Table 1. Chromatographic retention times and boiling points of various methyl esters of C_3 to C_5 carboxylic acids. A 5-percent SE-30 column at 50°C was used. All boiling points are at atmospheric pressure except that marked with an asterisk (13 mm-Hg).

Methyl ester	Boiling point (°C)	Retention time (min)
Propionic	79.7	1.4
Butyric	102	2.6
Isobutyric	93	2.0
Cyclopropane carboxylic	119	3.5
n-Valeric	127.3	5.0
Isovaleric	116	3.5
2-Methylbutyric	113*	3.5
Pivalic	100–102	2.4
Cyclobutane carboxylic	136	7.1

of authentic C_3 to C_6 fatty acids were examined. The spectrum of the secretion was closely similar to the spectra of the simple saturated acids, but differed distinctly from those of the unsaturated or cyclic acids. Authentic samples of methyl esters of every possible C_3 to C_5 saturated acid were prepared and subjected to direct comparison by gas-liquid chromatography with the esters from the caterpillar secretion (Table 1). From these comparisons it is clear that one of the acids in the secretion (retention time, $t = 2.0$ min) is isobutyric acid (I). The other acid ($t = 3.5$ min) could be cyclopropane carboxylic acid, isovaleric acid, or 2-methylbutyric acid. The cyclopropane structure was excluded by direct infrared comparison. The distinction between the remaining two possibilities was made by gas-liquid chromatography on a column of 15-percent Apiezon grease-L at 100°C, which separates methyl 2-methylbutyrate ($t = 7.5$ min) from methyl isovalerate ($t = 8.0$ min). On this column, the esterified secretion showed two peaks at 4.0 minutes (methyl-isobutyrate) and 7.5 minutes (methyl 2-methylbutyrate). The second acid in the secretion is evidently 2-methylbutyric acid (II).

Additional milkings were made from larvae that had been reared on two other umbelliferous plants, carrot (*Daucus carota*) and parsnip (*Pastinaca sativa*). In both cases the principal components of the secretion were again isobutyric acid and 2-methylbutyric acid, although the same chromatographic method showed the presence of additional minor components that were not pursued further. Evidently, the nature of the food plant does not grossly affect the constitution of the mixture. It might be added that neither of the two acids figures among the many compounds that have been isolated from fennel, carrot, or parsnip (*5*). There is certainly no reason to presume that the larvae need rely on any but readily available metabolic precursors for the synthesis of these simple aliphatic acids. The suggestion (*4*) that the gland provides a vehicle for the elimination of essential oils obtained with the diet is evidently contraindicated, at least for *Papilio machaon*.

The relative proportion of the acids in the secretion was estimated. This was done by comparison with gas-liquid chromatograms of known mixtures of the authentic methyl esters. Caterpillars fed on carrot and fennel produced isobutyric and 2-methylbutyric acid in the ratio of 70:30. For those fed on parsnip the ratio was 52:48.

Although neither of the acids produced by *Papilio* is known to occur in the secretions of other arthropods, it is interesting that certain beetles (Carabidae) (*6*) should produce a defensive spray containing methacrylic (III) and tiglic acid (IV), the unsaturated analogues of isobutyric and 2-methylbutyric acid. Additional acids secreted by arthropods include formic, acetic, and caprylic (*7*). Certain caterpillars of the moth family Notodontidae discharge a spray containing formic acide (*7, 8*). Their gland is entirely different from the osmeterium and is clearly no homologous to it.

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Lactate Dehydrogenase Isozyme Patterns of Human Platelets and Bovine Lens Fibers

Abstract. *Human platelets and bovine lens fibers contain lactate dehydrogenase-3 (LDH-3) as the predominant isozyme but only very faint traces of LDH-5. Since the platelets and lens fibers, like mature human erythrocytes, lack a nucleus, the results strengthen the case for a previously developed association between LDH-5 and the cell nucleus. These three cell types are mainly anaerobic, and therefore their isozyme patterns are incompatible with the theory that anaerobic tissues exhibit predominantly LDH-5 and aerobic tissues mainly LDH-1.*

The lactate dehydrogenase (LDH) isozyme pattern of platelets and bovine lens fibers is of interest in light of a previously developed association between LDH-5 and the cell nucleus or direct nuclear products such as RNA (*1*). Most tissues exhibit varying proportions of at least five LDH isozymes (*2*). The mature human erythrocyte, devoid of a nucleus, displays mainly LDH-1 and -2 with small amounts of LDH-3 and -4 (*3*). By use of a sucrose-saline lysing solution traces of LDH-5 have been identified on starch-gel electrophoresis (*4*). The association between LDH-5 and the nucleus was also suggested by a comparative study of LDH isozymes in erythrocytes from various classes of vertebrates in which only species with circulating nucleated erythrocytes revealed cathodal isozymes (*3*). Furthermore, LDH-5 appeared in hemolyzates from anemic patients with reticulocytosis and from guinea pigs developing reticulocytosis after administration of phenylhydrazine (*1*). Finally, the isozyme pattern of nuclei purified from duck erythrocytes showed predominantly LDH-5 whereas the cytoplasmic pattern exhibited mainly LDH-1, -2, and -3 (*1*). Since the human platelet and the bovine lens fiber also lack a nucleus, they provide a unique opportunity to investigate further this association between LDH-5 and the cell nucleus or direct nuclear products.

Platelets were prepared from each of six normal individuals. Citrated blood (20 ml) was centrifuged for 5 minutes at 600*g*, and the supernatant of platelet-rich plasma was centrifuged at 1200*g* for 20 minutes. The sedi-

mented platelets were washed three times in normal saline; direct platelet and leukocyte counts were performed. The platelets, resuspended either in distilled water or in sucrose (0.25*M*)-saline (0.15*M*) solution, were disrupted by ultrasonic vibration; the supernatant was assayed spectrophotometrically for LDH activity at 340 mμ (*3*) and subjected to vertical starch-gel electrophoresis at 4°C for 4 hours at 12 volt/cm (*5*). The gels were sliced horizontally, and sites of LDH activity were identified (*4*). In several experiments the platelet suspensions were divided into two equal parts and treated with high-frequency sound, one part in water, the other in the sucrose-saline solution. Twelve bovine lenses obtained within 6 hours of the animals' deaths were dissected and sectioned to yield a posterior portion containing cells without nuclei; these posterior parts were minced in the sucrose (0.25*M*)-saline (0.15*M*) solution. After ultrasonic disruption and centrifugation the supernatant was separated by starch-gel electrophoresis.

Figure 1 shows the LDH-isozyme patterns of human platelets prepared in different solutions. The most prominent isozyme is LDH-3; next in intensity are LDH-2 and -4; then LDH-1 and finally faint traces of LDH-5 occurred in the sucrose-saline lysing solution but not in water. These two media were also employed in the extraction of LDH activity from human psoas muscle, where LDH-5 is the predominant isozyme. The LDH activity in the supernatant of the various extracts was assayed before electrophoretic separation. The activity of the water extract was only two-thirds that of the sucrose-saline extract. Starch-gel electrophoresis revealed that the loss involved only LDH-5. No difference in total LDH activity existed between the water and the sucrose-saline extracts of platelets, probably because LDH-5 comprised so small a fraction of the total platelet-LDH activity. The platelet counts of each of six different human preparations were between 2 and 3 × 10^6 per cubic millimeter. The leukocyte counts ranged between 120 and 1200 per cubic millimeter. This is too low to contribute sufficient LDH-5 activity to account for the faint LDH-5 band, since electrophoretic separation of solutions containing this number of lysed leukocytes fails to produce any bands on the gel. Furthermore, the intensity of LDH-5 was not proportional to the leukocyte count. The total LDH activity of the platelet extracts prior to application on the gel was between 2200 and 3600 international units per milliliter.

Figure 2 shows the LDH isozymes in a sucrose-saline extract of bovine lens fibers. The pattern is similar to that observed in human platelets: LDH-3 is the most prominent band, then LDH-2 and -4, then LDH-1, and finally LDH-5. The bovine isozymes exhibit different electrophoretic mobility from the human isozymes.

Low LDH-5 activity in human platelets and bovine lens fibers is apparently not due to the presence of inhibitors, since platelet or lens extracts, when added individually to either purified preparations of human or rabbit muscle LDH-5, produced an arithmetic mean, rather than a decline, of the initial activities.

An LDH isozyme pattern of human platelets similar to that shown in Fig. 1 has been reported (*6*). However, the platelet extracts were obtained by repeated freezing and thawing. Since under these conditions LDH-5 is more labile than other isozymes (*7*), the interpretation could not be excluded that platelet LDH-5 had been destroyed during extraction. The substitution of sonication for freezing and thawing in the present experiments eliminates this possibility.

The isozyme pattern of bovine lens fibers shown in Fig. 2 reveals very faint LDH-5 in addition to distinct bands of activity of LDH-1, -2, -3, and -4. As yet only LDH-1 has been reported (*8*). A sucrose-saline lysing solution of high molarity, which was introduced by Starkweather *et al.* (*4*) for detection of LDH-5 in human erythrocytes (*3*), may have revealed LDH-5. The experiments in which equal portions of human muscle were extracted with either water or a sucrose-saline solution demonstrate that LDH-5 activity is appreciably decreased when water is used for extraction. These results agree with the sensitivity of LDH-5 to dilution (*9*).

Mature human erythrocytes, platelets, and bovine lens fibers lack the nucleus they possessed in earlier developmental stages. Decline of LDH-5 activity with loss of the nucleus, in addition to other evidence summarized at the outset, strengthens the association between the nucleus and LDH-5. In the apparent absence of LDH-5 inhibitors within platelets, erythrocytes,

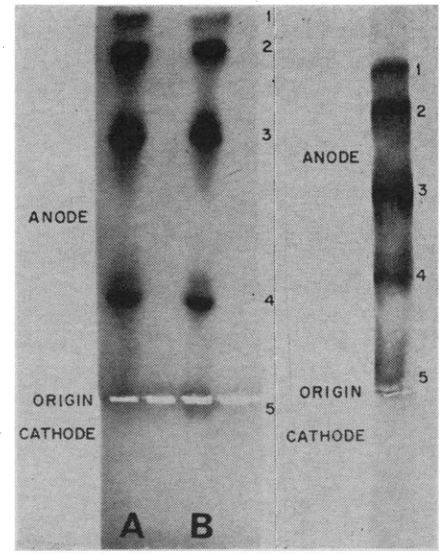

Fig. 1 (left). Starch-gel electrophoretic patterns of LDH isozymes in platelets extracted in (*A*) water and in (*B*) sucrose-saline. Numbers refer to isozymes. Fig. 2 (right). LDH isozyme pattern of bovine lens fibers.

and lens fiber cells, differences may possibly exist in the rate of intracellular catabolism of the various isozymes with increased lability of LDH-5. Rates of catabolism of individual isozymes may vary in different tissues, and this has been suggested as a possible explanation for the observation that various tissues of an organism have different isozyme patterns (*10*). Increased LDH-5 catabolism is consistent with differential sensitivity of LDH-5 to a variety of conditions including heating (*11*), freezing (*7*), and dilution (*9*). That purified rabbit muscle LDH-5 intravenously administered to normal mice was cleared from plasma more rapidly than purified LDH-1 from pig heart (*12*) would seemingly support this hypothesis. However, in these experiments the more rapid decline of LDH-5 activity may also have resulted from the increased heat sensitivity of LDH-5 as compared to LDH-1 (*13*). Differences in reactivity between LDH-5 and LDH-1 toward high concentrations of pyruvate at 25°C entirely disappear at 40°C (*13*). Loss at 40°C of resistance to substrate displayed at 25°C has additional theoretical implications. The hypothesis has been advanced that LDH-5, because of its resistance at 25°C to pyruvate concentrations to which LDH-1 is sensitive, occurs as the predominant isozyme in anaerobically metabolizing tissues (*14*). This hypothesis is incompatible with

the observation that, at the more physiological temperature of 40°C, LDH-1 and LDH-5 are indistinguishable in reactivity toward substrate (*13*). Furthermore, this theory is incompatible with the observations that three distinct cell types predominantly anaerobic in metabolism—mature human erythrocytes (*15*), platelets (*16*), and bovine lens fibers (*17*)—exhibit mainly isozymes 1–4 but only traces of LDH-5. According to this theory, liver, because it contains mainly LDH-5, is considered anaerobic; however, it has a very active aerobic metabolism and, unlike skeletal muscle, maintains a positive arteriovenous difference in lactate concentration.

According to current concepts of the structure of LDH isozymes, LDH-5 is composed of four identical A subunits, whereas LDH-4 contains one B and three A subunits, LDH-3 has two A and two B subunits, LDH-2 one A and three B subunits, and LDH-1 four B subunits (*18*). Zymograms of platelet and bovine lens fibers reveal highest activity in LDH-3 and, except for diminished LDH-5 activity, approach the pattern observed on random recombination of equal amounts of A and B subunits, that is, isozymes in a proportion of 1 : 4 : 6 : 4 : 1. Prominence of LDH-4 on the zymograms indicates that decreased LDH-5 activity cannot be ascribed to insufficient synthesis of the requisite A subunit. Possibly for the anuclear cell, isozymes composed of four A subunits are constructed from constituent subunits with more difficulty or catabolized more rapidly than isozymes composed of one B and three A subunits.

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Sex-Linkage of Erythrocyte Glucose-6-Phosphate Dehydrogenase in Two Species of Wild Hares

Abstract. *Glucose-6-phosphate dehydrogenase specific to the erythrocytes of each of two wild hares found in Europe was discerned by starch-gel electrophoresis at* pH *7.0 and* pH *8.6. The single, sharp band of the dehydrogenase of* Lepus europaeus *was faster than that of* L. timidus, *at both* pH *levels. The sex-linkage of this enzyme was tested through reciprocal hybrids between the two species. Each male hybrid had a single band of enzyme identical with that of its mother, while both parental types of this enzyme coexisted in female hybrids. Thus, sex-linkage of glucose-6-phosphate dehydrogenase has been suggested not only in man and in the family Equidae, but now in the family Leporidae of placental mammals as well.*

Cytological reasons for postulating the persistence of the entire primitive X-chromosome of a common ancestor, protoinsectivores, to various placental mammals of today (*1*) imply that divergent species share the same kinds of sex-linked genes. Among sex-linked traits known in man, hemophilia A and B and anhidrotic ectodermal dysplasia are also found in at least one other species of placental mammals (*2*).

The glucose-6-phosphate dehydrogenase (G-6-PD) present in the erythrocyte, known to be produced by a sex-linked gene in man, is sex-linked in the horse and donkey (*3*). In that the species-specific G-6-PD of these two interbreeding species had different electrophoretic mobilities, an opportunity was afforded to test the sex-linkage of this enzyme through reciprocal hybrids, mules and hinnies, in the family Equidae.

A parallel situation in the family Leporidae permitted the test for sex-linkage of erythrocyte G-6-PD. One of us (I.G.) successfully obtained reciprocal hybrids between two species of wild hares of Europe, *Lepus europaeus* and *L. timidus*. Both species have 48 chromosomes in their diploid complements, and, unlike mules and hinnies, male as well as female interspecific hybrids are fertile (*4*).

Vertical starch-gel electrophoresis was carried out at *p*H 8.6 in borate buffer and at *p*H 7.0 in phosphate buffer with resulting sharp delineation of G-6-PD bands. Electrophoresis was continued for 14 hours at 4°C with a gradient of 4 volt/cm (*5*).

From twice-washed erythrocytes, a hemolyzate (the cells were disrupted by freezing and thawing) of 1-to-100 dilution was prepared in 0.01*M* potassium phosphate buffer, *p*H 7.0, which contained ethylenediaminetetraacetate (EDTA) and β-mercaptoethanol. Each starch-gel plate contained 500 mg of EDTA and 5 mg of triphosphopyridine nucleotide (TPN). The staining solution was prepared by a modification of the spot-test reagent for G-6-PD (*6*). Ten milligrams of glucose-6-phosphate dipotassium salt (Sigma), 2 mg of 3(4,5-dimethylthiazolyl-1, 2)-2, 5-diphenyltetrazolium bromide, 2 mg of phenazine methosulfate, and 2 mg of TPN were dissolved in 10 ml of 0.1*M* tris-HCl buffer, *p*H 8.0, and poured over the sliced gel. The gel was then kept covered at room temperature for 2 hours.

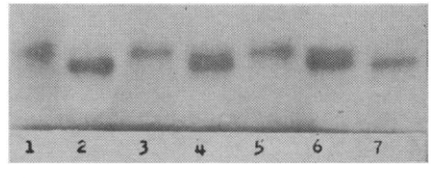

Fig. 1. Starch gel (*p*H 7.0) showing erythrocyte glucose-6-phosphate dehydrogenase of parental species and interspecific hybrids. Anodal direction is upward, and the distance between the slow-moving band of slots 2 and 4, and the starting point, measures 15 mm. Slot 1: female *Lepus europaeus*, the mother of four hybrids shown in slots 3, 4, 5, and 6. Slot 2: male *L. timidus*. Slots 3 and 5: hybrid sons. Slots 4 and 6: hybrid daughters of the mother shown in slot 1. Slot 7: male hybrid with the *L. timidus* mother.