bovine serum albumin (Sigma)

as the standard. To duplicate protein samples containing 20-200 µg of protein in 2.0 ml, 2.0 ml of solution C were added, mixed well, and allowed to stand for 30 min at room temperature. Then 0.2 ml of 1N Folin-Phenol reagent (Fisher) were added rapidly with immediate mixing. After 30 min, the optical density was read at 660 mµ. After isolation of the enzyme, protein concentration was determined spectrophotometrically by the ratios of absorption at 280 mµ and 260 mµ, as described by Warburg and Christian (1942).

Ion Exchange Chromatography and Gel Filtration

DEAE cellulose and phosphocellulose were prepared according to Peterson and Sober (1962), and equilibrated in the appropriate buffer, following alternate washings with NaOH and HCl. Columns were packed with 30-cm hydrostatic pressure, and a constant flow of buffer was maintained with a pump.

For the determination of molecular weights by gel filtration, Sephadex G-200 (particle size 40-120 µ) was packed into a 20 x 110 cm column under a 10-cm hydrostatic head of pressure. The column was calibrated with proteins of known molecular weights, and the elution volumes were correlated with molecular weights (Andrews, 1964; Determann and Michel, 1966) or Stokes' radii (Andrews, 1970).

Electrophoresis

Disc gel electrophoresis was performed according to Davis (1964) with the Canalco model 200 apparatus. Gels

were prepared with a 7.5% monomer concentration in Tris-glycine buffer, pH 8.8, and stained specifically for LDH by the method of Dietz and Lubrano (1967). A "negative" stain of the gels was prepared by incubating the gels at 37° in a solution containing 20 mg/ml sodium pyruvate and 2 mg/ml DPNH for 20 min and then placing the gels in a solution containing 0.6 mg/ml MTT tetrazolium and 0.18 mg/ml phenazine methosulfate for 10 min. The stained gels were fixed in 5% acetic acid.

Cellulose-acetate electrophoresis was carried out in a Gelman electrophoresis unit, using Gelman Sepraphore III strips, 2.5 x 18 cm, at pH 8.8 in 0.05 M Tris-barbital buffer. Samples were subjected to electrophoresis for 60 min at a constant current of 1.5 mA per strip. The strips were stained for lactate dehydrogenase activity by the method of Preston et al. (1965).

Sedimentation Velocity and Sedimentation Equilibrium

Ultracentrifugation experiments were performed in a Beckman-Spinco model E analytical ultracentrifuge. Schlieren patterns and Rayleigh fringes were measured from photographic enlargements. Sedimentation coefficients were converted to $s_{20,w}$ values after correction for temperature and solvent viscosity, and a molecular weight was determined by application of the Svedberg equation to the sedimentation velocity data. A molecular weight was also determined by

the equilibrium ultracentrifugation method described by Yphantis (1964).

Inhibitor Studies

Three 1.0 ml aliquots of H. diminuta extract in 10 mM Tris-chloride buffer (pH 7.5) were warmed to 25°. Each sample contained 8 units of lactate dehydrogenase activity. One 1.0 aliquot was used as the control and to it 0.1 ml of 10 mM Tris-chloride buffer was added. To another 1.0 ml sample, 0.1 ml of 1.1 mM N-ethyl maleimide in the Tris buffer was added to bring the concentration of the inhibitor to 0.1 mM. To the third 1.0 ml aliquot, 0.1 ml of 1.1 mM p-chloromercuribenzoic acid in the Tris buffer was added to bring the concentration of the inhibitor to 0.1 mM. The solutions were monitored for activity for a period of two hours. At this time, dithiothreitol (Sigma) was added to bring the concentration of this reducing agent to 78 mM. The activity of the various solutions was then monitored for 30 min.

RESULTS

Enzyme Purification Procedure

Extraction

Rats were killed by cervical dislocation. H. diminuta were expelled from the small intestine by cautious syringing with saline and washed in several changes of saline to remove possible contaminating bacteria (Campbell, 1963). All worms were taken from hosts fed ad libitum.

Eighty grams of worms were blotted on filter paper, weighed, and placed in an ice bath. All subsequent procedures were carried out 0-3°. The worms were then minced and homogenized in 10 mM Tris-chloride buffer (pH 8.5) containing 1 mM EDTA, with a w/v ration of 1:3 in a glass homogenizer with a motor driven teflon pestle. The mixture was then centrifuged for 15 min at 10,000 x g in a Sorvall Superspeed RC2-B centrifuge. The resulting milky supernatant solution was filtered through glass wool. The pellet was resuspended in a minimal amount of homogenizing medium and rehomogenized in a TenBroeck glass homogenizer. This homogenate was then centrifuged for 15 min at 10,000 x g. The resulting supernatant solution from this centrifugation was filtered through glass wool and pooled with the supernatant solution from the first centrifugation.

Heat Treatment and G-25 Filtration

Solid magnesium acetate was added to the extract and brought to a concentration of 0.01 M. The extract was divided into 15 ml samples and heated for 5 min at 58°, and then rapidly cooled to 0° in an ice bath. The solution was then centrifuged at 10,000 x g for 10 min. Due to the large volume of solution following the heat treatment, the supernatant solution was divided into 50 ml samples to be desalted on a 2.5 x 30 cm Sephadex G-25 column equilibrated with 10 mM imidazole buffer (pH 6.5) containing 1 mM EDTA. Fractions of 5 ml were collected. The fractions were monitored for protein spectrophotometrically at 280 mμ and for enzyme activity as described under "Materials and Methods." Fractions containing lactate dehydrogenase activity were pooled.

First Phosphocellulose Chromatography

The solution from the Sephadex G-25 column was applied to a phosphocellulose column (2.5 x 30 cm) which had been equilibrated in 10 mM imidazole buffer (pH 6.5) containing 1 mM EDTA, and the column was washed (flow rate, 0.5 ml per min) with the same buffer until the effluent was protein-free as determined by spectrophotometric measurement at 280 mμ. Thereafter, a linear sodium chloride gradient elution was used starting with 1 liter of 10 mM imidazole (pH 6.5) containing 1 mM EDTA in the mixing chamber and

1 liter of 10 mM imidazole (pH 6.5) containing 1 mM EDTA and 0.5 M NaCl in the reservoir. Fractions of 7 ml were collected and those containing lactate dehydrogenase activity were pooled. The enzyme activity appeared in fractions 105-122, as illustrated in Fig. 1.

DEAE Cellulose Chromatography

The phosphocellulose fraction was dialyzed overnight against 8 liters of 10 mM Tris-chloride buffer (pH 8.5) containing 1 mM EDTA. Then 100 ml of the solution was applied to a DEAE cellulose column (1.5 x 30 cm) equilibrated in 10 mM Tris-chloride buffer (pH 8.5) containing 1 mM EDTA and the column was washed (flow rate, 0.5 ml per min) with the same buffer until the effluent was free of protein. A linear NaCl gradient was used to elute the protein starting with 250 ml of 10 mM Tris-chloride (pH 8.5) containing 1 mM EDTA in the mixing chamber and 250 ml of 10 mM Tris-chloride (pH 8.5) containing 1 mM EDTA and 0.5 M NaCl in the reservoir. Fractions of 9 ml were collected and those containing lactate dehydrogenase activity were pooled. The enzyme activity appeared in tubes 60-67, as depicted in Fig. 2.

Second Phosphocellulose Chromatography

The DEAE cellulose fraction was concentrated against polyethyleneglycol (Fisher Scientific) from a volume of 50 ml to 10 ml. The concentrated enzyme was dialyzed

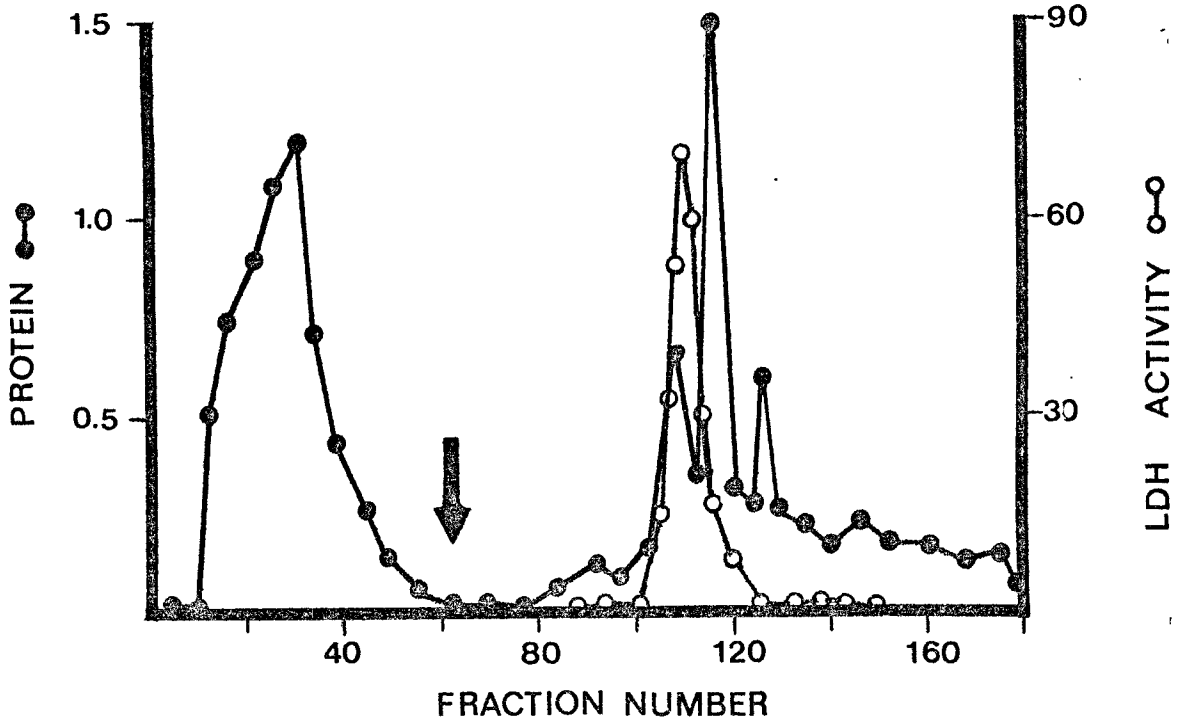


Fig. 1. Elution pattern of H. diminuta lactate dehydrogenase on phosphocellulose column. Experimental details are described in text. Protein concentration was determined by absorbance at 280 mμ. Enzyme activity is in terms of ΔA_{340} per min per ml. Arrow marks the application of the linear salt gradient.

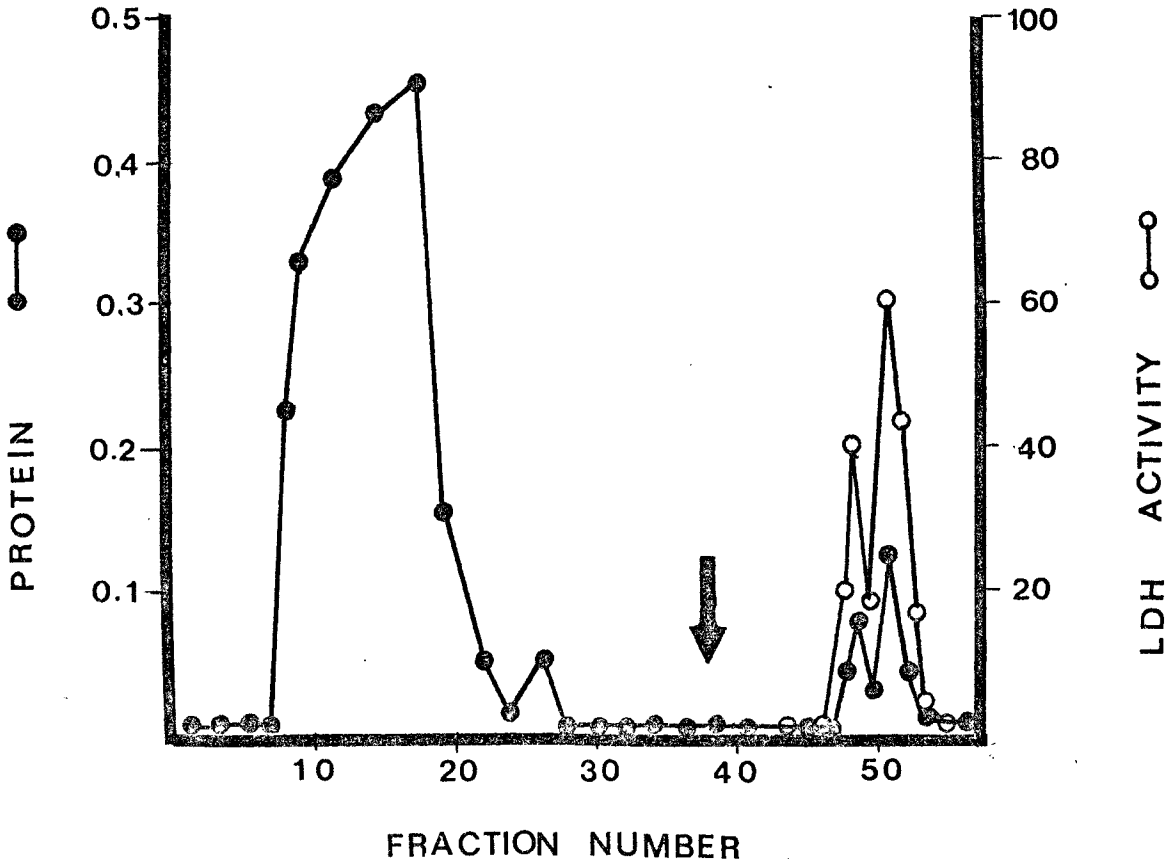


Fig. 2. Elution pattern of H. diminuta lactate dehydrogenase on a DEAE cellulose column. Experimental details are described in text. Protein concentration was determined by absorbance at 280 mμ. Enzyme activity is in terms of ΔA_{340} per min per ml. Arrow marks the application of the linear salt gradient.

overnight against 8 liters of 10 mM imidazole buffer (pH 6.5) containing 1 mM EDTA and 1 mM 2-mercaptoethanol. The solution was then applied to a phosphocellulose column (0.9 x 6 cm) equilibrated with 10 mM imidazole buffer (pH 6.5) containing 1 mM EDTA and 1 mM 2-mercaptoethanol. The column was washed (flow rate 0.25 ml per min) with the same buffer until the effluent was protein free. A linear salt gradient was started with 100 ml of 10 mM imidazole buffer (pH 6.5) containing 1 mM EDTA and 1 mM 2-mercaptoethanol in the mixing chamber and 100 ml of 10 mM imidazole buffer (pH 6.5) containing 1 mM EDTA, 1 mM 2-mercaptoethanol, and 0.50 M NaCl in the reservoir. Fractions of 3 ml were collected. Protein and catalytic activity appeared together in fractions 27 to 31, as shown in Fig. 3. A summary of the purification procedure is presented in Table I.

TABLE I

PURIFICATION OF H. DIMINUTA LACTATE DEHYDROGENASE

Fraction	Vol	Total Activity	Specific Activity	Purification	Recovery
	ml	units	units x 10^{-2}/mg		%
Homogenate	300	1,220	83		100
Sephadex G-25. . .	200	1,150	337	4	95
Phosphocellulose .	118	938	1,200	15	77
DEAE cellulose . .	62	370	8,500	103	40
Phosphocellulose .	15	111	10,600	128	9

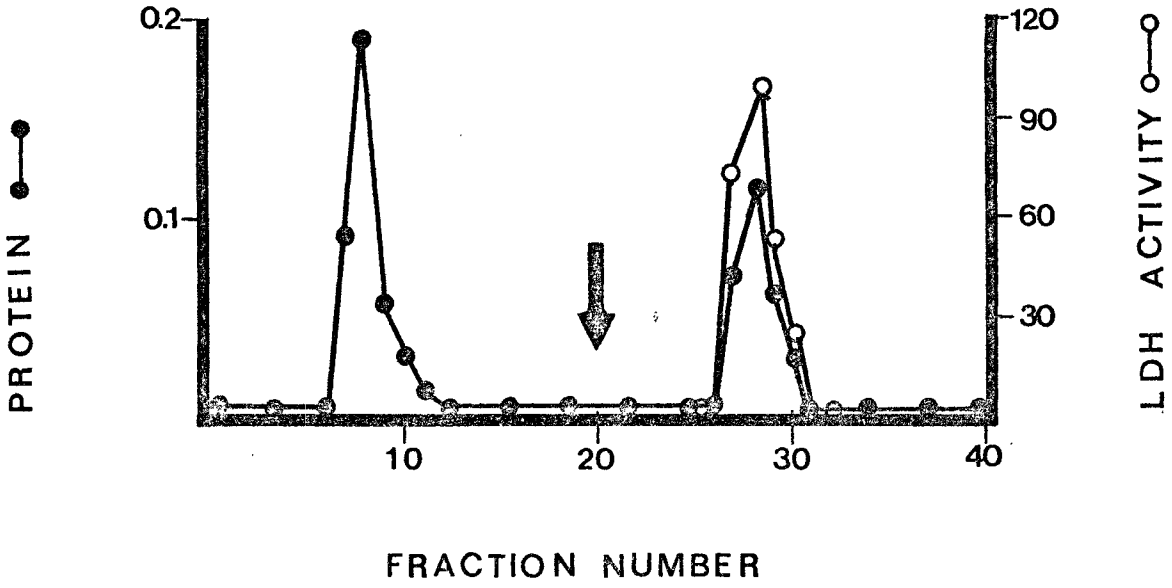


Fig. 3. Elution pattern of H. diminuta lactate dehydrogenase on second phosphocellulose column. Experimental details are described in text. Protein concentration was determined by absorbance at 280 mμ. Enzyme activity is in terms of ΔA_{340} per min per ml. Arrow marks the application of the linear salt gradient.

Physical Properties of H. diminuta Lactate Dehydrogenase

Electrophoretic Study

As seen in Fig. 4, Fig. 5, and Fig. 6, only one lactate dehydrogenase isoenzyme is detectable with cellulose-acetate and polyacrylamide gel electrophoresis of crude H. diminuta extract.

Molecular Weight Determination

Molecular weight determinations of the lactate dehydrogenase protein in a sample of crude extract of H. diminuta were performed by Sephadex gel filtration (Fig. 7). When the elution volume of lactate dehydrogenase was correlated with elution volumes and molecular weights of known proteins (Andrews, 1964), a value of 75,000 ± 10,000 was obtained for the molecular weight of lactate dehydrogenase. When the calibrated Sephadex column was used to correlate exclusion volume with the Stokes' radius (Andrews, 1970), a value of 31.5 Å was obtained (Fig. 8).

Sedimentation Velocity Experiment

When purified H. diminuta lactate dehydrogenase at a protein concentration of 4.0 mg per ml was subjected to ultracentrifugation at 60,000 rpm, the enzyme sedimented as a single, symmetrical boundary throughout the cell. A value for $s_{20,w}$ of 5.3×10^{-13} sec was obtained. The diffusion coefficient was estimated by the method of

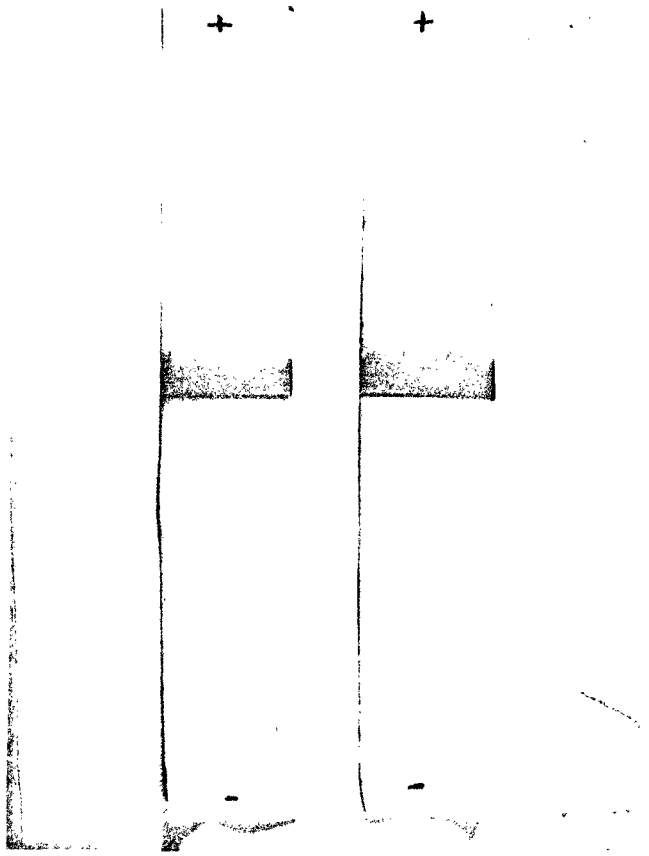


Fig. 4. Cellulose acetate electrophoresis of H. diminuta lactate dehydrogenase.

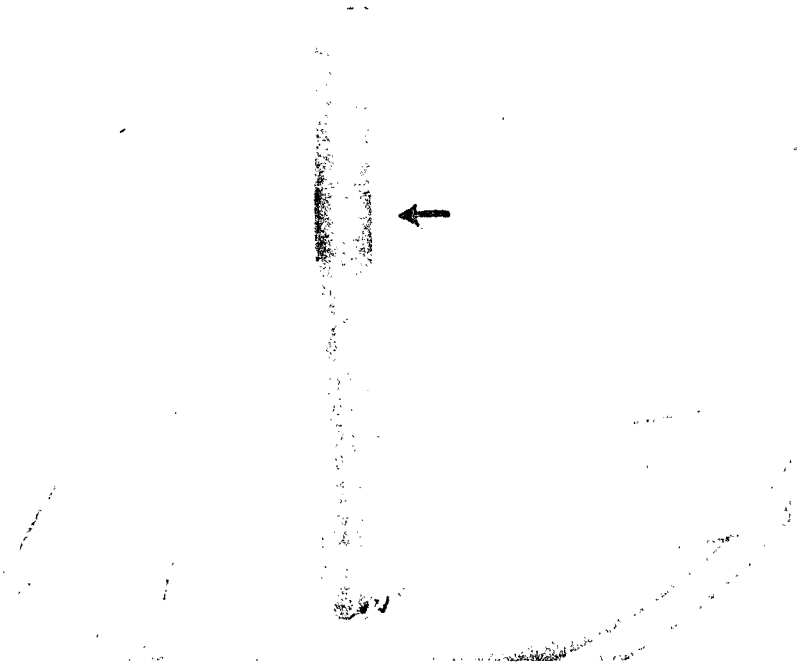


Fig. 5. Polyacrylamide gel electrophoresis of H. diminuta lactate dehydrogenase. Positive stain.

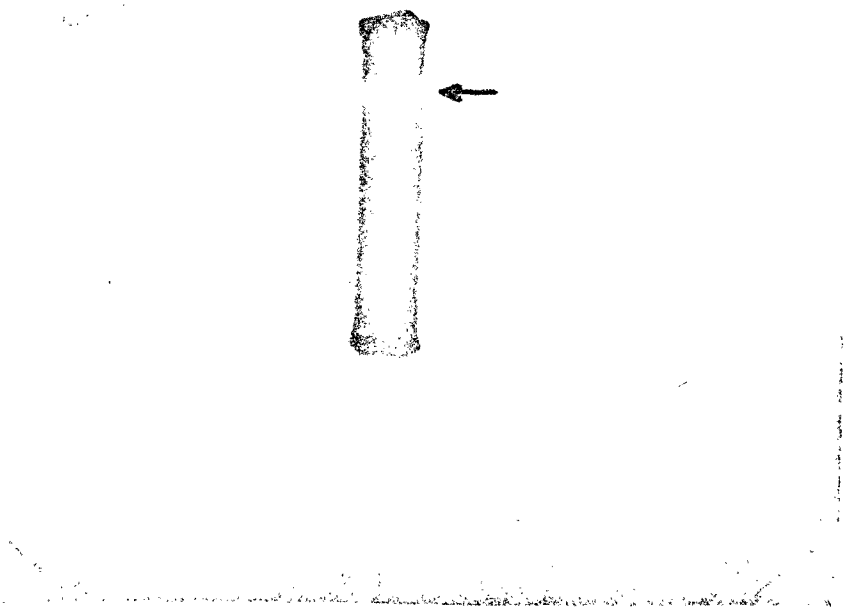


Fig. 6. Polyacrylamide gel electrophoresis of H. diminuta lactate dehydrogenase. Negative stain.

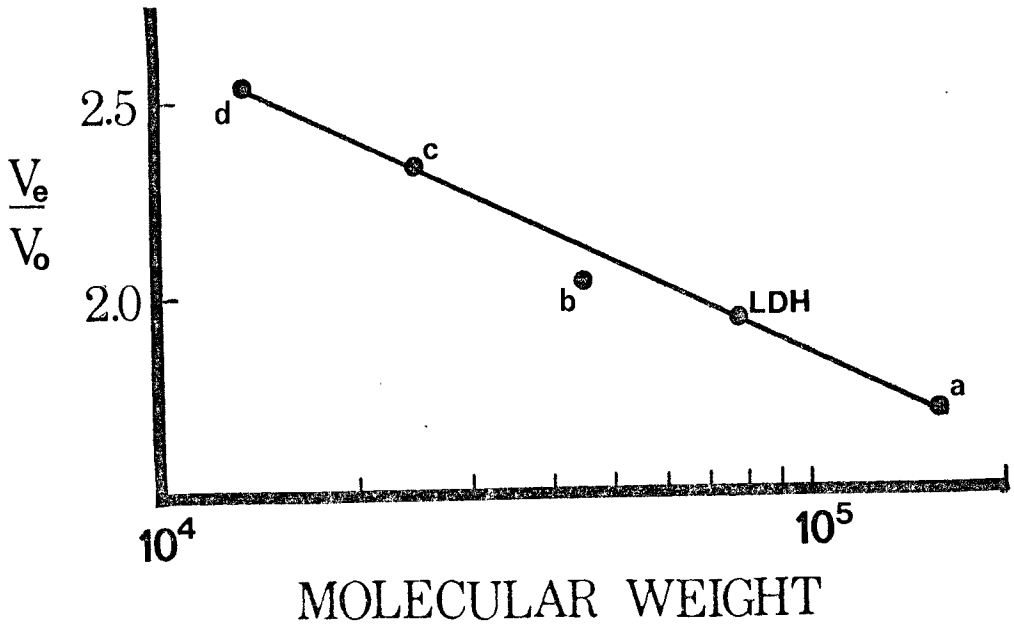


Fig. 7. Molecular weight of Hymenolepis diminuta lactate dehydrogenase on Sephadex G-200. The column was calibrated with aldolase (a), ovalbumin (b), chymotrypsinogen A (c), and ribonuclease A (d). Ve/Vo is the elution volume for the protein divided by the column void volume.

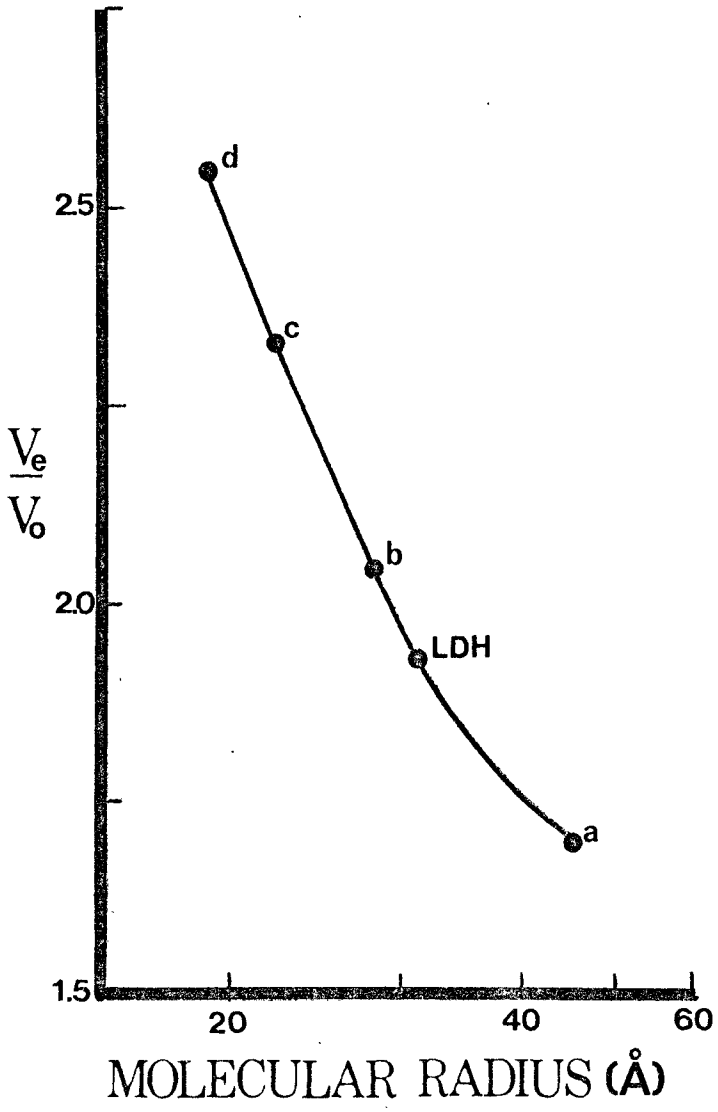


Fig. 8. Determination of Stoke's radius for Hymenolepis diminuta lactate dehydrogenase by Sephadex G-200 column chromatography. The column was calibrated with aldolase (a), ovalbumin (b), chymotrypsinogen A (c), and ribonuclease A (d). Ve/Vo is the elution volume for the protein divided by the column void volume.

Andrews (1970) by correlating the exclusion volume from the calibrated Sephadex G-200 column with the diffusion coefficient. A value of 7.15×10^{-7} cm^2 per sec was obtained for the diffusion coefficient ($D_{20,w}$) of the tapeworm lactate dehydrogenase. The partial specific volume of the protein was assumed to be 0.740 ml per g. When these values were substituted into the Svedberg equation, the molecular weight was determined to be 76,000.

Sedimentation Equilibrium Experiment

The purified enzyme was dialyzed for 48 hours against one liter of 10 mM Tris-chloride buffer (pH 7.5) containing 1 mM EDTA, 0.05% v/v 2-mercaptoethanol, and 0.1 M NaCl. The enzyme (0.47 mg per ml) was then subjected to high speed equilibrium ultracentrifugation at 36,000 rpm in a 12-mm double sector cell with quartz windows for 24 hours at 20°. The protein concentration was calculated from the fringe displacement in millimeters as measured from photographic enlargements. The results of high speed equilibrium ultracentrifugation indicate a weight average molecular weight determined to be 39,000 with larger molecular weight contamination (Fig. 9). At the end of the ultracentrifugation, no lactate dehydrogenase activity could be demonstrated. This value for the molecular weight is similar to the subunit molecular weight of beef heart lactate dehydrogenase as reported by Apella and Markert (1961).

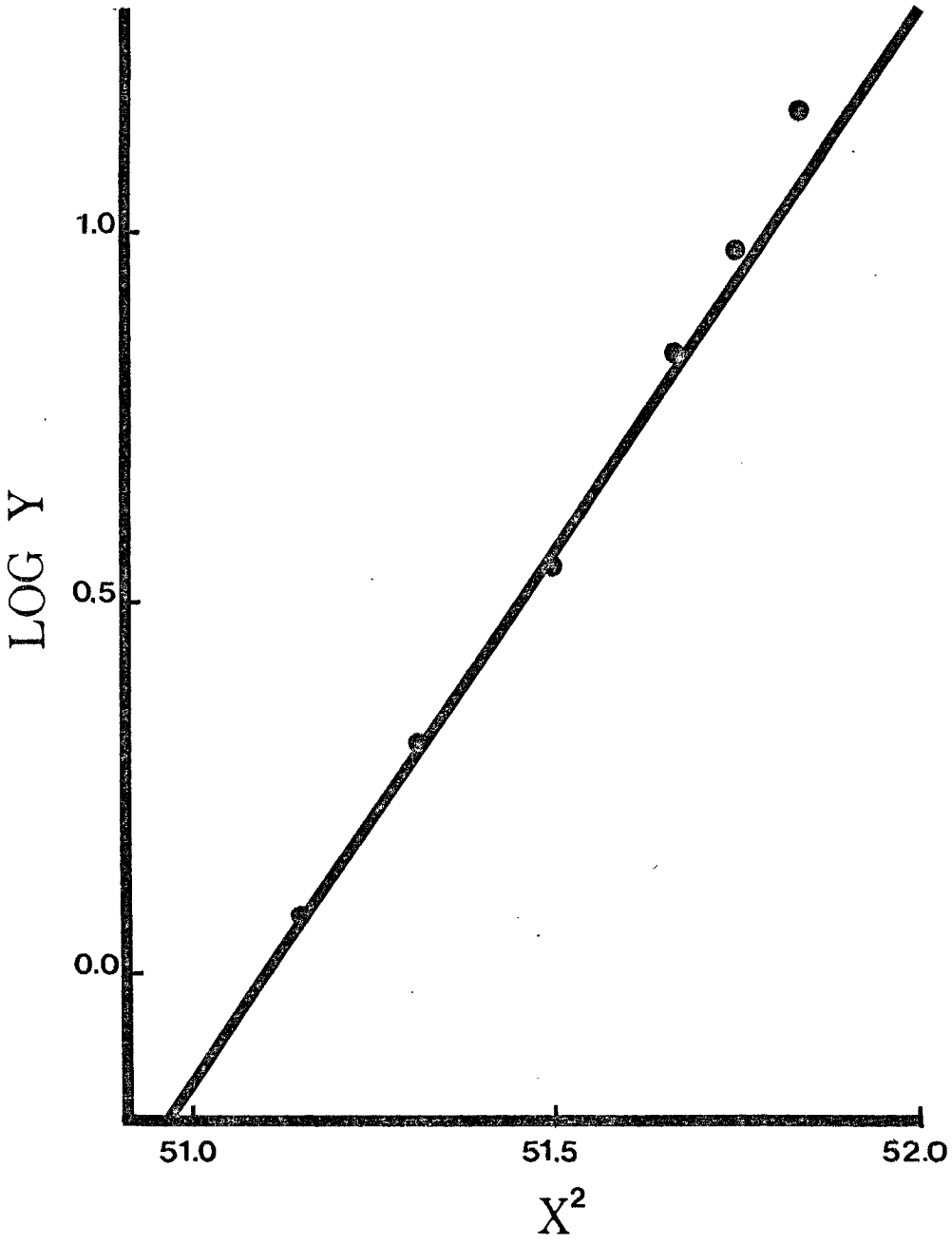


Fig. 9. Fringe displacement obtained from sedimentation equilibrium experiment with H. diminuta lactate dehydrogenase. The protein concentration was calculated from the fringe displacement (Y) in mm. The abcissa represents the square of the distance (cm) from the center of rotation.

Kinetic Properties of H. Diminuta Lactate Dehydrogenase

Catalytic Characteristics of Reverse Reaction

Kinetic studies of the approximately 100-fold-purified lactate dehydrogenase were performed. Pyruvate kinase (Bueding and Saz, 1968), malate dehydrogenase (Zee and Zinkham, 1968), and malic enzyme (Hsu and Lardy, 1967) could not be demonstrated in this purified extract. No catalysis was observed when TPNH (Sigma), 0.39 mM, was substituted for DPNH in the assay for the reduction of pyruvate to lactate. Michaelis constants were calculated from kinetic data by the method of Wilkinson (1961) as adapted for computer calculations by Cleland (1963), which is basically an application of weighted non-linear regression method. The Michaelis constant for pyruvate was determined to be 1.7×10^{-4} (Fig. 10). This K_m for pyruvate closely resembles the K_m for pyruvate of heart lactate dehydrogenase of vertebrate origin (Pesce et al., 1967). Plots of rate against substrate concentration yielded rectangular hyperbolas and had the usual characteristics of Michaelis-Menten kinetics (Fig. 11). Slight substrate inhibition was demonstrated at pyruvate concentrations above 1.0 mM.

Catalytic Characteristics of Forward Reaction

No activity was observed when D-lactate (Sigma) was substituted for L-lactate, or when TPN was substituted for DPN in the assay for the oxidation of lactate to pyruvate.

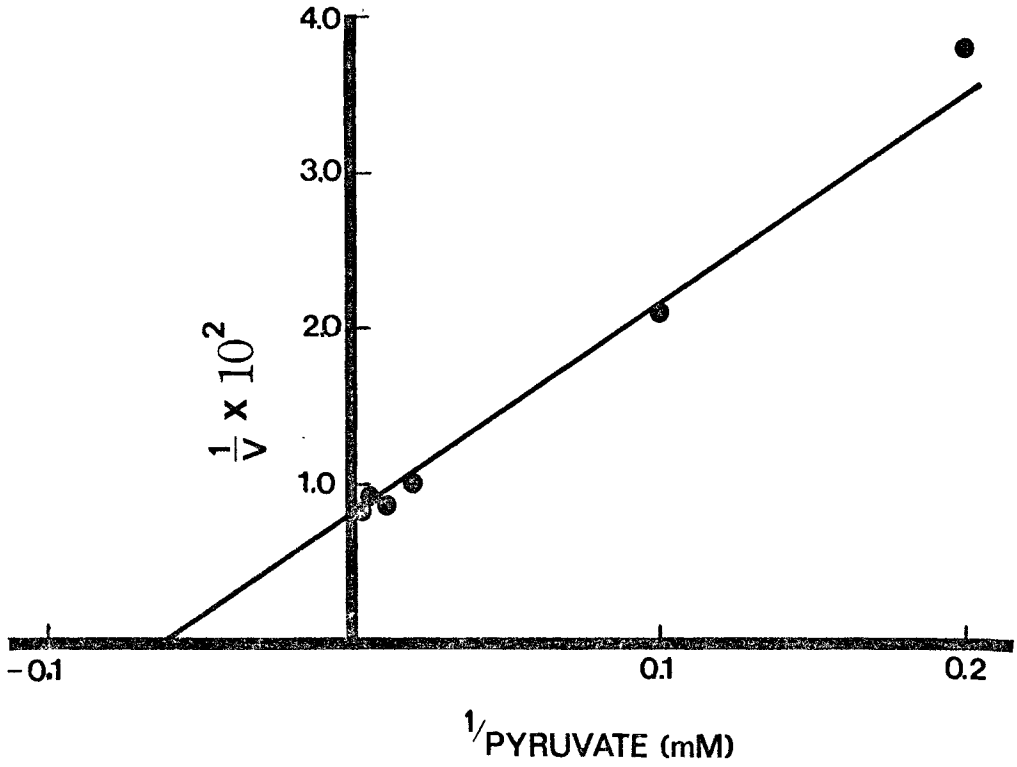


Fig. 10. Double reciprocal plot of initial velocity as a function of pyruvate concentration. Velocity is expressed as µmoles DPNH oxidized per min at 340 mµ. Experimental details described in text.

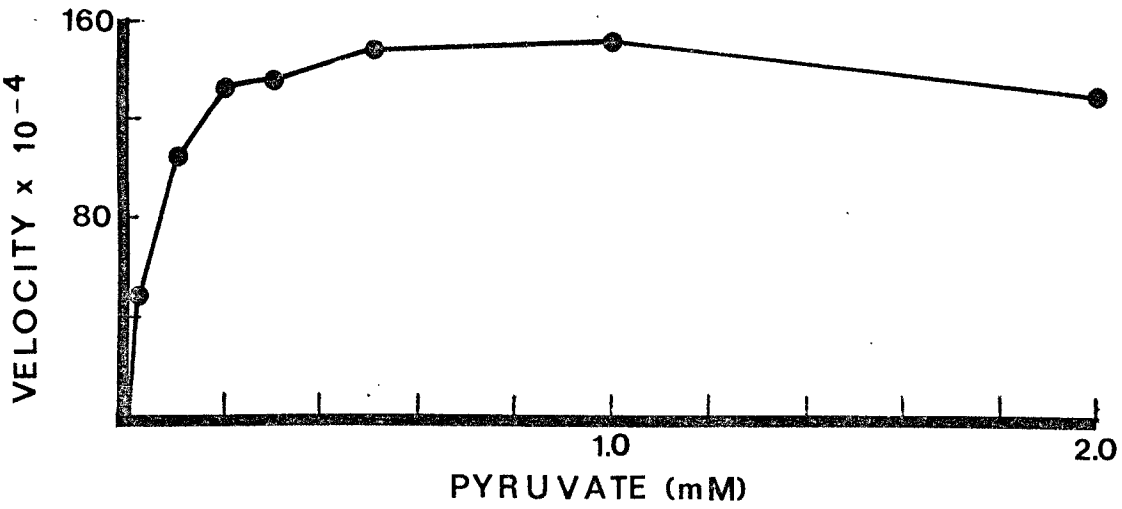


Fig. 11. Plot of initial velocity as a function of pyruvate concentration. The velocity is expressed as μmoles of DPNH oxidized per min at 340 mμ. Experimental details described in text.

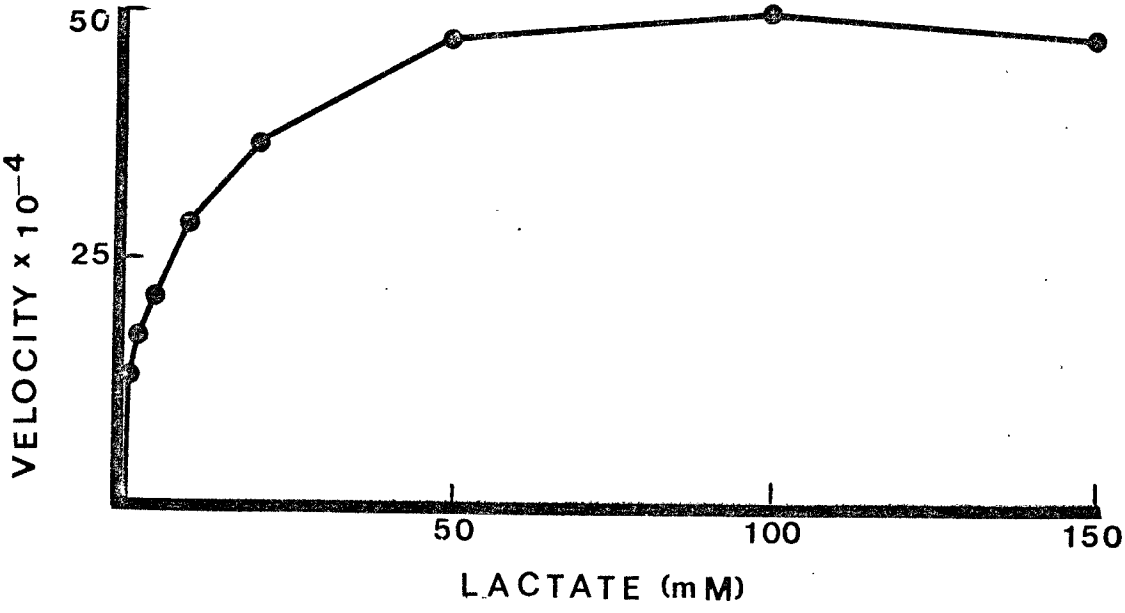


Fig. 12. Plot of initial velocity as a function of lactate concentration. The velocity is expressed as μmoles of DPN reduced per min at 340 mμ. Experimental details described in text.

Plots of rate against substrate concentration yielded rectangular hyperbolas and had the usual characteristics of Michaelis-Menten kinetics (Fig. 12). The K_m for lactate was determined to be 6.3×10^{-3} M (Fig. 13). This K_m for lactate also closely resembles the K_m for lactate of heart lactate dehydrogenase of vertebrate origin (Pesce et al., 1964; Pesce et al., 1967).

Abortive Complex Formation

An experiment was performed to determine if the tapeworm lactate dehydrogenase was capable of forming the abortive ternary complex of pyruvate, DPN, and enzyme. Two samples of crude extract were each incubated at 25° with 2.5 µM pyruvate and 2.0 µM DPN. Two additional samples were incubated at 25° with 2.0 µM DPN. A third set of samples was incubated at 25° containing no pyruvate or DPN. This set served as the control samples. After one hour, the extracts were tested for activity in the reverse direction as previously described. The results are presented in Table II.

TABLE II

EFFECT OF PRIOR INCUBATION WITH DPN

Sample	Percentage of Initial Activity After One Hour
LDH (Control)	100
LDH + DPN	98
LDH + DPN + Pyruvate	39

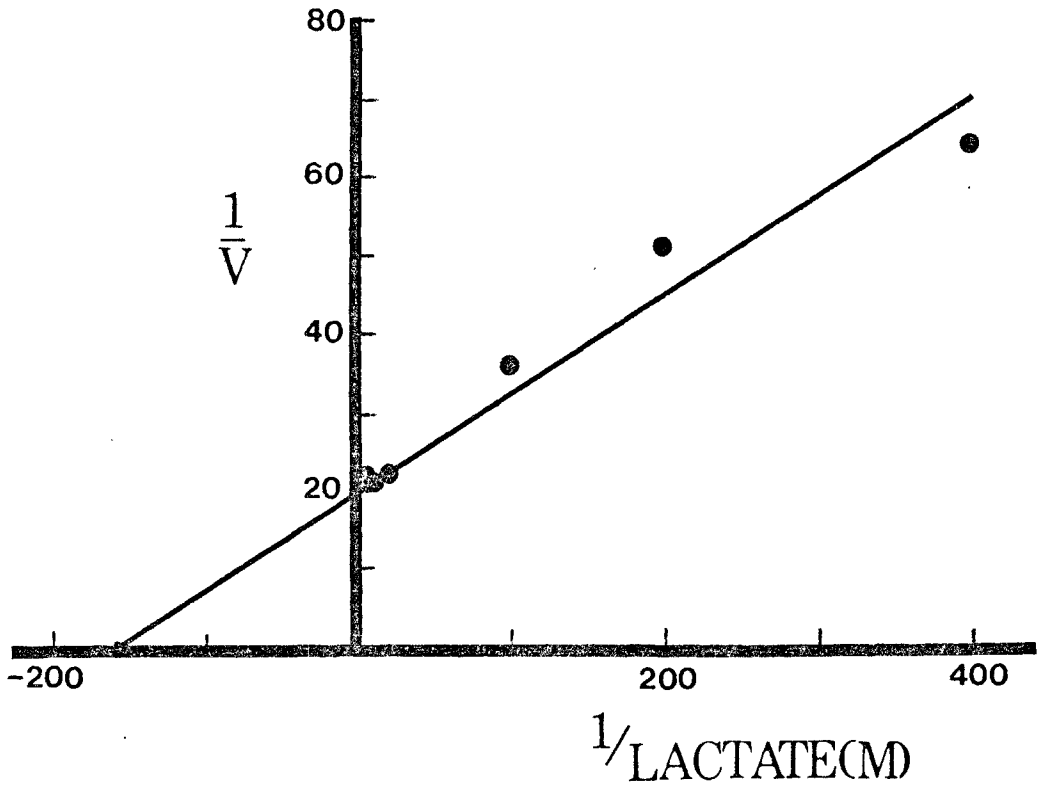


Fig. 13. Double reciprocal plot of initial velocity as a function of lactate concentration. Velocity is expressed as µmoles of DPN reduced per min at 340 mµ. Experimental details described in text.

The control samples demonstrated approximately 2.5 times the activity of the samples incubated with pyruvate and DPN. However, the controls demonstrated no greater activity than did the samples incubated with DPN alone. These data are similar to the results reported by Kaplan et al. (1968).

Inhibitor Studies

N-ethyl maleimide (NEM) and p-chloromercuribenzoic acid (p-CMB) were found to inhibit all lactate dehydrogenase activity in 10-fold-purified extract within 5 min at inhibitor concentrations of 10^{-4} M, as shown on Table III.

TABLE III

NEM AND P-CMB INHIBITION OF LACTATE DEHYDROGENASE

Sample	Activity (% of initial activity) 1 min	5 min
LDH (Control)	100.0	100.0
LDH + NEM	4.6	0.0
LDH + p-CMB	0.0	0.0
LDH + NEM + pyruvate	1.5	0.0
LDH + p-CMB + pyruvate	0.0	0.0
LDH + NEM + DPNH	100.0	91.0
LDH + p-CMB + DPNH	13.5	0.0

Pyruvate provided no protection against either NEM or p-CMB inhibition. DPNH protected the enzyme against total inactivation by NEM for up to 120 min (Fig. 14). Only slight protection was afforded the enzyme by DPNH against p-CMB inactivation. All activity was lost after 10 min in the presence of this inhibitor.

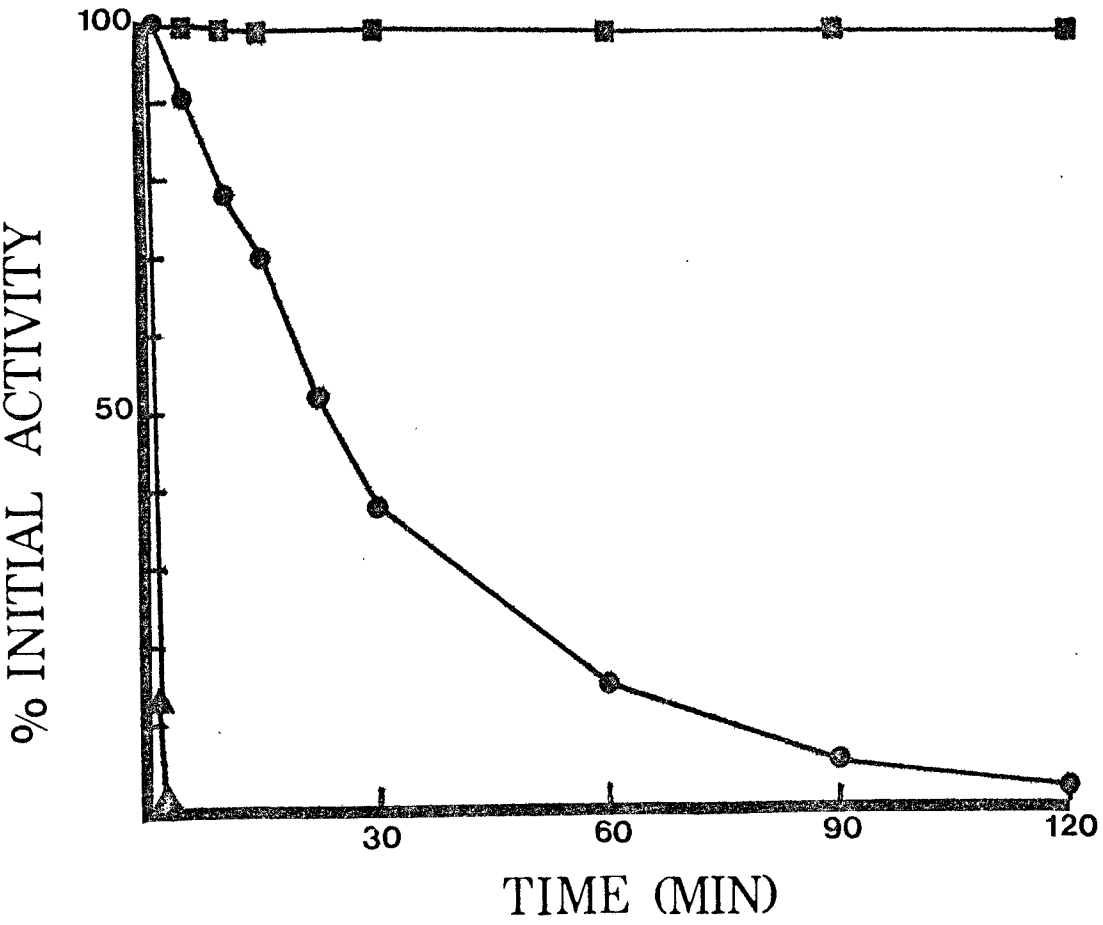


Fig. 14. Effect of NEM inhibition and p-CMB inhibition on DPNH protected enzyme. NEM = (●); p-CMB = (▲); control = (■). Initial reaction velocity, expressed as percentage of maximal activity, is plotted against time. Experimental details described in text.

As shown in Table IV, the addition of 78 mM dithiothreitol to the NEM-inhibited enzyme samples failed to restore activity. However, addition of the dithiothreitol to the p-CMB-inhibited enzyme samples restored as much as 75% of the initial lactate dehydrogenase activity after 30 min.

TABLE IV

RESTORATION OF ACTIVITY WITH DITHIOTHREITOL

Inhibited Sample	% Initial Activity Restored
LDH + NEM	0.0
LDH + NEM + pyruvate.	0.0
LDH + NEM + DPNH.	0.0
LDH + p-CMB	74.0
LDH + p-CMB + pyruvate.	73.8
LDH + p-CMB + DPNH.	75.0

DISCUSSION

The isolation procedure described here results in a 128-fold purification over the homogenate supernatant with a 9% recovery of total activity. This purification represents the first time that this enzyme has been isolated from H. diminuta. Although disc electrophoresis and Sephadex G-200 tests for purity could not be performed due to the small quantity of enzyme resulting from purification, it may be assumed that this enzyme was isolated in pure form. This statement is supported by two facts. First, the catalytic activity and protein appeared together in the last stage of purification. Second, the specific activity of the lactate dehydrogenase purified in this experiment is very similar to the specific activity of lactate dehydrogenase isolated from lobster tail muscle (Kaloustian et al., 1969), crayfish abdominal muscle (Urban, 1969), and rat liver (Hsieh and Vestling, 1966).

During the isolation procedure, several difficulties were encountered. The enzyme could not be purified by the method of Pesce et al. (1967), which is a standard method for lactate dehydrogenase isolation. The enzyme would not bind to the ion exchange celluloses at the ionic strength of the buffers proposed by Pesce et al. Lower ionic

strength was required for the enzyme to bind to the celluloses. Another difficulty was the increasing instability of the enzyme with purification and concentration. It was found that 10^{-3} M dithiothreitol or 2×10^{-3} M 2-mercaptoethanol greatly stabilized the enzyme after purification.

This instability may be due to the dissociation of the active oligomeric protein into subunits. Griffin and Criddle (1970) report that rabbit muscle lactate dehydrogenase dissociates significantly at protein concentrations below 0.15 mg per ml. Markert and Massaro (1968) have shown that dilution of the horse lactate dehydrogenase promotes dissociation into subunits and that this must disrupt the tertiary structure of such subunits because they do not readily reform functional tetramers. During many stages of the isolation procedure the protein concentration fell below this figure of 0.15 mg per ml.

The sedimentation equilibrium studies also provide some evidence of dissociation of the enzyme molecule. A molecular weight for an inactive form of the protein was determined to be approximately one half of the active enzyme molecular weight.

Only one form of lactate dehydrogenase was demonstrated from this tapeworm. This is of interest since isoenzymes of lactate dehydrogenase serve a regulatory function in vertebrates. Dawson et al. (1964) have suggested the following mechanism for isoenzyme regulation in mammals: the pure

H type of lactate dehydrogenase is found predominantly in tissues capable of a highly aerobic metabolism. It has a relatively low turnover number with pyruvate and is maximally active only at low concentrations of this substrate, which inhabits the enzyme strongly at higher concentration. In tissues of this sort, energy is required continuously, and this demand is met by complete combustion of pyruvate in mitochondria. In such tissues, pyruvate does not ordinarily accumulate, but when it does, the sensitivity of lactate dehydrogenase type H to this compound will assure that it is not channeled toward lactate but rather toward glucose formation. The physiology of skeletal muscle requires the release of energy in sudden, discontinuous burst under relatively anaerobic conditions. This energy demand is satisfied almost entirely by glycolysis, and as a result, pyruvate in high concentrations has to be reduced to lactate. The M type lactate dehydrogenase present allows this reaction to occur under these conditions. Once lactate is formed it has to be removed by the circulation, which transports it to tissues where it can be metabolized further and which contain enzymes of the H type.

It should be emphasized that both types of LDH will reversibly catalyze the oxidation of lactate to pyruvate in the presence of DPN. The control of the reaction is in the modification of the enzymes so that they can act in specific environments. In resting, voluntary, striated

muscle, most of the coenzyme is present in the oxidized form. During the course of sudden muscular activity, there is a rapid production of pyruvate. The H type enzyme forms an abortive ternary complex with pyruvate, DPN, and enzyme much more readily than the M type. If an H type enzyme existed in such muscles, then it would be possible for an abortive complex to be formed with the excess of oxidized coenzyme in the muscle. Such a complex would slow the rate of glycolysis by preventing the oxidation of generated DPNH. However, since it is more difficult to produce such a complex with the M type of the enzyme, it is distinctly an advantage to the animal to have the M type lactate dehydrogenase in its voluntary muscles and not the H form. The H type lactate dehydrogenase may be better geared for the oxidation of lactate since heart is known to use lactate readily as a substrate (Kaplan et al., 1968). The H type may function as a lactate dehydrogenase in the heart, whereas the M enzyme may be geared to operate as a pyruvate reductase in voluntary muscle (Everse et al., 1970).

Kaplan et al. (1968) have suggested that the type of lactate dehydrogenase present in a tissue may be related to the rate of glycolysis in the tissue. The H type would function optimally in cells with a constant rate of glycolysis, while the M type would function more suitably in cells with sporadically rapid glycolytic rates.

Multiple forms of lactate dehydrogenase have also been demonstrated in invertebrates, such as lobster (Kaloustian et al., 1968), grasshopper (Aronson, 1968), certain cockroaches (Gilbert and Goldberg, 1966), Ascaris suum (Nagase, 1968), and trypanosomes (D'Alesandro and Sherman, 1964; Bayne and Roberts, 1969). The fact that H. diminuta has only one form of lactate dehydrogenase is interesting in that it may indicate that this animal has lost the ability to produce multiple forms of the enzyme.

Other invertebrates such as certain species of crayfish (Urban, 1969), schistosomes (Conde-del Pino et al., 1966), and amoebas (Tilgner, 1968) possess only one form of this enzyme. Of the many bacteria examined for isoenzymes, none possess more than one form of L-lactate dehydrogenase (Detter and Rapp, 1969; Green and Goldberg, 1967; Stockland and San Clemente, 1969). These data suggest that, with a few exceptions, the ability to produce isoenzymes is characteristic of higher invertebrates and vertebrates. Therefore, it is possible that H. diminuta may never have possessed multiple enzyme forms.

This absence of an isoenzyme regulatory mechanism may also be indicative of a shift in regulatory importance from lactate dehydrogenase to another enzyme in carbohydrate metabolism, such as PEP carboxykinase.

Molecular weight determinations from Sephadex G-200 yielded a value of approximately 75,000 ± 10,000. This

is in agreement with the value obtained from the sedimentation velocity experiment. A similar value was obtained by Long and Kaplan (1968) for a D-lactate dehydrogenase from the horseshoe crab, Limulus polyphemus. Millar (1962) and Hathaway and Criddle (1966) reported a dimer molecular weight of beef L-lactate dehydrogenase of 72,000 which was formed following the dissociation of the tetrameric native enzyme. Chilson et al. (1966) have demonstrated, however, that this dimer is catalytically inactive.

Sedimentation equilibrium studies indicated a value of 39,000 for the molecular weight of the enzyme. However, because this value represents the molecular weight of an inactive form of the enzyme, it may represent the subunit molecular weight. This figure would be in agreement with a subunit molecular weight of an active dimer with molecular weight of 78,000.

Since the enzyme apparently dissociates rather readily, the question of the accuracy of the gel filtration method of molecular weight determination arises. Mammalian lactate dehydrogenases dissociate readily upon dilution (Griffin and Criddle, 1970). If a similar phenomenon occurs with the tapeworm enzyme, as the data indicate, then the molecular weight measured by gel filtration could be a value for a partially dissociated tetrameric lactate dehydrogenase. However, the evidence does not support this possibility. No enzyme activity whatsoever could be detected at elution

volumes which represented molecular weights above 100,000. It would seem probable that, considering the relatively large amount of enzyme used for the determination (25 units), some catalytic activity would be found at an elution volume corresponding to a molecular weight of approximately 150,000, indicating the tetrameric weight. Markert and Massaro (1968) reported the disruption of tertiary structure as a result of dissociation induced by dilution. For this reason, it would seem unlikely that dissociated subunits would reassociate into a functional enzyme. At no time during the purification was any increase in activity noted following concentration of the diluted enzyme.

It is, therefore, postulated that the active form of lactate dehydrogenase from H. diminuta is a dimer with a molecular weight of approximately 78,000. Although a dimeric D-lactate dehydrogenase with a molecular weight of approximately 69,000 has been reported from the horseshoe crab by Long and Kaplan (1968) and Selander and Yang (1970), this is the first reported L-lactate dehydrogenase to occur in dimer form. This lactate dehydrogenase from H. diminuta may represent an ancestral form of the modern vertebrate tetramer.

The Michaelis constants for this enzyme indicate that it is kinetically similar to the H form of mammalian lactate dehydrogenase (Pesce et al., 1964; Pesce et al., 1967). The cestode enzyme also exhibits some substrate inhibition

by pyruvate as does the H form of the mammalian enzyme (Stambaugh and Post, 1966). The mechanism by which excess pyruvate inhibits lactate dehydrogenase may be related to the formation of an abortive ternary complex between pyruvate, DPN, and the enzyme (Kaplan et al., 1968; Gutfreund et al., 1968). In the tapeworm, DPNH formed from glucose is generally oxidized by systems other than lactate dehydrogenase, such as malate dehydrogenase.

Bueding and Saz (1968) have demonstrated that the pyruvate kinase activity in H. diminuta is only one-fifth as active as the PEP carboxykinase activity in the worm. As a result, little pyruvate is produced in glycolysis while the PEP carboxykinase is fully active. In this way the phosphoenolpyruvate can be metabolized to succinate with the production of ATP. The pyruvate that is produced by pyruvate kinase may be prevented from being converted to lactate by the formation of an abortive ternary complex of pyruvate, DPN, and enzyme.

The ability of the cestode enzyme to form this abortive complex has been demonstrated. The pyruvate conserved in this manner could be used as a substrate by other enzymes in the tapeworm, such as glutamate transaminase.

However, when PEP carboxykinase activity is decreased, perhaps by a decrease in CO_2 concentration, there may be an accumulation of DPNH due to lack of substrate for malate dehydrogenase. At that time, the accumulated DPNH could

displace the abortive ternary complex and allow for the reduction of pyruvate to lactate as well as for the oxidation of DPNH. This would be similar to the regulatory operation of the H form of lactate dehydrogenase in aerobic mammalian tissue (Everse et al., 1970).

The inhibition studies using p-chloromercuribenzoate (p-CMB) and N-ethyl-maleimide (NEM) demonstrate the importance of two amino acids in the active site of this enzyme. It is well established that lactate dehydrogenases from mammalian sources contain 4 cysteine residues, and that when they are blocked by organic mercurials (Di Sabato and Kaplan, 1964) or by various maleimide derivatives (Holbrook and Pfleiderer, 1965), the protein loses its enzymatic activity. The importance of histidine residues in the active site of this enzyme have been indicated by Winer and Schwert (1959) and Woenckhaus et al. (1969).

Two reagents, p-CMB and NEM, have been traditionally used as sulfhydryl reagents. Inactivation of lactate dehydrogenase by these reagents has been attributed to blocking of an essential thiol group in the active site. However, both p-CMB (Barnard and Stein, 1958) and NEM (Smyth et al., 1960) react with the imidazole group of histidine. DPNH protects the essential thiol group against NEM (Holbrook and Stinson, 1970) and p-CMB (Holbrook, 1966). Adams et al. (1970) report that the essential thiol is located opposite the coenzyme binding site on the edge of

the central catalytic celft of the lactate dehydrogenase subunit. The action of NEM and p-CMB is to prevent the binary enzyme-nucleotide complex from binding substrate (Holbrook and Stinson, 1970). Since NEM- and p-CMB-inhibited lactate dehydrogenases are still capable of binding DPNH (Allison, 1968), it may be that NEM and p-CMB irreversibly modify an imidazole group of histidine. An imidazole group has been suggested by Winer and Schwert (1959) to be responsible for pyruvate binding.

The fact that pyruvate alone was unable to protect the enzyme from NEM and p-CMB inhibition can be explained if an obligatory binding order of coenzyme followed by substrate is assumed for cestode lactate dehydrogenase as it is for vertebrate lactate dehydrogenases (Gutfreund et al., 1968). The partial protection provided by DPNH to the enzyme against NEM and p-CMB inhibitions can be explained if it is assumed that the cestode enzyme is similar at the active site to vertebrate lactate dehydrogenases. Adams et al. (1970) report that the binding site for DPNH is at the edge of the catalytic cleft. If this is the case, the DPNH may protect simply by sterically hindering the inhibitors' entrance to the active site.

No activity could be restored to NEM inhibited enzyme by dithiothreitol. This could be explained by either of two possibilities. NEM binds essentially irreversibly and rapidly to cysteine and histidine (Smyth et al., 1960).

If either or both of these residues are important for catalytic activity, the irreversible binding of NEM to these amino acids could not be reversed by dithiothreitol.

As much as 75% of the initial activity of the p-CMB inhibited enzymes could be restored by the dithiothreitol. The inhibitor, p-CMB, also reacts with both cysteine and histidine (Barnard and Stein, 1958). The reaction of p-CMB with the sulfhydryl groups of proteins can be reversed by reducing agents. This explains the partial restoration of activity. However, the fact that only partial activity was restored can be explained by the slower reaction of p-CMB with histidine. During the 120 min time period, it is possible that some of the histidine groups in the active sites could have been irreversibly modified by p-CMB. The failure of dithiothreitol to restore all activity may also be due to irreversible denaturation of the protein by p-CMB (Riordan and Vallee, 1967).

It is therefore postulated that the H. diminuta lactate dehydrogenase has catalytically essential amino acids in its active site which are similar, or identical, to the catalytically essential amino acids in the active sites of vertebrate lactate dehydrogenases.

SUMMARY

Lactate dehydrogenase of Hymenolepis diminuta has been purified by heat treatment and column chromatography. The purified enzyme has a specific activity of 106 units per mg protein.

The pure enzyme has a molecular weight of 75,000 as determined by Sephadex gel filtration and sedimentation velocity. This value is approximately one half the reported molecular weight of tetrameric lactate dehydrogenases. An equilibrium ultracentrifugation study suggests a subunit molecular weight of 39,000, which is similar to published values for the subunit molecular weights of tetrameric lactate dehydrogenases. From these data, a dimer form of the native enzyme is proposed.

Only one molecular form of lactate dehydrogenase was demonstrated by cellulose acetate and polyacrylamide gel electrophoresis performed on crude extract.

The enzyme is very similar in all kinetic properties examined to the H type of the vertebrate lactate dehydrogenase. The reaction is slightly inhibited by high pyruvate concentrations. The enzyme is specific for L-lactate and DPN and not reactive with DPNH.

Inhibition studies using N-ethyl maleimide and p-chloromercuribenzoate indicate the possibility of an

essential histidine and/or cysteine residue(s) in the active site of the enzyme.

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