lytic enzymes. Szodoray's studies, generally overlooked by keratin chemists, indicate that skin sections treated with trypsin resulted in the digestion of the lower malpighian cells (*19*).

The disc-electrophoresis patterns of solubilized proteins indicate one major protein band for trypsin-solubilized proteins and two major bands for chymotrypsin-solubilized proteins. These proteins apparently resistant (P_R) to extensive enzymatic degradation, constitute about one-third of the total protein as judged by the amount of protein precipitable by trichloroacetic acid (*15*). Thus, about two-thirds of the total protein solubilized is not resistant (P_{NR}) but is enzymatically digested to peptide fragments.

Apparently some of the P_R remains firmly bound to membranous material after enzymatic action. The protein, solubilized by dilute alkali (0.05*M* NaOH), travels in polyacrylamide-gel electrophoresis similarly to P_R.

A model is tentatively suggested for the structure of epidermal keratin based on the experimental results of this work and the recent findings of Crewther and Harrap (*20*). These workers found that a low-sulfur protein fraction, isolated from wool, has a structure that is 50 percent helical. After treatment of thin protein with pronase and trypsin, the helix content of the protein became 84 and 60 percent, respectively.

In the model for epidermal keratin, if it is assumed that the P_R is helical and the P_{NR} is random-coiled protein, the insoluble nature of keratin may be based on the three-dimensional distribution of P_R and P_{NR} protein units. After enzymatic digestion of P_{NR} to peptide fragments, P_R units are soluble. Further study of the trypsin- and chymotrypsin-resistant protein, P_R, is required to establish whether its resistance to enzyme digestion is due to disulfide bonding, helical structure, or lack of specific sites for enzyme attack.

It has been suggested that lipid plays the role of "cementing substance" (*21*), bonding cellular keratin structures to one another. The results of our work suggests that this substance has no role in influencing the enzymatic solubilization of epidermal proteins. This may be inferred from the observations that epidermal insoluble residue is solubilized equally well by trypsin or chymotrypsin both before and after ether extraction.

The action of proteolytic enzymes on epidermal keratin in our study suggests the need for a better understanding of the role of proteases in epidermal keratinization.

At present the role of proteases in the keratinization process is unknown, although proteases have been found in skin (*18*) and in isolated epidermis (*22*). The action of proteases at specific sites in the epidermis and their regulation by inhibitory substances during keratinization may very well determine the nature of the keratin aggregate formed, and how this aggregate becomes a normal layered stratum corneum.

SIMON ROTHBERG
GERTRUDE D. AXILROD
Dermatology Branch, National Cancer Institute, Bethesda, Maryland 20014

References and Notes

1. As used in this text epidermal proteins are those, soluble and insoluble, that can be obtained from the epidermis; soluble proteins are those that can be solubilized from epidermis by homogenization, dialysis, or extraction with phosphate or tris buffer, *p*H 7.2; insoluble epidermal proteins are those in the insoluble residue, possibly keratin? (the insoluble residue remains after homogenization, dialysis, or extraction with phosphate or tris buffer, *p*H 7.2); solubilized proteins are those solubilized from insoluble epidermal protein by various chemical agents; epidermal keratin is the fibrous intermediate and end product of epidermal keratinization.
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21 November 1966

Esterase Polymorphism in Natural Populations of a Sulfur Butterfly, Colias eurytheme

Abstract. *Starch-gel electrophoresis of esterases of wild and laboratory-reared individuals of* Colias eurytheme *indicates that populations in central Texas are exceedingly variable, consisting almost entirely of heterozygotes formed from a large number of alleles at an autosomal locus controlling the production of an esterase designated EST E. Few wild individuals are genetically identical at this locus. The commonest member of the allelic series is an apparent null allele that results in no EST E activity.*

Genetic work on electrophoretic variation in Lepidoptera enzymes has dealt primarily with a few isozymes (*1*) of esterases (*2*) and acid phosphatases (*3*) in the domesticated silkworm (*Bombyx mori*). Developmental isozyme patterns have been described in saturniid silk moths (*Hyalophora cecropia* and *Samia cynthia*) (*4*) as well as in the silkworm (*5*).

Using starch-gel electrophoresis (*6*) to study samples from natural populations of a sulfur butterfly, *Colias eurytheme*, we found marked variation in two esterase systems, with an unusually large numbers of alleles apparently involved in the control of one of them (*7*). We now establish a genetic basis for the highly variable system and describe the complexities of the polymorphism in natural populations.

Sample preparation, starch-gel electrophoresis, and esterase assay have been described (*7*). We homogenized single live early-instar larvae and thick, transverse sections of late-instar larvae, pupae, and adults, used horizontal electrophoresis in a discontinuous system of buffers, and stained for esterase activity with α-naphthyl acetate and Fast Blue RR salt.

Adult butterflies were collected in 1966 at three localities in central Texas: 127 at Austin in May and early June (*7*); 13 at San Antonio on 22 May; and 17 in the Brazos River Valley west of Bryan on 23 July. They were usually analyzed promptly, but some females were maintained in the laboratory on a sucrose solution and induced to oviposit. Both lupine (*Lupinus texensis*) and white sweet clover (*Melilotus alba*) were used as substrates for oviposition, but all larvae were reared exclusively on *M. alba*.

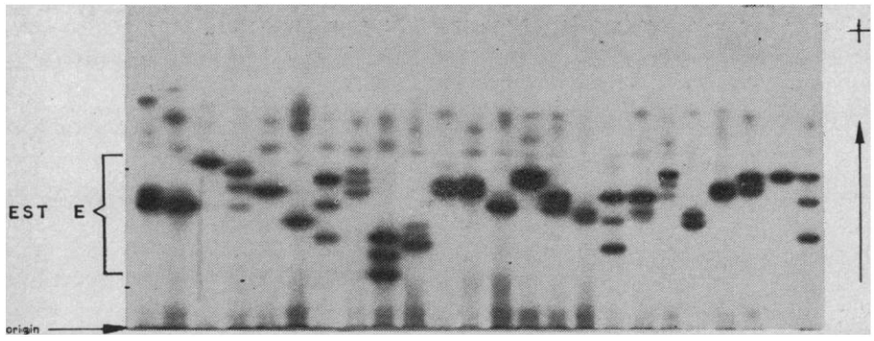

Fig. 1. Starch gel with numerous electrophoretic esterase variants in a sample of 24 adult males of *Colias eurytheme* collected in a single field in Austin, Texas, on the afternoon of 10 May 1966. In the EST E system, the bands are generally dark and are either single or triple. The arrow indicates direction of migration.

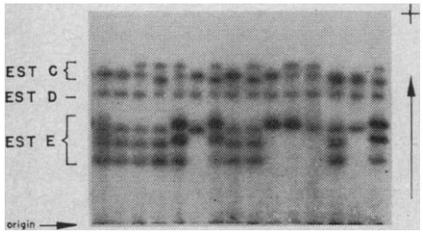

Fig. 2. Starch gel with (i) three esterase systems (EST C, EST D, and EST E), (ii) four EST C electrophoretic patterns, (iii) uniformity of EST D, and (iv) four EST E electrophoretic patterns appearing in the offspring of wild cross 66-4.

For genetic analysis, offspring were reared only from those wild females that were found by dissection to contain a single spermatophore and which therefore are assumed to have mated but once (*8*).

Figure 1 shows diverse esterase patterns in a sample of wild Austin adult butterflies. Variation is particularly evident in the system of prominent bands designated EST E, in which either one or three bands appear. Of the 127 wild specimens from Austin, 23 percent display one band, and 77 percent display three. Electrophoretic mobilities of both triplets and singles are so variable that, among the homogenates from 24 wild individuals that are normally run simultaneously on one gel, few produce identical EST E patterns. The smaller samples from other localities show similar degrees of variation.

Esterases migrating more rapidly toward the anode show variation unrelated to that in EST E (Figs. 1 and 2). Although several systems of bands apparently overlap, some of them—especially EST C—can often be read from gels. In this system there are either single or double bands (but not triplets), patterns that suggest homozygous and heterozygous genotypes, respectively, with several alleles involved.

On the assumptions that a single autosomal locus controls the expression of the EST E system, that the enzyme is a dimer, and therefore that a single band reflects a homozygous genotype while a triplet reflects a heterozygous one, the minimum number of alleles in the Austin population was estimated at 13 (*7*). This estimate is conservative; it was necessary to ignore numerous slight differences in electrophoretic mobility because reliable scoring between gels was not possible. Our

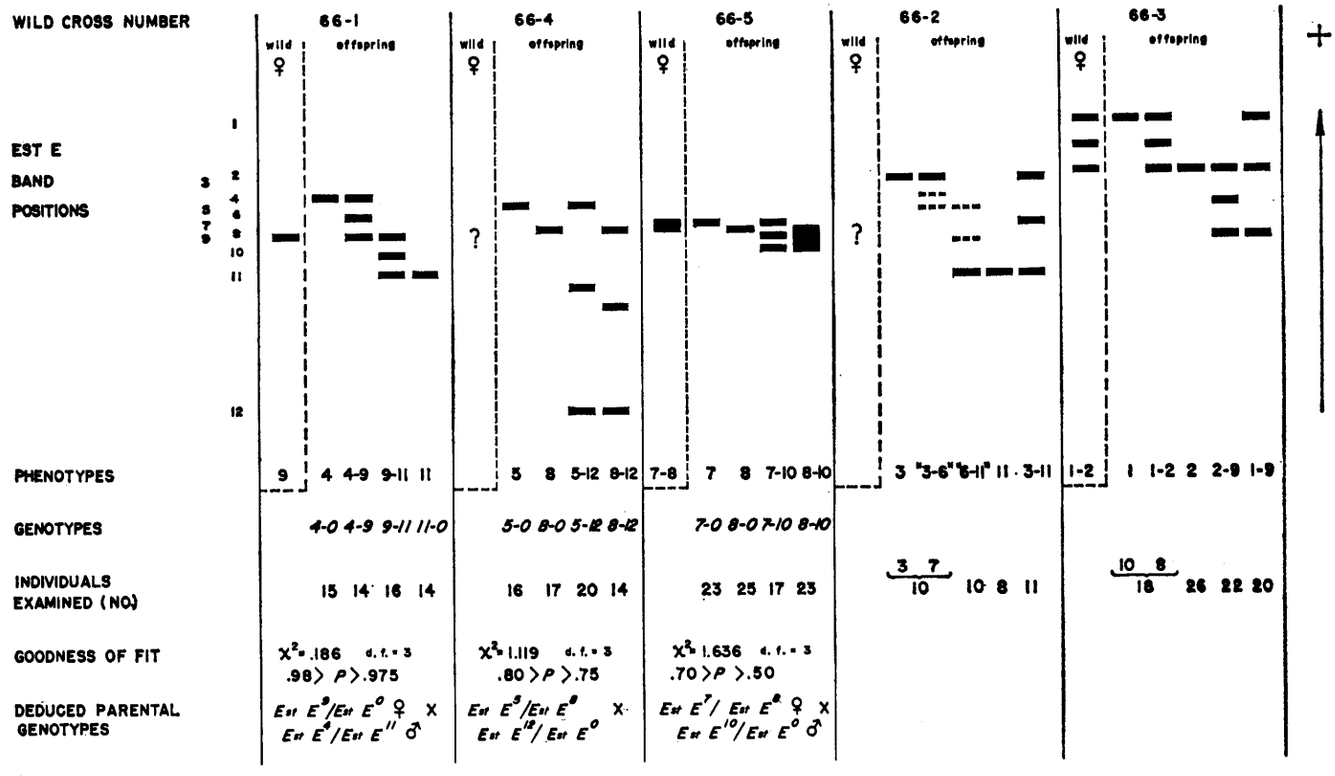

Fig. 3. Electrophoretic variants of EST E in the progeny of five wild females of *Colias eurytheme* from central Texas (66-1 from San Antonio; all others from Austin). Genotypes are not shown for two wild crosses in which incompletely analyzed secondary effects appear. Variable band expression of two variants in cross 66-2 is denoted by dashes.

genetic studies indicate that the allelic series must be considerably greater.

Genetics of EST E variation was determined (i) by assaying F_1 offspring (first instar larvae to adults) reared from wild females whose progeny represent single-pair matings (wild crosses), and (ii) by assaying F_2 offspring (larvae only) obtained from laboratory crosses. The EST E phenotypes of three of five wild female parents and of all laboratory parents were determined. Developmental changes in expression of EST E isozymes were not apparent, except that bands were sometimes faint or absent in first instar larvae.

Twelve different electrophoretic positions (not counting intermediate, that is, hybrid, bands in presumed heterozygotes) are represented in the offspring of the five wild females (Fig. 3). These positions (numbered consecutively from the anode in order of decreasing mobility) probably reflect the number of *Est E* alleles in individuals involved in the wild crosses. Different wild crosses produce identical individuals in only two instances. The progeny of crosses 66-4 and 66-5 both include the phenotype of EST E single band 8; and those of 66-1 and 66-2, the phenotype of EST E single band 11.

In each of three wild crosses (66-1, 66-4, and 66-5), four kinds of offspring appear (Fig. 3). With the postulated existence of an *Est E* allele that results in no EST E activity—a null allele, *Est* E^0—these three crosses are consistent with the hypothesis of multiple alleles at a single locus. On this basis, wild cross 66-1 is written *Est* $E^9/Est\ E^0$ ♀ × *Est* $E^4/Est\ E^{11}$ ♂; 66-4, *Est* $E^5/Est\ E^8$ × *Est* $E^{12}/Est\ E^0$; and 66-5, *Est* $E^7/Est\ E^8$ ♀ × *Est* $E^{10}/Est\ E^0$ ♂. Where determined (66-1 and 66-5), the phenotype of the wild female parent is in agreement with the postulated parental genotypes. Phenotypic ratios in the progeny of these three wild crosses are not significantly different from the expected 1 : 1 : 1 : 1 ratio.

The existence of a null allele is further suggested by F_2 data from laboratory crosses. The results of single-pair matings between 66-1 offspring are summarized in Fig. 4. In addition, one wild female in the Brazos River sample had no detectable EST E bands.

Interpretation of two wild crosses (66-2 and 66-3) is complicated by the occurrence of five kinds of offspring in each (Fig. 3); but, as in each of the previously discussed wild crosses, four alleles seem to be involved—three that

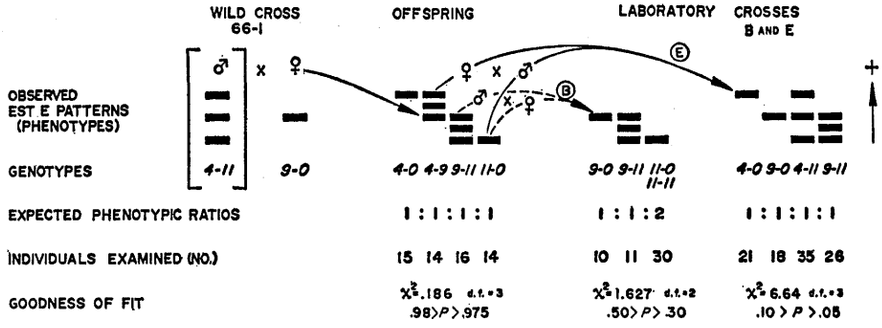

Fig. 4. Electrophoretic variants of EST E in F_1 and F_2 from wild female 66-1 of San Antonio, and genetic interpretation of the crosses involved. [Pattern and genotype of the wild male (given in brackets) are deduced.] These data illustrate the role of a postulated null allele, *Est* E^0.

determine different basic-band positions, in addition to the null allele. With multiple mating presumably ruled out, more than four offspring phenotypes are hard to explain by variation at a single locus. One can tentatively postulate that certain alleles at an independently segregating, modifier locus affect the expression of certain EST E genotypes. Or, noting that wild cross 66-2 could be written *Est* $E^3/Est\ E^{11}$ × *Est* $E^6/Est\ E^0$; and 66-3, *Est* $E^1/Est\ E^2$ ♀ × *Est* $E^9/Est\ E^0$ ♂; and noting that in each cross the unaccountable offspring category that appears is identical with the genotype of one parent (known to be the female in the case of 66-3), one can postulate some cytoplasmic effect. In any event, a secondary mechanism is apparently superimposed on the primary one.

Individuals homozygous at the *Est E* locus for any but the null allele produce a single band. But individuals heterozygous for the null allele also produce one band and would be classified, without progeny tests, as homozygotes, so that *Est E* variation in natural populations would be underestimated. Because one parent in each of the five wild crosses is deduced to be heterozygous for the null allele, most single-band types in central Texas populations are probably heterozygous. In population samples, triplet (heterozygous) individuals outnumbered single-banded ones by 3 or 4 : 1. Despite the complications, all five wild crosses appear to be between heterozygotes; and only two of the ten heterozygotes are the same. Altogether, the data suggest that the Texan populations of *C. eurytheme* consist almost entirely of heterozygotes among a large number of different alleles (of which the commonest is the null allele).

In central Texas, populations of *C. eurytheme* fluctuate seasonally in numbers and distribution, apparently in response to larval food resources rather than to weather. Most of the requisite leguminous food plants die in May and early June. Because the butterfly produces generations continuously (one every 4 to 6 weeks in warm or hot weather) without entering diapause, it disappears from central Texas in late spring, except for scattered populations that persist where sufficient food is maintained, as in cultivated fields of alfalfa (*Medicago sativa*). It usually returns by late October or November, remains through winter, and becomes common and widespread in a variety of habitats by spring. The extraordinary EST E polymorphism of *Colias eurytheme* may facilitate its rapid expansion into unoccupied regions in seasons when food plants become generally available. (Our sample taken in late July of a reservoir population in an alfalfa field along the Brazos River 120 km northeast of Austin showed undiminished EST E variation.)

Several factors may contribute to the observed high variability of these populations: (i) their alternately continuous and then extremely discontinuous distribution; (ii) the now unknown but probably diverse origins of repopulating immigrants that annually contribute to a new common gene pool; (iii) the introduction (due to pronounced mobility within the species population) of variation arising from extensive hybridization between *C. eurytheme* and *C. philodice* (*9*) over large areas of North America to the north; and (iv) the existence of heterotic allelic combinations (which may, especially, be those involving the null allele).

Because some enzyme alterations are not electrophoretically detectable, and because the number of females from which we bred is small, variation

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

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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