nature of networks. Might such a system prove useful as a model of ongoing fast-processing of information in the brain?

Molecular neurology, which is already on the way to becoming firmly established, together with molecular neuropsychology, which is emerging as a coherent field, seems destined to provide a powerful thrust in modern science. Society may well encourage, indeed demand, full speed ahead in these fields because of their important bearing on mental health, on the understanding of mechanisms of memory, learning, and other psychological parameters basic to science itself, and on man's deep personal concern about the nature of his being.

References and Notes

1. T. H. Huxley, *University Series, No. 1* (Chatfield, New Haven, Conn., ed. 2, 1870), p. 7.
2. T. Graham, *Phil. Trans. Roy. Soc. London* **151**, 183 (1861).
3. F. G. Hopkins, *Nature* **92**, 213 (1913).
4. E. B. Wilson, *Science* **57**, 277 (1923).
5. D. H. Hubel, *Sci. Am.* **209**, 54 (1963); ——— and T. N. Wiesel, *J. Neurophysiol.* **28**, 229 (1965).
6. J. Y. Lettvin, H. R. Maturana, W. S. McCulloch, W. H. Pitts, *Proc. IEEE (Inst. Elec. Electron. Engrs.)* **47**, 1940 (1959).
7. P. Weiss, *Regional Neurochemistry* (Pergamon, New York, 1961), p. 220; ——— and H. B. Hiscoe, *J. Exptl. Zool.* **107**, 315 (1948).
8. L. Lubińska, "Mechanisms of neural regeneration," *Progr. Brain Res.* **13**, 1 (1964).
9. K. S. Lashley, *Symp. Soc. Exptl. Biol.* **4**, 454 (1950).
10. Report of Work Session on "The Synapse: Morphological and Chemical Correlates of Function," *Neurosciences Res. Progr. Bull.* **3**, No. 4 (1965).
11. B. W. Moore, *Federation Proc.* **23**, 524 (1964).
12. L. Levine and B. W. Moore, *Neurosciences Res. Progr. Bull.* **3**, No. 1, 18 (1965).
13. F. O. Schmitt, *ibid.* **2**, No. 3, 44 (1964).
14. R. Levi-Montalcini, *Ann. N.Y. Acad. Sci.* **118**, 149 (1964); *Harvey Lectures Ser.* **1964-1965** (1965).
15. L. Mihailović and B. D. Janković, *Nature* **192**, 655 (1961); *Neurosciences Res. Progr. Bull.* **3**, No. 1, 8 (1965).
16. J. A. Stratton, *Technol. Rev.* **61**, 475 (1959).
17. M. Polanyi, *Personal Knowledge* (Univ. of Chicago Press, Chicago, 1958).
18. P. Horgan, "The abdication of the artist," paper presented before the American Philosophical Society, Philadelphia, 22 Apr. 1965.

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Electrophoretic Variation in Enzymes

Mutations which do not alter catalytic activity provide a major tool for biochemistry and genetics.

Charles R. Shaw

That genetic variation may be expressed through altered enzyme activity has been appreciated since Garrod developed the concept of inborn errors of metabolism. The concept was expanded and clarified concurrently with the one-gene, one-enzyme hypothesis elaborated by a number of workers in biochemical genetics. In the past two decades, a large body of knowledge on genetic variation in enzymes, both in haploid and diploid organisms, has accumulated at an accelerating rate. Most of this information is based on alteration in catalytic activity of the enzyme.

Technical developments have recently made possible the high-resolution "zymogram" display of a number of enzymes from whole-tissue extracts. These new techniques utilize zone electrophoresis (*1*) followed by histochemical staining methods to demonstrate the zones of enzyme activity directly in the electrophoretic medium (*2*). An important application of the zymogram method has been the investigation of genetic alterations which change the electrophoretic mobility of enzyme molecules. The genetic variants disclosed by these methods are enzymes in which activity is retained but structure is presumably altered. This is in direct opposition to the earlier studies by which enzyme variation could be detected only as a change (including absence) of total catalytic activity.

A large and rapidly increasing number of electrophoretic variants of enzymes have been discovered, most of them in the past 3 years. Because many of these are mutants which produce no apparent change in the action of the gene product, they represent a unique and important parameter in biological research. It therefore seems worthwhile to review the types and frequencies of such enzyme variants and to consider their significance for genetics and biochemistry.

This discussion will be limited to diploid organisms. To consider diploids and haploids together is scarcely valid, for the two appear to represent totally different approaches to evolution. Regulation of their metabolism and growth is generally different (*3*), in that microorganisms have many inducible enzymes and flexible systems of gene control, whereas diploids probably have mainly constitutive enzymes, with control mediated by feedback mechanisms affecting the enzyme activity rather than gene activity. (Knowledge on this subject is only preliminary.) Diploids, moreover, reproducing mainly through sexual mechanisms, utilize the vastly greater flexibility and polymorphism resulting from sexual recombination and, having most genes present in duplicate, they can "experiment" with mutations while continuing to produce a normal product from the nonmutant allele.

Nature of Electrophoretic Variation

The now classic studies of Ingram and his co-workers (*4*) on genetic variants of human hemoglobin demonstrated that changes in electrophoretic behavior result from substitutions of single amino acids in the polypeptide chain. The altered electrophoretic mobility reflects a change in the net charge of the protein molecule which occurs when the amino acid carries a

The author is director of the Biological Research Unit at Hawthorn Center, a children's psychiatric hospital in Northville, Michigan.

charge different from that of the one it replaces. The substitution presumably reflects a base-pair substitution in the DNA chain and thus represents a mutation in a structural gene.

Whether electrophoretically detectable mutants in the enzymes of diploid organisms actually result from single amino-acid substitutions has not, to my knowledge, yet been demonstrated. Sufficient amounts of purified material are not readily obtainable in most of the variants thus far described. One enzyme which has been studied in this respect is the human erythrocyte carbonic anhydrase described by Tashian *et al.* (*5*); recent work from their laboratory has shown that in the two variants studied, two different peptides are altered, and the evidence is good that each is the result of a single amino-acid substitution (*6*).

What is the probability that a mutation will produce an alteration in charge of the polypeptide? A tentative approach to this question is the calculation by MacCluer (*7*), based on the fact that of the 20 standard amino acids, 15 are neutral in charge, three have a positive net charge, and two have a negative net charge. By applying present (almost complete) knowledge of the DNA code, and assuming that all amino acids occur in the polypeptide in equal amounts, MacCluer has calculated that a single substitution in the nucleotide will produce a change in net charge in 27.56 percent of the cases. This means that, theoretically, almost three-fourths of all mutants will not be electrophoretically detectable. Amino-acid substitutions can, of course, have other effects on the molecule, and, so far as is now known, many or most such substitutions may so change the molecule as to destroy its activity. Thus the above calculations are at this time of little more than academic interest.

Another genetic mechanism known to alter net charge of a protein is nonhomologous crossing-over, resulting in either deletion or duplication of a segment of the DNA chain. This is reflected in a major alteration in the polypeptide product. Examples are the polymorphism of human serum haptoglobin (*8*) and the rare hemoglobin variant called Lepore (*9*). Nonhomologous crossing-over has not been demonstrated in any enzyme variant, but it also must be entertained as a possible cause of electrophoretic variation.

Mobility of a protein molecule through an electrophoretic medium such as starch gel is affected not only by net charge, but also by molecular size and configuration. Thus, a mutation may produce a change in the configuration of the molecule, with a resultant shift of the electrophoretic pattern, in the absence of alteration in net charge. A genetic alteration which apparently results in a molecule twice the normal size has been described in the hemoglobin variant known as Porto Alegre (*10*).

While this paper deals with electrophoretic variation, it should be noted that a few of these variant molecules also have altered enzyme activity; examples are an acid phosphatase (*11*) and a carbonic anhydrase (*12*) of human erythrocytes. Where ionic charge and catalytic activity are altered in the same variant, both effects are assumed to represent the same change in the molecule. Genetic variants which alter only catalytic activity may or may not be structural: a number of other mechanisms can affect enzyme activity, perhaps the most usual being one which controls the total amount of enzyme produced without changing the specific activity of the enzyme. Such control of enzyme production represents an altogether different approach to genetic variation, however, and is outside the scope of this paper.

Organisms and Enzymes Studied

The organisms which have been most extensively studied for electrophoretic enzyme variants are the house mouse (*Mus musculus*), the fruit fly (*Drosophila*), the deer mouse (*Peromyscus*), and man. For studying frequencies of variants, the latter two organisms, which are largely outbred, contribute most of the meaningful data. The laboratory populations of house mouse and fruit fly are principally inbred strains and are genetically more or less homogeneous; therefore, enzyme differences are usually demonstrated not between individuals but between strains.

The deer mouse remains outbred because sterility develops after a few generations of inbreeding; man is outbred largely for cultural reasons. Most population studies of human enzymes have been limited to blood cells and serum, since other tissues frequently are not readily obtainable. Our laboratory has been screening large numbers of enzymes from various tissues of the deer mouse, in cooperation with Elizabeth Barto.

The enzymes which can be studied by the combined zone-electrophoresis, histochemical-staining procedure are classified into two major groups, sometimes called "specific" and "nonspecific," terms which, as will be presently discussed, are inaccurate.

The nonspecific enzymes are demonstrated by the use of certain synthetic compounds as substrates, the action of the enzyme altering the substrate to produce a visible color change. Most widely used are the naphthol esters, which are hydrolyzed by a number of enzymes. When incubated with an appropriate diazonium salt, the naphthol released from its ester linkage by the enzyme then couples with the diazonium salt to produce a dark-colored, insoluble dye. The esters commonly used are those of the short-chain carboxylic acids such as naphthol acetate or butyrate, and naphthol phosphates. Enzymes hydrolyzing the former are called *esterases*, while those acting on the phosphate esters are termed *acid phosphatases* or *alkaline phosphatases*, depending upon the *p*H at which they are most active.

Obviously, there is some overlap among these three groups of "nonspecific" enzymes (some of the esterases are phosphatases). Also, the natural subtrates upon which most of them act are not known. Which of the many known hydrolytic enzymes are present among the nonspecific esterases and phosphatases is by no means completely determined. A tentative list would include the lipases, cholinesterases, nucleotidases, glycerophosphatase, creatine phosphatase, and probably many others. However, most of the nonspecific esterases and phosphatases have not yet been identified.

The presence of "specific" enzymes is demonstrated by the use of naturally occurring, specific substrates. Most of the enzymes in this group are dehydrogenases. The staining procedure for these is based on the principle that certain soluble tetrazolium compounds will be reduced to the diformazan state and become deeply colored and relatively insoluble in aqueous solution. Thus, to demonstrate a specific dehydrogenase, electrophoresis is performed and the gel is then incubated in a solution containing the appropriate substrate together with an electron-transfer agent, di- or triphosphopyri-

dine nucleotide where required, and a tetrazolium. Wherever in the gel a dehydrogenase for that substrate occurs, a zone of color is produced.

In addition to dehydrogenases, enzymes of the "specific" type include catalase, amylase, carbonic anhydrase, phosphoglucomutase, tyrosinase, and peroxidase.

The distinction between specific and nonspecific enzymes is an operational one, and not entirely valid or meaningful. For example, while "natural" substrates are used to demonstrate the presence of dehydrogenases, this is no proof that the metabolism of that particular substrate is the only or even the chief action of that molecule. Conversely, some of the esterases display activity toward a number of synthetic substrates in vitro, whereas in vivo they may have a high degree of specificity.

The total number of enzymes which can thus far be demonstrated in zymograms is relatively small. However, new and improved techniques are steadily being developed.

Results of Search for Variants

When the first electrophoretic variants were reported, they were regarded as rare events, and it was believed that most enzymes were probably uniform throughout a species. Now the opposite view is generally held, and the opinion has been expressed that if one looks hard enough, almost every enzyme will be found to show variation. Data pertinent to this point are scarce, because systematic surveys reporting both positive and negative results have not been published. Johnson (*13*) states that about half of all *Drosophila* enzymes studied in his laboratory have shown genetic variation; in our laboratory a survey of dehydrogenases of *Peromyscus* revealed variation in six out of eight; and of the three dehydrogenases surveyed extensively in man—lactate, glucose-6-phosphate, and 6-phosphogluconate—all have shown electrophoretic variation. Thus it would seem that enzymes which vary are the rule rather than the exception.

Table 1 is a compilation of all the reported, genetically determined electrophoretic variants in the enzymes of diploid organisms. The frequency of a variant within the population is indicated when known; in many cases, the occurrence is known only to be relatively rare or common, and is so designated.

Enzyme differences between inbred strains, such as a phosphatase of the protozoon *Tetrahymena* (*14*), obviously tell us nothing about population frequency of a variation, but only indicate which enzymes may have varied in the wild progenitors. In the case of intrastrain polymorphism, as in one of the *Drosophila* esterases (*15*), the frequency may or may not reflect the "natural" situation; this depends on a number of factors, including size of original colony, number of generations of inbreeding, and selective factors in the laboratory environment. Nonetheless, data on frequencies within inbred strains contribute to the general picture of wide variability in frequency of electrophoretic mutants.

Our laboratory *Peromyscus*, while not inbred, are many generations removed from the wild condition, and in many cases have been selected and bred for certain traits. They too may not accurately reflect frequencies of variations as they occur in nature, but, provided the enzyme under investigation has not been specifically bred for, and provided the population is of sufficient size, the frequency data from such populations may be significant. Although many of the selection factors present in the wild condition, specifically those related to food gathering and escape, are less operative in the laboratory, the single most potent one, fertility, remains in clear evidence (*16*). Thus, the enzyme variants demonstrated in these populations are those which can occur in organisms that survive, grow to adulthood, and produce viable offspring.

Unfortunately, frequencies of many of the variants are not known. There are several reasons for this. In many cases, the variant has been studied for other purposes, such as its use as a genetic marker, or in investigations of molecular structure. Many of the variants have been discovered in small or selected populations; in man, some were found in hospitalized patients.

A further difficulty in determining

Table 1. Electrophoretic variants in enzymes of diploid organisms.

Organism and tissue	Frequency	Remarks	Reference
		Acid phosphatase	
Man, erythrocytes	Gene frequencies: A, 0.36; B, 0.60; C, 0.04	Polymorphism in random English population; three-allele autosomal, associated quantitative differences	(*11*)
Tetrahymena pyriformis	Strain differences	Three hybrid enzymes in heterozygote, probably tetramer	(*14*)
Drosophila	Polymorphism	Hybrid enzyme in heterozygote, probably dimer; gene on Chromosome 3	(*33*)
		Alcohol dehydrogenase	
Drosophila	Polymorphisms in several strains	Hybrid enzyme in heterozygote, probably dimer; gene on Chromosome 2	(*34*)
Peromyscus, liver	Polymorphism	Probably dimer	(*26, 35*)
Man, liver	Polymorphism	No genetic data	(*36*)
		Alkaline phosphatase	
Man, serum and placenta	Polymorphism	Hybrid enzyme in heterozygote, probably dimer	(*37*)
Drosophila larva	Strain differences	Hybrid enzyme in heterozygote, probably dimer; gene on Chromosome 3	(*38*)
Chicken, serum	Polymorphism	Fast and slow bands; only fast band in heterozygote (dominance?)	(*39*)
		Aminopeptidase	
Maize	Strain differences	Four alleles	(*40*)
Drosophila	Polymorphism; gene frequencies 0.13, 0.53, 0.33	Three alleles, no hybrid enzyme in meterozygote; gene on Chromosome 3	(*41*)
		Amylase	
Drosophila	Strain differences and intrastrain polymorphisms	Several alleles; gene on Chromosome 2	(*42*)
Housefly	Polymorphism		(*43*)
House mouse, pancreas and saliva	1 : 23	Possible linkage between genes controlling pancreatic and salivary enzymes	(*44*)

Organism and tissue	Frequency	Remarks	Reference
		Carbonic anhydrase	
Man, erythrocytes	1 : 3000 (Caucasian) 4 : 490 (Micronesian) 1 : 600 (Amer. Negro)	Three variant alleles	(*5, 6*)
Lower primates	5 : 13 (orangutan) 26:338 (baboon) 1 : 112 (rhesus) 2 : 78 (cynomolgus) 1 : 13 (gibbon)	Two enzymes occur: CA I and CA II. Data given are for CA I; polymorphism of CA II also found in rhesus and cynomolgus. No genetic studies	(*6, 32, 45*)
		Catalase	
Maize	Strain differences	Three hybrid enzymes in heterozygote, probably tetramers	(*46*)
Man, erythrocytes	Rare (one family)		(*47*)
		Cholinesterase	
Man, serum	Gene frequency: 0.02 (also two other, rarer variants)	"Pseudocholinesterase," low activity in variants	(*48*)
Horse, serum	One family (total not given)		(*49*)
		Esterase	
Man, erythrocytes	Rare (2 : > 4000)	"A" esterases, three groups of isozymes, all doubled in variant	(*25*)
Housefly	Strain differences and intra-strain polymorphisms	Variations involving nine enzymes	(*43, 50*)
Rabbit, erythrocytes	Polymorphism	Hybrid bands in heterozygote	(*51*)
Tetrahymena	Strain differences	Two enzymes—both show variation	(*52*)
Drosophila adult	Polymorphism; frequency varies among strains	"Esterase 6"; gene on Chromosome 3	(*15*)
Drosophila	Strain and individual differences; frequency unknown	"C" and "E" esterases, both show variation	(*53*)
Maize	Strain differences	Hybrid band in heterzygote, probably dimer; three alleles	(*22*)
Birds, sera	Wide variability among species, probably polymorphisms		(*54*)
House mouse (inbred laboratory strains), serum	Strain differences		(*55*)
House mouse (feral population), serum	Probable polymorphism	"Esterase V" doubled in some animals (heterozygote?); other esterases show quantitative variation	(*56*)
House mouse, kidney and erythrocytes	Strain differences		(*57*)
Peromyscus, many tissues	Gene frequencies 0.26, 0.60, 0.14	Three alleles, "C, D, e"; C and D electrophoretically different, ee has no activity	(*30*)
Peromyscus, many tissues	Polymorphism	Several enzymes; over half show variation	(*58*)
Newt, liver	Species and individual differences	Multiple bands, additive in hybrids	(*19*)
		Glucose-6-phosphate dehydrogenase	
Man, erythrocytes	80–20 polymorphism in Amer. Negro; several other rare variants in Caucasian	X-linked	(*59*)
Drosophila	Strain differences	X-linked	(*60*)
Peromyscus, many tissues	Polymorphism	"B" enzyme, autosomal, four alleles; hybrid enzyme in heterozygote, probably dimer	(*24*)
		Isocitrate dehydrogenase	
House mouse, several tissues	Strain differences	Two forms, supernatant and mitochondrial; variant is supernatant; hybrid enzyme in heterozygote, probably dimer	(*61*)
Drosophila	Probable polymorphism	Hybrid enzyme in heterozygote, probably dimer	(*62*)
		Lactate dehydrogenase	
Peromyscus, many tissues	Polymorphism	Fifteen bands in heterozygote; tetramer. Variant involves only B subunit	(*17*)
Man, erythrocytes	15 : 5158	Heterozygotes involving either A or B subunits	(*63*)
Frog, several tissues	Species and individual differences; frequency not determined	Complex patterns, probably two isozymic systems. Variants indicate a tetramer in one system	(*20*)
Newt, liver	1 : 6	Probably heterozygote with hybrid enzymes, tetramer	(*19*)
Baboon, liver	13 : 6 : 1 (homozygote: heterozygote: homozygote)	A subunit	(*64*)
Pigeon, testis	Polymorphism with strain difference	Distinct from LDH of other tissues	(*65*)
		Malate dehydrogenase	
Newt, several tissues	Species differences and intra-species polymorphism	Several alleles; hybrid enzyme in probable heterozygote indicates dimer	(*19*)
House mouse	Strain differences	"Malic enzyme" requiring triphosphopyridine nucleotide; hybrid enzyme in heterozygote, probably dimer	(*66*)
		Phosphoglucomutase	
Man, several tissues	Polymorphism; gene frequencies 0.74, 0.26	Two alleles, English population	(*67*)
		6-Phosphogluconate dehydrogenase	
Man, erythrocytes	Polymorphism, 95 : 4 (plus several rare variants)	At least five alleles, some showing quantitative decrease in activity; hybrid enzyme in heterozygote, probably dimer	(*68*)
Peromyscus, many tissues	Polymorphism	Several alleles; hybrid enzyme in heterozygote, probably dimer	(*58*)
Drosophila	Strain differences and intra-strain polymorphism	X-linked, hybrid enzyme in heterozygote, probably dimer	(*69*)
Rat, erythrocytes	Polymorphism	Hybrid enzyme in heterozygote, probably dimer	(*70*)
Pigeon, erythrocytes	Polymorphism	Hybrid enzyme in heterozygote, probably dimer	(*71*)
		Xanthine dehydrogenase	
Drosophila	Strain and individual differences	At least three variants; hybrid enzyme in heterozygote, probably dimer	(*72*)

the frequency of a variant is that present methods do not disclose whether variants which occur in different families and appear to be the same are in fact the same. Presumably, substitutions of many different amino acids at either the same or different sites could produce identical alterations in the electrical charge of a protein. Thus the lactate dehydrogenase variants we found (*17*) in four different families of deer mouse may be identical variants, four different ones, or something between. This consideration obviously raises serious questions concerning frequency. There are no data on the question of identity of variants in the enzymes of higher organisms; however, a parallel is seen in the D variant of human hemoglobin, where the proteins from three unrelated individuals were indistinguishable electrophoretically, but each had a different amino-acid substitution (*18*).

Although our present knowledge of frequencies is thus only cursory, certain major trends have begun to emerge, and these provide a picture of a rather continuous distribution, extending from extreme rarity to true polymorphism.

Variation as a Research Tool

Electrophoretic variants of enzymes have a number of research applications in genetics and biochemistry. The variant molecules provide a natural "label" which makes them readily detectable in small quantities of biological material. Since catalytic activity is in most cases unaltered, these tagged molecules have no effect on biological activity, and the organism remains fertile and healthy. The enzyme is a direct product of the gene, which makes analysis of the gene action relatively straightforward. For biochemical research, many of the procedures can be carried out on crude tissue extracts, obviating the technical difficulties and molecular distortions sometimes attendant upon studies in which purified enzymes are required. Moreover, the zymogram method is applicable to mass screening of a number of different enzymes in large populations.

Major research applications of electrophoretic variants are: (i) as genetic markers in linkage studies, in the characterization of cultured cell lines, and in ontogenetic studies; (ii) in analysis of the subunit structure of complex enzymes; (iii) to clarify molecular relationships among isozymes; and (iv) to demonstrate multiple substrate specificities of enzymes.

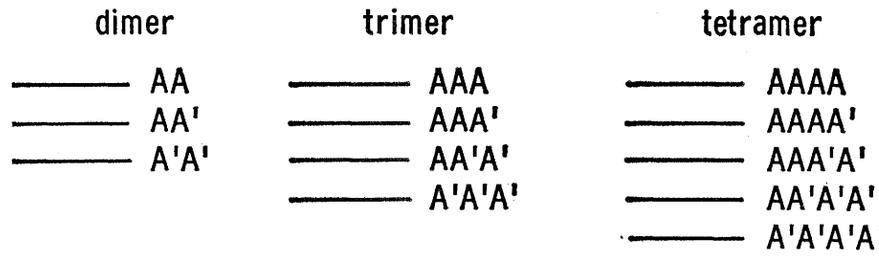

Fig. 1. Diagram of electrophoretic patterns produced by heterozygous variant forms of dimeric, trimeric, and tetrameric molecules. Normal and variant subunits are represented by A and A'.

As genetic markers, these variants are less cumbersome technically than certain other markers traditionally employed, such as drug sensitivity and specific enzyme deficiency. Also, as noted previously, the fact that enzyme activity remains normal precludes the difficulties encountered in some other markers, which often result in selective advantages or disadvantages due to nutritional differences, chemical sensitivities, and so on. A nice application as a marker is the ontogenetic work on hybrid embryos of newt, *Taricha* spp., (*19*) and frog, *Rana* spp. (*20*), where the stages at which paternally derived genes begin to operate could be demonstrated by appearance of the paternal forms of the enzymes.

In analysis of subunit structure of enzymes (*21*), the overwhelming advantage of the electrophoretic-variant technique is that studies can be carried out on small amounts of unpurified material. Conventional biochemical approaches to this problem require purified enzyme, which is then subjected to procedures designed to cleave the molecule at the positions of relatively weak bonding, dividing the molecule into its "natural" subunits. Schwartz (*22*) demonstrated a genetic variant of an esterase in maize, in which each of the homozygous forms occurred as a single band while, in the heterozygote, a third band appeared midway between the other two. Schwartz hypothesized that the enzyme was a dimer composed of two randomly associating subunits, the intermediate band representing pairs of the two unlike subunits.

A number of other such "hybrid" molecules have been found in electrophoretic mutants. In fact, approximately half of the enzymes listed in Table 1 demonstrate hybrids, indicating that polymer structure of enzymes is rather general. Most of these are similar in pattern to the heterozygote described above, but some produce a five-band pattern in which the three intermediate bands are hybrids, suggesting a tetrameric structure. Fig. 1 is a diagram of the number of bands expected in the heterozygous forms of dimeric, trimeric, and tetrameric molecules in which the subunits associate randomly; the normal and variant subunits are labeled A and A', respectively. No examples of a trimer variant are known. A more complex example is that of the lactate dehydrogenase (LDH) molecule, in which a genetic variant demonstrated 15 bands (*17*), whereas the normal LDH pattern consists of five; this evidence supports the hypothesis that LDH normally occurs as five tetramers formed from two different subunits controlled by two genetic loci.

Enzymes may occur in more than one molecular form. These multiple forms have been termed *isozymes*, and demonstration of their occurrence was one of the earlier fruits of the zymogram technique (*23*). An important question in the study of isozymes is whether multiple zones of activity in a zymogram represents "true" isozymes; that is, are they structurally similar, related molecules, or are they quite different molecules which have catalytic activity toward the same substrate? The study of genetic variants can clarify some of the relationships between isozymes. For example, if a variant shows alteration in one of two bands but not the other, then it is likely that the two are produced at different genetic sites. They may or may not be structurally similar, and to determine this would require further physicochemical studies. On the other hand, if in a genetic variant both bands of an isozymic pair are invariably al-

tered together, then they are probably true isozymes, with at least part of the molecule of each isozyme being produced by the same gene; they may be polymer forms of the same enzyme, or they may be complex proteins which share a common polypeptide subunit. Again, further chemical study is needed for final analysis. An example of the application of genetic variation in analysis of isozymes is the glucose-6-phosphate dehydrogenase system of *Peromyscus* (*24*). Three isozymes of this dehydrogenase commonly occur in certain *Peromyscus* tissues; a polymorphism was found involving only one of the three, indicating that it was an enzyme distinct from the other two. As another example, there are six major esterases of human erythrocytes, and a genetic variant was found (*25*) involving three of the six, indicating that those three are related and are distinct from the others.

In the study of multiple substrate specificities of enzymes, the same advantage applies as in analysis of subunit structure, namely, the feasibility of using small amounts of crude biological material. Standard biochemical methods for study of substrate specificity employ purified enzyme and quantitative measurement of activity on individual substrates. Such techniques are time-consuming and laborious, and as a result, only a relatively small number of possible substrates are usually tested. By the zone-electrophoresis technique, a variety of reactions can be tested quickly. Wherever an identical zone of activity occurs in two different reactant mixtures, one may suspect that the enzyme acts on two different substrates. However, this may simply indicate that there are two different enzymes which happen to have identical electrophoretic mobilities under those particular conditions. But if, in addition, a genetic variant of the enzyme is placed in the gel alongside the normal form, and if both forms show the dual activities, then the evidence is strong that a single enzyme is involved. Alternative but less likely possibilities are that two enzymes are present but attached to a common molecule which carries them along together, or that the two enzymes are simply attached to one another, or that the two separate enzymes share a polypeptide subunit which is produced by the mutant locus.

The above principle was used to demonstrate that the "nothing dehydrogenase" activity present in many tissues, long a puzzle to histochemists, is probably produced by alcohol dehydrogenase; both activities occurred at the same position in the gel on several different variant forms, and their identity was subsequently confirmed by quantitative study of pure alcohol dehydrogenase (*26*).

An additional advantage of this approach to the study of multiple specificities is that, since a mixture of many hundreds or thousands of different enzymes is present in the gel, unexpected relationships may be uncovered which otherwise would never have been looked for. As more methods are developed for demonstration of enzymes in the gels, mass screening studies for the elucidation of multiple specificities may assume increasing importance in enzymology.

Biological Significance

It was stated above that many of the electrophoretic variations of enzymes produce no apparent change in biological activity. The concept of genetic mutants which have activities identical with those of the wild type appears to be assuming greater importance in research as methods are developed for the detection of such mutants. The term "isokinetic," to denote similar activities, is proposed. Certain of the electrophoretic enzyme variants would thus represent one class of isokinetic mutants. Additional mutant forms of enzymes which are isokinetic but which are not detectable electrophoretically will doubtless be discovered by other methods of protein analysis. Other classes of isokinetic mutants or polymorphisms would perhaps include the serum proteins (*27*), transferrins, haptoglobins, Gc component, and some of the hemoglobin variants.

We do not know with certainty whether truly isokinetic mutations occur, but we can find, in many of the electrophoretic mutants, no detectable alteration in catalytic activity. Changes in the molecule may of course affect functions other than catalysis, such as attachment to the cell wall, binding of inhibitor or activator substances, antigenicity, or solubility; in most cases such effects are not known. It thus appears reasonable to conclude that isokinetic enzyme mutants do occur. However, until this can be definitely determined for at least several specific cases, discussion of the significance of these variants to genetics and evolution must remain speculative.

If these enzyme alterations *are* without biological effect, the compelling question is, why do they occur in such variable frequency? The rare ones presumably represent chance mutations, but others cannot be so easily explained, and their relatively high frequency suggests that natural selection may have been oversold in evolution theory. Mechanisms other than selection which can effect incorporation of a mutant form into a population include: (i) genetic drift; (ii) variable mutation rates for different genetic sites; (iii) close linkage with genes which do have selective value (*28*); and (iv) sharing of polypeptide subunits between two or more enzymes, one of which has selective advantage. Let us consider these further:

1) Genetic drift theoretically requires the introduction of a mutant into a relatively small population. It could certainly operate in some of the animals studied, such as the laboratory *Peromyscus*. Whether it could explain any of the human polymorphisms is controversial.

2) There is no direct evidence on mutation rates of electrophoretic variants. In the few large surveys that have been made, no new mutations have been reported. There is almost certainly variation in the frequency with which mutations occur at the loci controlling these enzymes, but whether any occur frequently enough to account for variants other than the rare, isolated ones remains to be demonstrated. Perhaps there are highly mutable "hot spots" along the genes of diploids, such as have been found in microorganisms (*29*). The methods used for the study of hot spots in microbiology, which require mass techniques with multiple linked markers, are scarcely applicable in higher organisms (unless the field of tissue culture soon begins to fulfill its earlier optimistic billing). Rather, a more likely approach to this question will probably be through physical-chemical study of the gene products. Such hot spots could account for the wide variety of mutant enzymes of, for example, glucose-6-phosphate dehydrogenase in man.

3) Few linkage studies have thus far been done with electrophoretic variants, and although close linkage to other genes has been reported in at

least two instances (*15, 30*), it remains to be shown that such is the explanation for the persistence of an electrophoretic variant.

4) Polypeptide subunits may occur in more than one enzyme, and conceivably a mutant of such a "shared" polypeptide might not affect the biological activity of the particular enzyme studied but would affect that of another enzyme in which it occurred. Shared subunits are known to occur in human hemoglobin: the alpha chain is the same in both adult and fetal hemoglobins, and an alpha mutant affects both molecules (*31*). Also, as described previously, Tashian has shown that three different human erythrocyte A esterases (*25*) apparently share a common polypeptide subunit; however, in the heterozygous mutant, in which all three esterases showed electrophoretic shifts, catalytic activity appeared normal in each. Nevertheless, such an explanation remains a theoretical possibility for the occurrence of electrophoretic polymorphisms.

Does the fact that some enzymes show little or no variation within a particular species suggest that these have achieved "perfection" at a particular point in time in the course of evolution? If so, what controls their constancy and prevents their changing? Or does this invariability indicate that such an enzyme cannot tolerate change, so that any mutation drastically alters its activity and renders the mutant lethal? Presumably, enzymes differ widely in the alterations which they can tolerate; papain, for example, can lose a major portion of its polypeptide chain without detriment. However, loss of activity is unlikely to be lethal in diploid heterozygotes, since the normal allele "covers" the loss.

It is important to know whether the frequency of variation is relatively constant for homologous enzymes among different species. There are few data on this point. Tashian's studies on erythrocyte carbonic anhydrase among primates (*6, 32*) indicate that the frequency of mutants for this enzyme varies considerably among different species; they are relatively rare in man and certain other primates, whereas in orangutan the same enzyme shows polymorphism. This finding suggests that if the varying frequencies are a reflection of mutation rate, then specific genetic sites vary among species in their mutability.

The demonstration of electrophoretic variants appears to raise more questions than it answers, and we must look to other methods to follow up these provocative leads. The most significant contribution of the zymogram method to the field of molecular genetics will perhaps be the discovery of variant enzymes, making it possible to select from a large population of polypeptides that small fraction whose intensive study will be most fruitful.

Conclusions

Electrophoretic techniques which facilitate mass screening for enzyme mutants provide a powerful research tool for biochemistry and genetics. These mutants give to the molecule a built-in "label," while preserving catalytic activity. Such tagged molecules have wide application as genetic markers and in biochemical analysis of isozyme relationships, of the structure of complex enzymes, and of multiple substrate specificities.

Almost all enzymes thus far studied have shown electrophoretic variation. The frequencies among populations vary widely, from extreme rarity to polymorphism. The fact that they generally appear not to have altered biological activity suggests some control other than one based on direct selective advantage. The accumulating mass of data on electrophoretic variations will gain added significance as more studies attend to frequency within natural populations, presence or absence of associated effects on biological activity, mutation rate, and the molecular basis for variation.

References and Notes

1. O. Smithies, *Biochem J.* **61**, 629 (19→ M. D. Poulik, *Nature* **180**, 1477 (1957); O. Smithies and M. D. Poulik, *ibid.* **177**, 1033 (1956).
2. G. Gomori, *Proc. Soc. Exp. Biol. Med.* **67**, 4 (1948); R. L. Hunter and C. L. Markert, *Science* **125**, 1294 (1957); M. Nachlas and A. Seligman, *J. Nat. Cancer Inst.* **9**, 415 (1949); C. L. Markert and R. L. Hunter, *J. Histochem. Cytochem.* **7**, 42 (1959); R. L. Hunter and M. S. Burstone, *ibid.* **8**, 58, (1960).
3. A. Pardee and A. C. Wilson, *Cancer Res.* **23**, 1483 (1963).
→ V. M. Ingram, *Nature* **180**, 326 (1957); J. A. Hunt and V. M. Ingram, *ibid.* **181**, 1062 (1958).
5. C. R. Shaw, F. N. Syner, R. E. Tashian, *Science* **138**, 31 (196→ R. E. Tashian, C. Plato, T. Shows, *ibid.* **140**, 53 (1963); R. E. Tashian, D. P. Douglas, Y.-S. L. Yu, *Biochem. Biophys. Res. Commun.* **14**, 256 (1964).
6. R. E. Tashian, unpublished.
7. J. MacCluer, personal communication.
8. O. Smithies, G. E. Connell, G. H. Dixon, *Nature* **196**, 232 (1962).
→ C. Baglioni, *Proc. Nat. Acad. Sci. U.S.* **48**, 1880 (1962).
10. D. L. Rucknagel, C. V. Tondo, F. M. Salzano, *Acta Genet. Med. Gemellol.* **9** (3), 29 (1963).
11. D. Hopkinson, N. Spencer, H. Harris, *Am. J. Human Genet.* **16**, 141 (1964).
12. C. R. Shaw, F. N. Syner, R. E. Tashian, *Science* **138**, 31 (1962).
13. F. M. Johnson, personal communication.
14. S. Allen, M. Misch, B. Morrison, *Genetics* **48**, 1635 (1963).
15. T. Wright and R. MacIntyre, *ibid.*, p. 1717.
16. B. D. H. Latter, *Symp. Roy. Soc. Victoria, Melbourne* (Dec. 1959), p. 191.
→ C. R. Shaw and E. Barto, *Proc. Nat. Acad. Sci. U.S.* **50**, 211 (1963).
18. S. Benzer, V. M. Ingram, H. Lehmann, *Nature* **182**, 852 (1958).
19. C. Speaker, M. A. thesis, Univ. of Illinois (1965); ——— and F. Moyer, in preparation.
20. D. Wright, M. A. thesis, Univ. of Illinois (1965); ——— and F. Moyer, in preparation.
21. C. R. Shaw, *Brookhaven Symp. Biol.* **17**, 117 (1964).
→ D. Schwartz, *Proc. Nat. Acad. Sci. U.S.* **46**, 1210 (19(→ *ibid.* **51**, 602 (1964).
23. R. L. Hunter and C. L. Markert, *Science* **125**, 1294 (195 → C. L. Markert and F. Møller, *Proc. Nat. Acad. Sci. U.S.* **45**, 753 (1959).
24. C. R. Shaw and E. Barto, *Science* **148**, 1099 (1965); ———, unpublished.
25. R. E. Tashian and M. W. Shaw, *Am. J. Human Genet.* **14**, 295 (1962); R. E. Tashian, unpublished.
26. C. R. Shaw and A. L. Koen, *J. Histochem. Cytochem.*, in press.
27. H. Cleve and A. G. Bearn, *Progr. Med. Genet.* **2**, 64 (1962); H. Cleve, J. Prunier, A. G. Bearn, *J. Exp. Med.* **118**, 711 (1963); H. E. Sutton, J. V. Neel, F. Livingstone, G. Binson, P. Kunstadter, L. Trombley, *Ann. Human Genet.* **23**, 175 (1959); O. Smithies and G. E. Connell, *Biochemistry of Human Genetics*, G. Wolstenholme and C. O'Conner, Eds. (Churchill, London, 1959), p. 178; O. Smithies, *Biochem J.* **61**, 629 (1955).
28. R. C. Lewontin, *Genetics* **50**, 757 (1964).
→ S. Benzer, *Proc. Nat. Acad. Sci. U.S.* **47**, 403 (1961).
30. S. Randerson, *Genetics* **50**, 277 (1964); S. Randerson, Ph.D. dissertation, Univ. of Michigan (1965).
31. V. Minnich, J. K. Cordonnier, W. J. Williams, C. V. Moore, *Blood* **19**, 137 (1962); D. J. Weatherall and S. H. Boyer, *Bull. Johns Hopkins Hosp.* **110**, 8 (1962).
32. R. E. Tashian, *Am. J. Human Genet.* **17**, 257 (1965).
33. R. MacIntyre, in preparation.
34. F. M. Johnson and C. Denniston, *Nature* **204**, 906 (1964); H. Ursprung and J. Leone, *J. Exp. Zool.*, in press; E. Grell, K. B. Jacobson, J. Murphy, *Science* **149**, 80 (1965).
35. C. R. Shaw, A. L. Koen, E. Barto, in preparation.
36. C. R. Shaw and A. L. Koen, unpublished.
37. S. H. Boyer, *Science* **134**, 1002 (1961).
38. L. Beckman and F. M. Johnson, *Genetics* **49**, 829 (1964).
39. F. H. Wilcox, *ibid.*, in press.
40. L. Beckman, J. Scandalios, J. Brewbaker, *ibid.* **50**, 899 (1964).
41. L. Beckman and F. M. Johnson, *Hereditas* **51**, 221 (1964); F. M. Johnson and R. Sakai, *Nature* **203**, 373 (1964).
42. H. Kikkawa and Z. Ogita, *Jap. J. Gent.* **37**, 394 (1962); H. Kikkawa, *Ann. Rep. Sci. Works, Fac. Sci. Osaka Univ.* **2**, 41 (1963).
43. Z. Ogita, *Jap. J. Genet.* **37**, 518 (1962).
44. K. Sick and J. T. Nielsen, *Hereditas* **51**, 291 (1964).
45. N. A. Barnicot and C. Jolly, *Nature* **202**, 198 (1964).
46. L. Beckman, J. G. Scandalios, J. L. Brewbaker, *Science* **146**, 1174 (1964).
→ E. W. Baur, *ibid.* **140**, 816 (1963).
48. W. Kalow, in *Biochemistry of Human Genetics*, G. Wolstenholme and C. O'Connor, Eds. (Churchill, London, 1959), p. 39; W. R. Horsfall, H. Lehmann, D. Davies, *Nature* **199**, 1115 (1963); J. Liddell, H. Lehmann, D. Davies, A. Sharih, *Lancet* **1962-I**, 463 (1962); H. Harris, D. Hopkinson, E. Robson, M. Whittaker, *Ann. Human Genet.* **26**, 359 (1963); N. Simpson and W. Kalow, *Am. J. Human Genet.* **16**, 180 (1964).
49. Y. Oki, W. Oliver, H. Funnell, *Nature* **203**, 605 (1964).
50. K. Van Asperen and M. Van Mazijk, *Nature* **205**, 1291 (1965).
51. A. Grunder, personal communication.
52. S. Allen, *Genetics* **45**, 1051 (1960).
53. L. Beckman and F. M. Johnson, *Hereditas* **51**, 212 (1964).

54. M. Kaminski and M. Jeanne-Rose, *Experientia* **20**, 286 (1964).
55. R. Popp and D. Popp, *J. Heredity* **53**, 111 (1962).
M. Petras, *Proc. Nat. Acad. Sci. U.S.* **50**, 112 (1963).
57. F. Ruddle and T. Roderick, *Genetics* **51**, 445 (1965); C. F. Pelzer, Ph.D. dissertation, Univ. of Michigan (1964); C. Pelzer, *Genetics*, in press.
58. C. R. Shaw and E. Barto, unpublished.
H. Kirkman, H. Riley, B. Crowell, *Proc. Nat. Acad. Sci. U.S.* **46**, 938 (1960); P. A. Marks and R. T. Gross, *J. Clin. Invest.* **38**, 2253 (1959); P. A. Marks, R. T. Gross, J. Banks, *ibid.* **40**, 1060 (1961); H. Kirkman, *Pediat. Clin. N. Am.* **10**, 299 (1963); I. Porter, S. Boyer, J. Schulze, V. McKusick, *Proc. Int. Cong. Human Genet., 2nd, Rome* (1961); T. Shows, R. Tashian, G. Brewer, R. Dern, *Science* **145**, 1056 (1964); A. Tarlov, G. Brewer, P. Carson, A. Alving, *Arch. Int. Med.* **109**, 137 (19(
S. H. Boyer, I. Porter, R. Weilbacher, *Proc. Nat. Acad. Sci. U.S.* **48**, 1868 (1962).
60. W. Young, J. E. Porter, B. Childs, *Science* **143**, 140 (1964).
61. N. Henderson, *J. Exp. Zool.* **158**, 263 (1965).
62. S. Watts, W. Young, B. Childs, in preparation.
63. S. H. Boyer, D. Fainer, E. Watson-Williams, *Science* **141**, 642 (1963); W. Nance, A. Claflin, O. Smithies, *ibid.* **142**, 1075 (196
A. P. Kraus and C. Neely, *ibid.* **145**, 595 (1964);
E. Vesell, *ibid.* **148**, 1103 (1965); R. E. Tashian, unpublished; E. Vesell, *Progr. Med. Genet.*, in press.
64. F. N. Syner and M. Goodman, in preparation.
65. W. H. Zinkham, A. Blanco, L. Kupchyk, *Science* **144**, 1353 (1964).
66. N. Henderson, in preparation.
67. N. Spencer, D. Hopkinson, H. Harris, *Nature* **204**, 742 (1964).
68. R. Fildes and C. Parr, *ibid.* **200**, 890 (1963); C. Parr, *ibid.*, in press; R. Dern, G. Brewer, R. Tashian, T. Shows, *J. Lab. Clin. Med.*, in press.
69. W. Young, *Genetics*, in press.
70. C. Parr, *Nature*, in press.
71. B. Childs, W. Young, H. Kazazaian, in preparation.
72. E. Glassman and P. Saverance, *J. Elisha Mitchell Sci. Soc.* **79**, 139 (1963); E. Glassman, unpublished.
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Of Whales and Whaling

The whaling industry is rapidly destroying the valuable natural resource on which its future depends.

Noel Simon

In recent years there has been increasing awareness of the need for wildlife and wild places. Man's preoccupation with problems of his own survival may have made him slightly more sympathetic toward the other animals with which he shares this planet. There is also perhaps a dawning realization that, while it is permissible to use the income derived from some of the things of beauty, interest, and value which are part of man's natural heritage, the capital must be handed down intact to future generations. Indicative of man's growing concern over the prodigal squandering of nature are a mirror and accompanying sign set up at the Bronx Zoo; the sign states, simply but emphatically, "You are looking at the most dangerous animal in the world. It alone of all the animals that ever lived can exterminate (and *has*) entire species. . . ."

The oceans of Antarctica sustain the largest animal the world has ever known—considerably larger than the most massive of the dinosaurs which dominated the Mesozoic era—and one which has earned a high place on the list of mammals in the service of man. An adult blue whale, up to 30 meters long and weighing perhaps as much as 160 tons (*1*), dwarfs any other animal in the whole of creation; even its newborn young are larger than a full-grown elephant and reputed to consume more than half a ton of milk a day. But it may not be long before the blue whale joins the dinosaurs in the museum of oblivion. The demise of the dinosaurs remains veiled in mystery and surmise, but there is no need to speculate on the reasons for the disappearance of the blue whale; the rapaciousness of man is wholly responsible. Seas and oceans comprise 70 percent of the earth's surface, and one would have thought this ample habitat allowed more than enough space for the whale's survival, but pursuit of the whale has been so persistent that nowhere on the face of the sea or in its uttermost depths, however remote or vast or forbidding, is there any longer true sanctuary beyond the reach of man's ruthless exploitation.

During the heyday of the old-time whaling industry, only the smaller whales, which could be pursued in open boats, were hunted. The speed and size of the large rorquals (the blue, fin, and humpback whales) rendered them safe, but in 1865 their natural immunity was lost through the invention of the harpoon gun and the development of the steam-powered catcher (*2*) as a vehicle for the new weapon. These inventions gave fresh impetus to a flagging industry but, at the same time, sealed the fate of the great rorquals, notably the blue whale, which is particularly valuable commercially since it yields about 140 barrels of oil, twice the yield of the fin whale.

Before 1904 whaling was almost entirely restricted to the Northern Hemisphere, and the Southern Hemisphere whale populations on the antarctic feeding grounds were free from human exploitation. Improved ocean-going catchers and the growing scarcity of whales in northern waters encouraged the industry to break new ground, and in 1904 the first ship started whaling from South Georgia in the South Atlantic. The commencement of deep-sea whaling in the Antarctic was followed by the development of new and increasingly efficient techniques. The factory ship, with a slipway built into the stern, up which whales could be winched for flensing on deck, greatly extended the radius of operations. Thenceforth expeditions could operate freely throughout the oceans of Antarctica, wherever whales were to be found, the covey of catchers in combination with the factory ship making virtually a miniature task force, the whale oil being transferred in bulk to attendant tankers for transportation to the home base.

Postwar refinements include the use of helicopters for spotting whales, sonar devices developed from wartime asdic, more efficient harpoons (both explosive and electric-powered), and the modern factory ship, which processes the carcass of a fin whale in half an hour (disposing of a blue whale takes a

The author is on the staff of the International Union for Conservation of Nature and Natural Resources, Morges, Switzerland.