Table 1. Activity of beta glucuronidase in skin components from 15 patients with cystic fibrosis (mean age 8.5 years) and 15 controls (mean age 11 years). Units are expressed as micrograms of phenolphthalein released per gram of dry tissue per hour of incubation; t is the difference between the means divided by the standard error of the difference. The t values (P associated with this t value): sweat gland, $t = 2.9$ ($P = .01$); epidermis, $t = 2.6$ ($P = .02$); dermis, $t = 0.77$.

Patients with cystic fibrosis			Controls		
Sweat gland (units $\times 10^3$)	Epidermis (units $\times 10^3$)	Dermis (units $\times 10^2$)	Sweat gland (units $\times 10^3$)	Epidermis (units $\times 10^3$)	Dermis (units $\times 10^2$)
1.2	1.8	1.8*	2.7*	2.0	0*
	2.7	5.9*	3.1*	3.2	0*
2.9	2.9	5.0	2.2*	2.2	0*
1.9	2.0	1.4	1.3*	3.3	0*
1.5*	2.0	2.5	4.2	2.6	2.7
	1.5	3.7*	2.2	2.6	5.0
0.5*	1.2	2.8	2.3	2.0	4.8
1.5	1.6	4.1	2.9	2.5	3.1
1.4*	1.7	3.2	2.2*	1.9	1.5
2.1	2.4	1.0*	2.2	3.3	3.2
3.3	1.9	4.9	2.7	2.6	5.7
1.4	2.2	3.3	2.8	3.4	5.3
2.2	1.9	4.3	1.7	3.2	2.7
1.6	1.8	2.2	4.1	1.5	5.2
1.8	2.5	4.0	1.9	1.7	2.8
		Means ± standard deviations			
1.8 ± 0.7	2.0 ± 0.46	3.3 ± 1.4	2.6 ± 0.8	2.5 ± 0.6	2.8 ± 2.1

* This figure is not the average of three separate determinations.

uronic acid and chondroitin sulfate. Others have implicated the enzyme both in the synthesis and degradation of acid mucopolysaccharides. Montagna (*3*) found high activities of beta glucuronidase in human sweat gland, and Ballantyne and Wood (*4*) observed increased activity of the enzyme in the actively secreting salt glands of ducks that had been given a load-dose of salt. Thus, beta glucuronidase may be a necessary component of the salt-secretion mechanism, and may have some role in mucopolysaccharide metabolism.

We have determined the activities of several enzymes, including acid and alkaline phosphatase, ouabain-sensitive adenosine triphosphatase activated by Na^+ and K^+, phosphatidic acid phosphatase, and lactate, malate, glucose-6-phosphate, isocitrate, and succinic dehydrogenases (*5*) in components of freeze-dried skin isolated by microdissection from children with cystic fibrosis. We found no differences in enzyme activities between normal children and those with cystic fibrosis, with respect to enzyme activities until we assayed beta glucuronidase activity.

Skin biopsies were taken with a high speed drill and imbedded in tragacanth gel. The samples were frozen, cut into thin sections on a microtome, and dried in a vacuum. Fragments of epidermis, sweat gland, and dermis were isolated by microdissection and weighed on a quartz-fiber balance (*6*). Average weights for dermis, epidermis, and sweat gland were 9, 6, and 3 μg, respectively. Beta glucuronidase activity of the fragments was determined with phenolphthalein glucuronide as the substrate (*7*). The liberated phenolphthalein was measured on a spectrophotometer after the mixture was incubated for 24 hours. Kinetic studies indicated that the rate of release of product during the entire incubation period was linear. There is a statistically significant ($P = .05$, Student's t-test) decrease in beta glucuronidase activity in the epidermis and sweat gland of patients with cystic fibrosis (Table 1).

We do not know whether this decrease in enzyme activity represents decreased synthesis, abnormal enzyme structure, or enzymatic inhibition by some other substance. The decreased activity of beta glucuronidase would seem to indicate some defect more fundamental than general debilitation, inasmuch as a number of other enzymes assayed had normal activities (*5*). The finding (*8*) of metachromatic material (presumably mucopolysaccharide) accumulating in cultured cystic fibrosis fibroblasts is interesting in view of the connection between beta glucuronidase and mucopolysaccharide metabolism.

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Horseshoe Crab Lactate Dehydrogenase: Tissue Distribution and Molecular Weight

Abstract. *Lactate dehydrogenase from* Xiphosura (Limulus) polyphemus *is* D(−)*-lactate specific. It does not use* L(+)*-lactate, α-hydroxybutyrate, α-hydroxyvalerate, or α-hydroxyisocaproate as substrate. In most tissues lactate dehydrogenase is composed of five isozymes with a molecular weight of 140,000 for each, as judged by gel-filtration chromatography. This suggests that the isozymes are tetramers comprised of varying amounts of two physicochemically distinct subunits.*

Long and Kaplan observed that horseshoe crab, *Xiphosura* (*Limulus*) *polyphemus,* lactate dehydrogenase (LDH) is D(−)-lactate specific (*1*). Also, they reported that the molecular weight of this enzyme, as judged by gel filtration and ultracentrifugation, was approximately 70,000 or one-half that of vertebrate lactate dehydrogenase [E.C. 1.1.1.27 : L-lactate : nicotinamide-adenine dinucleotide (NAD) oxidoreductase] (*2*). This report confirms that *Limulus* LDH is D(−)-lactate specific. However, its molecular weight, as judged by gel filtration, is essentially identical to that of vertebrate LDH (approximately 140,000).

Because of the discrepancy between the molecular weights obtained for *Limulus* LDH, I compared it with a D(−)-lactate specific LDH from another invertebrate. It has been reported (*1*) that a D(−)-lactate specific LDH is present in the moon snail (*Gastro-*

poda), but that LDH is lacking in other molluscan classes (*3*). However, the common edible clam *Mercinaria mercinaria* contains a D(−)-lactate specific LDH, albeit in low concentration (*4*). By means of gel-filtration chromatography, the molecular weight of this enzyme was estimated to be approximately 130,000 (*4*).

The specimens of *Xiphosura polyphemus* were obtained from various locations in Long Island Sound by commercial fishermen. Others were obtained from the Marine Biological Laboratory, Woods Hole, Mass. The organisms ranged in size (diameter of carapace) from 5 to 30 cm. Those that were not used immediately upon arrival at the laboratory were maintained in aerated seawater tanks. No more than 2 weeks elapsed between capture and tissue preparation.

Vertical starch-gel electrophoresis (Buchler Instruments, Fort Lee, N.J.) was carried out at 4°C with 14 percent gels (*5*) in a tris(hydroxymethyl)-aminomethane (tris)–borate–ethylenediaminetetraacetate (EDTA) discontinuous buffer system (*p*H 8.6) (*6*). Electrophoresis was conducted at a constant voltage gradient of 8 to 9 volt/cm or at a constant current of 15 ma for 14 to 17 hours. The gels were stained for LDH activity by the tetrazolium method (*5*, *7*) with lithium lactate and other α-hydroxy acids as substrates (*8*). In all cases, half of the gel served as the control and was incubated in the staining solution minus substrate to detect spurious bands of enzyme activity such as might result from the activity of "nothing" dehydrogenase (*9*).

With the exception of the hemolymph, all tissues were minced and homogenized at a ratio of 1.0 g of tissue per milliliter of buffer by repeated short bursts of blending in a Sorvall Omni Mixer (Ivan Sorvall, Inc., Norwalk, Conn.). Blending time, over a wide range, had no effect on the tissue-specific pattern of the isozymes. Hemolymph was collected from the heart with a syringe fitted with an 18-gauge needle; it was then diluted with buffer (1:1). The homogenates (*10*) and diluted hemolymph were centrifuged at 100,000*g* for 60 minutes. The enzyme was precipitated from the supernatant with ammonium sulfate between 40 and 60 percent of saturation.

Individual isozymes were isolated and purified by diethylaminoethyl (DEAE)-cellulose column chromatography. The ammonium sulfate precipitate was dissolved in a minimum amount of neutral 0.001*M* sodium phosphate buffer containing $5 \times 10^{-3}M$ β-mercaptoethanol and dialyzed against two changes (1000 volumes each) of the same buffer. The dialyzate was centrifuged at 100,000*g* for 60 minutes. The supernatant was applied to a DEAE-cellulose column equilibrated against the same buffer and eluted in a stepwise manner between 0.001 and 0.2*M*.

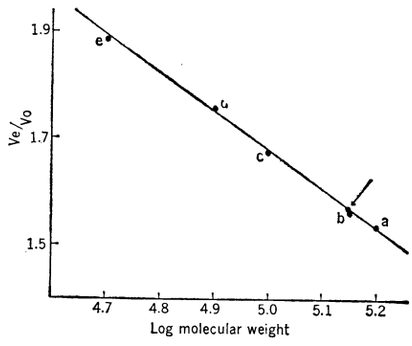

Fig. 1. Molecular weight of *Xiphosura polyphemus* lactate dehydrogenase by gel filtration on Sephadex G-150. The column was calibrated with rabbit muscle aldolase (*a*), horse LDH-5 (*b*), yeast hexokinase (*c*), bovine serum albumin (*d*), and ovalbumin (*e*). The elution volume (V_e) of ferritin (100 ml) was employed as the void volume (V_o) of the column. The arrow denotes horseshoe crab LDH.

The molecular weight of *Limulus* D(−)-lactate dehydrogenase was estimated by gel-filtration chromatography [Sephadex G-100, G-150, and G-200 (*11*) and Bio-Gel A-0.5 m, 100 to 200 mesh]. The dimensions of the columns were 2.5 by 35.0 cm, 2.5 by 50.0 cm, and 2.0 by 105 cm. Chromatography was carried out both at 4° ± 2°C and 23° ± 2°C with either the supernatant (100,000*g*) of the tissue homogenates or purified individual isozymes (*12*).

Under all conditions used, *Limulus* D(−)-lactate dehydrogenase was eluted from the columns between human γ-

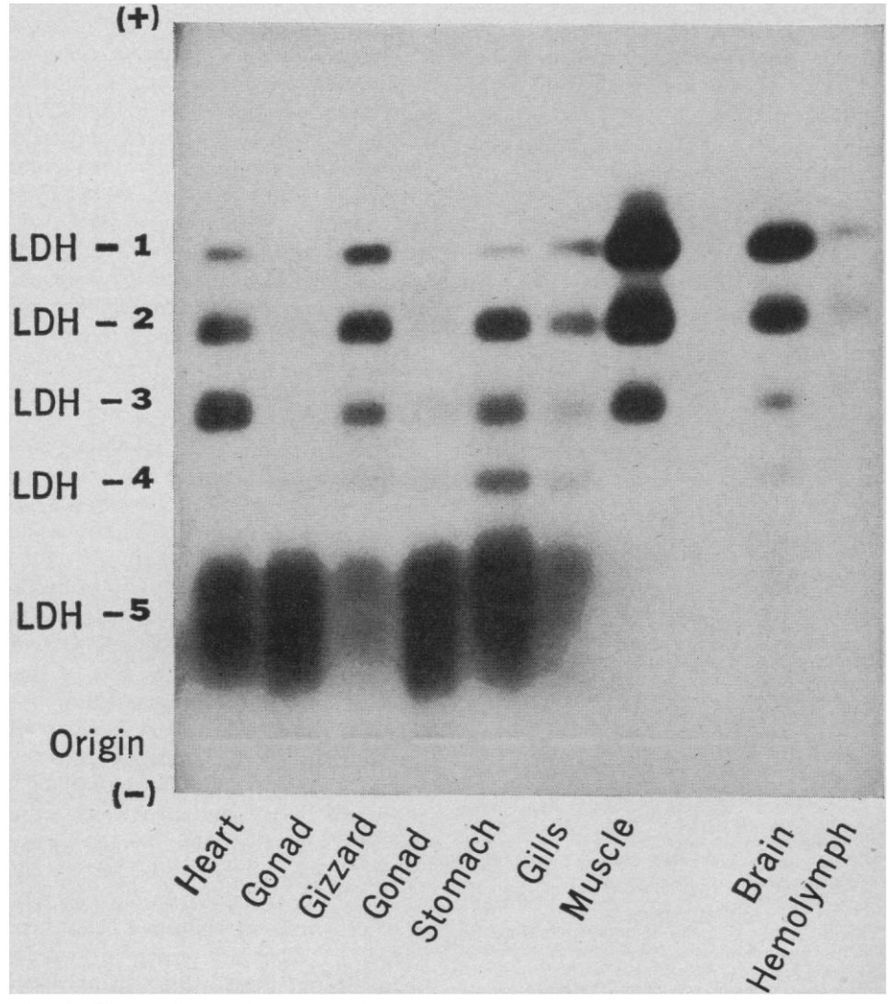

Fig. 2. Tissue distribution of *Xiphosura polyphemus* lactate dehydrogenase. Electrophoresis was conducted in 14 percent starch (gel) at *p*H 8.6 in 45 m*M* tris, 25 m*M* boric acid, and 1 m*M* disodium EDTA at 8 volt/cm for 16.5 hours at 4°C.

globulin or rabbit muscle aldolase and yeast hexokinase (Fig. 1). Also, it cochromatographed with beef, horse, or rabbit LDH. Thus, the molecular weight of *Limulus* LDH is similar to that of mammalian L(+)-lactate dehydrogenase (approximately 140,000).

The LDH appears to exist in the tissues of the horseshoe crab in five isozymic forms (Fig. 2). This zymogram is representative of tissue preparations from all specimens (several dozen) examined. Each tissue possesses a specific pattern of isozymes. Thus, as in vertebrates, it may be that *Limulus* LDH is a tetramer which exists in the form of two homopolymers, BBBB and AAAA (designated LDH-1 and LDH-5, respectively), and three heteropolymers, BBBA, BBAA, and BAAA (designated LDH-2, -3, and -4, respectively). This could be proved by molecular hybridization (*13*). However, it has not yet been possible to reversibly dissociate the subunits of horseshoe crab LDH (*14*).

The LDH isozymes of *Limulus* appear to be highly specific for D(−)-lactate. Neither α-hydroxyvalerate, α-hydroxyisocaproate, nor α-hydroxybutyrate could substitute for lactate in the staining mixture.

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Production of Mouse Urinary Bladder Carcinomas by Sodium Cyclamate

Abstract. *Sodium cyclamate was suspended in cholesterol pellets that were surgically implanted in the urinary bladders of mice. In duplicate experiments, incidences of mouse bladder carcinomas observed in animals exposed to these pellets were 78 and 61 percent compared with incidences of 13 and 12 percent in control mice exposed to pellets of pure cholesterol. The exposure of the mouse bladder to sodium cyclamate was very brief, as the time required for 50 percent of the compound to disappear from the pellets was about 1 hour. This experimental technique was found to be highly sensitive, reproducible, and predictive of the bladder carcinogenicity of orally administered cyclamate.*

Cyclamate has been employed as a sweetening agent for nearly 20 years, but only since 1961 has it enjoyed increasingly widespread consumption and popularity (*1*). Although studies of the potential carcinogenic activity of cyclamate have been conducted in animals, they have not been satisfactory. One deficiency of all these studies is that none of them have reported a thorough investigation of the urinary bladders of test animals for the presence of carcinoma. The mammalian urinary bladder is known to be carcinogenically sensitive to chemically defined organic compounds, and correlations of human and animal bladder carcinogenicity for several of these carcinogens have been made (*2*). The data presented here are the first published which demonstrate the carcinogenic activity of cyclamate. The technique of implantation of a pellet in the mouse bladder, used in this study, has been found to be highly sensitive, reproducible, and predictive of the bladder carcinogenicity of cyclamate administered orally.

The experimental details of our use of the above-mentioned technique have been reported (*2, 3*). Cholesterol, which was purified by recrystallization before use, and sodium cyclamate (*4*) were ground separately to a fine powder in an agate mortar. Sodium cyclamate was then mixed carefully with four times its weight of cholesterol before the mixture was compressed into spheroidal pellets, 5/32 inch (0.4 cm) in diameter and 20 to 24 mg in weight, with a standard face die (*2, 3*). The quantity of sodium cyclamate in representative pellets was measured by gas-liquid chromatography (*5*) prior to surgical implantation of the remaining pellets in the bladder lumens of Swiss female mice, 60 to 90 days old (*2, 3*). Pellets were removed from some mice at various periods of time after surgical implantation, and the quantity of sodium cyclamate remaining in these pellets was measured (*5*). These data were used to obtain an assessment of the rate of disappearance of sodium cyclamate from the cholesterol pellet—the elution-rate constant (K) and the time required for 50 percent of the sodium cyclamate to disappear from the pellet, the 50 percent elution time ($T½$)—in order to evaluate the probable exposure of the bladder to the test compound (*2, 3*). Pellets of identical shape and mass were made from purified cholesterol and surgically placed in the bladder lumens of another group of mice as comparative controls.

For the carcinogenicity experiments the following procedure was used. Pellets composed of either pure cholesterol or sodium cyclamate and cholesterol were surgically placed in the bladder lumens of duplicate groups of 100 mice (*2, 3*). The animals in each group were allowed to survive 13 months, and the bladders of animals surviving more than 175 days were microscopically evaluated for the presence of bladder carcinoma by use of the criteria of Bonser and Jull and of Roe (*6*). Carcinomas that were not observed to invade muscle were classified as stage I; those that were seen to invade muscle as stage II; and those that presented evidence of serosal spread or local pelvic metastases as stage III. Only the total incidence of carcinoma was used to assess carcinogenicity. Statistical comparison of the incidence of carcinoma related to the