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Urea-Requiring Lactate Dehydrogenases of Marine Elasmobranch Fishes

Paul H. Yancey and George N. Somero

Scripps Institution of Oceanography, University of California, San Diego, La Jolla, California 92093, USA

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Summary. The kinetic properties – apparent K_m of pyruvate, pyruvate inhibition pattern, and maximal velocity – of M_4 (skeletal muscle) lactate dehydrogenases of marine elasmobranch fishes resemble those of the homologous lactate dehydrogenases of non-elasmobranchs only when physiological concentrations of urea (approximately 400 mM) are present in the assay medium. Urea increases the apparent K_m of pyruvate to values typical of other vertebrates (Fig. 2), and reduces pyruvate inhibition to levels seen with other M_4-lactate dehydrogenases (Fig. 3). Urea reduces the activation enthalpy of the reaction, and increases V_{max} at physiological temperatures (Fig. 4).

The M_4-lactate dehydrogenase of the freshwater elasmobranch, *Potamotrygon* sp., resembles a teleost lactate dehydrogenase, i.e., although it is sensitive to urea, it does not require the presence of urea for the establishment of optimal kinetic properties.

Introduction

Marine elasmobranch fishes utilize urea to attain an isosmotic (or slightly hyperosmotic) state relative to sea water. Urea concentrations in the extra- and intracellular fluids range from 300 to over 500 mM (Smith, 1929; Forster and Goldstein, 1976; Chan and Wong, 1977). The reduction of osmotic gradients appears of obvious benefit to marine elasmobranchs, but the use of urea to achieve this end seems rather paradoxical. Urea is an effective protein denaturing agent, exerting its effects by interfering with hydrogen bonds (Hermans, 1966), hydrophobic interactions (Wetlaufer et al., 1964), and the structure of water (Finer et al., 1972). Indeed, concentrations of urea in the physiological range for marine elasmobranchs are known to have adverse effects on the structure and function of many proteins of non-elasmobranchs, including collagen (Fessler and Tandberg, 1975), mammalian hemoglobin (Rossi Fanelli et al., 1964), and numerous enzymes (Inagaki, 1959; Elödi and Jecsai, 1960; Rajagopalan et al., 1961; Wilk et al., 1969; Nandi, 1971; Yu and Gunsalus, 1974).

These findings raise the following question: Has the incorporation of urea as an osmoregulatory agent in the body fluids of marine elasmobranchs necessitated adaptations in elasmobranch proteins which counteract the disruptive effects of urea on these molecules? For two types of proteins, available data suggest that this is the case. Elasmobranch hemoglobin, unlike mammalian hemoglobin, is insensitive to urea at physiological concentrations (Bonaventura et al., 1974), and shark eye-lens protein actually requires urea for proper conformation (Zigman et al., 1965). However, in the case of enzymes, which represent the greatest number and diversity of proteins, essentially no information on urea adaptations is available. The studies discussed below were conducted to determine whether homologues of muscle-type (M_4) lactate dehydrogenase (LDH, E.C. 1.1.1.27; NAD^+:lactate oxidoreductase) from marine elasmobranchs, on the one hand, and freshwater elasmobranchs, teleosts and mammals, on the other, differ in their responses to physiological concentrations of urea. Our results show that the LDH's of marine elasmobranchs actually require the presence of urea for correct pyruvate binding ability, insensitivity to high substrate inhibition, and optimal catalytic activity.

Materials and Methods

The following animals were used in this study: Teleost fishes – *Trematomus* (=*Pagothenia*) *borchgrevinki* (Nototheniidae); *Sebastolobus alascanus* (Scorpaenidae); *Hippoglossus stenolepis*, pacific halibut (Pleuronectidae); *Gillichthys mirabilis*, mudsucker (Go-

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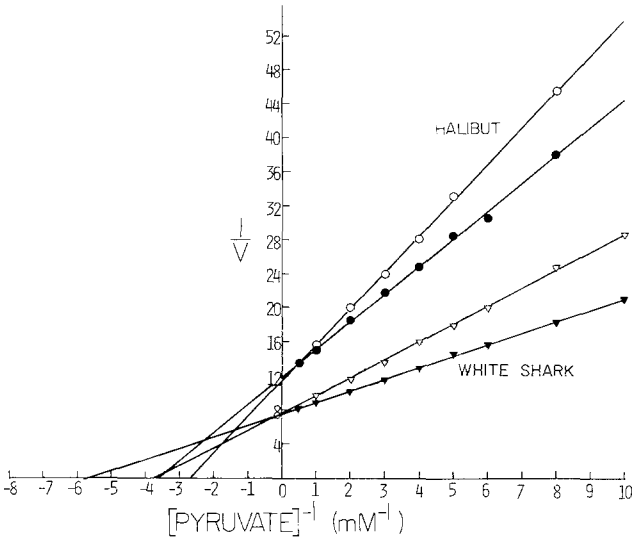

Fig. 1. Double reciprocal plot of reaction velocity versus substrate concentration, showing competitive inhibition of M_4-LDH's by urea. ●, ▼, assays without urea; ○, ▽, assays with 400 mM urea. Halibut enzyme was assayed at 10 °C, white shark enzyme at 20 °C

biidae); *Thunnus thynnus*, bluefin tuna (Scombridae); *Hypostomus plecostomus*, Amazon armored catfish (Loricariidae); Elasmobranch fishes – *Carcharodon carcharias*, white shark (Lamnidae); *Rhinobatos productus*, shovelnose guitarfish (Rhinobatidae); *Parmaturus xaniurus*, filetail cat shark (Scylliorhinidae); *Potamotrygon* sp. (freshwater stingray; Potamotrygonidae); Mammal – rabbit.

M_4-LDH's from halibut, bluefin tuna, and white shark were generously provided by the laboratory of Dr. N.O. Kaplan. Rabbit enzyme was purchased from Sigma Chemical Company, St. Louis, Mo. M_4-LDH's from the remaining organisms were purified on an oxamate-Sepharose affinity column as previously described (Yancey and Somero, 1978). Measurement of enzyme activity and calculation of apparent Michaelis constants (K_m[1]) for pyruvate and theoretical maximal velocities (V_{max}) also followed procedures previously described (Yancey and Somero, 1978), but using only 80 mM imidazole-HCl buffer, which mimics the observed dependency of intracellular pH on temperature (Rahn et al., 1975). When appropriate, assay solutions also contained 400 mM urea, an approximate average of reported urea concentrations in marine elasmobranchs (Smith, 1929; Forster and Goldstein, 1976).

All reagents were purchased from Sigma Chemical Company, St. Louis, Mo. Urea solutions were made fresh daily and treated with Amberlite, a mixed-bed ion exchanger, to remove any isocyanate and ammonium ions.

Results

Urea Inhibition

Urea was found to be a competitive inhibitor of pyruvate binding by teleost and elasmobranch M_4-LDH's (Fig. 1), as has been reported for rabbit enzyme (Rajagopalan et al., 1961; Withycombe et al., 1965). This inhibition is reversible, independent of time, and is more effective at lower temperatures; K_I's for urea average about 600 mM at 10 °C and about 1200 mM at 25 °C.

Apparent K_m Values

As has been previously demonstrated (Yancey and Somero, 1978), the K_m values for pyruvate of non-elasmobranch M_4-LDH's are very similar when measured at respective body temperatures and intracellular pH values, all K_m's falling in the range of about 0.15 to 0.35 mM in this assay system (Fig. 2). *Potamotrygon* sp., a member of the only family of elasmobranchs permanently adapted to freshwater and which cannot retain urea even if placed in sea water (Gerst and Thorson, 1977), also fits this pattern. In urea-free medium, its M_4-LDH has K_m values for pyruvate similar to those of teleost fish LDH's (Fig. 2). The white shark, guitarfish, and cat shark enzymes, on the other hand, do not fit this pattern if assayed without urea (dotted lines, Fig. 2). However, when determined with 400 mM urea in the assay mixture, the K_m's of the M_4-LDH's from these marine elasmobranchs fall completely within the range of the other vertebrate K_m's (open symbols).

Substrate Inhibition

At high pyruvate concentrations, LDH's become inhibited, probably by the formation of an enolpyruvate-NAD^+ abortive complex (Everse and Kaplan, 1973; 1975). Inhibition of H_4 (heart muscle) LDH's is much greater than that of M_4-LDH's, largely because of their much higher affinity for pyruvate (Everse and Kaplan, 1973). In this study, teleost, rabbit, and *Potamotrygon* enzymes showed a typical M_4 response to high pyruvate, while the enzymes of the marine elasmobranchs did not unless urea was present at 400 mM. Representative data are presented in Figure 3, showing that urea significantly reduces inhibition of guitarfish M_4-LDH by high concentrations of pyruvate. Thus a second important attribute of M_4-LDH function is present in marine elasmobranch LDH's only when physiological concentrations of urea are present.

Activation Enthalpies and Maximal Velocities

A careful examination of the data reveals another effect of urea on M_4-LDH's: a small but consistent reduction in activation enthalpies ($\Delta H^{\ddagger}$). This effect

[1] The symbol K_m has been used throughout this paper to signify apparent K_m

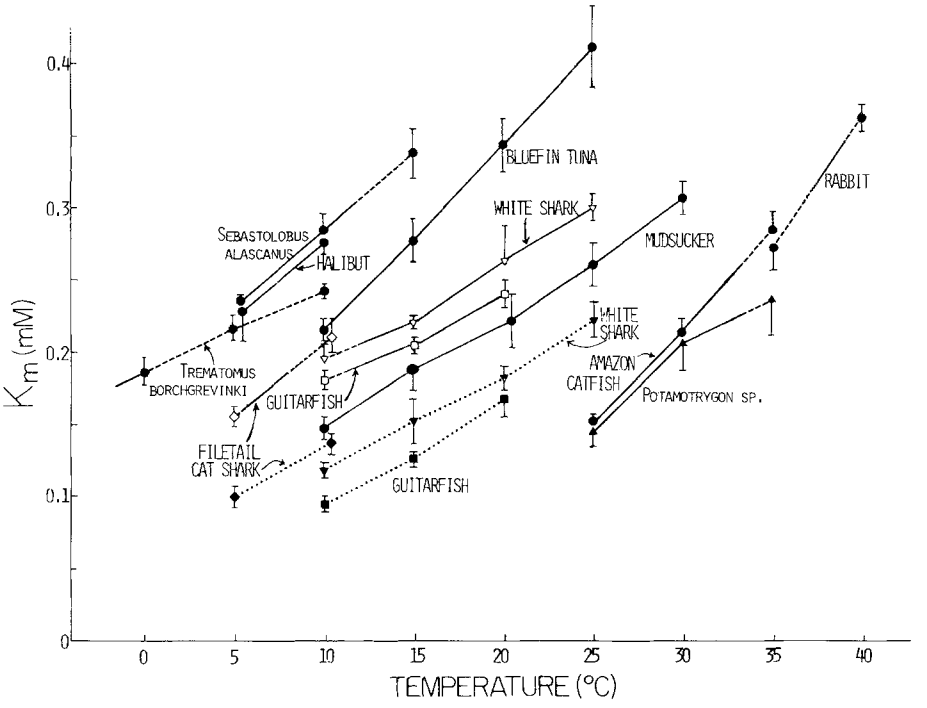

Fig. 2. Effect of temperature and urea on apparent Michaelis constants (K_m's) for pyruvate of M_4-LDH's, assayed at approximate physiological pH values as described by Yancey and Somero (1978). Assays without urea: ●, non-elasmobranch enzymes; ■, ▼, ▲, ◆, elasmobranch enzymes. Assays with 400 mM urea: ○, ▽, ◇, marine elasmobranch enzymes. Solid lines connecting K_m's represent approximate temperature ranges of these species (*Trematomus* plot is extrapolated below 0 °C). 95% confidence limits are indicated

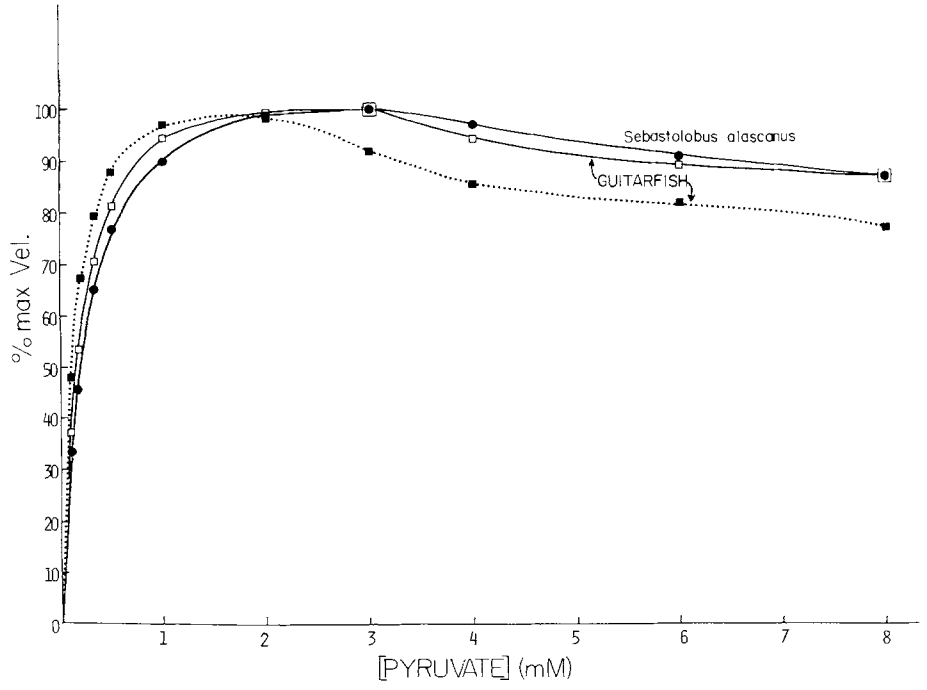

Fig. 3. Substrate saturation curves for M_4-LDH's: percent of highest experimental velocity versus pyruvate concentration. ●, ■, assays without urea; □, assays with 400 mM urea. Guitarfish enzyme was assayed at 15 °C, *Sebastolobus* enzyme at 5 °C

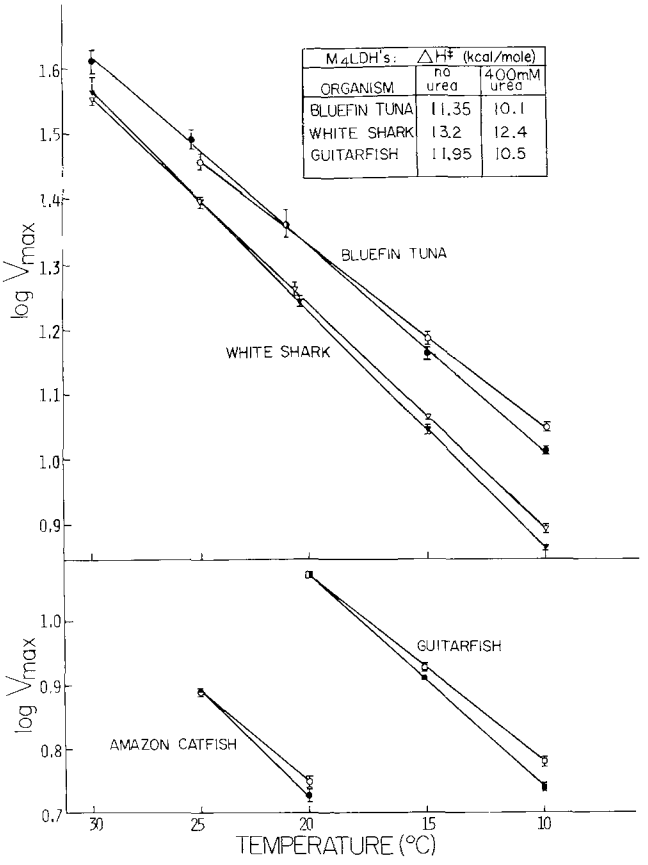

Fig. 4. Arrhenius plot of log V_{max} (not turnover number) versus the reciprocal of temperature, showing the effect of urea on activation energies (proportional to the slopes). ●, ■, ▼, assays without urea; ○, □, ▽, assays with 400 mM urea (V_{max}'s were found to be independent of pH in the range 6.6–7.5 and were determined in phosphate buffer, pH 7.4). 95% confidence limits are shown except where smaller than symbol size. Inset table shows activation enthalpies calculated from the data by linear regression

for several enzymes is shown in Figure 4, an Arrhenius plot of log V_{max} versus the reciprocal of temperature. Urea decreases the slope (and hence the $\Delta H^{\ddagger}$), resulting in higher catalytic rates at low temperatures and reduced rates at high temperatures. Similar results were found for every M_4-LDH used in this study. Thus, since urea changes V_{max}'s slightly, inhibition by urea is not strictly competitive except at a single temperature for each enzyme. However, competition with pyruvate and alteration of catalytic efficiency may be independent effects. We have found that, at temperatures below 25 °C, urea increases the apparent K_m of NAD^+ (unpublished data), and thus the increase in V_{max} due to urea may be the result of facilitation of NAD^+ release from the enzyme. The latter event is thought to be the rate-limiting step in the LDH reaction when conducted in the direction of pyruvate reduction (Everse and Kaplan, 1973).

Discussion

There is some evidence that marine elasmobranchs require elevated concentrations of urea at all times. Dogfish (*Mustelis canis*) eye-lens protein will precipitate at temperatures below 10 °C unless at least 250 mM urea is present (Zigman et al., 1965). The heart of another shark (*Squalus sucklii*) would not beat in vitro unless bathed with at least 200 mM urea (Simpson and Ogden, 1932). Elasmobranch embryos contain normal adult urea concentrations even when encased in impermeable egg membranes which prevent osmotic contact with seawater (Price and Daiber, 1967). Even euryhaline elasmobranchs retain a minimum 100–200 mM urea when adapted to freshwater (Smith, 1931; Thorson et al., 1973). The results of the present study demonstrate that marine elasmobranchs, which retain high concentrations of urea, actually require this urea for proper functioning of their M_4-lactate dehydrogenases. This is seen in at least two properties of this enzyme: 1) pyruvate K_m values, and 2) substrate inhibition.

Pyruvate K_m Values

The data of Figure 2 show that there is an optimal range of pyruvate K_m's for vertebrate M_4-LDH's, about 0.15 to 0.35 mM under the conditions of this study; e.g., teleost fishes living at 10 °C have enzymes with K_m's at this temperature similar to the K_m of the rabbit enzyme at 38 °C. The M_4-LDH of *Potamotrygon*, a freshwater elasmobranch which has lost the ability to retain high amounts of urea (Gerst and Thorson, 1977), fits this pattern without urea, indicating that this optimal range of K_m's defined by the teleost and mammalian M_4-LDH's is applicable to elasmobranch enzymes as well. Thus the K_m values for pyruvate of vertebrate M_4-LDH's appear to be under strict control by natural selection. Since urea is a strong competitive inhibitor of pyruvate binding by all vertebrate M_4-LDH's examined (Fig. 1; Whithycombe et al., 1965), marine elasmobranch homologues should therefore have some mechanism to compensate for this effect of urea. It is apparent from Figure 2 that the enzymes of marine elasmobranchs are adapted to urea by having higher affinities[2] for pyruvate (lower K_m's) which urea, through competitive inhibition, lowers to the appropriate functional level

[2] Use of the term "affinity" is not meant to imply that the apparent K_m of pyruvate is a quantitative measure of the dissociation constant of pyruvate, a relationship that has not been established for LDH's, but rather the term is employed as an index of an enzyme's capacity to initiate catalysis under conditions of non-saturating substrate concentrations (see Atkinson, 1976)

as judged by the affinities of other vertebrate M_4-LDH's. Without this inherently higher affinity for pyruvate, the marine elasmobranch homologues would have too weak pyruvate binding in the presence of urea.

Enzyme substrate affinities are likely to have selective value for several interrelated reasons (Atkinson, 1976; Somero and Low, 1977). In the case of M_4-LDH, a homologue with too high a K_m (low affinity) relative to intracellular concentrations of pyruvate may never use most of its catalytic potential. An M_4 with too low a K_m will be working at or near its maximum velocity all of the time; in a resting muscle it will be reducing pyruvate that should be channeled into the Krebs cycle. In either case, the enzyme could not quickly metabolize increasing amounts of pyruvate during anaerobiosis and thus would allow substrate concentrations to accumulate to dangerous levels (Atkinson, 1976). An "ideal" M_4-LDH will probably have a K_m somewhat greater than pyruvate concentrations in resting muscle; such an enzyme will not interfere with oxidative metabolism during resting periods, can respond quickly to increasing pyruvate levels and yet have substantial catalytic capacity in reserve for periods of extreme anaerobiosis.

This reasoning leads to two predictions: 1) K_m's of a single M_4-LDH should change with temperature in the direction that intracellular pyruvate concentrations change; and 2) organisms with similar intracellular pyruvate levels should have M_4-LDH's with similar K_m's. The enzymes examined in this study fit these predictions. Where they have been measured, steady-state pyruvate concentrations in skeletal white muscle increase with temperature in a single animal, about 6-fold (0.06 to 0.37 mM) from 5 °C to 25 °C in goldfish (calculated from Freed, 1971), and about 2-fold (0.05 to 0.11 mM) from 2 °C to 20 °C in frogs (calculated from Sacks et al., 1954). The K_m's in Figure 2 show similar changes with temperature; over a range of 20 °C, white shark K_m's would increase about 2-fold, bluefin tuna closer to 3-fold. Furthermore, pyruvate concentrations in resting white muscles are quite similar among vertebrates: 0.11 mM in trout, 11.5 °C (Black et al., 1962); 0.12 mM in carp, 8–14 °C (Wittenberger and Diaciuc, 1965); 0.14 mM in fed eels, 0.37 mM in starving eels, 15 °C (Mayerle and Butler, 1971); 0.06 to 0.37 mM in goldfish, 5 to 25 °C (Freed, 1971); 0.11 mM in frogs, 20 °C (Sacks et al., 1954); 0.33 mM in dogs, 38 °C (Vesell and Pool, 1966); 0.18 mM in rats (Bollman and Flock, 1939). Thus a similarity in K_m's of vertebrate M_4-LDH's is not surprising. In addition, since pyruvate levels appear to be higher in mammals than in most ectotherms, K_m's of mammalian M_4-LDH's should be at the higher end of the range in Figure 2; rabbit enzyme fits this prediction. Differences in K_m's of organisms adapted to similar temperatures (e.g., tuna and guitarfish at 15 °C) may reflect somewhat different steady-state rates of glycolysis or different levels of basal metabolism.

Substrate Inhibition

Inhibition by high concentrations of pyruvate probably has adaptive significance for the function of H_4-LDH's in aerobic tissues (Everse and Kaplan, 1973; 1975), but could critically impair the function of M_4-LDH's. If local intracellular concentrations of pyruvate ever reach 2 mM during anaerobiosis, marine elasmobranch enzymes without urea would be inhibited and in fact lose activity with any further increase in pyruvate levels, potentially resulting in a tremendous accumulation of this substrate (Atkinson, 1976). But with urea present the enzymes are not fully saturated until 3 or 4 mM, adding a large margin of safety similar to that possessed by other vertebrate M_4-LDH's (Fig. 3).

Urea does little to the substrate inhibition patterns of non-elasmobranch M_4-LDH's, which are already fairly insensitive to high pyruvate concentrations. Thus the increased sensitivity of marine elasmobranch M_4-LDH's to substrate inhibition is probably not a direct adaptation to urea, but may simply be a consequence of a reduced pyruvate K_m: a higher affinity for pyruvate, necessary as an adaptation to urea, also means a higher affinity for the enolpyruvate-NAD^+ abortive complex (Everse and Kaplan, 1973).

Activation Enthalpies and Maximal Velocities

The effect of urea on the $\Delta H^{\ddagger}$ values of the marine elasmobranch LDH reactions appears to be of some biological significance. Since the body temperatures of the guitarfish, cat shark and white shark lie below 20–25 °C, the temperatures at which the Arrhenius plots intersect (Fig. 4), the increased activity of the enzyme in the presence of urea at temperatures below 20–25 °C will enhance rates of pyruvate reduction in these three species.

It is interesting that, in the absence of urea, the catalytic efficiencies (measured as substrate turnover numbers) of marine elasmobranch M_4-LDH's may be intermediate between those of birds and mammals, on the one hand, and marine teleost fishes, on the other (Pesce et al., 1967). In the presence of urea, the activities of marine elasmobranch LDH's are closer to the activities characteristic of marine teleost M_4-

LDH's. Thus in a third manner, urea may be necessary to enable the M_4-LDH's of marine elasmobranch to resemble, in their functional properties, the homologous enzymes of marine teleosts.

Concluding Remarks

A wide variety of enzymes and substrates have been found to be competitively inhibited by urea (Inagaki, 1959; Rajagopalan et al., 1961; Nandi, 1971). The molecular basis of this competition is not known; urea is not a structural analogue of any of the affected substrates. Although a mechanism cannot be determined from kinetic analysis (Laidler and Bunting, 1973), the competitive nature of the inhibition suggests that urea blocks substrate binding at the active site of enzymes. As a hydrogen-bonding agent, urea may be able to replace water bound to important active-site amino acids and have to be displaced by the substrate (see discussion by Rajagopalan et al., 1961).

Conservation of apparent substrate K_m's has been demonstrated for many enzymes (Kobayashi et al., 1974; Wedler and Hoffmann, 1974), including vertebrate acetylcholinesterases (Baldwin, 1971) and pyruvate kinases (Low and Somero, 1976) as well as M_4-LDH's (Hochachka et al., 1976; present study). This indicates that the selective importance of substrate affinity is probably a general rule. Since competitive inhibition by urea appears to be a common phenomenon among enzymes, many elasmobranch enzymes may have adapted to urea by increasing their affinities for substrates. For this and no doubt other reasons, urea has become an essential requirement for marine elasmobranch biochemistry.

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