gave two major bands on SDS–gel electrophoresis at positions corresponding to proteins of 62,000 daltons (crm_{197}) and 45,000 daltons (crm_{45}) (Fig. 2A). Almost all of the enzymic activity was associated with the crm_{45} peak, but it is evident that a small amount of free fragment A (24,000 daltons) was also present. After removal of DTT and reoxidation, a significant proportion of the activity had moved over into the 62,000-dalton peak (Fig. 2B). In fact, the shift of enzymic activity into this peak corresponds to nearly 25 percent of all the crm_{45} originally added and is equivalent to approximately half of the activity to be expected if all the crm_{197} had been converted into active enzyme. The trypsin-treated dialyzed mixture was injected, intraperitoneally, into guinea pigs (250 to 280 g) in amounts corresponding to 10, 5, and 2.5 μg of the crm_{197} originally present. The survival times were 10 to 12, 14 to 16, and 20 hours, respectively. From the dose-survival curve of Baseman *et al.* (*4*), it may be calculated that the yield of reassembled toxin corresponds to between 40 and 80 percent of the crm_{197} originally added, a result in excellent agreement with the proportion of enzymic activity shifted into the 62,000-dalton peak (*9*). Antitoxin specifically neutralized the toxicity of the reconstituted mixture.

In conclusion, we have demonstrated that each of the two dissimilar fragments comprising the diphtheria toxin molecule has a separate and distinct function. Toxicity depends on the unique enzymic activity associated with fragment A. This activity is responsible for the arrest of polypeptide chain elongation in sensitive cells by specific ADP-ribosylation of translocase. But fragment A can only reach the sensitive cell cytoplasm when it is specifically associated with fragment B. The number of sites on HeLa cells available to react with toxin molecules is small (*8*). Since crm_{197}, but not crm_{45}, successfully competes with toxin in HeLa cell cultures, it seems most probable that attachment and penetration of toxin are specific processes that depend on the composition and conformation of the 17,000-dalton COOH-terminal amino acid sequence of the molecule.

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References and Notes

1. D. M. Gill and L. Dinius, *J. Biol. Chem.* **246**, 1485 (1971); R. J. Collier and J. Kandel, *ibid.*, p. 1496.
2. D. M. Gill and A. M. Pappenheimer, Jr., *ibid.*, p. 1492; R. Drazin, J. Kandel, R. J. Collier, *ibid.*, p. 1504.
3. T. Honjo, T. Nishizuka, O. Hayaishi, I. Kato, *ibid.* **243**, 3553 (1968); D. M. Gill, A. M. Pappenheimer, Jr., R. Brown, J. T. Kurnick, *J. Exp. Med.* **129**, 1 (1969).
4. J. B. Baseman, A. M. Pappenheimer, Jr., D. M. Gill, A. A. Harper, *J. Exp. Med.* **132**, 1138 (1970).
5. T. Uchida, D. M. Gill, A. M. Pappenheimer, Jr., *Nature* **233**, 8 (1971).
6. J. Everse, D. A. Gardner, N. O. Kaplan, W. Galasinski, K. Moldave, *J. Biol. Chem.* **245**, 899 (1970); R. S. Goor and E. S. Maxwell, *ibid.*, p. 616; A. M. Pappenheimer, Jr., and D. M. Gill, in *Symposium on Molecular Mechanisms of Antibiotic action on Protein Biosynthesis*, D. Vasquez, Ed. (Elsevier, New York, in press).
7. R. S. Goor and A. M. Pappenheimer, Jr., *J. Exp. Med.* **126**, 913 (1967); L. Montanaro and S. Sperti, *Biochem. J.* **107**, 730 (1967).
8. A. M. Pappenheimer, Jr., and R. Brown, *J. Exp. Med.* **127**, 1073 (1968).
9. It will be noted from Fig. 2B that, after treatment with trypsin, relatively little material remained in the 45,000-dalton band (despite the fact that crm_{45} was in twofold excess), possibly because fragment A has a higher affinity for intact fragment B derived from crm_{197} than for the corresponding 21,000-dalton fragment derived from crm_{45} or for another fragment A molecule to form a dimer. Because of the relative instability of fragment B, some degradation probably always occurs, and the reconstituted mixture therefore contains an excess of fragment A, seen as a fast-moving component.
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30 September 1971

Isozymes of Phenylalanine Hydroxylase

Abstract. *Three isozymes of phenylalanine hydroxylase exist in adult rat liver. They are chromatographically unique. Partial chracterization suggests that they are similar in chemical properties and differ only in charge. Estimation of the Stokes radii indicates that the isozymes have similar molecular weights of about 200,000. Two isozymes exist in human fetal liver. Alterations of the relative amounts of these isozymes may control the phenotype of the disease phenylketonuria.*

Phenylalanine hydroxylase (E.C. 1.14.3.1) catalyzes the irreversible hydroxylation of phenylalanine at the para position to yield tyrosine (*1*). The hydroxylating system consists of several protein components (*2*) and requires reduced nicotinamide adenine dinucleotide phosphate and pteridine as cofactors (*3*). Huzino and Bessman separated the hydroxylase component into two isozymes (*4*). Kaufman and Fisher also found two forms of the enzyme and further characterized them (*5*).

We have separated and characterized three isozymes from adult rat liver and have distinguished between two isozymes from human fetal liver. The three forms isolated from rat liver, which we have called pi, kappa, and upsilon, appear to be three distinct isozymes of phenylalanine hydroxylase and not conformational isomers.

All procedures were carried out at 4°C unless otherwise noted. Male rats (Sprague-Dawley strain), 150 to 250 g, were decapitated, and the livers were homogenized in two volumes of buffer that contained 0.025*M* K_2HPO_4, 0.15*M* KCl, and 5 m*M* dithiothreitol (DTT), *p*H 6.8. The homogenates were centrifuged at 100,000*g* for 30 minutes, and a volume of clear supernatant containing 30 mg of protein (estimated by the biuret method) was placed on a column of neutral calcium phosphate gel and wet cellulose powder (30:70, by volume). Column dimensions were 7 by 200 mm and bed volume was 10 ml. The sample was eluted with a linear gradient of K_2HPO_4, 0.025 to 0.2*M*, *p*H 6.8. The gradient also contained 0.15*M* KCl and 5 m*M* DTT; total volume was 50 ml. Flow rate was approximately 0.25 ml/min, and all of the recoverable activity was eluted in 100 0.4-ml fractions. The yield was 90 to 100 percent.

The specific activity of the most active fraction of the kappa peak was about tenfold greater than the specific activity of the crude supernatant.

Table 1. Properties of isozymes of phenylalanine hydroxylase; K_m is the Michaelis constant. Thermal inactivation (therm. inact.) is the percentage of enzyme inactivated by 8 minutes at 42°C. The inhibition (Inhib.) study used 10^{-3}*M* D,L-*p*-chlorophenylalanine (*p*-CPA).

K_m (mmole of tyrosine)	Optimum *p*H	Therm. inact. (%)	Inhib. by *p*-CPA (%)
	Pi		
0.87	7.2	40.0	43.1
	Kappa		
0.78	7.2	26.1	40.2
	Upsilon		
0.64	7.2	47.4	51.4

Frozen supernatant solutions retained about 80 percent of their activity for about a month. Three isozymes were also found in these preparations.

Fractions were incubated for 15 minutes at 37°C with 0.25 μmole of dimethyltetrahydropteridine ($DMPH_4$), 50 μmole of K_2HPO_4 at *p*H 6.8, 1 μmole of L-phenylalanine, 3 μmole of DTT, and 3800 units of catalase. Total volume was 0.5 ml. This reaction system was modified from that of Bublitz (*6*). Tyrosine formed was assayed by an automated method based on that of Hochella (*7*).

Frozen samples from livers of human fetuses (19 to 22 weeks of gestation) (*8*) were thawed and centrifuged, and the 100,000*g* supernatants were used as above.

Results of these experiments are shown in Fig. 1, A and E. The elution pattern for enzyme from rat liver was consistent for samples from more than 25 different rats. The ratio of total activity in the pi, kappa, and upsilon peaks was 1 : 8 : 6. The two-peak elution pattern for enzyme from human fetal liver was also consistent for samples from four different fetuses. These results suggested that three forms of the hydroxylase exist in adult rat liver and two forms exist in the human fetal liver, and that the forms from each species differ in charge.

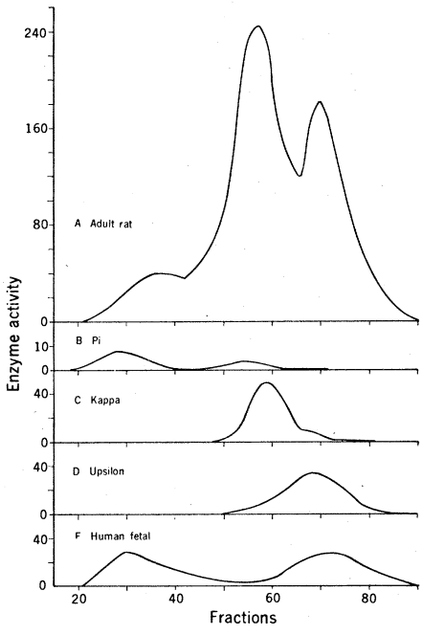

Fig. 1. Separation and rechromatography of isozymes on calcium phosphate gel. Of the fractions from adult rat (A), fractions 28 to 35 were pooled, dialyzed, and concentrated to give fraction pi. Fractions 55 to 62 were treated similarly to give fraction kappa; and fractions 75 to 82, to give fraction upsilon. These fractions were rechromatographed separately (B–D). The ordinal scale is expanded for the pi fraction. Crude supernatants from human fetal liver produced two peaks (E). Enzyme activity is expressed as nanomoles of tyrosine formed per fraction in a 15-minute incubation at 37°C.

For the rechromatography experiments and subsequent characterizations of the rat liver isozymes, a larger column was used to obtain an adequate amount of each enzyme fraction for study. These columns were 15 by 200 mm. Samples of the 100,000*g* supernatant which contained 200 mg of protein were placed on the column and eluted with the same linear gradient in 100 ml. The elution patterns from these columns were identical to those obtained from the smaller columns. Both the relative peak positions and magnitudes were the same for enzyme from approximately 50 different rats.

Pooled, dialyzed, and concentrated fractions containing each isozyme were rechromatographed separately and yielded individual peaks, each of which maintained both its relative position and magnitude. Results of the rechromatography are shown in Fig. 1, B to D.

Pooled fractions from single peaks were used for the estimation of several enzyme characteristics (Table 1). Michaelis constants (K_m's) and *p*H optima for the pi, kappa, and upsilon isozymes are consistent with reported values for phenylalanine hydroxylase (*6, 9*) and are essentially the same for each form. There were small but reproducible differences in the slopes of curves for temperature inactivation and inhibition by *p*-chlorophenylalanine. This suggested that the isozymes have slightly different chemical properties.

High-speed centrifugation of the crude homogenates yields an extract with a specific activity of 0.01 μmole of tyrosine formed per milligram of protein per minute at 37°C. The most active fractions from the phosphate gel column have a specific activity of 0.1 μmole of tyrosine formed per milligram of protein per minute. The preparations were tested for tyrosine hydroxylase by a modification of the method of Nagatsu *et al.* (*10*), and no activity was found. Tryptophan hydroxylase activity was assayed by two different methods (*11*), and activity was detected in all three peaks. The activity of tryptophan hydroxylase follows the same pattern as that for phenylalanine hydroxylase. The ratio of activity of phenylalanine hydroxylase to that of tryptophan hydroxylase is the same over the entire elution pattern of the enzyme from rat. The question of whether phenylalanine hydroxylase has activity toward two substrates or whether there are actually two enzymes has been studied by several workers (*9, 12*). Our data supply further evidence for a single enzyme with two activities.

We have examined the three forms separately on Sephadex G-200 columns (Fig. 2). All three forms appear in the same elution fraction near the catalase marker. Thus, the Stokes radii (*13*) of the pi, kappa, and upsilon forms are strikingly similar. These results point to a single molecular weight of about 200,000, the value ascribed to the tetrameric form by Kaufman and Fisher (*5*).

Huzino and Bessman (*4*) reported two isozymes of phenylalanine hydroxylase, and Kaufman and Fisher (*5*) also reported two. Both groups overlooked the pi isozyme because

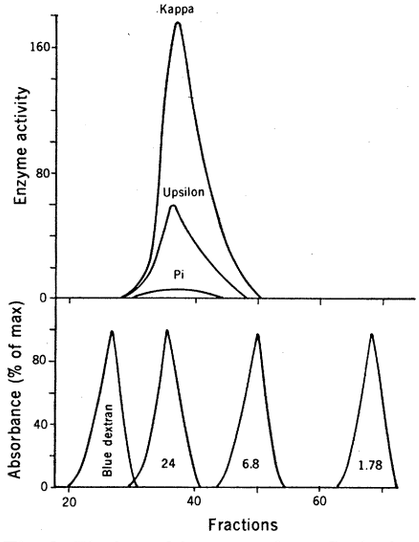

Fig. 2. Elution of isozymes from Sephadex G-200. (Top) Each isozyme was filtered separately on a column 2.5 by 20 cm at a flow rate of 7 ml/hour. The eluent contained 0.01*M* tris(hydroxymethyl)aminomethane, 0.15*M* KCl, and 5m*M* DTT. A total of 100 1-ml fractions were collected and assayed. Each isozyme produced a single activity peak with maximum activity in fraction 37. Enzyme activity is expressed as nanomoles of tyrosine formed per fraction in a 15-minute incubation at 37°C. (Bottom) The void volume was determined with blue dextran. Protein markers were used for calibration of molecular weight. In order of elution, proteins and their molecular weights were catalase, 240,000; hemoglobin, 68,000; and myoglobin, 17,800. For each marker, the number under the peak is 10^4 molecular weight. Absorbance is expressed as percentage of maximum absorbance at 280 nm.

they started the separation by discarding the material extracted by 0 to 10 percent ethanol. After we found the three isozymes in the crude supernatant, we examined the fraction extracted by 0 to 10 percent ethanol and found it to contain most of the pi isozyme. Since the pi isozyme constitutes only about 5 to 10 percent of the total activity, it was easily overlooked. The pi isozyme seems to represent about half of the enzyme activity in fetal liver, however, and therefore may be important.

The existence of three isozymes of phenylalanine hydroxylase may be important clinically. A genetic defect that results in the absence of the enzyme causes the well-known disease phenylketonuria (PKU) (*14*). As reported by Auerbach and his associates (*15*), PKU is not a simple all-or-none phenomenon. Varying degrees and combinations of temporary or permanent symptoms have been observed. This range, from a mild condition—characterized by a slight increase in phenylalanine in the blood, no mental retardation, and no phenylpyruvic acid in the urine—to classical PKU, prompted these investigators to classify PKU as a special case of "hyperphenylalanemias." Much of this clinical variation could be explained by these isozymes. For instance, genetic defects less extreme than that which causes complete loss of the enzyme might result in the absence or delayed maturation of one or more of the isozymes. This variation in genetic defects would produce varying degrees of total enzyme activity and variable symptomatology and disease in the adult.

Moreover, the possibility that fetal isozymes exist (as indicated by the pattern of enzyme activity from human fetal liver) or that isozymes appear at different ages may explain the occurrence of mental retardation. As Winick and his associates have pointed out (*16*), brain growth has a rapid perinatal phase. If a fetal isozyme were absent or if appearance of other isozymes were delayed during any part of that period of rapid growth, brain growth and development would be impaired (*17*). Further, as the other isozymes developed, the perinatal insufficiency of phenylalanine hydroxylase could go unnoticed.

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References and Notes

1. S. Udenfriend and J. R. Cooper, *J. Biol. Chem.* **194**, 503 (1952).
2. C. Mitoma, *Arch. Biochem. Biophys.* **60**, 476 (1956).
3. S. Kaufman, in *Oxygenases*, O. Hayaishi, Ed. (Academic Press, New York, 1962), p. 129; *J. Biol. Chem.* **226**, 511 (1957); *Methods Enzymol.* **17A**, 603 (1970).
4. A. Huzino and S. P. Bessman, *Fed. Proc.* **28**, 408 (1969).
5. S. Kaufman and D. B. Fisher, *J. Biol. Chem.* **245**, 4745 (1970).
6. C. Bublitz, *Biochim. Biophys. Acta* **191**, 249 (1969).
7. N. J. Hochella, *Anal. Biochem.* **21**, 227 (1967).
8. Livers from human fetuses were homogenized in five volumes of 0.15*M* KCl, frozen, and shipped. The four samples used were from fetuses of ages 19, 21, 21, and 22 weeks. Unfortunately it was not possible to assay activity before freezing and shipping the samples. We thank M. Towell for the gift.
9. W. Coulson, E. Wardle, J. Jepson, *Biochim. Biophys. Acta* **167**, 99 (1968).
10. T. Nagatsu, M. Levitt, S. Udenfriend., *Anal. Biochem.* **9**, 122 (1964).
11. S. Udenfriend, in *Fluorescence Assay in Biology and Medicine* (Academic Press, New York, 1969), vol. 2, p. 232; ———, H. Weissbach, C. T. Clark, *J. Biol. Chem.* **215**, 337 (1955).
12. J. Renson, H. Weissbach, S. Udenfriend, *J. Biol. Chem.* **237**, 2261 (1962); R. A. Freedland, I. Wadzinski, H. Waisman, *Biochem. Biophys. Res. Commun.* **5**, 94 (1961).
13. L. M. Siegel and K. J. Monty, *Biochim. Biophys. Acta* **112**, 346 (1966).
14. P. Justice, M. E. O'Flynn, D. Y. Y. Hsia, *Lancet* **1967-I**, 928 (1967); G. A. Jervis, *Proc. Soc. Exp. Biol. Med.* **82**, 514 (1953); C. Mitoma, R. M. Auld, S. Udenfriend, *ibid.* **94**, 634 (1957); S. Kaufman, *Science* **128**, 1506 (1958).
15. V. H. Auerbach, A. M. Di George, G. G. Carpenter, in *Amino Acid Metabolism and Genetic Varation*, W. L. Nyhan, Ed. (McGraw-Hill, New York, 1967), chap. 2, p. 11; G. G. Carpenter, V. H. Auerbach, A. M. Di George, *Pediat. Clin. N. Amer.* **15**, 313 (1968).
16. M. Winick, R. Rosso, J. Waterlow, *Exp. Neurol.* **26**, 393 (1970); I. Fish and M. Winick, *ibid.* **25**, 534 (1969); *Pediat. Res.* **3**, 407 (1969); M. Winick, *Pediat. Clin. N. Amer.* **17**, 69 (1970).
17. S. P. Bessman, *Amer. J. Dis. Child.* **113**, 27 (1967).
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Turnover of Molecules Which Maintain the Normal Surfaces of Contact-Inhibited Cells

Abstract. *In confluent cultures normal chick embryo fibroblasts become highly agglutinable by concanavalin A within 6 hours after their synthesis of protein is inhibited by cycloheximide, pactamycin, or emetine. When growing cells are similarly treated, they fail to become more agglutinable. Apparently, molecules which maintain the normal structure of the cell surface must be replaced continually when cell growth is contact inhibited.*

A difference between the surfaces of normal and of cancerous cells has been revealed by several carbohydrate-binding plant agglutinins, including concanavalin A (Con A) (*1*). Normal cells, which respond to cell contact in culture by limiting their growth, are not agglutinated by these substances, while cancer cells, which do not exhibit contact inhibition of growth, are agglutinated. The cell surface sites which react with Con A are involved in growth control; partially degraded Con A reacts with transformed cell surfaces and restores their ability to become contact-inhibited (*2*). We found that normal, nonagglutinable fibroblasts from chick skin became agglutinable by Con A when they were treated with cycloheximide, an inhibitor of protein synthesis. The experimentally induced change, from a normal to a malignant-like cell surface, suggests a turnover or a loss of specific molecules of the cell surface. This fact could be useful in the study of the cell surface alteration

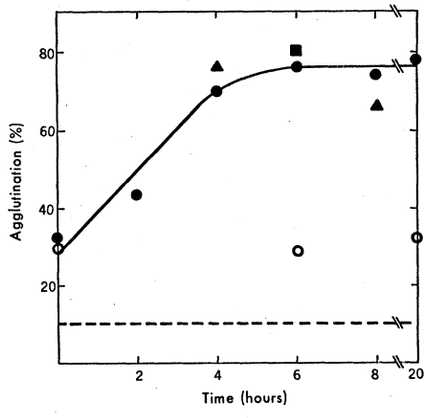

Fig. 1. The agglutinability of chick embryo fibroblasts with 200 μg of concanavalin A per milliliter of saline solution after inhibition of cellular protein synthesis. Contact-inhibited cells (closed symbols) and growing cells (open symbols) were incubated with cycloheximide (circles), emetine (triangles), or pactamycin (squares) for the time indicated. The cells were removed from plates and were shaken with concanavalin A; the percentage of cells agglutinated in clumps of two or more cells were then counted. The dashed line represents the constant background clumping of cells when concanavalin A was not present.