must significantly exceed what we have directly demonstrated. Instances of polymorphism for electrophoretic mobility variants at a variety of loci in each of several drosophila populations raise major questions concerning the maintenance of abundant genic heterozygosity in natural populations (*10*). But, even at a single locus, the evolution and maintenance of so lengthy a series of alleles as that occurring at the *Est E* locus of *Colias* is not easily explained.

In the vicinity of Middletown, Connecticut, 85 specimens of *Colias eurytheme* and *C. philodice* (and hybrids between the two) were collected on 3, 4, 8, and 9 October 1966 and were sent by air to the Austin laboratory, where the electrophoretic patterns of mid-abdominal homogenates were obtained. Striking EST E polymorphism, as extensive as that genetically analyzed in central Texas populations, was found.

The occurrence of molecular variation may be invaluable in analysis of possible rapid evolution in, for example, a currently invading Palearctic skipper butterfly (*Thymelicus lineola*) whose mode of colonization should promote population differentiation (*11*) but whose color pattern is so simple that few readily alterable superficial traits are available for study. Enzyme variation may yield characters for precisely separating recently evolved sibling species of food-plant specialists, such as *Erynnis lucilius* and *E. baptisiae* (*12*), and for analyzing natural hybridization between such closely related and still largely allopatric forms.

JOHN M. BURNS
Department of Biology, Wesleyan University, Middletown, Connecticut
F. M. JOHNSON
Genetics Foundation, Department of Zoology, University of Texas, Austin

References and Notes

1. C. L. Markert and F. Møller, *Proc. Nat. Acad. Sci. U.S.* **45**, 753 (1959).
2. M. Eguchi, N. Yoshitake, H. Kai, *Jap. J. Genet.* **40**, 15 (1965).
3. N. Yoshitake and M. Akiyama, *ibid.* **39**, 26 (1964).
4. H. Laufer, *Ann. N.Y. Acad. Sci.* **94**, 825 (1961).
5. M. Eguchi and T. Sugimoto, *J. Insect Physiol.* **11**, 1145 (1965); M. Eguchi, *Bull. Fac. Textile Fibers, Kyoto Univ. Ind. Arts Textile Fibers* **4**, 351 (1965).
6. O. Smithies, *Biochem. J.* **61**, 629 (1955).
7. F. M. Johnson and J. M. Burns, *J. Lepidopterists' Soc.* **20**, 207 (1966).
8. J. M. Burns, *Science* **153**, 551 (1966).
9. W. Hovanitz, *Evolution* **3**, 170 (1949).
10. J. L. Hubby and R. C. Lewontin, *Genetics* **54**, 577 (1966); R. C. Lewontin and J. L. Hubby, *ibid.*, p. 595; F. M. Johnson, C. G. Kanapi, R. H. Richardson, M. R. Wheeler, W. S. Stone, *Proc. Nat. Acad. Sci. U.S.* **56**, 119 (1966).
11. J. M. Burns, *Can. Entomol.* **98**, 859 (1966).
12. ———, *Univ. Calif. Publ. Entomol.* **37**, 1 (1964).
13. We thank W. S. Stone and R. K. Selander for providing laboratory space for this project; and R. O. Kendall, B. I. Kiefer, C. G. Kanapi, S. Rockwood, C. Greer, H. E. Sutton, R. K. Selander, S. N. Burns, and B. K. Johnson for advice and assistance. Supported in part by NIH research grant GM 11609 to W. S. Stone and M. R. Wheeler, and NIH training grants 2T1-GM-337-06 and GM 00337-07 to R. P. Wagner *et al.*

15 February 1967

Genetic Control of Lactate Dehydrogenase Formation in the Hagfish Eptatretus stoutii

Abstract. *The isozyme patterns of lactate dehydrogenases of various tissues were studied on 51 hagfish by starch-gel electrophoresis. Nine lactate dehydrogenase phenotypes were encountered, suggesting the coexistence of two alleles at each of the two separate gene loci. There apparently was no interaction between the products of these two separate loci. Even the products of two alleles at the same locus were apparently incapable of forming hybrid molecules, an indication of the possible monomeric nature of each lactate dehydrogenase molecule.*

It has been shown that, in higher vertebrates, two separate gene loci code for subunits of lactate dehydrogenase (LDH). The two polypeptides, A and B, are assembled to make five tetrameric molecules: A_4, A_3B_1, A_2B_2, A_1B_3, and B_4. This suggests that these genes arose by duplication. As LDH-5 (A_4) predominates in embryonic tissue, it is suspected that, of the two, the one which is producing a less negatively charged A polypeptide is ancestral to the other (*1*). It is of considerable interest to find out when in vertebrate evolution this gene duplication occurred. Markert and Faulhauber (*2*) studied 30 species of teleosts, most of which already had two LDH subunits and therefore at least two gene loci. The flatfish of the order Heterosomata was an exception in that it had a single LDH isozyme and therefore only one LDH gene locus. A single LDH isozyme of flatfish is analogous to LDH-5 (A_4) of higher vertebrates (*3*).

The hagfish is more primitive than teleost fish in that it represents the jawless state of vertebrate evolution. Yet it possesses (*4*) four separate gene loci for monomeric hemoglobin. Whether or not the hagfish already has two separate gene loci for LDH subunits is now being studied.

Under the direction of D. Jensen of the University of California at San Diego, two traps, about 100 m apart, were set on the ocean bottom (approximate depth was 220 m) about 9 km due west of Point Loma. About 200 live hagfish were caught. Because of the closeness of the two trapping sites, the fish may be regarded as members of one population occupying a particular niche. Of those, 51 were used for our study on LDH. Body length ranged from 27 to 47 cm, and weight from 35 to 130 g. The presence of an ovary was recognized in 21 and a testis in 8 of the fishes. It was not possible to locate a gonad in the remaining 22. Tissues used were branchial heart, brain, gill pouch, liver, skeletal muscle, ovary, and testis. The water temperature preferred by hagfish is about 4°C. Their LDH and other enzymes deteriorated very quickly even at room temperature. Individual fish were killed only a few hours before each electrophoresis. The entire procedure was carried out quickly at around 4°C. Minced pieces of tissue were homogenized in an equal volume of 0.12*M* KCl solution buffered at *p*H 7.6 with 0.02*M* tris buffer. The clear supernatant obtained after 2 hours of centrifugation at 15,000*g* (at 4°C) was used for electrophoresis. A discontinuous system of vertical starch-gel electrophoresis at *p*H 8.7 was used with a tris-borate buffer (*2*). Electrophoresis was continued for 18 hours at 4°C with a gradient of 6 volt/cm. Lactate dehydrogenase bands were visualized by incubating a gel plate for 2 hours at 37°C in 75 ml of staining solution. The latter consisted of 75 ml of 0.5*M* phospate buffered at *p*H 7.0, 1.6 g of lactate lithium salt, 100 mg of neotetrazolium chloride (Sigma), 60 mg of nicotinamide adenine dinucleotide (NAD), 1.3 g of hydrazine sulfate, and 4 mg of phenazine methosulfate.

Of the 51 hagfish, 14 showed identical LDH patterns. These were regarded as being homozygous for a wild-type allele at each of the two gene loci. The skeletal muscle of such a fish demonstrated a single LDH band at a position very near the starting point (Fig. 1a). As customary this less negatively charged LDH shall be called LDH-5 and its constituent polypeptide, A. The gill pouch of the same fish

Table 1. Classification of the 51 hagfish by nine LDH phenotypes.

LDH-5	LDH-1		
	A/A	A/A^f	A^f/A^f
B/B	14	8	1
B/B^s	5	8	5
B^s/B^s	5	3	2

demonstrated, in addition to LDH-5, the other LDH band at the position further toward the anode (Fig. 1b). This faster-moving LDH existed alone in the branchial heart (Fig. 1c). This isozyme is referred to as LDH-1, and its constituent polypeptide, as B. The constant coexistence in a nearly equal ratio of LDH-5 and LDH-1 was observed only in the gill pouch. In Fig. 1b, there are no intermediate bands which would correspond to three hybrid tetramers: LDH-4 (A_3B_1), LDH-3 (A_2B_2), and LDH-2 (A_1B_3). The LDH-1 isozyme was also predominant in the brain, liver, and ovary. In two testes containing spermatozoa, upon electrophoresis, in addition to LDH-1, another faintly stained band was visualized at the position further toward the anode. Whether this faintly stained LDH band indicates the presence of a third gene locus coding for a C-subunit cannot be ascertained at the moment. In mammals and birds, this third gene locus expresses itself only in sexually mature testes (*5*). A slower-moving variant of LDH-1 from the branchial heart of a hagfish of unknown sex is illustrated in Fig. 1d. If this fish is presumed to be homozygous for a mutant allele which produces a less negatively charged B^s-polypeptide, then Fig. 1e illustrates the separation by electrophoresis of wild-type and variant LDH-1 in the same tissue from a female heterozygote (B/B^s). In the heart of such a heterozygote, the wild-type LDH-1 band always stained more intensely than the variant band, but, in the liver of the same fish, two bands were stained in about the same degree (Fig. 1f). In Fig. 1, e and f, here again there are no intermediate bands between a wild-type and a variant LDH-1 band. Thus even the products of two alleles at the same gene locus are incapable of forming demonstrable hybrid molecules in the hagfish. A faster-moving variant of LDH-5 found in skeletal muscle of the same female is shown in Fig. 1g. This fish was apparently homozygous for a mutant allele which produces A^f-subunit which is more negatively charged than A. Heterozygotes (A/A^f) gave one broad, intensely stained band of LDH when an undiluted extract of skeletal muscle was used. By appropriate dilution, however, this broad band was delineated as two sharply defined bands, one corresponding to a fast-moving variant and the other to the wild-type LDH-5. The coexistence of two alleles at each of the two separate gene loci should give rise to nine phenotypes. All nine phenotypes expected were actually encountered (Table 1).

Among many teleosts that apparently had two separate gene loci for LDH subunits, some, such as whiting (*Merluccius bilinearis*), presented a five-isozyme pattern similar to that of mammals, whereas others such as porgy (*Stenotomus versicolor*) presented only two isozymes. Markert and Faulhauber (*2*) thought that the two-isozyme pattern of the latter was due to preferential formation of autotetramers, A_4 and B_4. In the hagfish, hybrid isozymes were not formed between A and B polypeptides nor between the products of two alleles of the same locus, namely A and A^f as well as B and B^s. The most logical explanation of the situation encountered in hagfish appears to be that each LDH molecule of this primitive vertebrate is monomeric like its hemoglobin molecule. The following experiments appear to confirm the above view. Filtrate from a thin-layer gel was used to estimate the molecular weight of LDH-1 and LDH-5 from the gill pouch. Extracts were run with a series of marker proteins of known molecular weight on a layer of superfine Sephadex G-75 or G-150 (*4*). An LDH spot on the plate was visualized by application of the staining solution. The molecular weight of the LDH from the hagfish was about 30,000, a value close to that expected for monomeric subunits of tetrameric LDH of higher vertebrates. Gill pouch extracts were assayed spectrophotometrically for LDH activity. Extracts in 1*M* NaCl, after being frozen and thawed, still retained 22 percent of their original activity.

The gene duplication may be a consequence of unequal crossing-over during meiosis. If this was a cause, the duplicates should be extremely closely linked. The gene loci for β- and δ-chains of human hemoglobin obviously arose by this mechanism (*6*). Similarly, man and the mouse appear to have several very closely linked gene loci coding for heavy chains of γ-globulin molecules (*7*). Conversely, duplication of every gene locus within the genome can be accomplished by the process of polyploid evolution. The duplicates produced in this manner should originally have been on two separate chromosomes. The gene loci for α- and β-chain of human hemoglobin are not linked and might have arisen in this way.

In the hagfish, the two alleles coexisted at each of the two gene loci. Thus, our result provides some insight into the mechanism that has endowed this primitive vertebrate with two separate gene loci for LDH subunits. The fact that all nine expected phenotypes were encountered reveals that the two gene loci are not in very close linkage. If they are, only three phenotypes such as AB/AB, AB/A^fB^s, and A^fB^s/A^fB^s should be found. This seems to indicate that the two separate gene loci for LDH subunits of vertebrates did not arise as a result of unequal crossing-over.

Previously, we have presented evidence to suggest that different degrees of polyploidization of the original vertebrate genome took place more than 300 million years ago when vertebrates were still aquatic. Of all the verte-

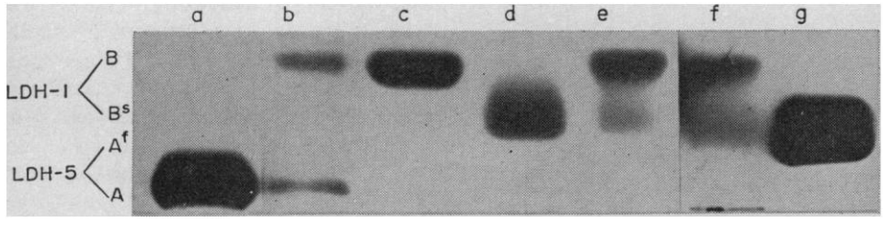

Fig. 1. Starch-gel electrophoretic pattern of different tissues from three different fish. Anodal direction is upward; the starting point is at the bottom; a, b, and c are from a male fish of the phenotype LDH-5 (A/A) and LDH-1 (B/B). (a) Wild-type LDH-5 in skeletal muscle; (b) LDH-5 and LDH-1 in gill pouch; (c) wild-type LDH-1 in branchial heart; (d) slower moving variant LDH-1 from branchial heart of a fish of unknown sex (B^s/B^s); (e and f) from branchial heart and liver of a female B/B^s heterozygote (in the heart, the wild-type LDH-1 predominates over the slower moving variant, while in liver the two appear to exist in equal amounts); (g) for a faster moving variant LDH-5 (A^f/A^f) from a homozygous female, as seen in skeletal muscle.

brates, flatfish of the order Heterosomata are poorest in DNA (only 20 percent that of mammals). They are regarded as the retainer of the original diploid genetic content (*8*), and it is not surprising that they alone have only a single locus coding for LDH (*2*, *3*). The hagfish, on the other hand, is rather rich in DNA (as much as 75 percent that of mammals) (*9*). In this light, the revelation that they already possess four gene loci for hemoglobin polypeptides (*4*) as well as two and possibly three gene loci for LDH subunits is not unexpected (*10*).

SUSUMU OHNO, JURAJ KLEIN
JACKIE POOLE, CEPHAS HARRIS
ANTONIA DESTREE, MARTIN MORRISON
Departments of Biology and
Biochemistry, City of Hope
Medical Center, Duarte, California

References and Notes

1. C. L. Markert and H. Ursprung, *Develop. Biol.* **5**, 363 (1962).
2. C. L. Markert and I. Faulhauber, *J. Exp. Zool.* **159**, 319 (1965).
3. N. O. Kaplan, M. M. Ciotti, M. Hamolsky, R. E. Bieber, *Science* **131**, 392 (19[illegible] R. D. Cahn, N. O. Kaplan, L. Levin, E. Zwilling, *ibid.* **136**, 962 (1962).
S. Ohno and M. Morrison, *ibid.* **154**, 1034 (1966).
A. Blanco and W. H. Zinkham, *ibid.* **139**, 601 (19[illegible] W. H. Zinkham, *ibid.* **144**, 1353 (1964).
6. R. Ceppellini, *Biochemistry of Human Genetics* (Churchill, London, 1959), pp. 133–138.
7. H. G. Kunkel, "Myeloma proteins and antibodies," *Harvey Lecture* **59**, (1963–64); L. Herzenberg, *Cold Spring Harbor Symp. Quant. Biol.* **19**, 455 (1964).
S. Ohno and N. B. Atkin, *Chromosoma* **18**, 455 (1966).
9. N. B. Atkin, personal communication.
10. Supported in part by PHS grant CA-05138-07. Contribution No. 1-67, Department of Biology, City of Hope Medical Center. We thank L. Andersen for editorial assistance. We are also grateful for criticism offered by M. Cohn of the Salk Institute and by E. Beutler, Burroughs Hill, and E. Roberts of this institute.

7 February 1967

Transferrin Polymorphism and Population Differences in the Genetic Variability of Chimpanzees

Abstract. *Genetic divergencies between chimpanzee populations, not only between* Pan panicus *and* Pan troglodytes *but also between different groups of the latter, are revealed by typing of transferrin. In particular, differences in the incidence of polymorphic transferrins occur between the groups formed by subdividing a large captive chimpanzee colony of heterogeneous geographic origins into racial types solely on the basis of morphological traits. Genetic variability is extremely high in one of these groups, intermediate in another, and relatively low in a third, with the pattern of changing frequencies of allelic genes at the* T_f *locus following the pattern of geographic distribution of the actual conspecific populations or races for which the groups are named.*

Chimpanzees have a very broad geographic distribution that extends for thousands of miles through the rain forests of central Africa from the Atlantic coast of Gambia to the mountains of Uganda and Tanganyika. Noting that chimpanzees vary sharply in morphological appearance from one part of their range to another, Hill (*1*) distinguishes at least four types which he considers races of *Pan troglodytes: P. t. verus* (Ptv), *P. t. troglodytes* (Ptt), *P. t. koolokamba* (Ptk), and *P. t. schweinfurthi* (Pts), in addition to *Pan paniscus,* the pygmy chimpanzee which is found on the south bank of the Congo river. At the far western periphery of the genus and progressing eastward, the race of *P. t. verus* is followed, after the Dahomey gap, by that of *P. t. troglodytes* and by *P. t. koolokamba*; the latter in the Cameroons meets the race of *P. t. schweinfurthi* which extends for over 1600 km through the Congo to Uganda and Tanganyika (*1*). A study of the genetic structures of the natural chimpanzee populations throughout their geographic range would clarify the process of evolution in a primate group which, among nonhuman forms, provides the closest homolog of the genus *Homo*. The potentialities of such a study are indicated by the data being gathered at the Holloman Air Force Base in New Mexico, where over 170 captive chimpanzees were obtained from a variety of sources. Hill and Fineg, on the basis of morphological criteria, classified the members of the colony into the four racial types: Ptv, Ptt, Ptk, and Pts. Marked differences in gene frequencies were then found between the Ptv and Pts groups when the colony was typed for various erythrocyte isoantigens (*2*).

Our study, which is concerned with the transferrin polymorphism of chimpanzees, describes further genotypic differences between the Ptv and Pts groups of the Holloman colony. The most striking finding is that, whereas transferrin monomorphism or homozygosity at the T_f locus predominates in the Ptv group, heterozygosity at this locus predominates in Pts chimpanzees. The degree of such genetic variability appears to be higher in *Pan troglodytes* as a whole than in humans and may be especially intense in chimpanzees from particular geographic areas.

The different molecular forms of transferrin, the serum iron-binding protein, were detected by vertical starch-gel electrophoresis (*3*) and autoradiography of serum samples treated with Fe^{59}, by the procedure of Giblett *et al.* (*4*). Five molecular forms and eight phenotypes of transferrin were found when serums from 111 chimpanzees of the Holloman colony were analyzed (Fig. 1). From the transferrin of least anodic mobility to those of faster mobility, these transferrins are labeled D, C, E, B, and A. Boyer and Young (*5*) had previously discovered and named transferrins D, C, B, and A in 25 chimpanzees of another group. Goodman *et al.* (*6*) also previously

Table 1. Distribution of racial groups and transferrin phenotypes in the older animals (66) and newer animals (66) of the Holloman chimpanzee colony. In the parentheses, the first number is the total number of animals in each group, and the second number is the number of animals typed for transferrins.

Transferrin phenotypes	Animals (No.) classified into racial groups and typed for transferrins							
	Older				Newer			
	Ptv (26/20)	Ptt (13/9)	Ptk (1/0)	Pts (26/23)	Ptv (37/34)	Ptt 14/14)	Ptk (1/1)	Pts (14/10)
DD	1	1		4				
DC				9				
DE		1						
DA	1	2		2				
CC	17	3		1	30	11		9
CB	1			1	4	3		1
CA		2		4			1	
AA				2				