Genetic Variation and Evolution of the Carboxylic Esterases and Carbonic Anhydrases of Primate Erythrocytes

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COMPARATIVE STUDIES of proteins at the molecular level are beginning to provide a chemical basis for the analysis of phylogenetic relationships among primate species. The major portions of such investigations have been devoted to the study of proteins found in the various constituents of blood (e.g., transferrins, albumins, haptoglobins, hemoglobins), utilizing for the most part the methods of zone electrophoresis and immunodiffusion. Several reports on this general subject have recently appeared as articles in symposia proceedings and treatises dealing with primate evolution (Buettner-Jansuch, 1962, 1964; Washburn, 1963). Interspecies analysis of the tryptic peptide patterns and amino acid composition of hemoglobin from a number of primate species comprise the furthest extension to date of the application of comparative protein methods to the study of primate phylogeny (Zuckerkandl, Jones, and Pauling, 1960; Zuckerkandl and Schroeder, 1961; Hill, Buettner-Janusch, and Buettner-Janusch, 1963).

Another technique which is proving useful for comparative protein studies is the procedure whereby soluble enzyme forms are separated by zone electrophoresis and then stained by a variety of histochemical procedures (e.g., Burstone, 1962; Wróblewski, 1961). The distinctive enzyme patterns, called zymograms (Hunter and Markert, 1957), obtained by these methods have the advantage of simultaneously characterizing both the electrophoretic migration and staining intensity of enzyme forms. The nonspecific carboxylic esterases (Tashian, 1961; Tashian and Shaw, 1962) and carbonic anhydrases (which also have esterase activity [Tashian, Douglas, and Yu, 1964]) of red blood cells are well suited for studies of this type because: (1) they are of homogeneous tissue origin, (2) they are relatively stable and readily separated and stained, (3) they show inter- and intraspecies variation, and (4) several distinct enzymes may be screened at the same time with a single synthetic substrate. Variations in the plasma esterase zymograms of man, chimpanzee, gorilla, and three species of macaque monkeys have been reported (Arfors, Beckman, and Lundin, 1963).

In this article, the application of the zymogram method to the comparative study of esterase forms from the hemolyzates of 23 primate species is described. These species (see Table 2) include members of the infraorder Lorisiformes (prosimian primates) and the three anthropoid superfamilies: Ceboidea

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TABLE 1. SUBSTRATE AFFINITIES AND DISTINGUISHING FEATURES OF ESTERASE TYPES FROM PRIMATE ERYTHROCYTES

Esterase types	α- and β-naphthol esters hydrolyzed: C_2 = acetate, C_3 = propionate, C_4 = butyrate	Diagnostic features
Butyrylesterase		
B esterase	$\alpha\text{-}C_4 > \beta\text{-}C_2 > \alpha\text{-}C_3 > \alpha\text{-}C_2$	Activated by 10^{-3} M CMB,* inhibited by 10^{-4} M DFP†
Propionylesterase		
P esterase	$\alpha\text{-}C_3 > \alpha\text{-}C_2 > \alpha\text{-}C_4$	Inhibited by 0.01 M eserine
Acetylesterases		
C esterase	$\alpha\text{-}C_2 > \beta\text{-}C_2 > \alpha\text{-}C_3 > \alpha\text{-}C_4$	Activated by 10^{-3} M CMB, *not* inhibited by 10^{-4} M DFP
A esterase	$\alpha\text{-}C_2 > \beta\text{-}C_2 > \alpha\text{-}C_3 > \alpha\text{-}C_4$	Inhibited by 0.02 M CMB
Carbonic anhydrase	$\beta\text{-}C_2 > \alpha\text{-}C_2 > \beta\text{-}C_4 > \alpha\text{-}C_4$	Inhibited by 10^{-4} M acetazolamide

Test compounds were either added to hemolyzates and incubated for 15 min. at 37° C prior to insertion in gels (A esterase, C esterase) or added directly to incubation mixtures with gels containing the separated esterases (B esterase, P esterase, CA I, CA II). In the latter process, one half of a gel was treated with test reagent and the other half served as control; gels were incubated in these mixtures for 30 min. before addition of histochemical substrates. Control gels were treated in the same way but without the test compound.

*p-Chloromercuribenzoate.

†Diisopropylfluorophosphate.

(New World monkeys), Cercopithecoidea (Old World monkeys), and Hominoidea (great apes and man). The results indicate that the degree of variability among these species in the electrophoretic patterns of at least eight distinct red cell esterases provides a useful supplementary biochemical tool for the study of primate evolution.

CHARACTERIZATION OF THE ESTERASES

Although all the enzymes which will be discussed here exhibit carboxylic esterase activity toward certain aromatic esters, they can be separated into five distinct esterase types on the basis of their characteristic electrophoretic migration, *in vitro* substrate preferences, and selective responses to activator and inhibitor compounds. According to their substrate preference, they have been classified into three main groups: butyrylesterase (B esterase), propionylesterase (P esterase), and acetylesterase (C esterase, A esterase, carbonic anhydrase). Table 1 lists some substrate affinities for the various esterases and summarizes those properties which can be used to identify the different types. The technical details regarding the methodology for preparing the zymograms and identifying the different esterases will be found in a footnote for Table 1 and the legend for Fig. 1.

The terminology which has been applied in the literature to the nonspecific esterases which catalyze the hydrolysis of aromatic carboxyl esters has been varied. The terms A esterase or aromesterase (Aldridge, 1953) and arylester-

ase (Augustinsson, 1961) have been used to designate plasma acetylesterases; the term C esterase has been applied to an acetylesterase of kidney (Bergmann, Segal, and Rimon, 1957) which is activated by low concentrations of sulfhydryl reagents; and an organophosphate sensitive and eserine resistant butyrylesterase of plasma has been designated B esterase (Aldridge, 1953). Some of the properties of plasma arylesterase and B esterase and kidney C esterase are similar to those described in this study for red cell A esterase, B esterase, and C esterase, respectively.

The physiological roles of these erythrocyte esterases are almost entirely unknown; and with the exception of carbonic anhydrase, attempts in our laboratory to identify them with hydrolases which are either known or postulated to occur in red cells have been unsuccessful (Tashian, unpublished results). For recent surveys of the chemical and histochemical literature on carboxylic esterases see Myers (1960), Hofstee (1960), and Pearse (1961).

INTERSPECIES VARIATION

A judgement as to which of the esterase forms of one species corresponds to those of another is based on the similarities of their electrophoretic patterns, histochemical staining properties, and responses to activator and inhibitor compounds. In addition, immunochemical procedures were employed with carbonic anhydrase I (Tashian and Shreffler, 1963). Figure 1 is a photograph of typical esterase zymograms from two starch gel preparations. The characteristic esterase zymogram for each species is diagrammed in Figs. 2 and 3, and the estimated quantitative variation between species of the different esterase types is summarized in Table 2.

B esterase. This butyrylesterase occurs as a narrow, well-defined band in all fresh hemolyzates. No interspecies variation in electrophoretic mobility could be detected. This characteristic lack of variation is useful in establishing the relative interspecies migration rates of the other esterase types.

P esterase. This eserine-sensitive propionylesterase is resolved as one or two bands and is always the most rapidly-migrating esterase form. It was found in one of the three lorisiform species tested and in four of six ceboid species; in the higher species, it was found only in gibbon. However, the P esterase of gibbon appears to be less sensitive to eserine than that of the other species. P esterase thus seems to be more characteristic of lower primate species; its apparent retention in gibbon erythrocytes suggests an early evolutionary divergence from the pongid line. The only other eserine-sensitive esterase known to occur in mammalian red cells is acetylcholinesterase which, if present, is bound to the stroma.

C esterase. Next to P esterase, this acetylesterase is the most rapidly migrating esterase type. It usually occurs as a rather weak, diffuse series of three or possibly four adjacent narrow bands in cercopithecoid and hominoid species. Acetylesterases which are activated by iodoacetamide are also found in the three lorisiform species, and they have been tentatively classified here as C-type esterases on this basis. However, with the possible exception of the squirrel monkey, no clear-cut example of C esterase was detected in the

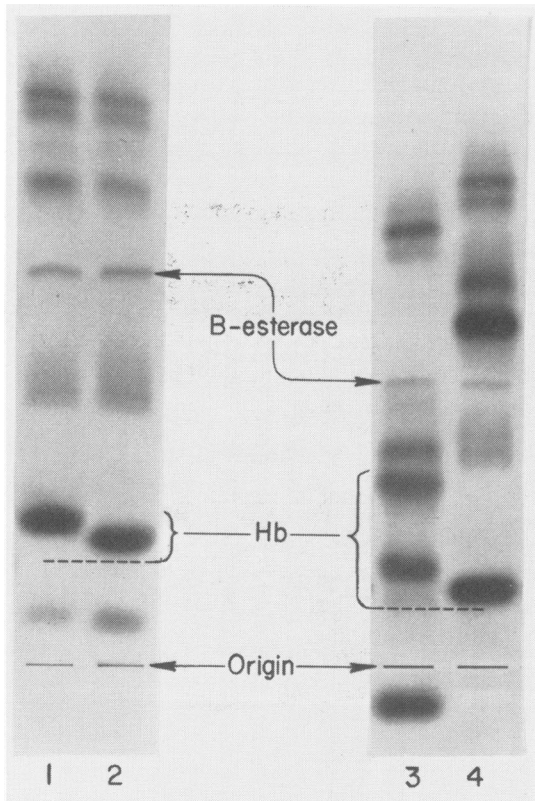

FIG. 1. Photographs of erythrocyte esterase zymograms from (1) gorilla *(Gorilla gorilla),* (2) man *(Homo sapiens),* (3) rhesus *(Macaca mulatta),* and (4) gibbon *(Hylobates lar).* Vertical starch gel electrophoresis carried out for 18 hrs. (man, gorilla) and 14 hrs. (rhesus, gibbon) at 4–5° C and 8–9 volts/cm in 0.02 M borate/NaOH gel buffer, pH 8.6, and 0.3 M borate/NaOH bridge buffer, pH 8.0, containing 0.03 M NaCl. Preparation of hemolyzates, tray dimensions, and coupling azo dye staining procedures are described elsewhere (Tashian and Shaw, 1962). C esterases stained too weakly under the test conditions and are not detectable in these photographs.

zymograms from the six ceboid species tested; whether in these species this esterase is absent or undetectable cannot be determined by present techniques.

A esterase. In addition to their characteristic migration patterns, the A esterase types may also be distinguished by their differential response to certain sulfhydryl reagents and urea. For example, in man, chimpanzee, orangutan, and gorilla, they are inhibited by iodoacetate in the order: A_2 esterase (0.02 M), A_3 esterase (0.04 M), and A_1 esterase (0.06 M); and by *p*-chloromercuribenzoate: A_2 and A_3 esterase (5×10^{-3} M), and A_1 esterase (0.02 M). A_1 and A_2 esterase are more rapidly inactivated by urea (2.6 M) than A_3 esterase (3.8 M).

It was usually possible to demonstrate an A_3-type esterase for almost all species at a position either immediately anodal or cathodal to B esterase. The multiple bands which comprise A_3 esterase are often diffuse and poorly de-

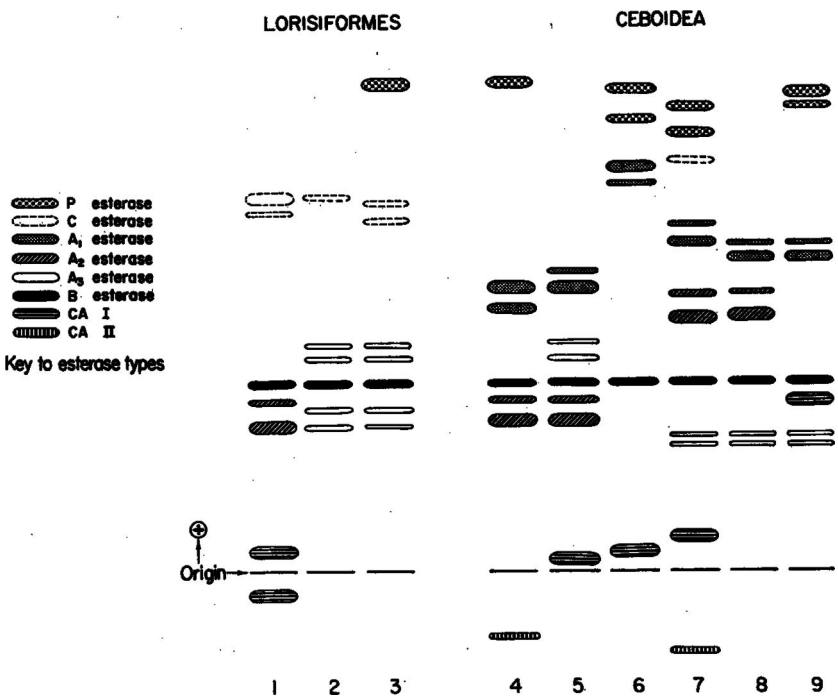

FIG. 2. Diagrams of erythrocyte esterase zymograms for some lorisiform and ceboid species: (1) *Nycticebus coucang,* (2) *Perodicticus potto,* (3) *Galago crassicaudatus,* (4) *Callimico goeldi,* (5) *Callicebus cupreus,* (6) *Aotes trivirgatus,* (7) *Saimiri sciurea,* (8) *Cebus capucinus,* (9) *Ateles belzebuth.* Positions of the hemoglobins have been omitted for the sake of clarity. When a zone of esterase activity is obscured or overlapped by hemoglobin, the electrophoresis time (usually 17–18 hrs.) or pH of the gel buffer (usually 8.6) are appropriately altered to reveal the underlying esterase band; otherwise conditions are the same as those given in Fig. 1.

fined at pH 8.6 but are better resolved at a somewhat lower pH (Tashian and Shaw, 1962). Because of this difficulty in resolution and the relative instability of A_3 esterase, it was not always possible to make adequate interspecies comparisons. This esterase, however, appears to be expressed as two or three narrow bands in Lorisiformes, Ceboidea, and Cercopithecoidea and by three or four bands in Hominoidea. The A_3 esterase of hominoid species migrates somewhat faster than that of the other primate species.

What appears to be a corresponding A_1 esterase was found in all hemolyzates with the exception of the three lorisiform species. It is characteristically present as a couplet of two molecular forms which shift anodally to form new altered forms in ageing or after freezing and thawing of the hemolyzates (Tashian and Shaw, 1962).

A_2 esterase also appears as a double band which, in the hominoid species, characteristically migrates immediately cathodal to the A_1 esterase. In gibbon, squirrel monkey, and capuchin monkey, an A_2-like couplet is observed to migrate midway between A_1 and A_3 esterase; in titi monkey and slow loris, an A_2-like esterase pair migrates immediately cathodal to B esterase. These

TABLE 2. VARIABILITY IN THE OCCURRENCE OF STAINING INTENSITY OF ESTERASE TYPES FOUND IN PRIMATE ERYTHROCYTES

Staining intensity for interspecies comparisons: (+) estimated unit, (tr) trace, (0) no activity detected, (?) detection not clear.

Species and common names	No. of individuals tested	B esterase	P esterase	C esterase	A esterase			Carbonic anhydrase	
					A_1	A_2	A_3	CA I	CA II
Suborder Prosimii									
Infraorder Lorisiformes									
Nycticebus coucang (slow loris)*	1	+	0	++	0	++	?	++	?
Perodicticus potto (potto)	3	+	0	++	0	0	+	0	?
Galago crassicaudatus (bush baby)	3	+	+	++	0	0	+	0	?
Suborder Anthropoidea									
Superfamily Ceboidea									
Callimico goeldi (Goeldi's monkey)	3	+	+	?	++	+	0	0	+
Callicebus cupreus (titi monkey)*	2	+	0	?	++	+++	+	+	?
Aotes trivirgatus (night monkey)*	1	+	+	?	++	?	?	+	?
Saimiri sciurea (squirrel monkey)	4	+	+	?	++	++	+	++	+
Cebus capucinus (capuchin monkey)	1	+	0	?	++	+++	?	0	?
Ateles belzebuth (spider monkey)*	2	+	+	?	++	?	?	++	?
Superfamily Cercopithecoidea									
Macaca speciosa (stump-tailed macaque)*	1	+	0	+++	++	0	?	0	?
Macaca mulatta (rhesus macaque)	149	+	0	+++	++	0	+	++++	+
Macaca irus (cynomolgus)	78	+	0	+++	++	0	+	+++++	+
Papio doguera (baboon)†	75	+	0	+++	++	0	+	++++	?
Cercopithecus aethiops (green monkey)	64	+	0	+++	++	0	+	tr, +++§	+
Cercopithecus mitis (Sykes' monkey)*	1	+	0	+++	++	0	+	0	?
Erythrocebus patas (patas monkey)*	1	+	0	+++	++	0	+	0	?
Presbytis entellus (leaf monkey)*	1	+	0	?	++	0	+	++	?
Superfamily Hominoidea									
Hylobates lar (gibbon)	13	+	+	?	++	+++	+	0, +, ++§	?
Pongo pygmaeus (orangutan)	13	+	0	+	++	+	+	++++	+
Pan troglodytes (chimpanzee)	47	+	0	++	+	0	+	++	+
Pan paniscus (lesser chimpanzee)*	2	+	0	++	++	++	+	++	+
Gorilla gorilla (gorilla)	2	+	0	++	++	++	+	++	+
Homo sapiens (man)‡	4,117	+	0	+++	++	++	+	+++	+

*Hemolyzates tested after storage at −15° C; other zymograms were prepared from fresh hemolyzates.

†A series of 62 additional baboons (*Papio* spp.) representing crosses among *P. hamadryas, P. cynocephalus,* and *P. doguera* were examined and found to have patterns similar to normal *P. doguera.*

‡Breakdown of ethnic types: Caucasian (European origin), 2,638; American Negro, 623; American Indian, 366; Micronesian (Mariana Is.), 490.

§See text (*Interspecies Variation*) for details on variability of CA I in green monkey and gibbon.

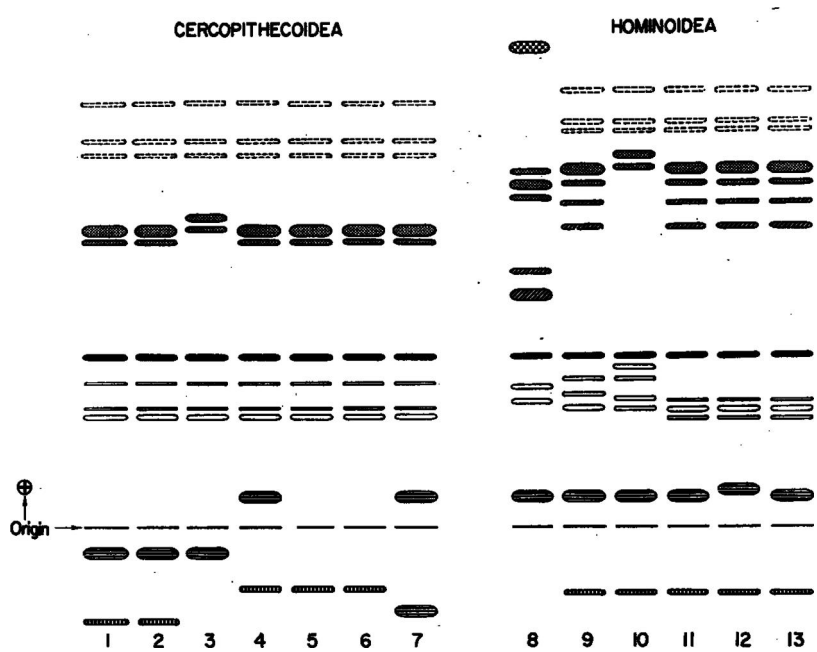

FIG. 3. Diagrams of erythrocyte esterase zymograms for some Cercopithecoid and Hominoid species: (1) *Macaca mulatta,* (2) *Macaca irus,* (3) *Papio doguera,* (4) *Cercopithecus aethiops,* (5) *Cercopithicus mitis,* (6) *Erythrocebus patas,* (7) *Presbytis entellus,* (8) *Hylobates lar,* (9) *Pongo pygmaeus,* (10) *Pan troglodytes,* (11) *Pan paniscus,* (12) *Gorilla gorilla,* (13) *Homo sapiens.* Conditions same as in Fig. 2.

A_2-like pairs resemble typical A_2 esterase of Hominoidea in their patterns and chemical responses, and they have been so designated for this reason.

Because the correspondence (homologies?) among the various A esterases is often uncertain, only in those species most closely related to man (Cercopithecoidea, Hominoidea) does it appear reasonable to assign an A esterase to a specific type. In Figs. 1 and 2 and Table 2, therefore, the assignment of A esterase types for lorisiform and ceboid species is tentative.

Carbonic anhydrases. These are usually the slowest migrating of the red cell esterases and the slowest to stain. They stain more intensely with β-naphthyl acetate than with α-naphthyl acetate, and when both substrates are added to the same incubation mixture, carbonic anhydrase stands out as a dark pink or purple band (β-naphthol/Blue RR complex) as compared to the dark gray stain of the α-naphthol/Blue RR complex of most of the other esterases. In many species they are present as two distinct forms designated carbonic anhydrase I (CA I) and carbonic anhydrase II (CA II). Other investigators (Nyman and Lindskog, 1964; Rickli *et al.,* 1964) use the notations CA B for CA I and CA C for CA II.

The considerable quantitative and qualitative variability of CA I is shown in Figs. 3 and 4 and Table 2. It appears to be absent in potto, galago, Goeldi's monkey, capuchin monkey, stump-tailed macaque, Sykes' monkey, and some gibbons; when present in gibbon it varies from low to moderate activity. The

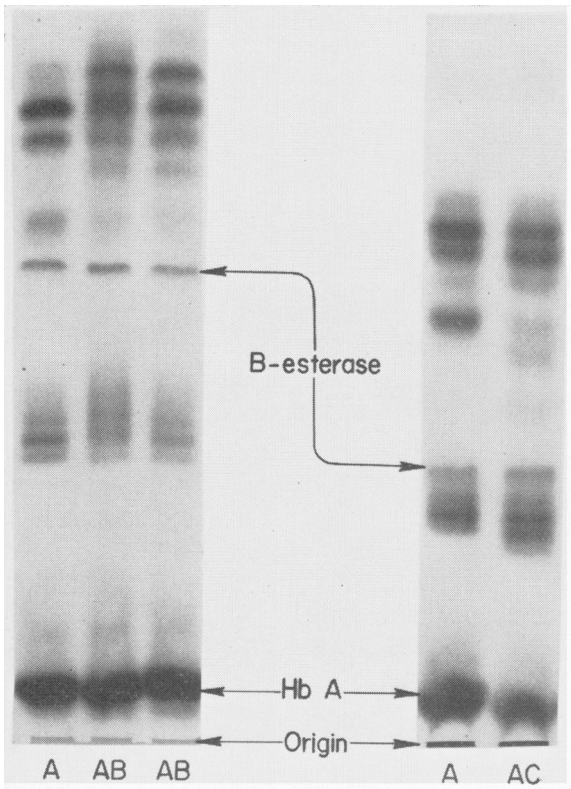

FIG. 4. Photographs of human erythrocyte zymograms showing homozygous normal (A) and heterozygous variant (AB, AC) A esterase patterns; Negro AB (left), Caucasian AB (right). Conditions are similar to those given in Fig. 1 except (for better resolution of variant patterns) the gels were prepared in pH 8.55 buffer; in addition, the gel with the AC variant was run for 15 hrs. and no NaCl was added to the bridge buffer to increase the migration of B esterase (Tashian and Shaw, 1962).

highest specific esterase activities are found in rhesus, cynomolgus, baboon, and green monkey. The highly variable expressivity of CA I cautions against its rigid use as an indicator of taxonomic relationships when only a limited number of individuals or species have been tested. A case in point is the stump-tailed macaque *Macaca speciosa* which (on the basis of one specimen) has no CA I while other members of the same genus, as well as *Papio* spp., show the highly active cathodal CA I typical of macaques and baboons. Also, marked quantitative intraspecies variation in CA I is observed in gibbon and green monkey (see following section).

Because CA II is usually poorly defined, it was difficult to determine the extent of variation in this enzyme form.

INTRASPECIES VARIATION

Significant amounts of data on intraspecific variability are thus far limited to man, baboon, chimpanzee, rhesus, cynomolgus, and green monkey (see

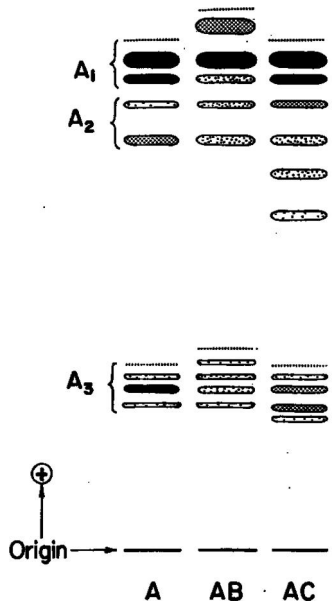

FIG. 5. Diagrams of the normal (A) and variant (AB, AC) A esterase patterns shown in FIG. 4. Positions of the other esterases and hemoglobins are omitted for clarity.

Table 2); in all other species, the number tested ranged from one to 13 individuals.

A esterase. Only three isolated variants of A esterase have been discovered during a survey of some 4,100 human hemolyzates (for breakdown of ethnic types see legend, Table 2). The variant A esterase zymograms are shown in Fig. 4 and diagrammed in Fig. 5. One example of the B variant was found during the screening of about 2,600 Caucasian samples, and one type B and one type C were discovered on testing some 600 Negro bloods. The Negro A esterase variants have not been previously described. Genetic control of the Caucasian B variant has been established (Tashian and Shaw, 1962), and the Negro B variant was reproducible on repeated testing. No genetic information is as yet available for type C.

The findings that these variants of A esterase show electrophoretic alterations in all three A esterase isozymes (A_1, A_2, and A_3) suggest that they share a common polypeptide subunit. Thus, we could postulate that these variants have resulted from amino acid substitutions which render the common subunit either more electronegative (B variant) or electropositive (C variant). The A esterase variants are a good example of how different molecular forms of an enzyme (isozymes) with similar, but distinct, chemical properties can be demonstrated to be related on genetic evidence. This condition may be likened to that found in lactate dehydrogenase where the different isozymes exhibit different catalytic properties (Cahn *et al.*, 1962) but where there is evidence that the subunits which make up the enzyme molecules are under the control of two or more genetic loci (Shaw and Barto, 1963).

Carbonic anhydrase I. Three variants of CA I have been found in the same

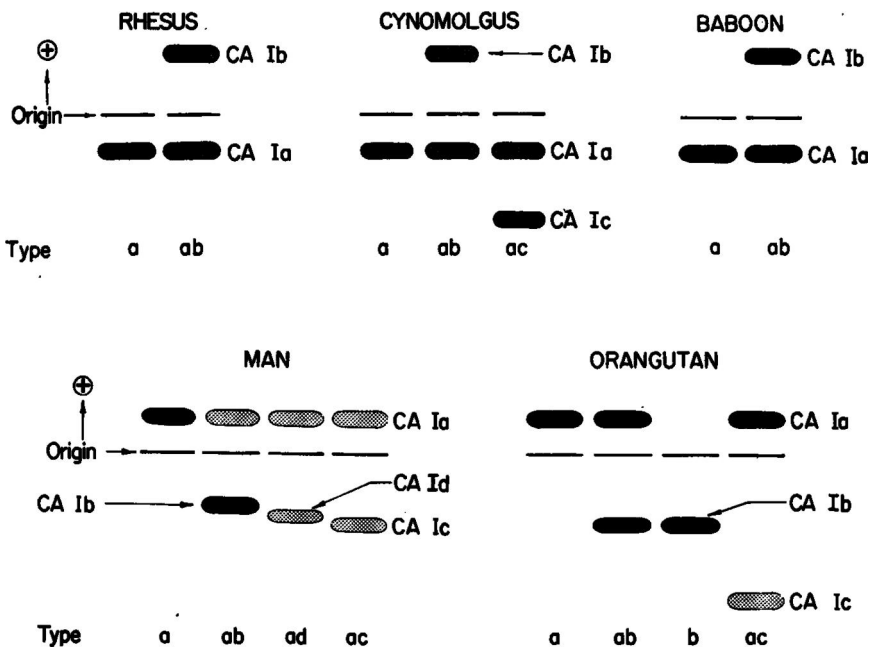

FIG. 6. Diagrams of electrophoretic phenotypes of red cell carbonic anhydrase (CA I). Top (left to right): rhesus, homozygous normal (a), heterozygous b variant (ab); cynomolgus, homozygous normal (a), heterozygous b variant (ab), heterozygous c variant (ac); baboon, homozygous normal (a), heterozygous b variant (ab). Bottom (left to right): man, homozygous normal (a), heterozygous b variant (ab), heterozygous c variant (ac), heterozygous d variant (ad); orangutan, homozygous normal (a), heterozygous b variant (ab), homozygous b variant (b), and heterozygous c variant (ac).

series of human samples described in the preceding section, and their electrophoretic phenotypes are diagrammed in Fig. 6. One example of the b variant (Shaw, Syner, and Tashian, 1962) was found in the Caucasian sample, four examples of the c variant (Tashian, Plato, and Shows, 1963) in the Micronesian survey, and one example of the d variant in the American Negro sample. The d variant, which has not been previously reported, was found in a Negro woman and her only child (daughter). The b and c variants have been shown to be under the control of single autosomal genes; all three variants probably represent allelic products.

Two variants of CA I have been observed in orangutan (Fig. 6) and these appear to make up a polymorphic system which may be under the control of at least three alleles (*a*, *b*, and *c*). Of 13 amimals examined, three were (on biochemical evidence alone) genotype *a/c*, one was *a/b*, one was *b/b*, and eight were *a/a*. No genetic evidence is available for these variants, but by analogy with the human CA I variants, autosomal, single gene control is indicated.

In the baboon series a variant of CA I was also discovered which was more electronegative than the normal form (Fig. 6). Of the 141 baboons tested (see legend, Table 2), seven showed the trait. Evidence that the baboon

variant is inherited is based on the reproducibility in repeat blood samples from all seven animals who showed the ab type and on the observation of one mother-to-offspring transmission of the trait. This is very likely the same variant described from baboon by Barnicot, Huehns, and Moor-Jankowski (1964).

Anodally-migrating variants of CA I, electrophoretically indistinguishable from the baboon variant just described, were also found in one individual from both the rhesus series and the cynomolgus series. In addition, one cathodally-migrating variant of CA I was discovered in the cynomolgus sample. These variants are diagrammed in Fig. 6.

Some qualitative and quantitative variation of CA I was observed in the few gibbons examined. As mentioned in the section on interspecies variation, no CA I was detected in 10 of 13 animals tested, either by immunodiffusion tests or by esterase activity. (Immunodiffusion is being carried out in collaboration with Dr. Donald C. Shreffler utilizing rabbit antihuman carbonic anhydrase I sera. A complete account will be the subject of another report.) However, a CA I band (see Fig. 3) was observed in two animals, one of which showed moderate esterase activity while the other stained weakly. A third gibbon displayed an apparently heterozygous condition by virtue of having two lightly-staining bands of equal intensity, one in the normal anodal position and the other at approximately the position as the b variant of orangutan (Fig. 6).

Two of the 64 green monkeys tested showed an almost complete absence of CA I; in both of these cases a normal CA II band was detectable. These findings, along with the quantitative variability found in the gibbon sample, indicate that variation in the synthesis of CA I may be under genetic control in some individuals. An almost complete absence of CA I has recently been reported in the hemolyzate from a 31 year old Negro woman (Rieder and Weatherall, 1964).

The double CA I pattern found in the single samples of slow loris *Nycticebus coucang* (Fig. 2) and leaf monkey *Presbytis entellus* (Fig. 3) suggest that CA I may also be polymorphic in these species.

SOME TAXONOMIC CONSIDERATIONS

Primate species within any one of the four main higher categories share certain features of their zymogram patterns which can, for the most part, be used to place them in a particular systematic group. As examples, lorisiform species have a unique C-type esterase but apparently lack A_1 esterase; in Ceboidea, P esterase is a common type and C esterase is either weakly defined or missing; in Cercopithecoidea, A_2 esterase is absent; and in hominoid species (except gibbon), the proximity of the A_1 and A_2 esterase patterns is diagnostic. There still remain, however, enough differences between species within each group to help shed light on some phylogenetic problems. In the following discussion, the technique of comparing zymograms is used to examine the taxonomic position of several species.

There has been some controversy among primate systematists as to whether

Goeldi's monkey *Callimico goeldi* belongs with the marmosets and tamarins in the ceboid family Hapalidae (= Callithricidae) or in the family Cebidae. On the basis of its chromosomal pattern, Chu and Bender (1962) consider *Callimico* more similar to the cebid species than to the two hapalid species they examined. Unfortunately, the only hapalid species whose esterase pattern was tested in the present study was *Callimico;* nevertheless, it is notable that the zymogram of *Callimico* shares many features in common with the cebid species.

On morphological evidence, gibbons are usually classified with the great apes (orangutan, chimpanzee, and gorilla) in the family Pongidae. This position has recently been questioned, however, because both the karyology (Klinger *et al.,* 1963) and serology (Goodman, 1963) of gibbon (*Hylobates* spp.) are quite different from the great apes. It has even been observed (Chu and Bender, 1962; Klinger *et al.,* 1963) that the chromosomal pattern of gibbon shows a greater similarity to the patterns of some specialized cercopithecoid monkeys than to any hominoid species. The distinct pattern of gibbon found in the present study (Figs. 1 and 3) favors the hypothesis that *Hylobates* separated early from the pongid line and therefore might best be placed in a separate family, Hylobatidae. This unique pattern, however, is not very helpful in relating gibbon to members of the living primate families tested; if anything, it bears a superficial resemblance to the ceboid patterns, and such a relationship is not very probable.

Goodman (1963) suggests that the highly similar immunological nature of the serum proteins of man, gorilla, and chimpanzee warrants their grouping into a single family, Pongidae. With the exclusion of gibbon, the esterase patterns for man and the great apes are similiar with the following exceptions: CA I for gorilla is slightly more anodal than the others, CA I of orangutan has high specific esterase activity, and the A esterase pattern of the chimpanzee *Pan troglodytes* is altered with an apparent absence of A_2 esterase. These biochemical differences among man, gorilla, chimpanzee, and orangutan, however, are not as great as those between any one of these species and gibbon or any cercopithicoid monkey examined. This is convincing evidence of the close kinship between man and these great apes and indicates that their presumed common ancestor evolved after the divergence of the Old World monkeys and the gibbons from the line leading to orangutan, gorilla, chimpanzee, and man.

SOME EVOLUTIONARY IMPLICATIONS

The interspecies variability in erythrocyte enzymes observed in this study demonstrate that, during the course of evolution within a higher taxonomic category, the proteins synthesized in any one tissue may (1) undergo little quantitative or qualitative change (B esterase, CA II, A_3 esterase), (2) be polymorphic in one species and rarely variant in others (CA I), and (3) be present in some species and absent in others (P esterase, CA I, A_2 esterase).

Is there any evidence that the basis for new proteins has arisen in consequence of a process of gene duplication? Of the two forms of carbonic anhy-

drase present in the red cell, CA I has more variant forms than CA II. The implication here is that CA II may represent the evolutionally older and more conservative molecule and that CA I has evolved by duplication and further changes of the CA II locus in a manner similar to that postulated for the evolution of the different haptoglobins (Smithies, Connell, and Dixon, 1962) and hemoglobin chains (Ingram, 1961). There are several lines of evidence that support this hypothesis. Although the two forms are immunologically distinct (Tashian and Shreffler, 1963; Fine *et al.*, 1963; Micheli and Buzzi, 1964) and have different amino acid compositions, they have similar molecular weights and both have a single cysteine residue and single zinc atom per mole (Laurent *et al.*, 1963; Nyman and Lindskog, 1964; Rickli *et al.*, 1964) and there is some preliminary evidence that portions of their primary structures have similar amino acid sequences (Nyman and Lindskog, 1964). The freedom of expressivity for CA I in structure and activity is seemingly "allowed" so long as the enzymic function of CA II remains relatively unaltered. As yet no mammalian order other than Primates has been reported to have two genetically distinct forms of red cell carbonic anhydrase.

It is tempting to speculate why some esterases are not present in some species. Possibly the deletion of the P esterase and A_2 esterase loci early in the evolution of the cercopithecoid line would explain their apparent absence in the Cercopithecoidea. On the other hand, the absence of CA I (one third of the species examined) is more sporadic, and it is more likely that the CA I locus is "suppressed" in some species by certain environmental (intracellular) factors as suggested by Zuckerkandl and Pauling (1962) in their "dormant" gene hypothesis. Ontogenetic evidence that intracellular factors can indeed control the synthesis of CA I comes from the observations that only trace amounts of CA I are detectable in human and rhesus cord bloods, as well as the fact that CA I has as yet been found to occur only in red blood cells (Tashian, unpublished observations). Another esterase which shows sporadic occurrence between species is P esterase, and it would be interesting to see whether this esterase is absent in the fetal blood of those species where it is characteristically present in the adult.

Although the invariability of B esterase limits its value as an indicator of evolutionary change, it is possibly an example of protein inviolability wherein no amino acid substitution is "tolerated" regardless of its location in the primary structure of the molecule.

As more and more comparative molecular data become available for a greater number of proteins, it will eventually be possible to classify an increasing number of specific protein characters for a given species. This would provide taxonomic units which would be eminently suitable for a numerical approach to primate taxonomy, perhaps along the lines set forth in the stimulating work of Sokal and Sneath (1963).

SUMMARY

Examination of the species-specific esterase zymograms from the hemolyzates of 23 primate species shows that the similarities and differences in their

esterase patterns are useful indicators of phylogenetic relationships. The significance of intraspecies variation of certain esterase forms and some of the evolutionary implications of interspecies variation are discussed.

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ADDENDUM

Since this article was submitted, one A esterase variant affecting both A_1 and A_3 esterases (a presumed heterozygote similar to the AB variant of man) was discovered on testing 42 additional rhesus hemolyzates. Also, the application of an improved technique for the resolution of CA II to fresh hemolyzates of 17 green monkeys, 17 cynomolgus monkeys, and 42 rhesus macaques has revealed a presumed polymorphism of CA II in the cynomolgus and rhesus samples; the three electrophoretic phenotypes (A, B, and AB) are apparently under the control of two codominant alleles. Examination of one fresh spider monkey hemolyzate showed a single CA II band in a position cathodal to the CA II of squirrel monkey.

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