determining spore viability, the ability of spores to germinate was adopted instead. As shown in Table 2, uracil at a concentration of 0.25 mg/ml is far most efficient in inducing spore maturation than either uridine or cytidine, and the mutant, therefore, might more properly be designated a uracil auxotroph. Ascospores that were tested had already been discharged and had accumulated on the inner side of the petri dish lids in cultures that were 2 weeks old. Maturation of spores can also be induced by adding the supplement in solution to cultures (6 days old) that have already produced perithecia, containing asci and immature ascospores, on the usual cornmeal-dextrose agar medium. On cytosine-supplemented medium only protoperithecia were formed at the highest concentration. The highest concentration of pyrimidines used in the experiment on growth was the same as the lowest concentration used in the experiment on ascospore germination.

Since the *pyr-3a* mutants in *Neurospora* can be suppressed either by certain arginine mutations or by an unlinked suppressor (*7*) that affects ornithine transcarbamylase and hence reduces the concentration of arginine in the mycelium (*8*), it seemed appropriate to search for such an interaction between *st-59* and *pyr*. Analysis of 40 asci from the cross *st-59* × *pyr* (Fig. 1d) yielded 6 parental ditype, 25 tetratype, and 9 nonparental ditype asci, which indicates that the two loci are not linked. The double mutants *st-59 pyr* recovered from this cross were deficient for both arginine and a pyrimidine and grew only in the presence of the two supplements. Lack of interaction between the two mutant loci is not surprising, since the *pyr-3a* mutants in *Neurospora* represent a minority among the *pyr-3* group (*9*). Furthermore, the *pyr-3a* mutants are blocked at an early step in the synthesis of pyrimidines prior to the appearance of ureidosuccinic acid (*8*), whereas the *pyr* mutant in *Sordaria fimicola*, unresponsive to orotic acid, is blocked at a later step. Regardless of whether interaction between arginine and pyrimidine mutants can be detected on the genetic level, arginine and pyrimidines are synthesized from a common precursor, carbamyl phosphate, along two divergent pathways, one leading to arginine by way of ornithine transcarbamylase and the other to pyrimidines by way of aspartate transcarbamylase. The role of amino acids and pyrimidines in ascosporogenesis and self-fertility of homothallic ascomycetes should be investigated further.

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References and Notes

1. A. S. El-Ani, L. S. Olive, Y. Kitani, *Amer. J. Bot.* **48**, 716 (1961).
2. A. S. El-Ani and L. S. Olive, *Proc. Nat. Acad. Sci. U.S.* **48**, 17 (1962).
3. A. S. El-Ani, *Science* **145**, 1067 (1964).
4. H. S. Loring and J. G. Pierce, *J. Biol. Chem.* **153**, 61 (1944).
5. N. Fries, *Ark. Bot.* **33a**, 1 (1946).
6. D. Bonner, E. L. Tatum, G. W. Beadle, *Arch. Biochem.* **3**, 71 (1943).
7. M. B. Mitchell and H. K. Mitchell, *Proc. Nat. Acad. Sci. U.S.* **38**, 205 (1952).
8. R. H. Davis, *Science* **134**, 470 (1961).
9. ——— and V. W. Woodward, *Genetics* **47**, 1075 (1962).
10. Supported in part by NIH grant AI-04425.

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Enzymatic Solubilization of Insoluble Proteins at Neutral pH

Abstract. *Insoluble epidermal proteins (possibly keratin), previously considered inert to enzyme action, were solubilized by either trypsin or chymotrypsin. Cleavage of the disulfide bonds prior to enzymatic action is not necessary. In addition, the enzymatic action on intact epidermis is not influenced by the presence or absence of endogenous lipids, soluble proteins, peptides, or amino acids. Solubilization of epidermal protein by chymotrypsin is inhibited by the supernatant solution of the homogenized epidermis.*

In normal epidermal keratinization, the epidermal proteins are considered to aggregate, forming tonofilaments, tonofibrils, and finally mature keratin. The study of epidermal proteins (*1*) and of the pathway by which these proteins become aggregate protein structures has been difficult because epidermal proteins (keratin?) are not easily solubilized, and when they are solubilized they are difficult to relate to the end product of keratinization (*2*). Soluble proteins have been obtained from insoluble epidermal proteins with urea (*3*), dilute alkali (*4*), LiBr (*5*), or citric acid (*6*).

Trypsin has been used to separate skin into its component parts, epidermis and dermis (*7*). The enzymatic action on the intracellular structures of the epidermis is thought to be minimum as the epidermis, after trypsin treatment, retains its initial ability to grow and keratinize in culture (*8*). Further evidence that viability of the epidermis is unaffected by enzyme action is found in experiments of Weiss and James (*9*). They treated chick embryo epidermis with trypsin and found that the dispersed cells, when incubated on dermis in culture, were able to reassemble into a normal growing and keratinizing epidermis. Many investigators routinely disperse epidermal cells by treatment with trypsin in order to obtain cells for culture studies.

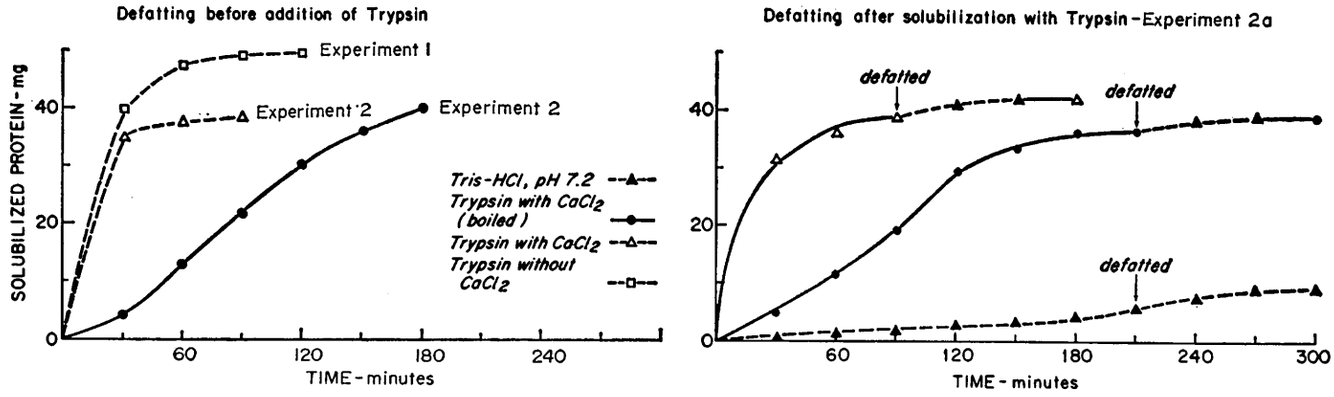
In our work both trypsin and chymotrypsin separately have been used to solubilize the insoluble epidermal proteins of the epidermis. The enzymatic attack brings into solution more than 65 to 75 percent of the original insoluble epidermal protein. About 15 to 25 percent of the original epidermis remains insoluble after final enzyme action.

Whole normal human epidermis was pooled from abdominal skin of autopsy specimens 12 hours or less after death. The epidermis was separated from the corium by the stretch method (*10*).

Several different substrate preparations were tested for each of the enzyme reactions. These included homogenized residue, R_{II}; epidermis before and after defatting, E_N and E_D, respectively; and whole skin.

In some experiments the pooled epidermis was lyophilized, extracted with ether (*11*), and then homogenized in 0.1*M* phosphate buffer, *p*H 7.2 (*12*), with a Teflon Potter-Elvehjem tissue grinder. The epidermis, after three homogenizations at 0°C, was separated from the residue by centrifugation at 3000*g*. The final homogenate supernatant contained negligible amounts of protein, as measured by absorption at 280 mμ. The residue, R_{II}, was washed twice with water, dialyzed (two ½-hour periods), and then used as a substrate for enzymatic action.

In other experiments the lyophilized epidermis was used as a substrate before and after extraction with ether. When the undefatted epidermis was the substrate, it was reacted with chymotrypsin until no additional protein was solubilized, and then it was extracted with ether. Several additional enzyme incubations were then made until no further protein, as measured by absorb-



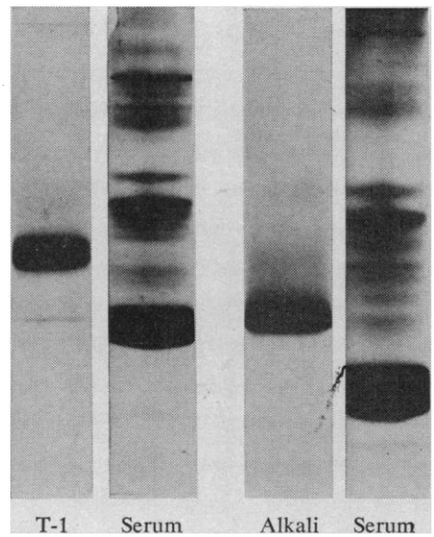


Fig. 1 (above). Solubilization of epidermal proteins by the action of trypsin, experiment 1; trypsin in 0.01*M* $CaCl_2$ on defatted, homogenized, insoluble, epidermal residue, experiment 2; trypsin in 0.01*M* $CaCl_2$ on undefatted, homogenized, insoluble, epidermal residue, experiment 2a.

Fig. 2 (left). Disc electrophoresis of epidermal proteins solubilized by the action of trypsin (T-1), and by the action of 0.05*M* NaOH on enzyme-treated insoluble epidermal residue.

ance at 280 mμ, was present in the separated supernatant.

The action of chymotrypsin on whole skin was tested on sections (0.2 mm thick) of skin prepared with a Storz dermatome (*13*). After initial incubations of the sections at 37°C with tris buffer, 0.05*M*, *p*H 7.2, to remove soluble proteins, peptides, amino acids and other soluble materials, the tissue was incubated (30 minutes) with chymotrypsin. Additional periods of incubation for 30 minutes with fresh enzyme were made until no additional soluble protein was detected in the separated supernatant.

Solutions of trypsin (Worthington, twice crystallized) and chymotrypsin (Worthington, three times crystallized) were prepared in 0.05*M* tris buffer, *p*H 7.2, the concentration being 1 mg/ml. To prevent autodigestion of trypsin and chymotrypsin, the enzyme solution was made 0.01*M* in $CaCl_2$ (*14*).

The starting material was 100 mg of dry, defatted epidermis or an equivalent of that amount of fresh epidermis. The substrate was incubated at 37°C for 30-minute periods with 3 ml of trypsin solution. Immediately afterward the reaction mixture was placed on ice and centrifuged in the cold at 10,000 rev/min for 15 minutes. Protein was determined (*15*) on portions of the supernatant containing the solubilized protein. The controls differed from the experimentals only in that the enzyme solution was boiled for 15 minutes prior to its addition to the substrate.

Disc electrophoresis (*16*) was carried out on epidermal proteins solubilized by enzyme action. The proteins were dialyzed at 0°C against tris-glycine-sucrose buffer, *p*H 8.3, and electrophoresis was conducted on 7½ percent polyacrylamide gel in tris-glycine buffer, *p*H 8.3.

The action of trypsin on homogenate residue resulted in the maximum rate and the greatest solubilization during the first 30-minute incubation (Fig. 1, experiment 1). After the enzymatic reaction was complete and no additional proteins were solubilized, the remaining residue was about 15 to 25 percent of the original 100 mg of undefatted dry epidermis.

The solubilized proteins were further resolved by disc electrophoresis. The solubilized epidermal proteins, T-1, of the first 30-minute trypsin digestion, appear predominantly as a single broad band (Fig. 2).

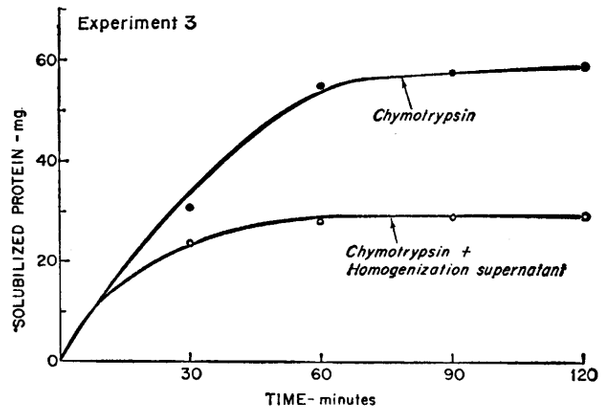
In the 30-minute incubation, 90 percent of the trypsin was autodigested. The trypsin was stabilized by the addition of 0.01*M* $CaCl_2$ to the trypsin solution (Fig. 1, experiment 2). The trypsin–$CaCl_2$ solution was reversibly denatured upon boiling (*17*), as indicated by its action on the insoluble residue from the homogenate in solubilizing epidermal protein. The net result of the boiled enzyme solution when compared to the active enzyme solution was solubilization of equal quantities of epidermal protein, but the rate was slower (Fig. 1, experiment 2).

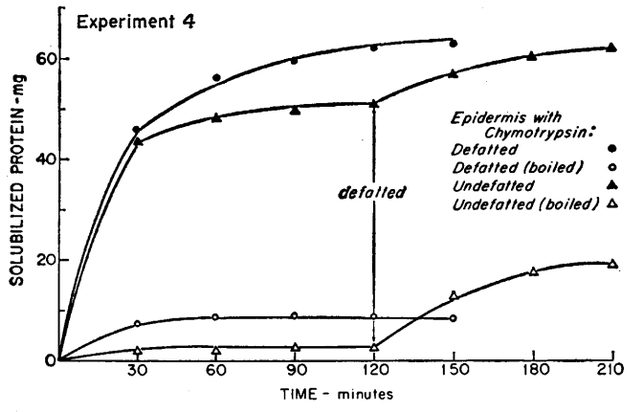
The digested residue gave no additional soluble protein after extensive reaction with trypsin, but yielded a small amount of solubilized protein when further treated with 0.05*M* NaOH for 20 hours. This alkali-solubilized protein, upon disc electrophoresis (Fig. 2), had a major peak similar to that obtained by trypsin solubilization (T-1) of insoluble residue.

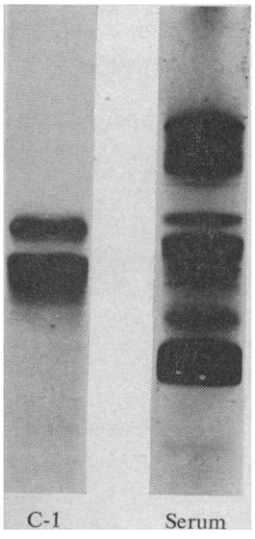
To determine whether only trypsin acts on the insoluble residue, or whether other proteolytic enzymes can use epidermal keratin as a substrate, the action of chymotrypsin was tested with the insoluble residue and epidermis as substrates. Chymotrypsin action on these substrates solubilizes epidermal protein at a slower rate than trypsin does (Fig. 3, experiment 3).

The disc-electrophoresis pattern for the protein solubilized by chymotrypsin is slightly different from that for proteins obtained after trypsin action. In addition to the major peak found in the trypsin reaction, a slower-traveling peak is present after chymotrypsin action (Fig. 4).

Enzymatic solubilization by chymo-







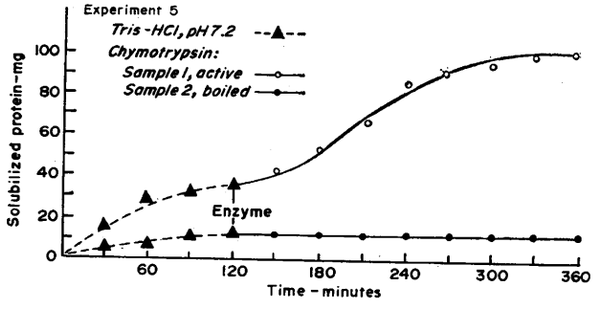


Fig. 3 (above). Solubilization of epidermal proteins by action on chymotrypsin in 0.01*M* $CaCl_2$ on defatted homogenized epidermal residue inhibited by homogenate supernatant (experiment 3); defatted and undefatted epidermis (experiment 4).

Fig. 4 (far left). Disc electrophoresis of epidermal protein solubilized by the action of chymotrypsin (*C-1*) on defatted homogenized epidermal residue.

Fig. 5 (left). Solubilization of epidermal proteins by the action of chymotrypsin in 0.01*M* $CaCl_2$ on skin (0.2 mm thick).

trypsin was inhibited when a portion (one-third of the supernatant obtained after the first homogenization of defatted epidermis) of the supernatant from the homogenate was added to the insoluble residue from the homogenate (Fig. 3, experiment 3).

To find out whether epidermis extracted with ether was more responsive than normal epidermis to proteolytic attack, experiments were carried out with undefatted epidermis. Figure 1, experiment 2, and Fig. 3, experiment 4, indicate that the digestions with trypsin or chymotrypsin proceeded equally well in both defatted and undefatted epidermis. The amount of soluble protein obtained by defatting before enzyme action, when compared to the protein obtained on defatting after enzyme action, was about equal. These experiments also indicate that there was another group of proteins, soluble only after defatting but not solubilized by chymotrypsin.

Chymotrypsin solubilized equal amounts of protein when either defatted epidermis or insoluble residue from the homogenate was used as substrate (see Fig. 3, experiments 3 and 4).

To test whether dermal influences affect the solubilization of epidermal proteins by protease, the action of chymotrypsin on whole skin (epidermis and dermis) was evaluated. The pattern of solubilization of epidermal proteins when whole skin is the substrate is different from that found with epidermis alone (Fig. 5). Instead of the rapid solubilization of epidermal protein in 30 to 60 minutes—as found with insoluble residue (epidermal or homogenate)—in the whole skin maximum epidermal protein solubilization occurs only after 180 minutes. This may either reflect an inhibition of the enzyme by the dermis, or it may show that the time interval for enzyme diffusion through the dermis to the epidermis does not permit immediate enzyme action on the epidermis substrate.

Upon testing whether lipid sites in the epidermis, altered by ether extraction, influence susceptibility of the epidermis to enzyme attack, it was found that equal amounts of protein were solubilized regardless of whether the epidermis was defatted before or after enzymatic treatment.

The question of inhibitor or inhibitors of protease activity (*18*) in the epidermis was examined. Removal of the homogenate supernatant solution from the homogenate residue does not result in solubilization of epidermal protein different from that found for intact epidermis. However, when the supernatant was added to the residue the enzymic action on the residue was inhibited, decreasing the amount of epidermal protein solubilized (Fig. 3, experiment 3). Our results suggest that a normally inactive protease inhibitor was released upon homogenization of the defatted epidermis.

That 75 percent of the insoluble residue was solubilized by trypsin and chymotrypsin suggests that the epidermal protein solubilized bears some relation to what is commonly referred to as keratin. If indeed it is keratin that is being solubilized, then our observations do not support the popular view that keratin is inert to protoe-

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.

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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.
2. S. Rothberg, in *The Epidermis*, W. Montagna and W. Lobitz, Eds. (Academic Press, New York, 1964), chap. 17.
3. K. M. Rudall, *Advance. Protein Chem.* **7**, 253 (1952).
4. C. Carruthers, D. Woernley, A. Baumler, B. Kress, *J. Invest. Dermatol.* **25**, 89 (1955); A. G. Matoltsy and C. A. Balsamo, *J. Biophys. Biochem. Cytol.* **1**, 191 (1955); S. Rothberg, *J. Invest. Dermatol.* **34**, 197 (1960).
5. D. A. Roe, *J. Invest. Dermatol.* **27**, 319 (1956).
6. A. G. Matoltsy, *Nature* **201**, 1130 (1964).
7. P. B. Medawar, *ibid.* **148**, 783 (1914); J. Fan, *J. Invest. Dermatol.* **30**, 271 (1958).
8. N. K. Wessells, *Develop. Biol.* **4**, 87 (1962).
9. P. Weiss and R. James, *Exp. Cell Res.* **3** suppl., 381 (1955).
10. E. J. Van Scott, *J. Invest. Dermatol.* **18**, 377 (1952).
11. In this text defatted epidermis refers to epidermis extracted for three 1-hour periods with ether. This is not equivalent to or meant to infer that the epidermis is totally defatted.
12. All *p*H's were measured at 25°C unless otherwise indicated.
13. Storz Instrument Co., St. Louis, Mo.
14. J. H. Northrop, M. Kunitz, R. Herriott, *Crystalline Enzymes* (Columbia Univ. Press, New York, 1948), p. 125; F. F. Nord and M. Bier, *Biochim. Biophys. Acta* **12**, 56 (1953).
15. O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
16. L. Ornstein and B. J. Davis, unpublished communication; preprint by Distillation Products Industries, Division of Eastman Kodak Co., Rochester, N.Y.
17. J. Mellanby and V. J. Woolley, *J. Physiol.* **46**, 159 (1913); J. H. Northrop, *J. Gen. Physiol.* **16**, 323 (1932).
18. C. J. Martin and A. E. Axelrod, *J. Biol. Chem.* **224**, 309 (1957).
19. L. Szodoray, *Arch. Dermatol. Syphilol.* **150**, 605 (1930).
20. W. G. Crewther and B. S. Harrap, *Nature* **207**, 295 (1965).
21. G. Swanbeck and N. Thyresson, *Acta Dermato-Venereol.* **41**, 289 (1961).
22. G. C. Wells and C. Babcock, *J. Invest. Dermatol.* **21**, 459 (1953).

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*.