Variations in the Lactic Dehydrogenase of Vertebrate Erythrocytes

ELLIOT S. VESELL and ALEXANDER G. BEARN

From The Rockefeller Institute

ABSTRACT Erythrocytes from representatives of the 5 classes of vertebrates revealed a marked species variation in the number of LDH isozymes, in the distribution of the total LDH activity among these isozymes, and in their electrophoretic mobilities. Starch gel electrophoresis of hemolysates followed by direct histochemical demonstration of LDH activity with nitro blue tetrazolium as dye and phenazine methosulfate as electron transporter showed that closely related species exhibited similar LDH patterns. The *rhesus* monkey had LDH isozymes of similar pattern to those of human hemolysate but slightly slower in electrophoretic mobility. The goat and sheep each had 1 band of LDH activity in their erythrocytes of identical electrophoretic mobility, whereas the single band in steer hemolysate migrated slightly faster. The 5 bands of chicken hemolysate were quite similar in pattern to the 5 bands of duck hemolysate but migrated slightly faster and exhibited a different distribution of the total LDH activity. The 2 species of snake each had 1 band of LDH activity with identical mobility. Staining occurred with the levorotatory form of the substrate and not with the dextrorotatory form. Examination of more than 380 human hemolysates failed to reveal any differences among individuals in the main LDH bands. The genetic basis for the species differences in erythrocyte lactic dehydrogenases is discussed.

Heterogeneity of lactic dehydrogenase (LDH) activity in human erythrocytes was first demonstrated by electrophoretic separation of hemolysate on a starch-supporting medium (1). Four peaks of LDH activity have been observed (1–7). Human serum contains LDH enzymes with electrophoretic mobilities similar to those in hemolysate; in myocardial infarction, leukemia, and hepatitis individual peaks of serum LDH activity are elevated (1–7). Other enzymes exhibiting multiple molecular forms in human tissues include malic dehydrogenase (2, 8), ceruloplasmin (9), cholinesterase (10), and alkaline phosphatase (11). Markert and Møller first employed the term isozyme to designate multiple molecular forms of an enzyme and cautioned against applying it to different enzymes with overlapping substrate specificities

(12). By means of starch gel electrophoresis they demonstrated differences between fetal and adult pig tissues in the complement of LDH isozymes (12).

Another method of identifying different molecular forms of an enzyme has been developed by Kaplan and his associates and is based on the use of diphosphopyridine nucleotide (DPN) analogs (13, 14). By comparing LDHs in tissue homogenates from various species with regard to kinetic behavior with DPN analogs, Kaplan and his associates detected subtle alterations in LDHs from numerous species and postulated a scheme of the phylogenetic evolution of the enzyme based on these differences (13).

The approach of comparing lactic dehydrogenases from various species by electrophoretic separation of tissue extracts on starch gels and by direct histochemical visualization of the enzyme activity is utilized in the present report. In the vertebrates examined, there was a widespread variation in both the number and the electrophoretic mobility of erythrocyte lactic dehydrogenases.

MATERIALS AND METHODS

Materials One to 4 ml of blood was taken from living adult members of each of the 5 classes of vertebrates. Mammals studied included human subjects, *rhesus* monkeys, Berkshire pigs, Hartley albino guinea pigs, albino rabbits, Sprague-Dawley rats, C-57 black mice, mongrel dogs and cats, non-thoroughbred horses, mixed breed of goats and of sheep. From the avian class roosters and ducks were bled; from the reptilian class 2 snakes, *Coluber constrictor* and *Drymarchon corais*, were used. *Rana pipiens* was selected from the Amphibia and carp from the Pisces. Blood was obtained by venesection except from the rats, mice, fish, and snakes on which cardiac puncture was performed. Three or more members of each species were bled except for the snakes and dogs from which 2 specimens of each were taken. Blood was obtained from more than 380 human individuals. 5 specimens of human cord blood were analyzed.

Preparation of Hemolysates Within 6 to 96 hours after blood was obtained hemolysates were prepared according to the method of Drabkin (15). After the removal of plasma by centrifugation the erythrocytes were washed thoroughly three times in normal saline and then ruptured osmotically by the addition of one and one-half volumes of distilled water and one-half volume of toluene. An additional procedure employed in some experiments was freezing the mixture of red cells, distilled water, and toluene in acetone and dry ice, followed by thawing. The hemolysates were centrifuged at 15,000 *G* for 30 minutes at 4°C to remove the ghosts.

Electrophoresis Vertical starch gel electrophoresis of the hemolysates was performed according to the method of Smithies (16). Gels were prepared from commercially available starch (starch-hydrolysed, Connaught Medical Research Laboratories, Toronto, Canada) in phosphate–citric acid buffer, 0.01 M, pH 7.0. The greatest separation of LDH activity from the hemoglobin could be achieved when electrophoresis of the hemolysate was carried out near the isoelectric point of hemo-

globin, whereas at higher pH the hemoglobin migrated toward the anode and interfered with staining by distorting the shape of the enzyme bands. The molarity of the phosphate–citric acid buffer in the proximal and distal vessels was 0.2. A potential gradient of 6 v per cm. was employed for 12 hours at 21°C. After electrophoresis the tray was placed in the refrigerator for 30 minutes to facilitate slicing, the vaseline covering of the gel was removed, and the gel was sliced into 2 sections. The top half was stained for protein with amidoblack 10B for 3 minutes and then decolorized with 10 per cent acetic acid. The bottom half was stained for LDH activity.

LDH Assay. (*A*) *Histochemical Method* LDH activity was identified directly on the gel utilizing nitro blue tetrazolium (Nutritional Biochemicals) according to methods developed by Rutenburg *et al.* (17). Phenazine methosulfate (Aldrich Chemical Co.) served as the electron transporter (18). A brilliant purple color produced by the reduction of the tetrazolium salt to the formazan appeared at the site of LDH activity on the starch gel. The nitro BT, relatively insoluble in water, was readily dissolved in a small volume of diethylene glycol. The incubating medium for the gel consisted of (19):

DL-Lactic acid 0.05 M (Allied Chemical and Dye Corp.) adjusted to pH 7.0	12.0 ml
DPN 10 mg/ml (Mann Fine Chemicals) adjusted to pH 8.0	2.4 ml
Nitro BT 1 mg/ml	6.4 ml
Tris buffer pH 8.3 or phosphate–citric acid buffer pH 7.4	62.0 ml
Phenazine methosulfate 1.6 mg/ml	7.2 ml
	90.0 ml

Phenazine methosulfate, the final reagent, was added in the dark because of its instability in light. The bottom half of the gel was overlaid directly with the solution described above and incubated at 37°C for 1 to 1½ hours. Some gels were also incubated in solutions containing either D-lactate (Mann) or L-lactate (Mann).

Controls consisted of incubating the gel in solutions without substrate and of incubating the gel in solutions without coenzyme.

(*B*) *Spectrophotometric Method* LDH activity of hemolysates was also determined spectrophotometrically according to modifications of the method devised by Kubowitz and Ott (20) in which the decrease in optical density at 340 mμ is measured as the reduced diphosphopyridine nucleotide (DPNH) is oxidized to DPN. A suitable dilution of each hemolysate was incubated for 20 minutes at room temperature with 0.2 ml of 0.003 M DPNH and 2.7 ml of phosphate buffer, pH 7.5, ionic strength 0.1. The mixture was transferred to a Beckman cuvette of 1 cm path length; 0.1 ml of 0.001 M sodium pyruvate, adjusted to pH 7.0, was added; and the decrease in absorption at 340 mμ was measured every 15 or 30 seconds for 2 minutes. One unit of dehydrogenase activity was defined as a decrease in the optical density at 340 mμ of 0.001 per minute per ml of hemolysate. The volume of hemolysate used in each assay was adjusted according to the total LDH activity of the hemolysate in order to limit the decrease in optical density to 0.100 per minute. Accuracy of measure-

ment was compromised when alterations in optical density exceeded 0.100 per minute. The LDH activity of the hemolysate was measured within 1 week after it was obtained and during this period of storage at 4°C there was no appreciable loss in activity for any of the species examined.

DPN Analogs Three analogs of DPN: deamino-DPN and 3-acetylpyridine–DPN (Nutritional Biochemicals) and thionicotinamide-DPN[1] were added in a concentration of 0.003 M to human hemolysates just prior to their separation electrophoretically.

Hemoglobin Determination The hemoglobin concentration of the hemolysates was determined, twice on each specimen, by the method of Drabkin (21).

RESULTS

Electrophoretic separation of hemolysates from 19 vertebrate species revealed a widespread variation in both the number and mobility of lactic dehydrogenases. Considerable species variation was observed in the distribution of the total LDH activity in the multiple bands. More than 40 LDHs could be distinguished by their differences in electrophoretic mobility alone (Figs. 1 and 2). Figs. 1 and 2 also show that not all the vertebrates examined have multiple molecular forms of LDH in their erythrocytes. In hemolysates from the rat, pig, steer, sheep, goat, frog, and snake only 1 band of LDH activity was observed. Limitation in the sensitivity of the histochemical technique could account for failure to observe bands of low LDH activity and the 5 isozymes of LDH activity known to be present in human serum (3, 6) could not all be visualized on the starch gel by the tetrazolium method. The LDH activity of human hemolysate is approximately 100 times greater than that of an equal volume of serum; and therefore, as suggested by Table I, in most species the red cell contains a sufficiently high LDH activity to serve as a suitable cell for the histochemical demonstration of LDH activity with the technique employed. It was estimated that approximately 200 units of LDH activity was the limit of visual resolution by the histochemical technique. Some species such as the steer, goat, sheep, and horse exhibited a considerably lower LDH activity per gram of hemoglobin than others (Table I).

A comparison of the bands of LDH activity observed after electrophoretic separation of hemolysates from various species revealed that closely related species had patterns which were quite similar (Figs. 1 to 3). Each of the 2 species of snake had a single band of erythrocyte LDH activity and the band in the *Coluber constrictor* had an electrophoretic mobility identical to that of the *Drymarchon corais*. Similarly the 2 mongrel dogs have identical patterns of erythrocyte LDH activity, each possessing 2 bands of activity. The goat and sheep each have 1 band of LDH activity with identical mobility, whereas the

[1] Kindly supplied by Dr. N. O. Kaplan, Brandeis University, Waltham, Massachusetts.

LDH of the steer migrates slightly faster and the main LDH band of the cat migrates slightly slower than the LDH of the goat and sheep. The duck and the chicken each have 5 LDH isozymes in their erythrocytes, but there are several distinguishing features. In duck hemolysates the LDH activity bands that migrate toward the cathode contain higher activity than those migrating toward the anode, whereas in chicken hemolysate the anodal bands have

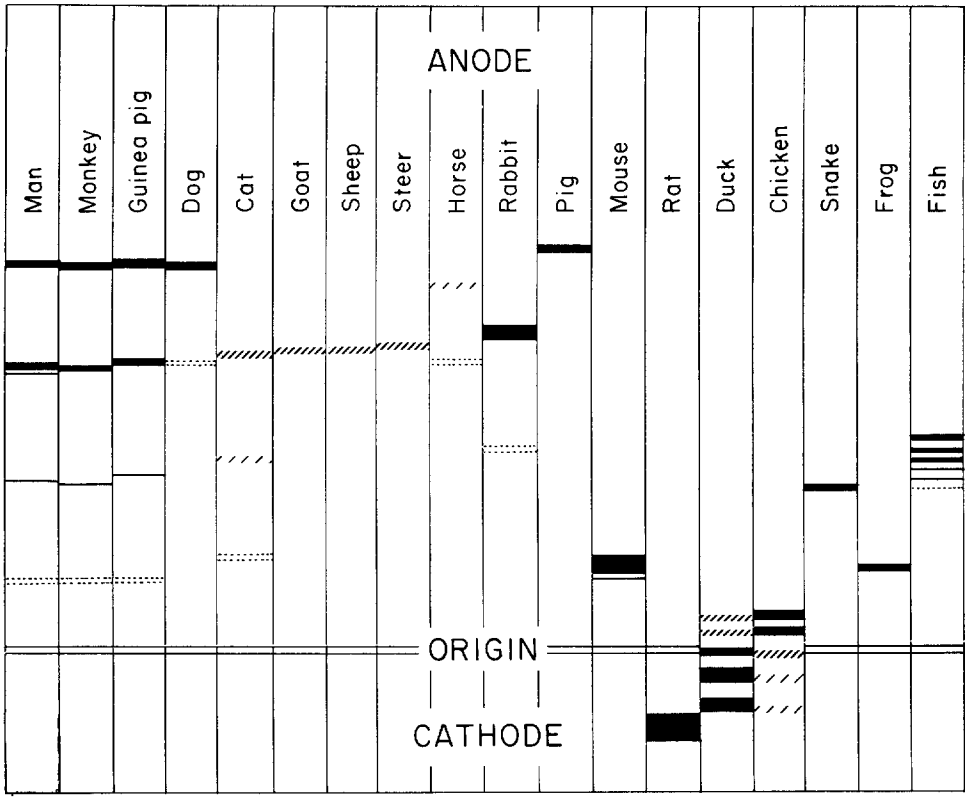

FIGURE 1. Diagram of the intensity, size, and localization of LDH isozymes from erythrocytes of 18 vertebrate species separated by starch gel electrophoresis.

more activity than the cathodal ones (Figs. 1 and 3). Furthermore, there are differences between the duck and chicken hemolysate in the electrophoretic mobility of each of the LDH isozymes. The isozymes of chicken hemolysate which migrate toward the anode are faster in mobility than those of duck hemolysate and the isozymes of chicken hemolysate which migrate toward the cathode are faster in mobility than those of duck (Figs. 1 and 3).

Comparison of the LDH isozymes in the erythrocytes of man and the monkey reveals similar patterns (Figs. 1 and 3). However, the 3 main bands of monkey hemolysate migrate slightly slower than do those of human he-

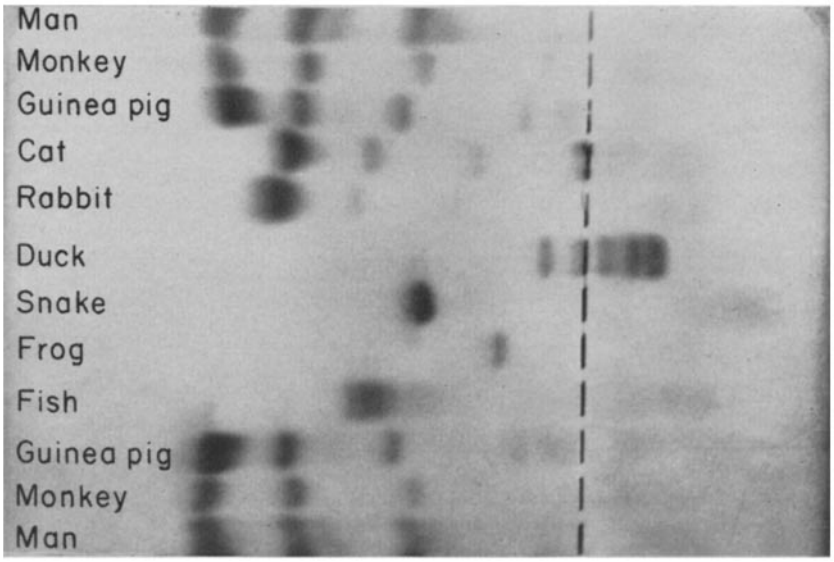

FIGURE 2. Photograph of a starch gel stained histochemically for LDH activity after electrophoretic separation. Hemolysates from representatives of the 5 classes of vertebrates are shown.

molysate (Figs. 1 to 3). Moreover, the doubling seen in the human hemolysate in the second fastest migrating isozyme has never been observed in hemolysates from 6 monkeys (Figs. 1 to 3). This band of low activity, migrating slightly

TABLE I

LACTIC DEHYDROGENASE ACTIVITY OF WHOLE HEMOLYSATES FROM VARIOUS SPECIES

Species	LDH activity/ml hemolysate	LDH activity/gm hemoglobin
Man	8,900	91,000
Monkey	8,500	95,000
Guinea pig	10,800	121,000
Dog	4,000	80,000
Cat	4,200	110,000
Goat	4,800	43,000
Sheep	4,000	35,000
Steer	4,900	64,000
Horse	5,500	45,000
Rabbit	9,000	109,000
Pig	6,500	69,000
Mouse	11,500	108,000
Duck	10,000	119,000
Chicken	8,000	123,000
Snake	7,800	98,000
Frog	4,500	72,000
Fish	5,500	78,000

The experimental error in the determination of each value is approximately 10 per cent.

slower than the second fastest LDH isozyme, can be seen in human hemolysates separated on the gel under ideal conditions (Figs. 1 to 3) although it has not been observed thus far in hemolysates separated by starch block electrophoresis (1–3). The slowest migrating isozyme, seen only occasionally in preparations of human and *rhesus* monkey hemolysate, is identical in mobility in man and monkey (Fig. 1). The electrophoretic mobility of the LDH isozymes in hemolysates prepared from 5 specimens of human cord blood was identical to that of the LDH isozymes in hemolysates from adults.

Of all the species examined only 1, the pig, exhibited a band of LDH activity with faster mobility than that of the most rapidly migrating band in

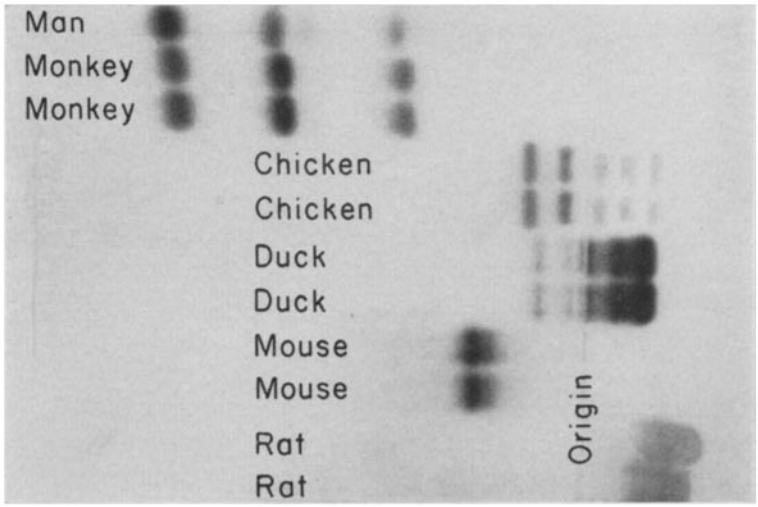

FIGURE 3. Photograph of a starch gel stained directly for LDH activity to illustrate similarities and differences in the isozyme pattern of hemolysates from closely and distantly related species.

human hemolysate (Fig. 1). Only 1 mammal, the rat, had a band of LDH activity which migrated toward the cathode (Figs. 1 and 3). In the species studied the number of LDH bands varied from 1 in the goat, sheep, steer, pig, rat, snake, and frog to 6 in the carp (Fig. 1).

Of more than 380 human hemolysates examined 3 main bands of LDH activity were observed in each specimen (Figs. 2 and 3). No alterations in the electrophoretic mobility of these 3 main bands occurred in any of the human hemolysates. However, in many preparations a fourth band of slower mobility than the 3 major bands appeared (Fig. 1). This band was very faint, and in those specimens in which it was not detected was either absent or present in too low a concentration to be visualized by the histochemical technique. The band is not regularly observed on the starch block where it was detected only once in 5 different hemolysates, and found to contain approximately 1 per

cent ± 1.8 per cent of the total LDH activity of human hemolysate (3). Therefore, the data presented cannot settle the question of possible variation among individuals in this slowest migrating LDH isozyme. However, in those preparations in which it was observed it exhibited no alterations in its electrophoretic mobility.

The addition of DPN analogs to human hemolysate prior to application to the gel produced alterations in electrophoretic mobility. With either 3-acetylpyridine–DPN or thionicotinamide–DPN the mobility of each of the isozymes was increased. There was no differential change in mobility of any 1 isozyme, the mobility of each isozyme being altered to the same extent.

Protein stains of the starch gel revealed that the hemoglobins, all of which migrated toward the cathode at pH 7.0, contained most of the protein in the hemolysates. The hemoglobin bands varied among species in their number and mobility. A few very faint bands appeared in some hemolysates in the anodal regions, but these protein bands did not correspond in mobility to to the bands of LDH activity.

In control experiments in which the incubating medium had been altered by eliminating either the substrate lactate or the coenzyme DPN, it was shown that no bands of LDH activity appeared when lactate was omitted. Furthermore, staining occurred only with L-lactate and was not observed with the dextrorotatory form of the substrate. However, 3 very faint bands of LDH activity were consistently observed when DPN was omitted from the solution bathing the gel. This phenomenon, noted previously (22, 23), has been attributed to non-specific reduction of the DPN followed by production of formazan from the tetrazolium salt and is supposedly intensified as the pH is increased. The possibility that the staining occurs because of the presence in the tissue extract of endogenous DPN which becomes bound to LDH was investigated by the addition of a preparation of diphosphopyridine nucleotidase from *Neurospora*[2] to a bathing solution from which DPN had been omitted. No bands appeared on the gel stained with the solution containing DPNase, whereas they appeared consistently on gels stained in solutions without DPN or DPNase.

The possibility that the multiple bands of LDH activity resulted from the formation of metastable polymers during electrophoresis was eliminated by the observation that the LDH peaks isolated from electrophoretic separation of human hemolysates on a starch block did not give rise to additional peaks when rerun on a starch gel (24). Further evidence against aggregation is the monodispersion in the ultracentrifuge of whole serum LDH activity (25).

DISCUSSION

Direct histochemical visualization of lactic dehydrogenase activity on the starch gel revealed wide variations among vertebrate erythrocytes in both

[2] Kindly supplied by Dr. David C. White of The Rockefeller Institute.

number and electrophoretic mobility of LDH bands. In their investigation of LDH isozymes from heart homogenates of 5 mammalian species Markert and Møller described a band of LDH activity with identical electrophoretic mobility in each species (12). Moreover, each of the 5 species exhibited heterogeneity of LDH activity, and each species had an LDH zymogram which differed from the other 4 either in the number or mobility of LDH heart isozymes (12). The data presented in this paper indicate that many species, including the swine, goat, sheep, snake, frog, and rat, exhibit homogeneity of erythrocyte LDH activity. Although there is marked variation among certain species in the number and mobility of erythrocyte lactic dehydrogenase, other species which are more closely related phylogenetically have identical patterns. For example, the chicken and the duck, both members of class Aves, each have in their erythrocytes 5 LDH isozymes. The electrophoretic mobility of the 5 LDH isozymes in duck hemolysate differs only slightly from that of the 5 isozymes of chicken hemolysate. The 2 species of snake each possess 1 band of erythrocyte LDH activity, the band of 1 species having a mobility identical to that of the other species. The sheep and the goat also have a single LDH band, the band of sheep having a mobility identical to that of the goat. And finally, man and the monkey exhibit LDH isozyme patterns which resemble each other closely but differ in that the 3 main isozymes of monkey hemolysate migrate slightly more slowly than do those of man.

Molecular variations in the proteins of man, as in hemoglobins, haptoglobins, and transferrins, probably arose through mutations, and the frequency of these variations in any given population is largely determined by natural selection. The possibility of the existence of such a genetic polymorphism in the LDH isozymes was investigated by starch gel electrophoresis of hemolysates from 380 individuals. With the techniques employed, there appeared to be no variation among individuals either in the number or mobility of the 3 main LDH isozymes.

Techniques other than electrophoresis have been utilized to demonstrate the species specificity of lactic dehydrogenase. Antiserum prepared to rabbit muscle LDH reduced the activity of the rabbit muscle LDH to a much greater extent than it did the activity of rat muscle LDH, whereas it had no effect on the LDH of schistosomes (26). Immunologic techniques have also been employed to demonstrate that the LDHs of various human tissues differ (27, 28). The use of DPN analogs by Kaplan has achieved a partial distinction among the LDHs from various species. This approach, however, has the limitation of dealing with a mixture of enzymes which remain unresolved; and the kinetic behavior of the extract is a mean effect of all the isozymes present. Employing DPN analogs, Kaplan's group reports that heart homogenates from several species exhibit almost identical kinetic behavior (13), whereas by starch gel electrophoresis Markert and Møller have shown that heart homogenates from 5 mammalian species differ in the number of LDH

isozymes, in the distribution of total LDH activity among them, and in their electrophoretic mobility (12). Furthermore, the present study demonstrates that the isozymes in human hemolysate are not readily distinguished by the use of DPN analogs, which affect the mobility of each isozyme to an equal extent. Previous studies have shown that human isozymes 4 and 5, the two present in highest concentration in human erythrocytes, are identical in their kinetic behavior toward several DPN analogs (24).

If the nature of the chemical differences among the LDHs from various species and among LDH isozymes is to be defined, isolation and chemical analysis of the separated LDHs will have to be accomplished. Steps in this direction have been taken by Wieland and Pfleiderer (29), who investigated the amino acid composition of the 5 isozymes of rat heart. They described a higher content of acidic charges due to an increasing number of aspartic acid residues in the more rapidly migrating isozymes (29). Other physicochemical studies on the electrophoretically isolated isozymes from human tissues include differences of isozymes 1 and 2 from isozymes 3, 4, and 5 in Michaelis-Menten constants and kinetic behavior with DPN analogs (3, 24). However, without the amino acid sequence of the isozymes and of the LDHs from various species, a discussion of the chemical basis for the differences observed in such properties as electrophoretic mobility becomes highly speculative, as does any attempt to fit the patterns of erythrocyte LDHs from different species into a phylogenetic classification. Species specificity of other proteins such as insulin, ACTH, and ribonuclease has been studied by comparison of the polypeptide chains (30); and, on the basis of this, the hypothesis has been advanced that the variations in structure occur at non-critical points in the molecule termed "filler sequences" (31) and do not affect the active center (30, 31). It is reasoned that the active center must remain unchanged from one species to another if the molecule is to retain its full function.

One explanation for the presence of multiple molecular forms of an enzyme involves the mechanism of chromosomal duplication in which a piece of a chromosome is broken off during crossing-over and repeated later in the sequence of genes. The repeated genes would produce proteins identical to those produced by the parent genes and mutation affecting one of the repeated genes would alter the protein formed by that gene. The altered protein might differ from the protein made by the gene not undergoing mutation in just such ways as the LDH isozymes of human erythrocytes differ.

Recently Allen has elucidated the genetic control of class 1 esterases of *Tetrahymena pyriformis* by demonstrating through crosses of 4 different strains that the esterase isozymes in group B and the esterase isozymes of group C are determined by alleles at a single locus, designated the E-1 locus (32). Strain B of *T. pyriformis* which produces only the group B esterase isozymes is homozygous for the $E\text{-}1^{B}$ allele; strain C of *T. pyriformis* which produces only the

group C esterase isozymes is homozygous for the E-1^{C} allele; and the heterozygote (E-1^{B}/E-1^{C}) has the potentiality for producing both groups of isozymes (32).

Species differences in erythrocyte lactic dehydrogenases, like alterations among species in other properties of erythrocytes (33–35), are probably attributable to the action of natural selection on variations in protein structure. The interest of studies on a specific protein in a wide range of organisms is both to localize points in phylogeny at which successive mutations took place in the course of biochemical evolution and to indicate what variations in protein structure are compatible with efficient maintenance of function.

The authors wish to thank Dr. Herndon G. Dowling, Associate Curator of Reptiles at the New York Zoological Park, for his aid in obtaining blood from snakes, and Dr. Richard E. Shope of The Rockefeller Institute for obtaining blood from pigs.

These studies were supported in part by a grant-in-aid, A-1542 (C4), from the United States Public Health Service.

Received for publication, June 23, 1961.

BIBLIOGRAPHY

1. VESELL, E. S., and BEARN, A. G., Localization of lactic dehydrogenase activity in serum fractions, *Proc. Soc. Exp. Biol. and Med.*, 1957, **94**, 96.
2. VESELL, E. S., and BEARN, A. G., Observations on the heterogeneity of malic and lactic dehydrogenase in human serum and red blood cells, *J. Clin. Inv.*, 1958, **37**, 672.
3. VESELL, E. S., and BEARN, A. G., Isozymes of lactic dehydrogenase in human tissues, *J. Clin. Inv.*, 1961, **40**, 586.
4. SAYRE, F. W., and HILL, B. R., Fractionization of serum lactic dehydrogenase by salt concentration gradient elution and paper electrophoresis, *Proc. Soc. Exp. Biol. and Med.*, 1957, **96**, 695.
5. HESS, B., DPN-dependent enzymes in serum, *Ann. New York Acad. Sc.*, 1958, **75**, 292.
6. WIEME, R. J., Studies on Agar Gel Electrophoresis, Brussels, Arscia Uitgaven N. V., 1959.
7. PLAGEMANN, P. G. W., GREGORY, K. F., and WRÓBLEWSKI, F., The electrophoretically distinct forms of mammalian lactic dehydrogenase. I. Distribution of lactic dehydrogenases in rabbit and human tissues, *J. Biol. Chem.*, 1960, **235**, 2282.
8. VESELL, E. S., and BEARN, A. G., The heterogeneity of lactic and malic dehydrogenase, *Ann. New York Acad. Sc.*, 1958, **75**, 286.
9. BROMAN, L., Separation and characterization of two coeruloplasmins from human serum, Nature, 1958, **182**, 1655.
10. WHITTAKER, V. P., Specificity, mode of action and distribution of cholinesterases, *Physiol. Rev.*, 1951, **31**, 312.
11. KOWLESSAR, O. D., PERT, J. H., HAEFFNER, L. J., and SLEISENGER, M. H.,

Localization of 5-nucleotidase and non-specific alkaline phosphatase by starch gel electrophoresis, *Proc. Soc. Exp. Biol. and Med.*, 1959, **100**, 191.

12. MARKERT, C. L., and MØLLER, F., Multiple forms of enzymes: Tissue, ontogenetic, and species specific patterns, *Proc. Nat. Acad. Sc.*, 1959, **45**, 753.
13. KAPLAN, N. O., CIOTTI, M. M., HAMOLSKY, M., and BIEBER, R. E., Molecular heterogeneity and evolution of enzymes, *Science*, 1960, **131**, 392.
14. KAPLAN, N. O., and CIOTTI, M. M., Heterogeneity of the lactic dehydrogenases of new-born and adult rat heart as determined with coenzyme analogs, *Biochim. et Biophysica Acta*, 1961, **49**, 425.
15. DRABKIN, D. L., Spectrophotometric studies. XIV. The crystallographic and optical properties of the hemoglobin of man in comparison with those of other species, *J. Biol. Chem.*, 1946, **164**, 703.
16. SMITHIES, O., An improved procedure for starch-gel electrophoresis: further variations in the serum proteins of normal individuals, *Biochem. J.*, 1959, **71**, 585.
17. RUTENBURG, A. M., GOFSTEIN, R., and SELIGMAN, A. M., Preparation of a new tetrazolium salt which yields a blue pigment on reduction and its use in the demonstration of enzymes in normal and neoplastic tissue, *Cancer Research*, 1950, **10**, 113.
18. KEARNEY, E. B., and SINGER, T. P., Studies on succinic dehydrogenase. I. Preparation and assay of the soluble dehydrogenase, *J. Biol. Chem.*, 1956, **219**, 963.
19. APPELLA, E., and MARKERT, C. L., personal communication.
20. KUBOWITZ, F., and OTT, P., Isolierung und Kristallisation eines Gärungsfermentes aus Tumoren, *Biochem. Z.*, 1943, **314**, 94.
21. DRABKIN, D. L., The standardization of hemoglobin measurement, *Am. J. Med. Sc.*, 1949, **217**, 710.
22. ZIMMERMANN, H., and PEARSE, A. G. E., Limitations in the histochemical demonstration of pyridine nucleotide–linked dehydrogenases ("nothing dehydrogenase"), *J. Histochem. and Cytochem.*, 1959, **7**, 271.
23. RACKER, E., Mechanism of action and properties of pyridine nucleotide-linked enzymes, *Physiol. Rev.*, 1955, **35**, 1.
24. VESELL, E. S., Significance of heterogeneity of lactic dehydrogenase activity in human tissues, *Ann. New York Acad. Sc.*, 1961, **94**, 877.
25. HILL, B. R., KUFF, E. L., and HOGEBOOM, G. H., Sedimentation behavior of serum lactic dehydrogenase, *Proc. Soc. Exp. Biol. and Med.*, 1956, **92**, 430.
26. MANSOUR, T. E., BUEDING, E., and STAVITSKY, A. B., The effect of a specific antiserum on the activities of lactic dehydrogenase of mammalian muscle and of *Schistosoma mansoni*, *Brit. J. Pharmacol.*, 1954, **9**, 182.
27. GREGORY, K. F., and WRÓBLEWSKI, F., Preparation and properties of purified antilactic dehydrogenase, *J. Immunol.*, 1958, **81**, 359.
28. NISSELBAUM, J. S., and BODANSKY, O., Reactions of human tissue lactic dehydrogenases with antisera to human heart and liver lactic dehydrogenases, *J. Biol. Chem.*, 1961, **236**, 401.
29. WIELAND, T., and PFLEIDERER, G., Chemical differences between multiple forms of lactic acid dehydrogenases, *Ann. New York Acad. Sc.*, 1961, **94**, 691.

30. ANFINSEN, C. B., The Molecular Basis of Evolution, New York, John Wiley and Sons, 1959, 142–163.
31. HOFMANN, K., Preliminary observations relating structure and function in some pituitary hormones, *Brookhaven Symp. Biol.*, 1960, **13**, 184.
32. ALLEN, S. L., Genetic control of the esterases in the protozoan *Tetrahymena pyriformis*, *Ann. New York Acad. Sc.*, 1961, **94**, 753.
33. PROSSER, C. L., Comparative Animal Physiology, Philadelphia, W. B. Saunders Co., 1950.
34. GRATZER, W. B., and ALLISON, A. C., Multiple haemoglobins, *Biol. Rev.*, 1960, **35**, 459.
35. PRANKERD, T. A. J., The Red Cell; An Account of Its Chemical Physiology and Pathology, Oxford, Blackwell Scientific Publications, 1961.