D-Lactate Specific Pyridine Nucleotide Lactate Dehydrogenase in Animals

Abstract. *A survey of representative invertebrates has revealed the presence of pyridine nucleotide-linked* D-*lactate dehydrogenase in a number of groups. All species studied contained either* D- *or* L-*lactate dehydrogenase, but no species contained both enzymes. The* D-*lactate dehydrogenase from* Limulus polyphemus *has been purified and has a molecular weight of 65,000.*

All animals have been assumed to possess a specific diphosphopyridine nucleotide-linked L-lactate dehydrogenase. The vertebrate enzymes which have been studied are L-lactate specific (*1*). An enzyme crystallized from lobster tail muscle also is L-lactate specific (*2*). Pyridine nucleotide-linked lactate dehydrogenases, which are D-lactate specific, occur in bacteria (*3*), in certain slime molds (*4*), and in a number of fungi (*5*).

During a purification of the lactate dehydrogenase from *Limulus polyphemus* (the horseshoe crab), it was noted that this enzyme was D-lactate specific and not L-lactate specific, as assumed in earlier studies. This was ascertained when the purified enzyme did not react with L-lactate, although it did react with a routine assay substrate consisting of a racemic mixture of lactate isomers.

The enzyme from *Limulus* muscle appears homogeneous as judged by sedimentation in the ultracentrifuge, starch-gel electrophoresis, and immunological properties. Molecular weight determined by the approach-to-equilibrium method (*6*) was 65,000, a value consistent with that obtained on Sephadex G-100 (*7*); values on three different preparations ranged from 62,000 to 70,000. Hence, the enzyme is quite different in size from the L-lactate specific enzymes purified from invertebrates and vertebrates, which have been found to have weights of 140,000 to 150,000 (*1, 2*).

The purified *Limulus* enzyme reduces pyruvate with reduced diphosphopyridine nucleotide stoichiometrically to form D-lactate. There is no indication of the formation of any L-lactate. Furthermore, L-lactate is not oxidized by either the purified enzyme or in the crude extracts from muscle.

Multiple forms of the D-lactate specific enzyme have also been found in *Limulus* (Fig. 1). The enzyme that has been purified from muscle is the major form in muscle and is the form which migrates farthest to the positive pole. The muscle forms apparently are also present in heart, but the major types in this tissue are quite different, as indicated by their much slower electrophoretic migration. The heart types are also strictly D-lactate specific and can be distinguished from muscle types by their difference in inhibition by excess pyruvate (Fig. 2). Hence, as for the vertebrate L-lactate dehydrogenase, there appear to be two major types of D-lactate dehydrogenase that can be distinguished by their catalytic and physical properties.

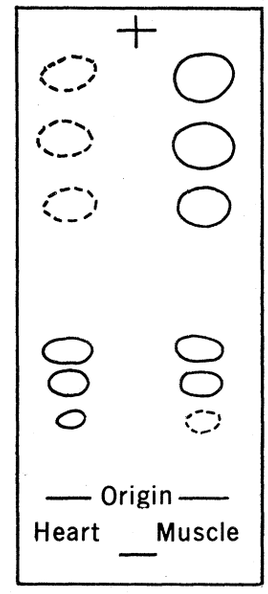

Fig. 1. The electrophoretic mobilities of heart and muscle lactate dehydrogenases from *Limulus polyphemus* on starch gel (*9*). Solid-lined circles represent bands which stain more intensely than bands represented by broken-line circles.

Table 1. Summary of invertebrate lactate dehydrogenase stereospecificities. Centrifuged supernatant fractions were assayed as described in the text.

Phylum	Subphylum	Class	Common name (*10*)	Lactate specificity	
				D-	L-
Arthropoda	Chelicerata	Meristomata	Horseshoe crab (heart, muscle)*	+	
		Arachnida	Spider A (legs, bodies) †	+	
			Spider B (bodies) †	+	
			Tarantula (legs) *	+	
			Wolf spider *	+	
			Scorpion (legs, bodies) †	+	
	Mandibulata	Crustacea	Shrimp (tail muscle) ‡		+
			Lobster (tail muscle) *		+
			Sow bug *		+
		Insecta	Grasshopper (legs) *		+
			Gypsy moth (bodies) *		+
		Chilopoda	Centipede *		+
		Diplopoda	Millipede*		+
Mollusca		Gastropoda ‖	Moon snail *	+	
Annelida		Polychaeta	Marine clam worm §	+	
		Oligochaeta	Earthworm §		+
			Common pond leech §		+
Coelenterata		Hydrazoa	Hydra *		+

* Organs or whole animals were ground in two volumes (g/ml) of 0.1*M* potassium phosphate buffer (*p*H 7.0) containing 1.0 m*M* β-mercaptoethanol and 1.0 m*M* ethylenediaminetetraacetate (EDTA). † Organs or whole animals were ground in 5.0 m*M* tris(hydroxymethyl)aminomethane–hydrochloride (tris-HCl) (*p*H 7.5) containing 1.0 m*M* β-mercaptoethanol and 1.0 m*M* EDTA (*11*). ‡ Organs or whole animals were ground in three to five volumes of 0.25*M* sucrose, 0.01*M* tris-HCl (*p*H 7.0), and 1 m*M* EDTA (*12*). § Whole animals were ground in two volumes of a 1 : 1 mixture of glycerol and 0.1*M* potassium phosphate (*p*H 7.0) containing 1.0 m*M* β-mercaptoethanol and 1.0 m*M* EDTA. ‖ Other classes of mollusks have been reported lacking in lactate dehydrogenase (*13*).

Extracts of a number of different invertebrates were assayed for lactate dehydrogenase stereospecificity by three methods: (i) direct assay with D- or L-lactate as substrate; (ii) stereochemical analysis of the products formed from pyruvate and reduced diphosphopyridine nucleotide; and (iii) reactions on starch-gel electrophoresis (Table 1).

It was observed that no animal possessed lactate dehydrogenases for both stereoisomers of lactate. In the arthropods, all the chelicerates (arachnids) studied possessed D-lactate enzymes, whereas the mandibulates (crustaceans, myriapods, and insects) have L-lactate specific dehydrogenases. The polychaete *Nereis* has a D-lactate specific catalyst, whereas the earthworm and leech have the L-lactate dehydrogenase. These results are consistent with earlier findings on lactate dehydrogenases using coenzyme analogs (*8*). Marked kinetic

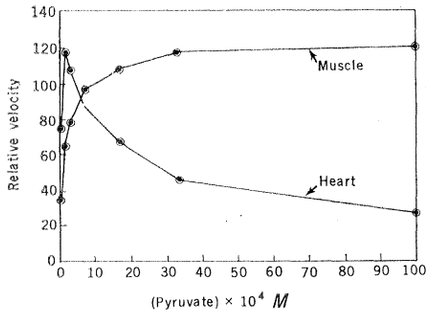

Fig. 2. The effect of pyruvate concentration on *Limulus* D-lactate dehydrogenase catalyses. The initial rate of loss of reduced diphosphopyridine nucleotide (DPNH) absorption at 340 nm was measured as a function of varying pyruvate concentration. Three-milliliter reaction mixtures contained the following concentrations: 0.117 m*M* DPNH (from P-L Biochemicals), and 13.9 m*M* potassium phosphate, *p*H 7.5.

differences were observed between chelicerates and the other arthropods, and between *Nereis* and other annelids.

Analyses of the molecular weights of the various D-lactate enzymes by the Sephadex G-100 method indicate that their weights are close to that of the purified enzyme from *Limulus* muscle, whereas those of the L-lactate enzymes of the invertebrates are roughly the same size as those of the vertebrate lactate dehydrogenases.

In each animal, the lactate dehydrogenases are specific for only one isomer of lactate. This suggests that both the L- and D-lactate enzymes may have arisen from mutation of one gene locus; and we hope to determine this development by further study. The finding of the D-lactate enzyme in invertebrates may add a useful parameter for evaluating phylogenetic and taxonomic schemes for invertebrates.

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References and Notes

1. N. O. Kaplan, in *Evolving Genes and Proteins*, V. Bryson and H. J. Vogel, Eds. (Academic Press, New York, 1965), p. 243.
2. H. D. Kaloustrian and N. O. Kaplan, in preparation.
3. D. Dennis and N. O. Kaplan, *Fed. Proc.* **18**, 213 (1960).
4. R. C. Garland and N. O. Kaplan, *Biochem. Biophys. Res. Commun.* **26**, 679 (1967).
5. F. H. Gleason, R. A. Nolan, A. C. Wilson, R. Emerson, *Science* **152**, 1272 (1966).
6. D. Yphantis, *Biochemistry* **3**, 297 (1964).
7. P. Andrews, *Biochem. J.* **91**, 222 (1964).
8. N. O. Kaplan and M. M. Ciotti, *Ann. N.Y. Acad. Sci.* **94**, 701 (1961).
9. I. H. Fine and L. Costello, *Meth. Enzymol.* **6**, 968 (1963).
10. The scientific names of identified specimens are: horseshoe crab, *Limulus polyphemus*; spider B, *Pholcus phalangioides;* tarantula, *Dugesiella hentzi;* wolf spider, *Geolycosa*; scorpion, *Centruroides sculpturatus*; shrimp, *Penaeus seriferus;* lobster, *Homarus americanus*; sow bug, *Oniscus*; grasshopper, *Melanopus bruneri* (obtained from F. T. Cowan); gypsy moth, *Porthetria dispar*; clam worm, *Nereis virens*; earthworm, *Lumbricus*; centipede, *Scolopendra polymorpha;* millipede, *Spirobolus*; leech, *Haemopsis grandis*; moon snail, *Polinices heros.*
11. Extracts were obtained from S. L. B. Chasalow.
12. Extracts were obtained from A. C. Wilson.
13. Y. Robin and N. Van Thoai, *Biochim. Biophys. Acta* **52**, 233 (1961).
14. Aided by grants from NASA (NSG 375-S4), National Institutes of Health (CA 3611-11), and American Cancer Society (77J). G.L.L. is a NDEA title IV graduate fellow. This is publication No. 609 of the Graduate Department of Biochemistry, Brandeis University.

12 August 1968

Mycobacterium tuberculosis in Macrophages: Effect of Certain Surfactants and Other Membrane-Active Compounds

Abstract. *Some compounds, not directly inhibitory or enhancing, nevertheless influence growth of tubercle bacilli in macrophages in cell culture. They include certain surfactants whose effects can be varied by their structural design. The compounds are probably stored in cell lysosomes. They can interact with various membranes to affect permeability. The anti- and protuberculous surfactants differ in such interaction and also in effect on lysosomal enzyme activity in infected macrophages. A link between the effect on lysosomal membranes and on tuberculous infection is suggested.*

Some compounds suppress or enhance experimental tuberculosis through host-mediation; correlated effects on bacterial multiplication in cell-free medium have not been shown. These compounds include cortisone (*1*), the trypanocidal sulfonated naphthylamine derivative suramin (*2*), and certain nonionic surface-active polyoxyethylene ethers (*3*, *4*) analogous to Triton WR-1339 (*5*). The members of one series of the surfactants [prepared by Cornforth and his colleagues (*3*)] are polyethylene glycol ethers of a *p-tert*-octylphenol-formaldehyde cylic tetramer, and are referred to as HOC-*x* (HOC indicates the constant phenolic nucleus and *x* the average number of ethylene oxide units per phenolic group, which can be varied). The value of *x* and the consequent lipophilic-hydrophilic balance is crucial in determining whether the compound shall suppress (low values) or enhance (high values) murine tuberculosis, or be inactive (intermediate values).

Since this type of surfactant can enter macrophages in the living animal (*6*), and these white cells are a main site of attack in tuberculosis infection, I have now studied whether the contrasting effects could be reproduced when the compounds were applied directly to isolated macrophages infected in tissue culture, or whether participation of the intact host (for example, through the known complex disturbances in lipid metabolism) was essential. Growth of tubercle bacilli in macrophages had been unaffected if WR-1339 was added to the cell culture, although it was inhibited if the cells were from animals that had been treated previously with this compound (*7*); but I suspected that this might be due to the low concentration tolerated in the culture. I used the less toxic HOC-12½ (Macrocyclon) and HOC-60 (mean molecular weights about 3,100 and 11,500), representative respectively of antituberculous and protuberculous (growth-accelerating) activity in the animal (*8*).

Macrophages were obtained from the unstimulated peritoneal cavities of normal mice (P strain) and were maintained as monolayers on glass coverslips in Leighton tubes containing 1 ml of medium suitable for long-term survival (*9*). The tubes were gassed with 5 percent CO_2 in air, closed tightly with silicone rubber bungs, and incubated at 37°C. The medium was changed (with gassing) every 10 to 14 days. Generally, a few days after start of incubation, the external medium was withdrawn and the cell layers were infected by exposure, in the Leighton tubes, to a well-dispersed suspension of bacilli for 2 hours at 37°C. *Mycobacterium tuberculosis,* human strain H37Rv, whose original virulence had become somewhat attenuated, was the strain used mainly, well-grown surface subcultures on Proskauer and Beck liquid medium being homogenized and filtered through paper. The concentration of the suspension was adjusted so as to give a light infection initially, with a mean of 0.5 to 1.0 bacillus per cell. After infection, free bacilli were removed by washing, and the wash fluid was replaced by the usual medium—with the addition of streptomycin (5 unit/ml), to prevent extracellular bacterial multiplication, and of one of the agents (Macrocyclon,