PHYSICAL AND KINETIC PROPERTIES OF LACTATE DEHYDROGENASE IN THE HEART, LEG AND CLAW TISSUES OF THE DUNGENESS CRAB CANCER MAGISTER DANA

by

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TABLE OF CONTENTS

ILLUSTRATIONS

INTRODUCTION

Most organisms, vertebrate and invertebrate, can convert glucose to a more readily utilizable form of energy. Lactate dehydrogenase (LDH) is one of the glycolytic enzymes that facilitate this process. Specifically, LDH catalyses the reversible reaction

$$\text{pyruvate} + \text{NADH} \rightleftharpoons \text{lactate} + \text{NAD}^+$$

which is the determinant step in the production of lactate by anaerobic glycolysis. The $\Delta G^{o'}$, the standard free energy change at pH 7.0 and $0^{o}C$, for this reaction is -6 Kcal/mole, meaning that the overall equilibrium for this reaction is far to the right. The final concentrations of lactate and pyruvate however will be determined by the intracellular environment of the cells in the tissue under consideration. If a group of cells in a tissue experiences a decrease in oxygen tension, or a decrease in pH, or an increase in pyruvate concentration, or a combination of these events, the above reaction will produce more lactate. If the same group of cells experiences an increase in oxygen tension, or an increase in pH, or a decrease in pyruvate concentration, or a combination of these events, then the reaction will favor production of more pyruvate.

LDH is found in most organisms in a variety of tissues, such as the heart, liver, limbs, nervous system, eye lens, and many others (Kaplan, et al, 1960, Kaplan & Ciotti, 1962, Kaplan, et al, 1963, Fonner, et al, 1969, Horowitz & Whitt, 1972). In all organisms studied

to date the enzyme is either a dimer or a tetramer of subunits, whose molecular weights are about 35,000. Throughout the first 25 years of study on LDH, it was assumed that the enzyme always consisted of four subunits, but recently several organisms have been shown to possess an active enzyme consisting of only two subunits (Long & Kaplan, 1968). In either case, tetramer or dimer, the subunits are of two types, designated A and B, or H and M. The latter nomenclature came into use when it was found that one homotetramer (H_4) occured mainly in heart tissue, and the other homotetramer (M_4) occured mainly in peripheral muscle (Kaplan, 1964). These two types of LDH, H_4 and M_4, are chemically and electrophoretically distinguishable, as are the three possible heterotetramers H_3M, H_2M_2, and HM_3 (Wilson, et al, 1963, Fondy & Kaplan, 1965). Often individual cells of a tissue produce both subunits, resulting in five possible combinations, or isozymes of LDH in the cells of that tissue. The number and type of isozymes present in a tissue is largely dependent on the type of environment surrounding the tissue. If the normal flow of energy in a tissue is through aerobic metabolism, as it is in the heart of most organisms, then the isozyme(s) present are those with mostly H subunits (H_4 and H_3M). Whereas, if the normal flow of energy in a tissue is through anaerobic metabolism, then the predominant isozyme(s) present are those with mostly M subunits (M_4 and M_3H) (Wilson, et al, 1963, Dawson, et al, 1964, Fondy & Kaplan, 1965).

Although the presence of five isozymes is the common finding in the various organisms studied (Markert & Møller, 1959), only one form

has been found in many flatfishes (Kaplan, 1964), while as many as 14 forms have been found in the brook trout, Salvelinus fontinalis (Hochachka, 1966). In order for a tissue to possess more than five isozymes, there must be multiple forms of one or both of the basic subunits.

Early studies on LDH showed that the enzyme specifically catalysed only the oxidation of L-lactate. Recently it was demonstrated that several invertebrates in different phyla possess a D-lactate specific enzyme (Long & Kaplan, 1968, Selander & Yang, 1970, Gleason, et al, 1971, Long & Kaplan, 1973). Although there is still some controversy over the molecular weight of LDH found in Limulus polyphemus, the generalization seems to be that all mollusks, polychaete annelids, and chelicerate arthropods studied to date possess a D-lactate specific enzyme which is a dimer (m.w. 70,000) and that most of the other groups possess an L-lactate specific enzyme which is a tetramer (m.w. 140-150,000). Exceptions to this "rule" are 1) the tetrameric D specific LDHs of all barnacles studied to date[1], 2) the sea urchin Arbacia lixula, which possess an enzyme that can oxidize both isomers of lactate at equal rates (Hammen & Lum, 1972), and 3) the two distinct forms of LDH in Lactobacillus plantarum, one that is D-lactate specific and one that is L-lactate specific (Dennis & Kaplan, 1960).

The catalytic properties of LDH have been studied mainly in vertebrate organisms; not many invertebrate LDHs have been studied in detail. A summary of the properties of vertebrate and some invertebrate LDHs

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[1] Chthamalus depressus, however has an L-lactate specific LDH (Hammen 1969).

are given in Table 1. Basically, properties such as the optimal pyruvate concentration, the Michaelis-Menten constant (K_M), and the concentration of pyruvate causing significant inhibition are distinctive for closely related organisms. These properties are a function of the subunit composition of the active enzyme. One of the distinguishing differences among the homotetramers found in the vertebrates is their relative tolerances of high concentrations of pyruvate. In Table 1 it can be seen that the H_4 isozyme operates optimally at much lower concentrations of pyruvate than does the M_4 isozyme. The same relationship is found when lactate is the substrate.

The effect of temperature on the catalytic properties of LDH has been studied mainly in fish. At alkaline pH values, above about 7.5, the K_M for pyruvate increases with temperature, but at lower pH values the K_M for pyruvate is essentially independent of temperature within the normal habitat range of the organism (Hochachka & Lewis, 1971, De Burgos, et al, 1973). At lower pH values the K_M has a minimum near the lower end of the organism's thermal range and increases at temperatures outside of this range (Hochachka & Somero, 1968, Somero, 1973). At low pyruvate concentrations, i.e. those close to physiological conditions, it appears that the enzyme-substrate affinity is more important than the effect of other thermodynamic factors on the reaction velocity (Hochachka & Somero, 1968, De Burgos, et al, 1973). At these low pyruvate concentrations, the enzyme is operating at a concentration of substrate below the K_M for that substrate; then an increase in enzyme-substrate affinity compensates at least partially for the decrease in thermal motion of the reactants. It has also been noted that the

pH of body fluid of poikilotherms varies inversely with temperature (Rahn, 1965). A decrease in temperature brings about an increase in pH, which by itself, would cause an increase in K_M (Hochachka & Lewis, 1971). But the same decrease in temperature causes a temperature-dependent decrease in K_M, so these two effects would tend to offset each other. Hence the reaction rate would remain constant, as long as the temperature was within the normal environmental range for that organism (Somero, 1969). These effects have been shown to occur in long term acclimation in the rainbow trout, Salmo gairdnerii, and the brook trout, Salvelinus fontinalis (Somero & Hochachka, 1969, Hochachka & Lewis, 1971).

A different mechanism has been postulated for the short term compensation in the Alaskan king crab, Paralithodes camtschatica. It has two kinetically active isozymes: one with a high K_M for pyruvate in the 10°- 15°C range and one with a low K_M for pyruvate in the same temperature range. At normal habitat temperatures, 0°- 5°C, both LDHs are active at physiological pyruvate concentrations. But at 10°- 15°C, the "high K_M" LDH is no longer active, since its K_M is two orders of magnitude greater than the pyruvate concentration in the cell. Therefore, the "low K_M" LDH is active at all temperatures, while the "high K_M" LDH is active only at normal habitat temperatures (Somero & Hochachka, 1969).

The Dungeness crab, Cancer magister Dana, is found in the shallow water of bays and estuaries and in the open ocean to a depth of 100 meters from Unalaska to Magdalena Bay, Baja California. This crab has been reported to prefer sand and mud bottoms (Schmitt, 1921). In early

summer large numbers of juvenile crabs have been found completely buried in the sand, leaving only their eyes and antennae exposed (McKay, 1942). A small cavity is left in front of the crab for water circulation. The sides of the carapace and the legs are covered with fine hairs, which filter out fine sand particles in the inhalent stream. Juvenile crabs will also burrow in the sand when disturbed. At low tide mature C. magister have been personally found stranded on mud flats, exposed to the ambient air temperature. The average habitat temperature for C. magister in Coos Bay is 10°C, with a range of 5°- 20°C over the period of a year (Oregon Fish Commission records). Since this animal is a large and abundant ectotherm, experiencing a wide temperature change in its natural environment, it might prove to be an interesting organism to investigate some structural and kinetic properties of LDH.

The following questions were asked in this thesis: 1) What are some of the structural properties of the LDH of C. magister, a) what is the molecular weight of the catalytically active form, b) what is the molecular weight of the smallest subunit-- is the enzyme a dimer or a tetramer, c) what is the isozyme composition of heart and peripheral muscle tissues; 2) What are some of the kinetic properties of the LDH enzyme, a) what stereospecificity for lactate does this enzyme possess, b) how do the apparent K_M values for pyruvate for the enzyme isolated from heart and peripheral muscle compare, c) do the LDHs isolated from these tissues incur substrate inhibition at high pyruvate concentrations, d) does the enzyme from these tissues follow Michaelis-Menten kinetics; 3) How does temperature affect the apparent K_M for pyruvate

of the LDHs from heart, leg, and claw tissues? The physical, chemical, and kinetic properties of Cancer magister LDH are compared with the corresponding properties of LDHs from crustaceans and other organisms.

MATERIALS AND METHODS

Male Cancer magister Dana were caught in Coos Bay and kept in sea water tanks until used. The holding tank water temperature was not monitored throughout the temperature experiments; however, since the experiments were performed during winter months (November through January), the holding tank temperature range can be estimated. The temperatures recorded in the lower Coos Bay by the Oregon Fish Commission for the period November 1, 1973 to January 31, 1974 ranged between 7.6°C and 11.6°C, with an average of about 10.3°C. The temperature of the holding tank water was measured at 4.3°C after a cold spell in early January, while the water temperature in the bay was measured at 6°C (surface) and 8.5°C (bottom)(personal measurements). The holding tank temperature during the temperature experiments, then, could have ranged from 4°C as a minimum to 12° or 13°C as a maximum. Fluctuations from day to day were not determined.

Individual legs and claws were removed through autotomy; the muscle was excised and placed in a mortar resting in an ice bucket. Sand was added to the mortar along with a volume of buffer (in mls) equal to twice the weight of the muscle. The buffer used was 0.1 M $NaPO_4$, pH 7.4, and 0.1 M in NaCl. Hearts were removed from live crabs and homogenized immediately in the same manner as the leg and claw tissues. The homogenate was then spun in a Sorvall RC2-B refrigerated centrifuge at 15,000 g for 10- 15 minutes. The supernatant was used

directly as a crude preparation of LDH.

The enzyme was partially purified by bringing the supernatant slowly to 35% saturation with ammonium sulfate and then slowly to 65% saturation with ammonium sulfate. The sample was centrifuged at 15,000 g for 15 minutes after each addition. After centrifugation of the 65% saturated solution, all of the enzyme activity was contained in the precipitate, which was redissolved in a minimum amount of 0.1 M $NaPO_4$, pH 7.4, and 0.1 M in NaCl. The enzyme solution obtained in this manner will be referred to as the purified preparation.

Enzymatic activity was determined by following the oxidation of β-Diphosphopyridine nucleotide (NADH) at 340 nm on a Perkin-Elmer DB recording spectrophotometer. The reaction mixture contained 0.1 ml of 2.56 mM NADH, 0.1 ml of an LDH sample, 0.1 ml of pyruvate of varying concentrations, and 1.7 mls of 0.1 M $NaPO_4$, pH 7.4, and 0.1 M in NaCl. All reaction components were kept on ice before being used.

The temperature study of the pyruvate to lactate reaction was accomplished by using a Haake temperature regulation unit and a thermal cell holder to control the temperature of the reaction mixture. The reaction mixture was the same as that described above. First 1.7 mls of 0.1 M $NaPO_4$ buffer was allowed to equilibrate to the chosen assay temperature. The NADH and LDH aliquots were then added. The reactants were mixed, the cuvette was placed in the thermal cell holder, and the recorder was activated to indicate the presence of other dehydrogenases. Finding no oxidation of NADH, the reaction was started by the addition of 0.1 ml of pyruvate, varying in final concentration from 10^{-5} M to 10^{-2} M. The initial velocities were obtained by drawing a

straight line tangent to the beginning of the recording for that assay.

Five separate experiments were performed on both crude and purified preparations of leg, claw, and heart tissues at 10°, 15°, and 20°C. All experiments conducted at a given temperature for either the crude or the purified form of the enzyme of all tissues were done on different preparations, e.g. once a given enzyme solution was used at a given temperature, it was not used again to do the same run in the same state of purity. The Michaelis-Menten constant, K_M, and the maximum velocity were obtained from Eadie-Hofstee plots of the data.

The stereospecificity of the enzyme was determined by monitoring the reaction converting lactate to pyruvate. The reaction mixture contained 1.7 mls of 0.1 M $NaPO_4$, pH 7.4, and 0.1 M in NaCl, 0.1 ml of the enzyme preparation (crude or purified), and one of the two following pairs of reactants: 1) 0.1 ml of 8×10^{-1} M lactate (D, L, or DL isomer) plus 0.1 ml of 8×10^{-2} M NAD^+, or 2) 0.1 ml of 4×10^{-1} M lactate (D, L, or DL isomer) plus 0.1 ml of 4×10^{-2} M NAD^+. These assays were carried out at room temperature (20°C). The lactate isomer was added last to start the reaction.

The pH dependency of the pyruvate to lactate reaction was studied by homogenizing five legs from the same crab in 0.1 M Tris-HCl, pH 7.6, and 0.1 M in NaCl. Before removing the legs, the hemolymph was collected from the live crab. The pH was measured at 10°C (holding tank temperature was 9.5°C). After spinning the remove cell debris, the supernatant was used as a source of the enzyme. 4.5 ml aliquots of the enzyme were titrated to the following pH values: 6.0, 6.5, 7.0, 7.6, 8.0, 8.6, 9.0. The volumes were then adjusted to 10.0 mls with

distilled water; the pH values were checked again and adjusted where necessary. The titrations were done one at a time so that each enzyme solution could stand for 10 minutes on ice at the adjusted pH before the assays were performed. Three reactions were run at each of the above pH values, using 0.5, 2.5, and 5.0 mM pyruvate. The pyruvate and NADH solutions were made in 0.1 M Tris-HCl at the above pH values. The reactions were carried out in the respective 0.1 M Tris-HCl buffer. The pH below 7.0 was closely monitored. All assays were carried out at 10°C using the same reactant volumes as described above.

Beef heart LDH was obtained from Sigma Chemical Company. The assays for the inhibition study were performed as described above, except that varying amounts of pyruvate were used, while keeping the assay volume constant at 2.0 mls. All assays with the beef heart LDH were performed at room temperature (22°C).

The statistical analysis of the effect of temperature on the apparent K_M for pyruvate was done by applying an F-test to a single classification analysis of variance, as described by Sokol and Rohlf (1969).

The molecular weight of the native LDH molecule was obtained using a 1.8 x 50 cm column of Sephadex G-200 (Fine) equilibrated with 0.1 M $NaPO_4$, pH 7.4, and 0.1 M in NaCl. The following calibrants were passed through separately or two at a time: blue dextran (m.w. ca. 2×10^6), catalase (m.w. 195,000 (Andrews, 1965)), rabbit muscle aldolase (m.w. 160,000), beef heart LDH (m.w. 131,000), bovine serum albumin (m.w. 68,000), catalase subunit (m.w. 60,000), α-chymotrypsinogen (m.w. 25,700), and sperm whale myoglobin (m.w. 17,800). 3.2 ml

fractions were collected in the cold for both the calibrants and the LDH samples. The LDH peak was located by monitoring the oxidation of NADH at 340 nm as described above.

To obtain the molecular weight of the LDH subunit, heart and leg samples were first purified as follows: The 65% ammonium sulfate saturated enzyme (the "purified" preparation) was dialysed in 1 liter of 0.01 M Tris-HCl, pH 7.6, with two changes. The sample was then applied to a 1.8 x 50 cm column of Sephadex G-200 equilibrated with the same Tris-HCl buffer. 3.4 ml fractions were collected and the enzyme peaks were located using the assay method described above. The peak samples were pooled and applied to a 1.8 x 10 cm column of DEAE-Cellulose in the same Tris-HCl buffer used earlier. 3.4 ml fractions were collected, using 165 mls of 0.01 M Tris-HCl buffer as the eluent. At this point no LDH activity was detected in the fractions. A gradient was then started with 100 mls of 0.1 M NaCl in 0.01 M Tris-HCl, pH 7.6, in one reservoir and 100 mls of 0.01 M Tris-HCl, pH 7.6, in the other reservoir.

The elution peak from the DEAE-Cellulose column was pooled. After lypholysing the sample the crystals were dissolved in distilled water and dialysed exhaustively against 500 mls of distilled water. The sample was lypholysed again; the resulting 2mg of material was used as a source of the LDH subunit.

The subunit molecular weight of C. magister LDH was determined by sodium dodecyl sulfate (SDS) electrophoresis as described by Weber and Osborn (1969), with the following changes: 1) Instead of incubating the proteins at 37°C for two hours, the LDH sample and the standards

were incubated at 100°C for three minutes and then dialysed for two hours (Pringle, 1970), and 2) the gels were destained for several days without the aid of electrophoresis. The standards used for this determination were ovalbumin (m.w. 43,000), pepsin (m.w. 35,000), and sperm whale myoglobin (m.w. 17,800).

Disc gel electrophoresis was performed according to Davis (1964), without spacer gels. The samples were applied directly to the 7% gel. Human serum was obtained from Kaiser Hospital in North Bend, Oregon. Purified heart, leg, and claw samples were electrophoresed concurrently, in separately tubes. Samples from different tissues were not electrophoresed together in the same gel. All experiments were performed in the cold.

Starch gel electrophoresis was performed using the procedures of Fine and Costello (1963). Heart, leg, and claw tissues were homogenized in a pH 7.0 buffer of 32 mM Na_2HPO_4 and 3 mM citric acid. After centrifugation, the crude supernatant was used as a source of the enzyme. Beef heart LDH, mentioned earlier, was diluted 1:100 using the same phosphate-citrate buffer. Thirteen grams of hydrolysed starch was dissolved in 100 mls of a pH 7.0 buffer of 56 mM Na_2HPO_4 and 9 mM citric acid. Electrophoresis was carried out for 16 to 23 hours in the cold. Half of the gel was stained for LDH activity using the stain described by Dietz and Lubrano (1967); the other half of the gel was used as a control to locate "nothing" dehydrogenases, by omitting lactate from the staining solution.

RESULTS

Molecular Weight

The molecular weight of LDH from C. magister leg and heart tissues was determined using a Sephadex G-200 column. In Fig. 1 the elution volumes obtained for the standards and the LDH samples were plotted versus the log of the respective molecular weights. A 65% saturated sample from leg tissue produced an elution pattern as shown in Fig. 2.

The results of two separate experiments indicate that the apparent molecular weight of lactate dehydrogenase in Cancer magister is 137,000.

The molecular weight of the LDH subunit was determined by SDS electrophoresis. Fig. 3 depicts the mobilities of the LDH subunit and several standards plotted as a function of the log of the molecular weight. The results of two separate experiments indicate an apparent subunit molecular weight of 40,000 for C. magister LDH.

DEAE-Cellulose chromotography

Figure 4 shows the elution profile for LDH from C. magister heart tissue on DEAE-Cellulose. Figure 5 is the elution profile for LDH from C. magister leg tissue. In both tissues there is one main isozyme, with a possible "second" isozyme present in the leg preparation. The minor peak in the leg LDH elution profile, however, contains only 5% of the total activity eluted for that leg sample. The total activity present in the heart sample LDH elution peak is approximately 13% of

that present in the main peak for the leg sample. The same wet weight of leg and heart tissue were used for these experiments.

Starch gel electrophoresis

Electrophoresis of crude heart, leg, and claw samples of LDH from C. magister at pH 7.0 and pH 8.0 produced only one band. Fig. 6 is a sketch of an experiment performed at pH 8.0. The isozyme from all tissues migrated the same distance, hence they appear to be the same isozyme. To demonstrate the separation ability of this technique, beef heart LDH was electrophoresed along with a crude leg LDH sample. Fig. 7 is a sketch of the results of this experiment. It is clear that this technique can separate different LDH enzymes. The C. magister LDH isozyme migrated 38% of the distance traversed by the beef heart LDH.

Disc gel electrophoresis

Purified samples from heart, leg, and claw tissues produced only one band in the gels. When human serum was electrophoresed at the same time as purified heart and claw samples from C. magister, the human serum LDH separated into four bands, as found by Dietz, et al (1970), while C. magister heart and claw tissue samples again displayed a single band. No results obtained could suggest if the single bands found for all C. magister tissue preparations were indeed the same isozyme.

pH effect on the reaction rate

Figure 8 depicts the pH dependency of the pyruvate to lactate reaction, assayed at 10°C, for a crude preparation of C. magister leg tissue. The initial velocity was fairly constant over the pH range 6.0 to 7.6 using 0.5 to 5.0 mM pyruvate as the substrate. The decrease in activity at pH values above 8.0 and the absence of activity at pH 9.0 was observed at each pyruvate concentration used. The pH of the hemolymph of the crab used for this experiment was 8.4 (measured at 10°C in vitro).

Temperature effect on apparent K_M for pyruvate

The results of the relationship between the apparent K_M and temperature are given in Figures 9 and 10. Each point is an average apparent K_M, with the standard error represented by the error bars. Although most of the plots suggest an increase in apparent K_M for pyruvate with an increase in temperature over the range studied, none of these trends are statistically significant at the $P = .05$ level. From this data it appears that the apparent K_M for pyruvate of C. magister LDH is temperature independent over the normal habitat range. The data from the three purified tissue preparations, when assayed at 10°C, do show a highly significant difference ($P < .005$) in apparent K_M; whereas the crude tissue preparations do not show any statistical difference, when assayed at 10°C. The enzyme isolated from all tissues studied exhibited hyperbolic Michaelis-Menten kinetics. This applies to both crude and purified preparations.

Stereospecificity

The activity of the leg and heart tissue preparations with the D- and L-lactate isomers is summarized in Table 2. These are the results of the assays using the higher concentrations of the reactants, lactate and NAD^+. The assays using the lower concentrations of these reactants gave the same results. It is apparent that the enzyme present in the leg and heart tissues are specific for L-lactate. The slight activity with the D isomer in the crude preparations could be due to residual L-lactate in the solution from the ruptured cells, since no activity was found in the purified preparations. The amount of activity found with the L isomer was not always consistent with that found using the DL mixture. This may be due to the hydroscopic nature of the L-lactate crystals which made exact weighings difficult.

Substrate inhibition

Inhibition by high pyruvate concentrations on the reaction rate was found by examining the data obtained from the temperature dependency experiments. It was noted that the maximum rate was usually obtained by the assay using 5×10^{-3} M pyruvate. So, the reaction velocity found using 10^{-2} M pyruvate divided by the reaction velocity found using 5×10^{-3} M pyruvate gives the percent of the maximum rate obtained using 10^{-2} M pyruvate. Out of a total of 47 K_M determinations, 55% of these experiments showed inhibition. But, of those runs showing some inhibition, the average percent inhibition, e.g. one minus the 10^{-2} M to 5×10^{-3} M ratio, was only 5.2, 7.8, and 7.3% for the claw, leg, and heart tissues respectively. The averages were computed for each tissue

by combining the results of the crude and purified preparations at the three experimental temperatures.

The purified preparations of all tissues showed a decrease in the amount of inhibition with an increase in temperature. Further, the claw and leg preparations showed no inhibition at 15° and 20°C, while the heart samples showed only 3.3% and 2.6% inhibition at 15° and 20°C respectively. However, only 37% of the experiments with the purified heart preparation showed any inhibition at these temperatures. It can be stated that there was essentially no inhibition in the purified sample at 15° and 20°C. At 10°C the purified leg preparation exhibited 2% inhibition and purified heart preparation exhibited 8% inhibition. Actual experiments that display these inhibition patterns are shown in Fig. 11 and Fig. 12. Also included in Fig. 11 are results of a series of assays personally performed using the beef heart isozyme. Notice the percent inhibition incurred by pyruvate concentrations above 1.0 mM by the beef heart isozyme.

In Fig. 12 the same two plots of C. magister LDH from leg and heart tissues are shown along with plots of heart and muscle isozymes from Limulus polyphemus (Long & Kaplan, 1968). Again,notice the inhibition exhibited by the Limulus heart isozyme and the lack of inhibition in the Limulus muscle isozyme and C. magister enzyme. The plots for the enzymes from C. magister leg and heart tissues and for Limulus muscle tissue are represented by one line in both figures, since the kinetic parameters of the three preparations are almost indistinguishable from each other.

DISCUSSION

Lactate dehydrogenase isolated from the heart and leg tissues of the Dungeness crab, Cancer magister Dana, has an apparent molecular weight of about 137,000. LDH found in the tail muscle of Homarus americanus has a molecular weight of 143,000 (Kaloustian, 1969) and the LDH found in Artemia salina has a molecular weight of 140,000 (Ewing & Clegg, 1972). The active enzyme in C. magister consists of four subunits (m.w. ca 40,000), while the subunit molecular weight of H. americanus and A. salina LDHs is 35,000. The molecular weight of other tetrameric LDHs also fall within the range of 130- 150,000 (Kaplan, 1964). The quaternary structure of C. magister LDH is similar to that of other LDHs. In contrast, Limulus polyphemus and all other chelicerates studied possess a catalytically active form of LDH with a molecular weight of about 70,000 (a dimer) (Gleason, et al, 1971). LDH from Nereis virens has been shown to have a molecular weight of 78,000, also a dimer (Long & Kaplan, 1973a).

LDH from leg tissue of C. magister shows an L-lactate stereospecificity. This is true for all other crustacean LDHs studied to date with the exception of most barnacle LDHs. Barnacle LDH has a molecular weight of 140,000 but is D-lactate specific. Chthamalus depressus is L-lactate specific (Hamren, 1969). The tetrameric forms of LDH found in all other mandibulates are L-lactate specific, while the dimeric forms of LDH found in chelicerates studied to date have a

D-lactate specificity. It is interesting to note that the dimeric form, specific for D-lactate, is found only in modern members of the phylogenetically primitive arthropods, the chelicerates, while the tetrameric form, specific for L-lactate with one exception, is found only in modern members of the phylogenetically advanced arthropods, the mandibulates.

Disc gel electrophoresis resolved only one isozyme for heart, leg, and claw tissues from C. magister. Starch gel electrophoresis demonstrated the presence of only one identical isozyme in each tissue. These results indicate the presence of only one form of LDH in C. magister, when the LDH composition is determined under the conditions employed in this study. The finding of a single isozyme in all tissues of an organism has been reported by several researchers. Gleason, et al (1971) found only one isozyme in Cancer antennarius, Callianassa affinis, Emerita analoga, and Orconectes propinquus by starch gel electrophoresis. Ewing & Clegg (1972) found only one isozyme in Artemia salina using a variety of techniques. Also, only one form of LDH has been found in the tissues of several flatfishes (Kaplan, 1964). The presence of one form of LDH in C. magister is consistent with data for other crustaceans, but is uncommon for most organisms studied to date.

The existence of a possible second isozyme in the leg tissue of C. magister is suggested by the elution pattern obtained from chromotography with DEAE-Cellulose. The small peak eluted by the NaCl gradient appears to be distinct from the major peak eluted by buffer alone (see Fig. 5). But since the major peak did not stick to the column before elution and the small peak represented only a small fraction of the

total activity eluted from the column, the presence of a second isozyme is uncertain at this time. It is possible that a second isozyme present in the leg tissue, comprising only about 5% of the total LDH, is not electrophoretically different enough or present in a sufficient quantity to produce a second band in the gel experiments.

When the major isozyme of leg tissue was electrophoresed on starch gel with beef heart LDH, the leg isozyme of C. magister moved 38% of the distance from the origin traversed by the beef heart LDH. Gleason, et al (1971) found that the LDH from C. antennarius moved 29% as far as beef heart LDH on starch gel using the same buffers, current, and migration time as in these experiments. Phylogenetic relationships can not be drawn from the relative mobilities of various LDH isozymes, though, since Gleason, et al (1971) also found that one LDH isozyme from Alpheus armillatus migrated 37% of the distance traversed by beef heart LDH.

Kinetic properties of LDH from various tissues of C. magister are consistent with the finding of only one form of LDH in C. magister. The apparent K_M for pyruvate of the crude preparations studied ranged from approximately 3×10^{-4} M to 5×10^{-4} M. These values are not significantly different at the P .05 level. The apparent K_M values for pyruvate of purified leg and claw tissue samples were not significantly different from those values found for the crude samples. However, the apparent K_M for pyruvate of the purified heart preparation ranged from 9.0 to 11.5×10^{-4} M, significantly higher than any other apparent K_M found. Apparently the tissue source and the state of purity of the LDH did not affect the apparent K_M for pyruvate at 10°,

15°, and 20°C, except for the purified heart sample. If the enzyme found in the heart and peripheral muscle tissues is indeed the same enzyme, then it would follow that the apparent K_M for pyruvate should be the same for the purified heart sample as for the purified leg and claw samples. However, a regulator might be present in the cells of one tissue type and not in the other. If such a regulator existed, it might affect both crude and purified heart samples, but not the purified sample alone.

Another hypothesis is that a second isozyme is present, which would alter the kinetics found in the tissue possessing it. The existence of this second isozyme in leg muscle is suggested by DEAE-Cellulose chromotography (see Fig. 5). However, it can not be postulated at this time how this second isozyme could alter the apparent K_M values for pyruvate of a purified sample without altering the apparent K_M values for pyruvate of a crude sample of LDH from the same tissue.

A third hypothesis is that the purified heart solution was too dilute. Markert and Massaro (1968) demonstrated that certain LDHs will dissociate and lose some catalytic capability when the enzyme concentration is less than 0.5 mg/ml. The LDH concentration in all purified heart samples was much less than that for purified claw and leg samples, never exceeding 0.1 mg/ml. It has been shown in vertebrate LDHs that a decrease in LDH concentration in the range used in the present experiments will cause a decrease in the apparent K_M for pyruvate (Wuntch, et al, 1970). However, the effect of low enzyme concentrations on invertebrate LDHs has not been studied. Further experiments would have to be performed in order to provide an explanation for the increase

in apparent K_M for pyruvate after the purification of a heart sample from C. magister.

The maximum velocity of the pyruvate to lactate reaction was attained at 5 mM pyruvate at 10°C and 10 mM pyruvate at 20°C for all samples studied. This value could be higher for the leg and claw samples, but 10 mM pyruvate was the highest concentration used in the experiments. The amount of inhibition incurred by the various samples at 10 mM pyruvate ranged from 5.2 to 7.8% when all of the data for a tissue was averaged together. The percent inhibition at 15° and 20°C was not significant for any heart, leg, or claw sample, while the percent inhibition at 10°C for these preparations was only slight (5 to 8%) at 10 mM pyruvate. The LDH enzyme in C. magister therefore appears to show slight inhibition at high pyruvate concentrations at the average habitat temperature (10°C).

C. magister LDH is similar to H. americanus LDHs with regards to catalytic properties (see Table 1). The apparent K_M for pyruvate and the concentration of pyruvate causing significant inhibition are almost identical for the enzymes of both species, while the optimal pyruvate concentration is five times greater for C. magister LDH at 20°C than for H. americanus LDHs at 25°C. The kinetic parameters for C. magister LDH are all twice the magnitude for the corresponding values for the LDH of Artemia salina. The properties of the LDHs of Paralithodes camtschatica are also different from those of C. magister LDH. The apparent K_M for pyruvate of Paralithodes LDH at 10°C is 3.0 mM, slightly less than the value for C. magister LDH, while the optimal pyruvate concentration and pyruvate concentration causing significant

inhibition are five and three times greater, respectively, for Cancer magister. The properties of the LDH in C. magister, then, more closely resemble the properties of the enzymes found in Homarus tail muscle than the enzymes found in Artemia and Paralithodes.

The LDH found in the peripheral muscle tissues of Limulus polyphemus operates maximally at approximately the same concentrations of pyruvate as C. magister LDH, but the apparent K_M for pyruvate is much lower. LDH from Limulus heart tissue can operate at a much lower range of pyruvate concentration than can LDH from C. magister heart tissue.

The optimal pyruvate concentration, e.g. the concentration of pyruvate at which the reaction rate is a maximum, and the pyruvate concentration causing significant inhibition are much higher in C. magister than in the LDH of organisms of other phyla listed in Table 1, while the values for the apparent K_M values for pyruvate of the other LDHs are both higher and lower than the value for C. magister LDH. With the exception of Crassostrea virginica, all of the non-crustacean invertebrate LDHs appear to be suited for catalysis at much lower concentrations of pyruvate than is the LDH from C. magister. This does not necessarily mean that the concentration of pyruvate is consistently lower in the tissues of non-crustacean invertebrates. It does mean, though, that the LDH enzyme in C. magister can operate less effectively at all levels of pyruvate than the LDHs of non-crustacean invertebrates.

The pH profile of the reaction pyruvate to lactate for C. magister LDH shows a maximum at pH values below 7.6. A rapid decline in reaction rate is observed at pH values above 8.0. Similar results were reported

for beef heart LDH (Winer & Schwert, 1957), rabbit LDH-1 (Fritz, 1967), and pig heart and muscle LDHs (Jaenicke, et al, 1971). In contrast, rabbit LDH-5 has a definite maximum at pH 6.4, with a substantial decrease in reaction rate below pH 6.2 and above pH 7.2. No pH profiles for invertebrate LDHs have been studied. The LDH in C. magister appears to have similar charged groups participating in catalysis at the active site as the vertebrate LDHs. However, the physiological pH of C. magister hemolymph is more alkaline than the blood of most vertebrates (Johansen, et al, 1970).

The kinetic experiments conducted over the 10°- 20°C range demonstrated a temperature independence for the K_M for pyruvate of C. magister LDH. A constant K_M for pyruvate, independent of temperature over an organism's habitat temperature range, has been shown for several fishes (Hochachka & Somero, 1968, Somero, 1973), the Alaskan king crab (Somero & Hochachka, 1969), and a snake (De Burgos, et al, 1973). The temperature independence in the king crab is explained by the presence of two LDH isozymes. Both forms are active at low temperatures, while only one isozyme, the "low K_M" LDH is active at higher temperatures. C. magister LDH is probably not operating in this manner, since only one major isozyme of LDH has been detected in this organism. No mechanisms of temperature adaption by LDH enzymes were postulated by the authors of the studies of trout and ophidian LDHs.

Many marine invertebrates are eurythermal, meaning that through genetic changes they have been able to adapt to wide changes in temperature. Control of its metabolic rate over a range of temperatures is one problem faced by such an organism. Although LDH is only one of

the many enzymes involved in the utilization of metabolites, the role of LDH in anaerobic metabolism is well established (Wilson, et al, 1963, Dawson, et al, 1964, Fondy & Kaplan, 1965). C. magister LDH has an apparent K_M for pyruvate which is constant over the physiological temperature range. For C. magister, then, the temperature independence of lactate production may be a way to maintain homeostasis during a temperature change.

Hochachka and Somero (1973) have analysed their results by equating K_M values for pyruvate with LDH-pyruvate affinities. The constant K_M may not be equal to the dissociation constant K_S for LDH and pyruvate, since the K_M for pyruvate involves the dissociation of the LDH-lactate complex to LDH plus lactate. It may be assumed by Hochachka and Somero that the K_M for pyruvate is a reflection of the enzyme-substrate affinity, expressed as K_S; however, Morris (1968) notes that this assumption is unwise, due to the results obtained by Chance and others on a variety of enzymes. In light of this information, the results of the experiments with C. magister LDH can only be interpreted as indicating a constant rate for the total catalytic process involved-- binding the substrate pyruvate to LDH and then releasing the product lactate. The mechanism responsible for this temperature independence can not be postulated at this time.

The existence of only one form of LDH in C. magister is not unique for a crustacean, but the kinetic properties of this enzyme, when found in heart tissue, is quite contrary to the findings in other organisms studied (see Table 1). All LDH isozymes isolated from heart tissues studied to date, with the possible exception of LDH from flatfishes,

have a lower K_M for pyruvate, operate optimally at lower concentrations of pyruvate, and show greater inhibition at elevated pyruvate concentrations than do the LDH isozymes found in the peripheral muscle tissues of the same organisms. In vertebrates, these findings have been associated with the type of environment surrounding the cells of the tissue. Skeletal muscle often experiences anaerobic periods during short bursts of activity, while heart tissue is usually found in an aerobic environment, due to the constant inflow of freshly oxygenated blood or hemolymph and the rhythmic contractions characteristic of heart tissue. The partial pressure of oxygen in the hemolymph of C. magister is very high when compared with the hemolymph of other decapod crustaceans. The arterial partial pressure of oxygen in C. magister hemolymph is 91 mm Hg, while the venous partial pressure of oxygen is 21 mm Hg (Johansen, et al, 1970). The hemolymph of most decapod crustaceans has a P_aO_2 of 5-29 mm Hg and a P_vO_2 of 2-18 mm Hg (Jones, 1972). Therefore, C. magister would be expected to possess different LDH isozymes in the heart and peripheral muscles, as is the case in the vertebrates (Dawson, et al, 1964). The partial pressure of oxygen in the hemolymph of Limulus is equal to or less than the partial pressure of oxygen in the hemolymph of C. magister. But even in Limulus the heart and peripheral muscles have kinetically distinct LDH isozymes, similar to those found in many vertebrate LDH systems.

Wuntch, et al (1970), however, has shown that LDH from heart and skeletal muscle in pigs, rats, and rabbits does not exhibit any substrate inhibition when assayed at physiological concentrations of LDH. They concluded that substrate inhibition by pyruvate may not occur

in vivo in many vertebrates. Similar evaluations of invertebrate LDHs have not been made. There is a possibility that the kinetic properties of LDH are not that important in *C. magister* tissues, and that the lack of inhibition by high pyruvate concentrations exhibited by LDH from heart tissue is of no consequence to the crab. Nevertheless, it would still be interesting to determine the kinetic properties of the LDH found in the heart tissue of *H. americanus*, in order to see if the homologies between the peripheral muscle LDHs of *H. americanus* and *C. magister* extends to the LDHs found in the heart tissues of these organisms.

Table 1. Kinetic properties of several vertebrate and invertebrate lactate dehydrogenases.

TABLE 1*

Organism	Optimal pyruvate	K_M (pyruvate)	Significant inhibition
Vertebrate range[1-4]	4 - 7 (H_4) 20 - 30 (M_4)	0.9 - 1.4 (H_4) 1.3 - 32 (M_4)	4 - 8 (H_4) 20 - 40 (M_4)
Arthropods			
Homarus americanus[5]	20 (A) 21 (B)	4.1 (A) 4.0 (B)	100 (A) >100 (B)
Artemia salina[7]	25	1.2	40
Paralithodes camtschatica[8]	10	0.8 - 3.0	40
Limulus polyphemus[6]	1.6 (heart) 40 (muscle)	--- 0.7	2 >100
Polychaetes			
Nereis virens[6]	8	1.3	8
Mollusks			
Loligo pealei[9]	---	6.4	----
Crassostrea virginica[10]	30	2.0	200
Tapeworms			
Hymenolepis diminuta[11]	---	1.7	10
Parasitic nematodes			
Ascaris suum[12]	---	2.8 - 5.4	---
Sponges			
Scypha lingua[10]	10	1.0	20
Bacteria			
Lactobacillus plantarum[13]	--- ---	3.7 (L) 4.7 (D)	--- ---

* All values are given in units of 10^{-4} M pyruvate.

Footnotes for Table 1

[1]From Wilson, et al (1963).

[2]From Pesce, et al (1964).

[3]From Fondy & Kaplan (1965).

[4]From Pesce, et al (1967).

[5]From Kaloustian, et al (1969).

[6]From Long & Kaplan (1973).

[7]From Ewing & Clegg (1972).

[8]From Somero & Hochachka (1969).

[9]From Roberts, et al (1958).

[10]From Hammen (1969).

[11]From Burke, et al (1972).

[12]From Langer & Smith (1971).

[13]From Dennis & Kaplan (1960).

Table 2. Stereospecificity of lactate dehydrogenase from Cancer magister leg and heart tissues.

TABLE 2

Preparation	Lactate isomer	△OD/min.*
Leg crude	DL	0.36
	L	0.18
	D	0.02
Leg purified	DL	0.30
	L	0.30
	D	0.00
Heart crude	DL	0.06
	L	0.11
	D	0.01
Heart purified	DL	0.02
	L	0.09
	D	0.00

*Results from assays using 4×10^{-2} M lactate and 4×10^{-3} M NAD^+; assays at 20°C. Activity monitored at 340 nm.

Figure 1. Molecular weight determination of C. magister LDH on Sephadex G-200 (Fine). The standards are, from top to bottom: catalase, catalase (m.w. determined by Andrews (1965)), rabbit muscle aldolase, beef heart LDH, bovine serum albumin, catalase subunit, α-chymotrypsinogen, and sperm whale myoglobin. Procedure described in text. Closed circle denotes C. magister LDH sample.

Figure 1

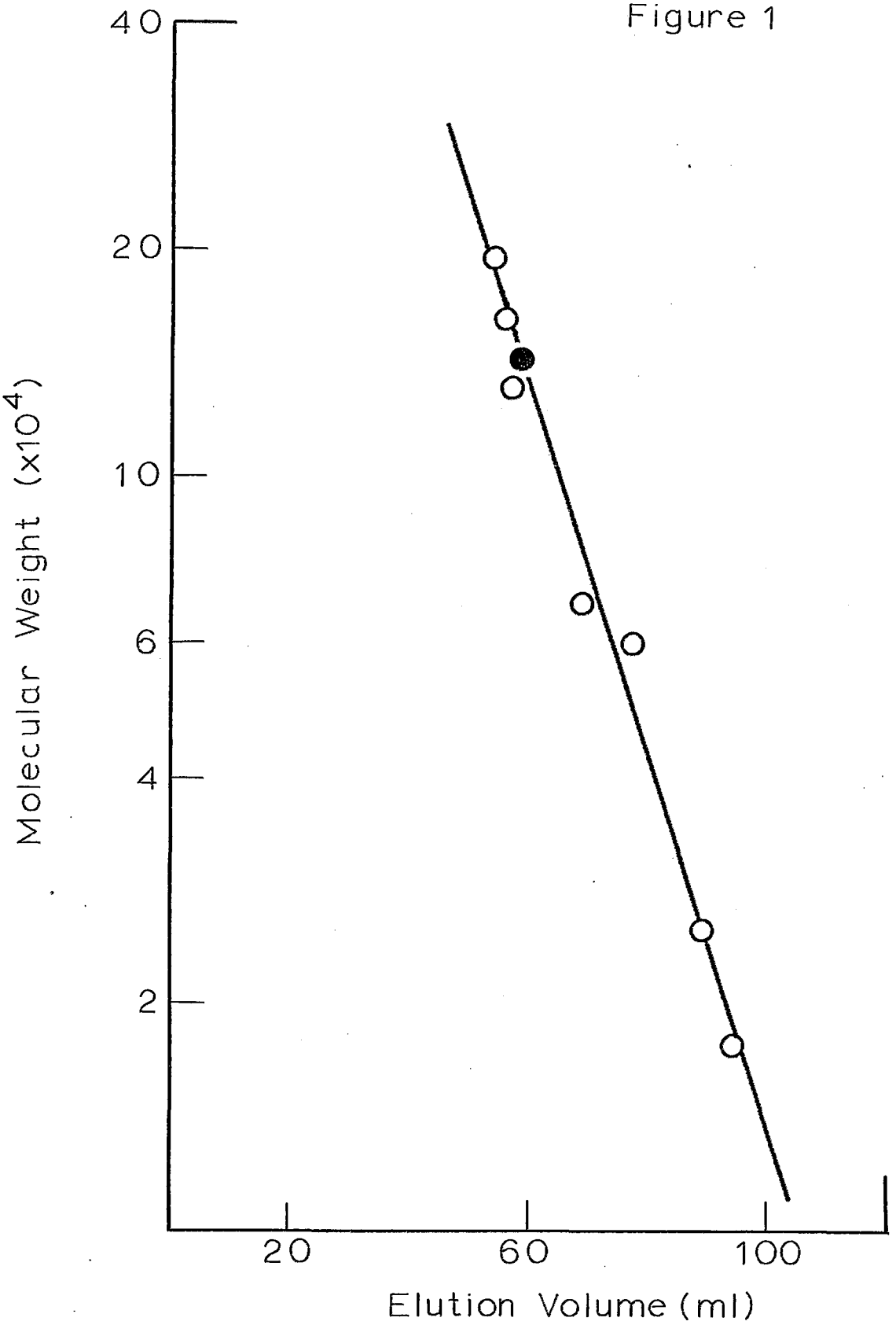

40
20
10
6
4
2
Molecular Weight (x10⁴)
20
60
100
Elution Volume (ml)

Figure 2. Elution pattern of LDH from C. magister leg tissue. ○, absorbance 280 nm; ●, enzyme activity, expressed as ΔOD_{340}/ min. Buffer: 0.1 M $NaPO_4$, pH 7.4, and 0.1 M in NaCl. Procedure described in text.

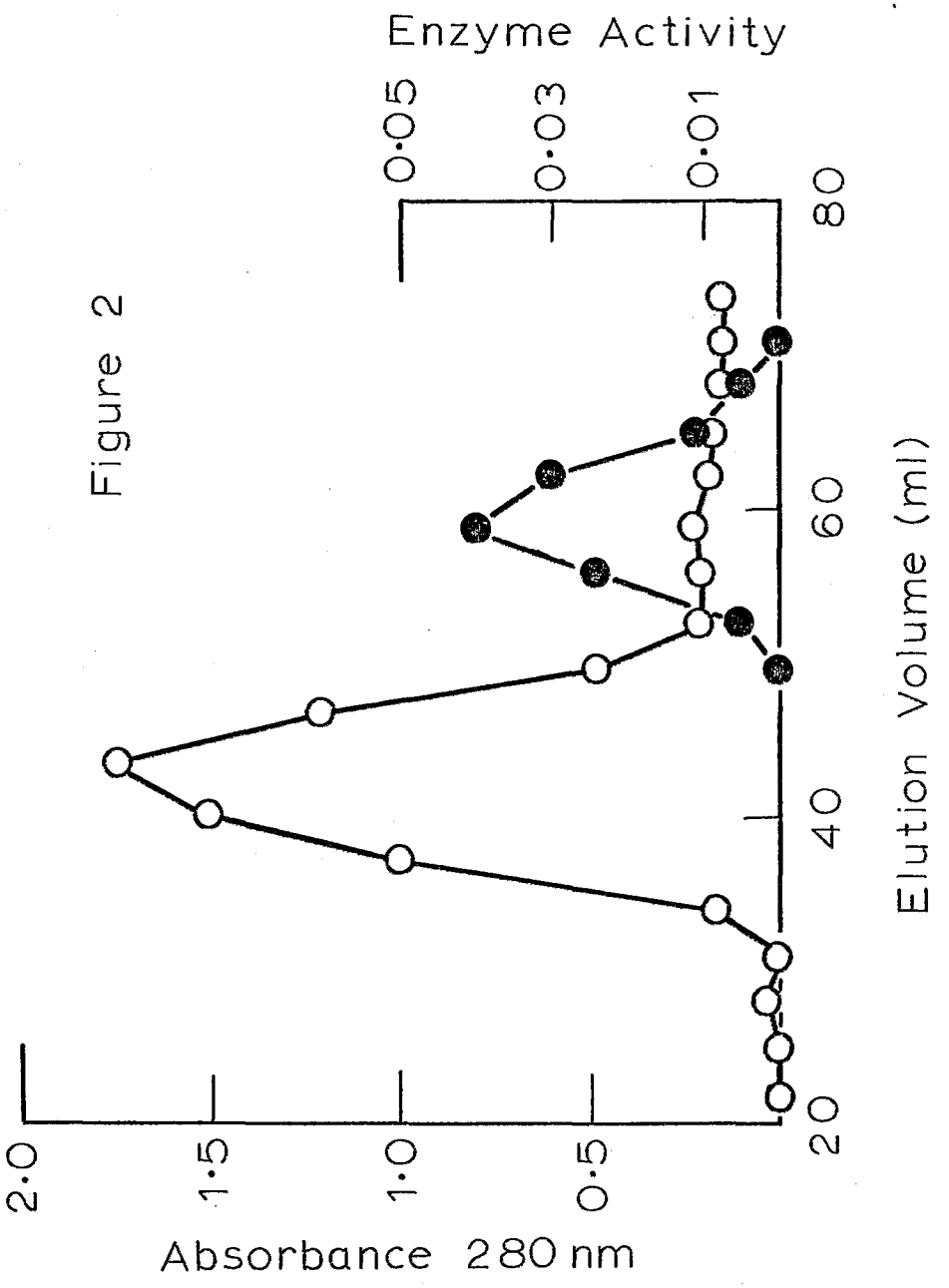

Enzyme Activity
0·05
0·03
0·01
80
60
40
20
Elution Volume (ml)
2·0
1·5
1·0
0·5
Absorbance 280 nm

Figure 2

Figure 3. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate of purified heart LDH from C. magister. Standards, ● , from top to bottom: ovalbumin, pepsin, and sperm whale myoglobin. Open circle denotes LDH sample. Procedure according to text.

Figure 3

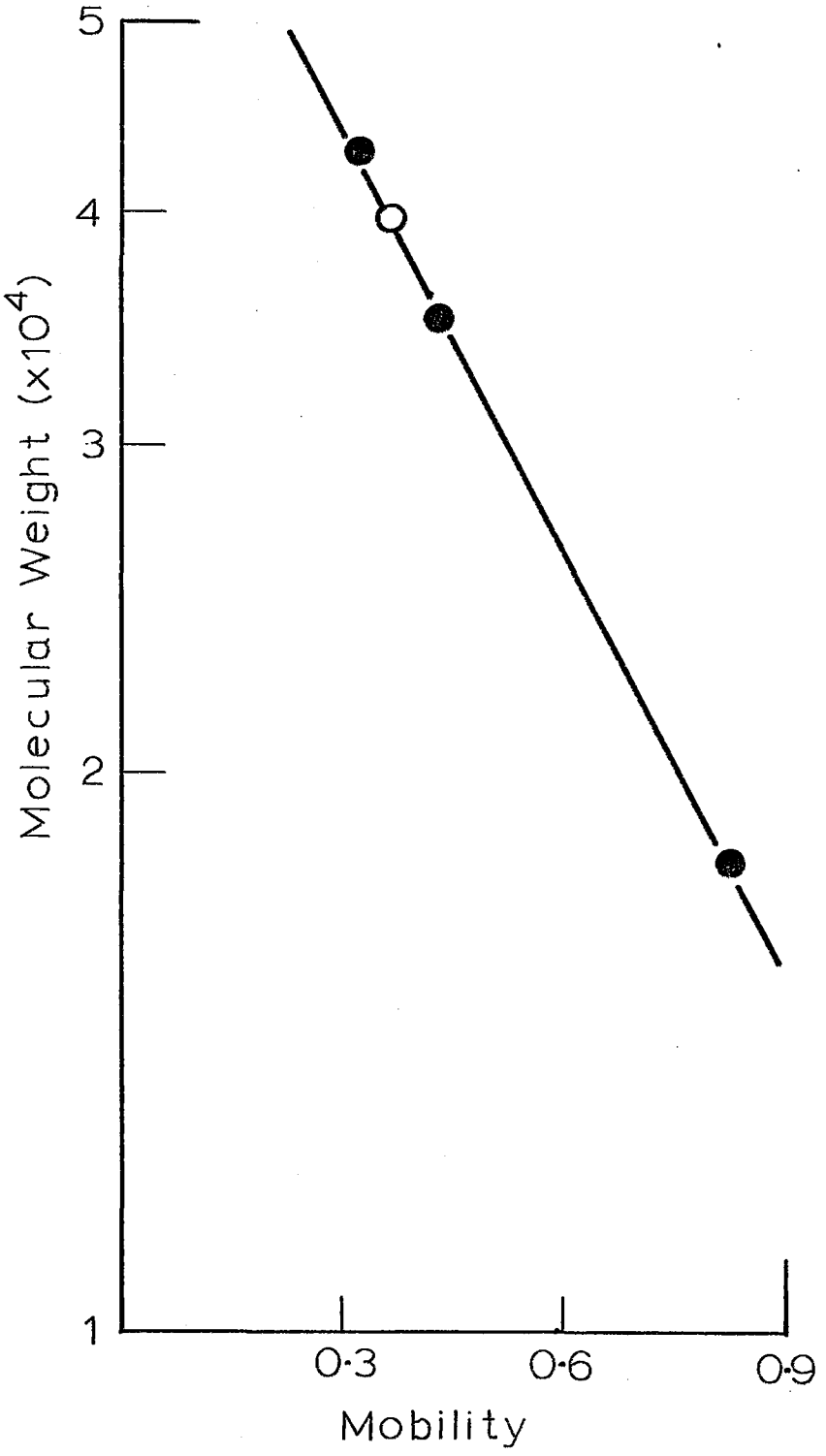


Figure 4. DEAE-Cellulose chromotography of a purified LDH sample from C. magister heart tissue. Enzyme activity expressed as ΔOD_{340}/min. Buffer used: 0.01 M Tris-HCl, pH 7.6. Procedure described in text.

Figure 5. DEAE-Cellulose chromotography of a purified LDH sample from C. magister leg tissue. Enzyme activity expressed as ΔOD_{340}/min. Buffer used: 0.01 M Tris-HCl, pH 7.6. Procedure described in text.

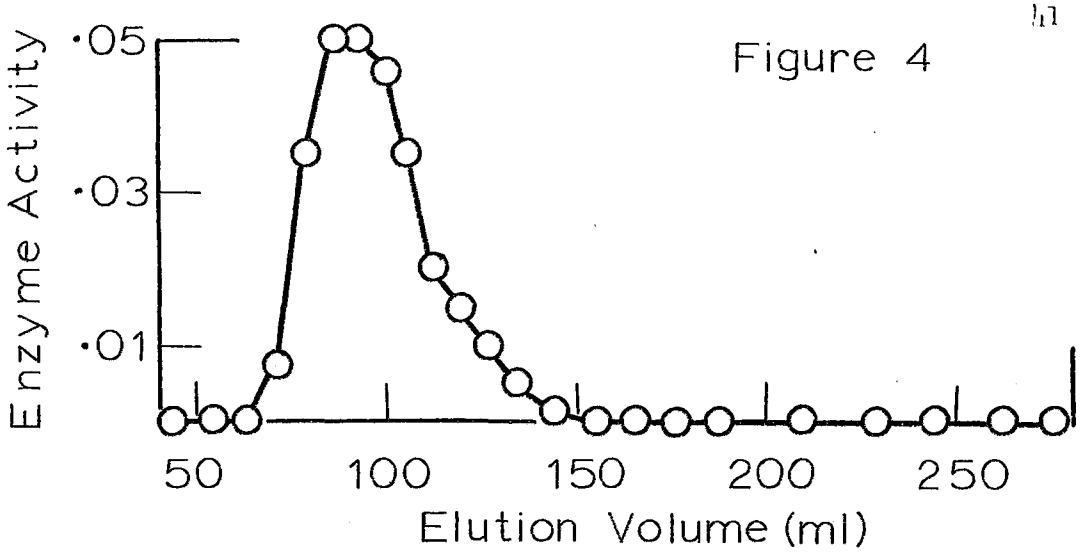


Figure 4

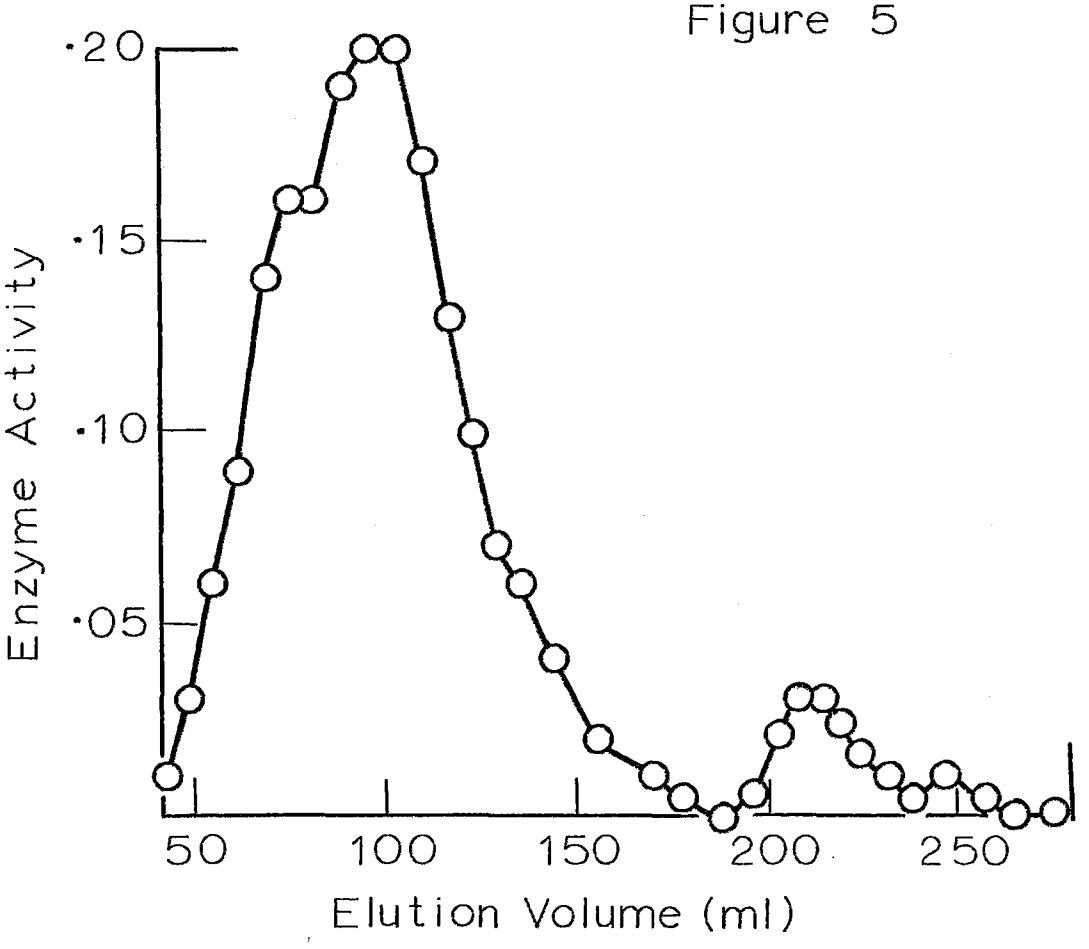


Figure 5

Figure 6. A sketch of starch gel electrophoresis of crude leg (L), heart (H), and claw (C) tissue samples from C. magister. 13% starch gel (w:v) in 56 mM Na_2HPO_4 and 9 mM citric acid, pH 8.0. Electrode buffer: 32 mM Na_2HPO_4 and 3 mM citric acid, pH 8.0. 200 volt current at 8-15 ma applied for 23 hours.

Figure 7. A sketch of starch gel electrophoresis of a crude leg tissue sample from C. magister (L), purified beef heart LDH (BH), and a mixture of the two samples (M). Same buffers and gel as in Figure 6, except both buffers at pH 7.0. 200 volt current at 7-12 ma applied for 16 hours. ◯ spots denote weak staining.

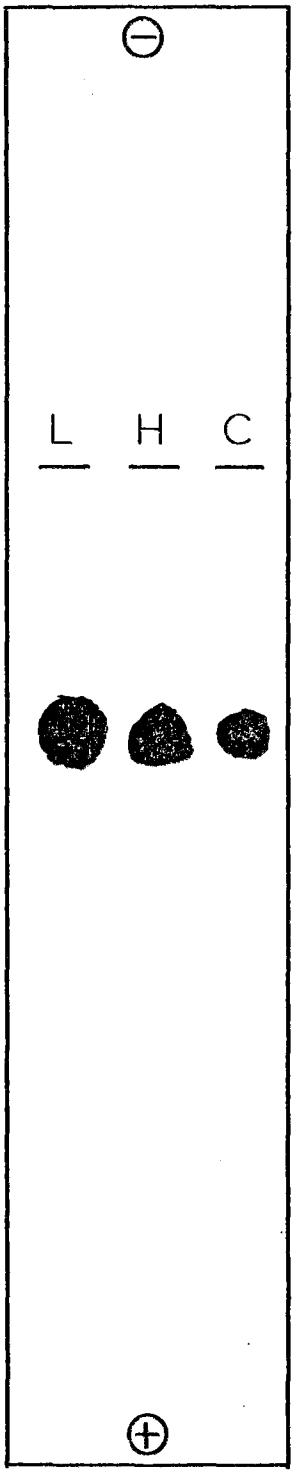


Figure 6

origin

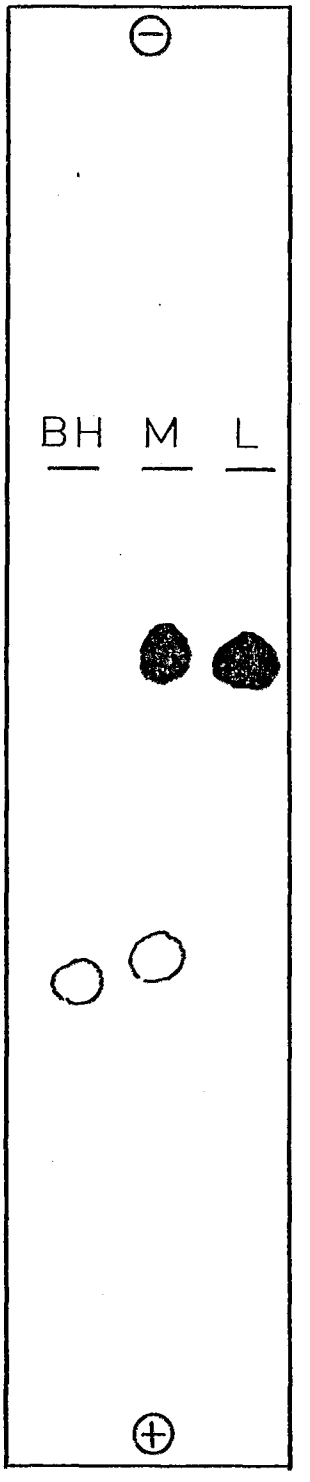


Figure 7

Figure 8. The effect of pH on the initial velocity of the reaction pyruvate to lactate for a crude LDH sample from C. magister leg tissue. Pyruvate concentrations used: □ 0.5 mM, △ 2.5 mM, and ○ 5.0 mM. All assays performed at 10°C. Procedure described in text.

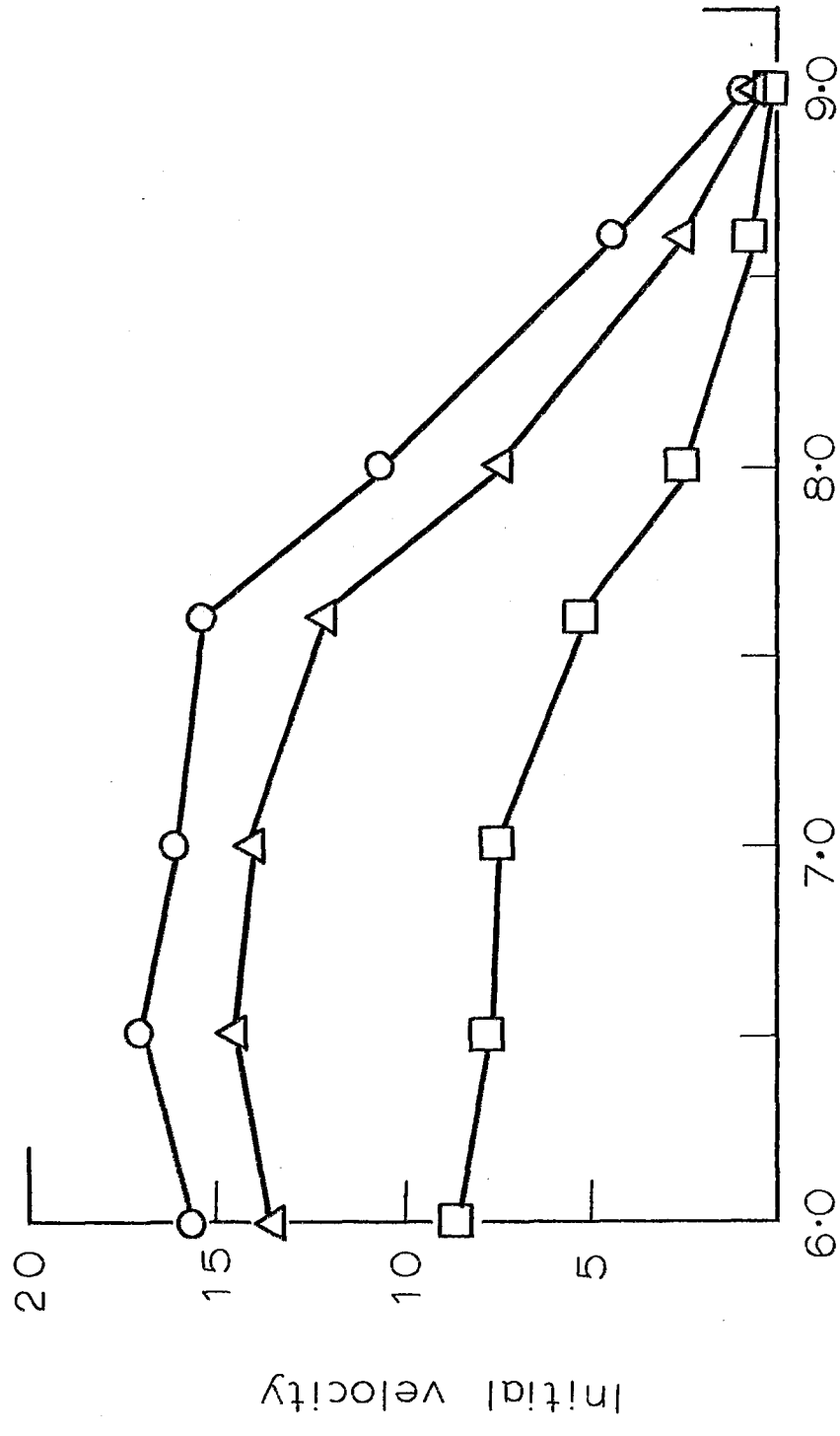


Figure 8

Figure 9. The effect of temperature on the apparent K_M for pyruvate of crude LDH samples from C. magister heart, leg, and claw tissues. All points are an average value for five different experiments, with the standard error represented by error bars. Procedure outlined in text.

Figure 9

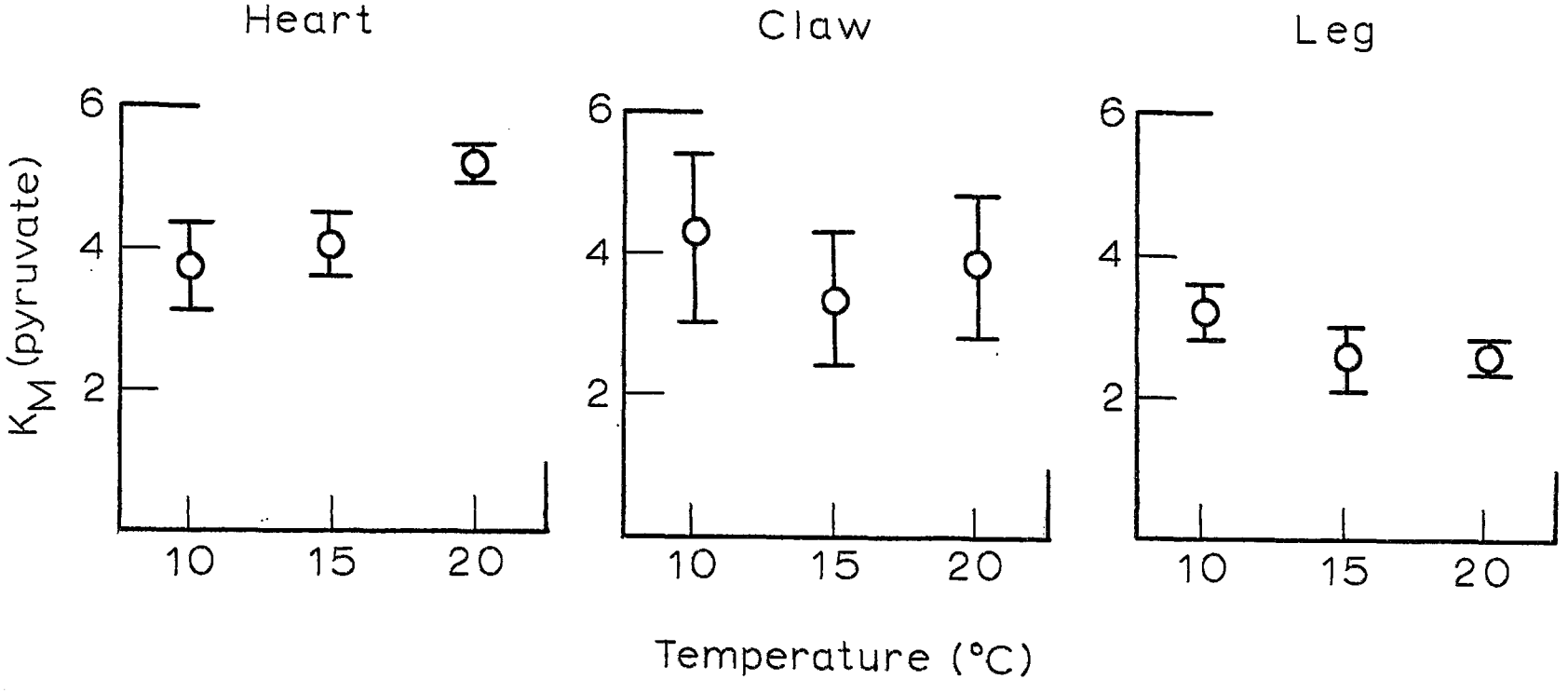


Figure 10. The effect of temperature on the apparent K_M for pyruvate of purified LDH samples from C. magister heart, leg, and claw tissues. All points are an average value for five different experiments, with the standard error represented by error bars. Procedure described in text.

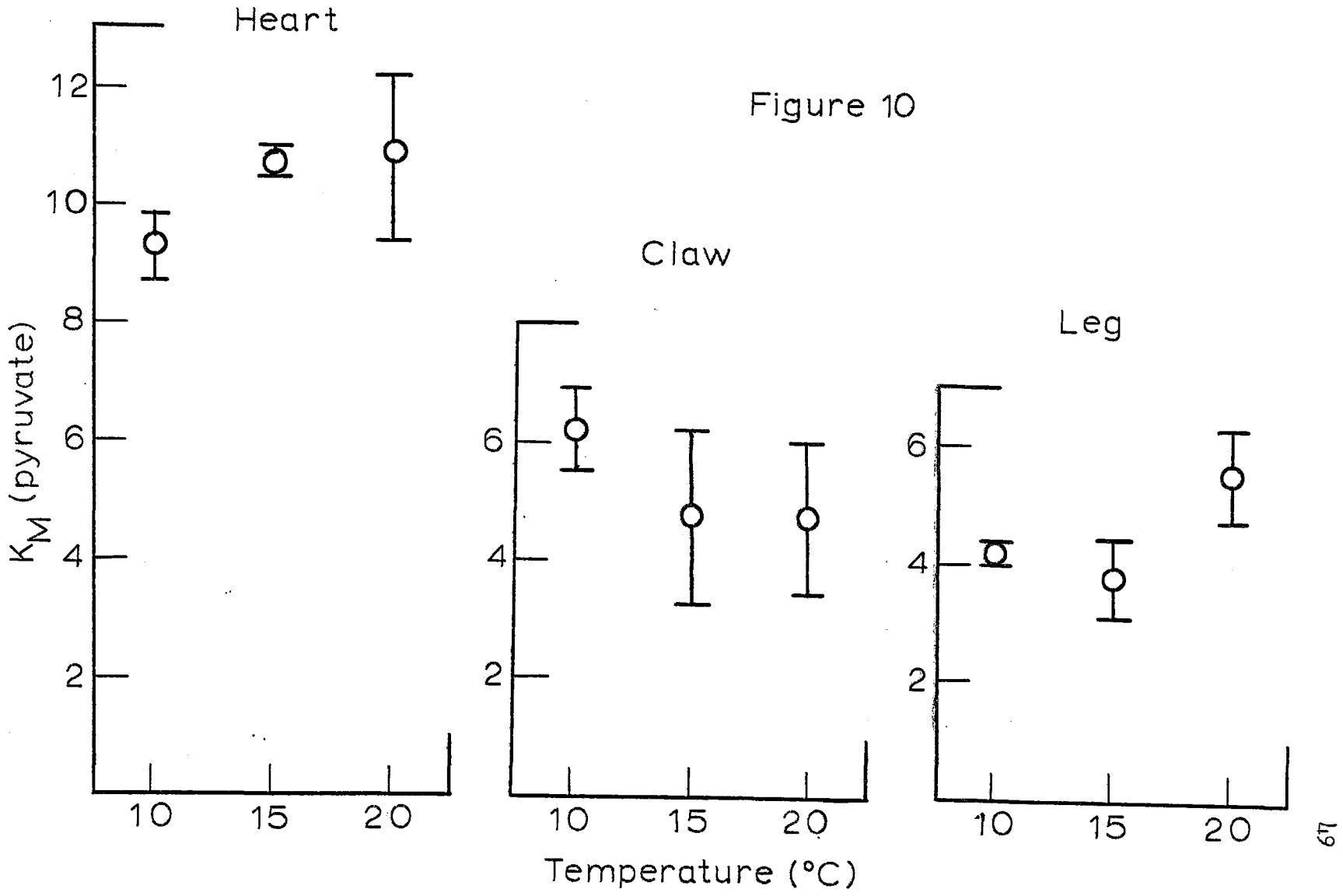


Figure 10

Figure 11. The percent of the maximum rate as a function of pyruvate concentration for beef heart LDH △ and C. magister LDH from leg ● and heart ○ tissues. Procedure described in text.

Figure 11

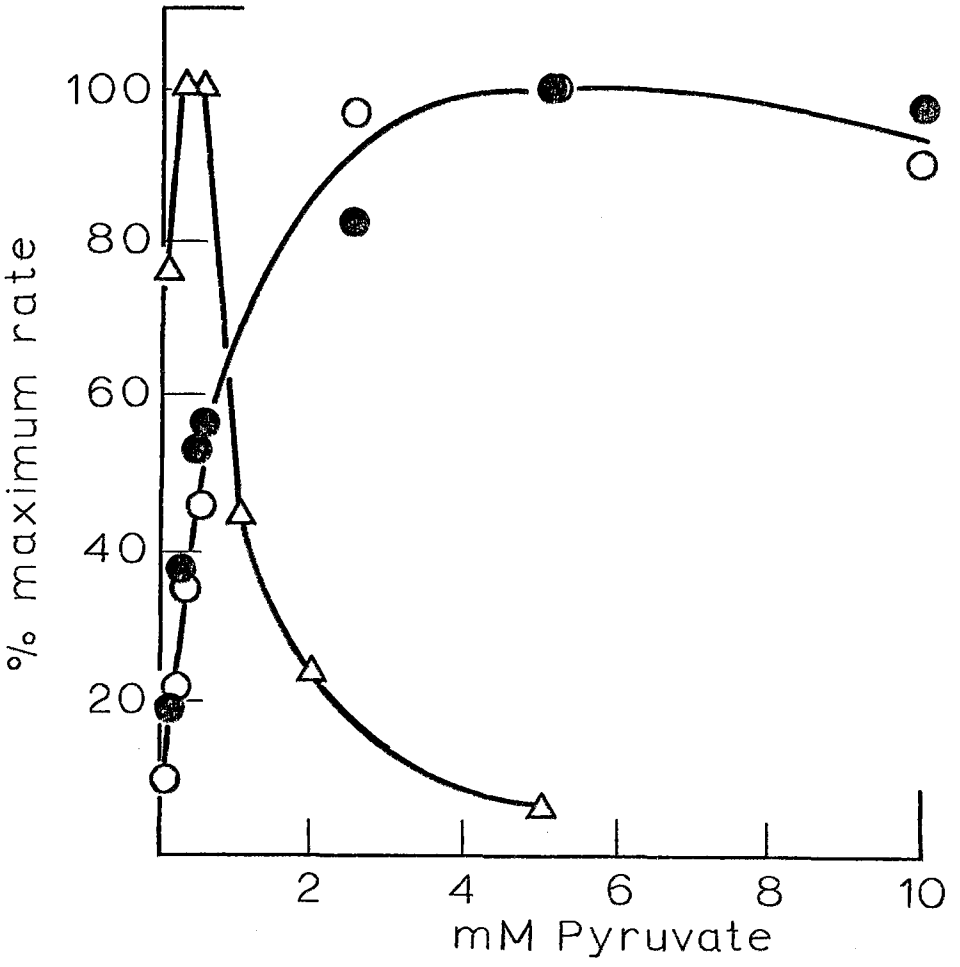
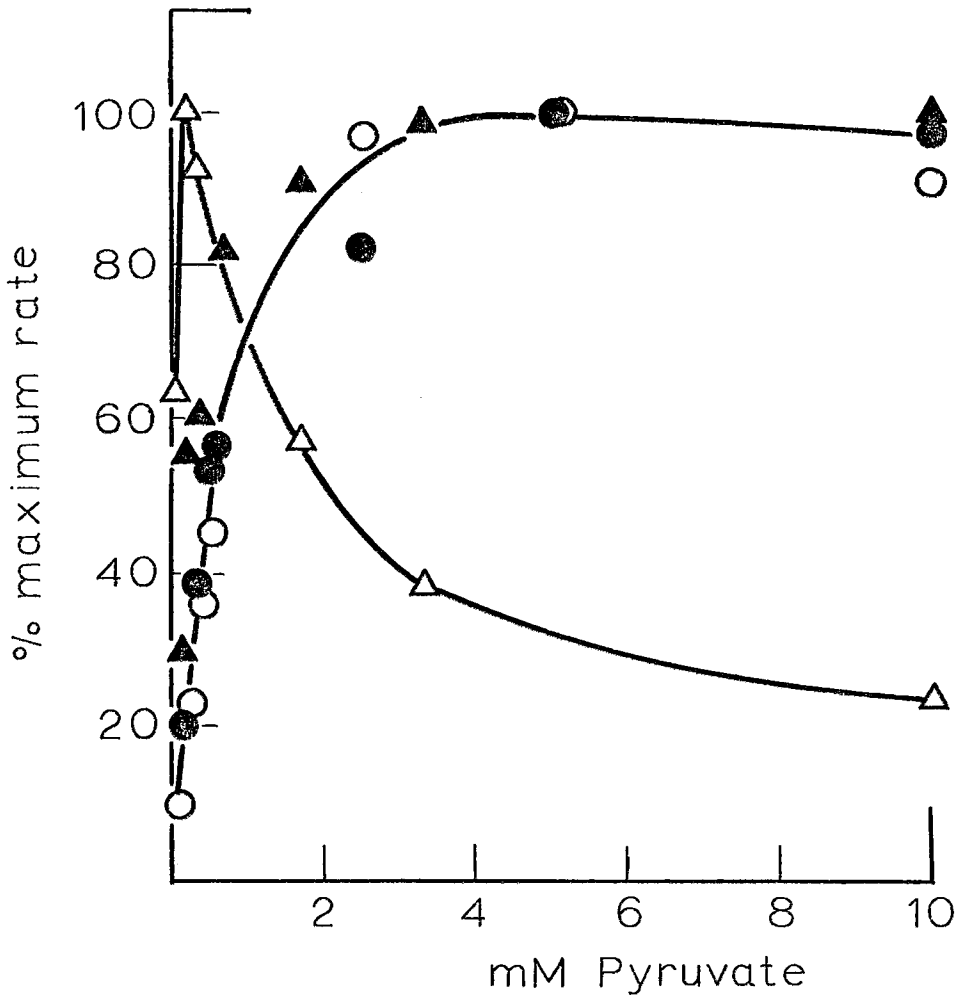


Figure 12. The percent of the maximum rate as a function of pyruvate concentration for the following tissues: C. magister leg ● and heart ○ , and Limulus polyphemus peripheral muscle ▲ and heart muscle △ . Procedure for C. magister samples described in text. Data for Limulus samples from Long & Kaplan (1968).

Figure 12



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