GENETIC AND PHYLOGENETIC VARIATION IN THE DIFFERENT MOLECULAR FORMS OF MAMMALIAN ERYTHROCYTE CARBONIC ANHYDRASES*

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Mammalian erythrocytes are known to possess either one or two molecular forms of carbonic anhydrase (*cf.* Tashian;[1] Armstrong *et al.*;[2] Duff and Coleman;[3] Nyman and Lindskog;[4] and Edsall, preceding paper). In the present report, we will compare some genetic and chemical aspects of the presumed homologous forms of red cell carbonic anhydrase from various mammalian species. This type of comparative study gives us an unusual opportunity to analyze the changes that have occurred in two enzyme molecules which have, in all probability, originated by a process of gene duplication (*cf.* Nyman *et al.*[5]), and have been "evolving" since that time in the same, or a similar, cytoplasmic environment. If this duplication occurred during the evolution of mammals, it might even be possible to locate its approximate phylogenetic position; and, by so doing, determine which of the two molecules represents the original form.

The enzyme forms will be referred to in this paper as carbonic anhydrases I and II (CA I and CA II) which are identical to the designations CA B and CA C, respectively, used by other investigators.[2-5]

EXPERIMENTAL

The methods used for the purification and assay of the enzymes, tryptic peptide analysis, determination of amino acid composition, and preparation of the electrophoretic patterns, are as described elsewhere,[6,7] unless otherwise specified.

Antisera against the two purified forms of human CA I and CA II were prepared by injecting (intraperitoneally) rabbits with the purified enzymes (5.0 mg in 1.0 ml of 10^{-3} M Tris buffer, pH 7.5, and 1.0 ml Freund's adjuvant). The same procedure was followed after two and four weeks, except that on the fourth week 1.0 ml of 0.85% NaCl was substituted for Freund's adjuvant, and on the following day, 3.5 mg of enzyme in 1.0 ml saline was injected intravenously. Antiserum was obtained from blood collected on the seventh and ninth

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TABLE 1
ELECTROPHORETIC VARIANTS OF ERYTHROCYTE CARBONIC ANHYDRASES IN PRIMATE AND RODENT SPECIES

Species	Number examined	Number of different variants	
		CA I	CA II
Great apes and man			
Man *(Homo sapiens)*	6,275	4	0*
Chimpanzee *(Pan troglodytes)*	92	0	0†
Orangutan *(Pongo pygmaeus)*	13	2‡	n.t.
Old World primates			
Baboon *(Papio cynocephalus)*	75	1‡	0
Stump-tailed macaque *(Macaca speciosa)*	11	1‡	n.t.
Rhesus macaque *(Macaca mulatta)*	159	1	1‡
Cynomolgus macaque *(Macaca irus)*	78	2	1‡
Green monkey *(Cercopithecus aethiops)*	64	0	0
New World primates			
Titi monkey *(Callicebus cupreus)*	4	0	1
Spider monkey *(Atles belzebuth)*	6	1	1
Capuchin *(Cebus capuchinus)*	3	1	0
Prosimian primates			
Slow loris *(Nycticebus coucang)*	5	1	1
Rodents			
Deer mouse *(Peromyscus maniculatus)*	115	0	0
House mouse *(Mus musculus)*	15	0	0
	Total	14	5

*200 tested
†50 tested
‡polymorphic (frequency > 10%)
n.t. = not tested

days after the final injection. Immunodiffusion tests were carried out on agar-coated slides essentially as described by Niswander *et al.*[8]

Results and Discussion

Genetic Variation

Those species in which either proved or assumed genetic variation in electrophoretic patterns have been observed for CA I and CA II are listed in TABLE 1, as well as species in which more than ten individuals were examined. No variation was observed in 18 other primate species in which fewer than ten animals were tested. Three of the four human CA I variants (CA Ib, CA Ic, and CA Id) have been demonstrated to be under the control of single autosomal genes,[9-11] and it is highly probable that variations in the homologous enzymes observed in other species are under similar genetic control.

The data in TABLE 1 suggest that there is a tendency for greater electrophoretic variation in CA I than in CA II; a total of 14 distinct variants were found for CA I, and five for CA II. In most species, the variants were either rare or occurred in low frequencies; however, variant types occurring in frequencies greater than ten percent (polymorphic) were found for CA I of orangutan (FIGURE 2), stump-tailed macaque, and baboon, and for CA II of rhesus and cynomolgus macaques (FIGURE 3). Several other species might also fall into this category (e.g., capuchin, spider monkey); however, not enough individuals were examined to be certain. Obviously, only a limited number of individuals and species from one order of mammals were tested; perhaps a different pattern will emerge when more nonprimate species are examined.

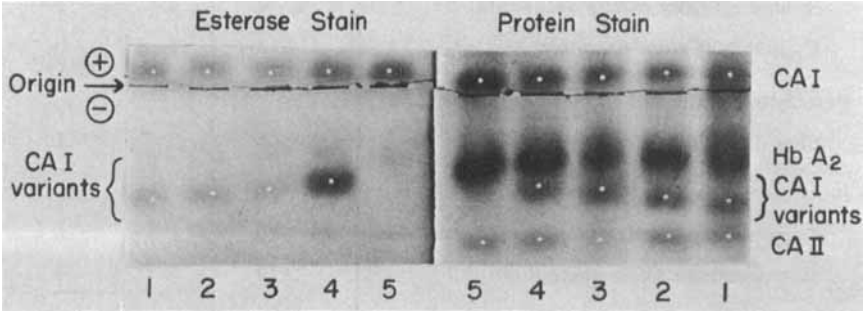

FIGURE 1. Electrophoretic patterns of heterozygous human variants of red cell carbonic anhydrase I (CA I) after esterase and protein staining. CA Ic Guam, 1; CA Ie Michigan, 2; CA Id Michigan, 3; CA Ib Michigan, 4; normal CA I, 5. White dots indicate the carbonic anhydrases. Esterase activity of carbonic anhydrase II (CA II) is too weak to be seen in photograph. Electrophoresis was carried out on a vertical starch gel, 0.02 M borate buffer, pH 8.8, and 0.3 M borate bridge buffer, pH 8 containing 0.03 M NaCl, 17 hr, 9 v/cm, 1-3° C. Enzyme activity visualized with Blue RR salt as dye coupler and β-naphthyl acetate as substrate; protein stain is nigrosin. See Reference 7 for details of methodology.

Some representative, rare, or polymorphic, electrophoretic patterns of both CA I and CA II from various primate species are shown in FIGURES 1–3. In FIGURE 1, both esterase and protein stains of the four human variants of CA I are illustrated. The CA II patterns are not altered in the human hemolysates containing the CA I variants; and alternatively, in the rhesus and cynomolgus hemolysates containing the CA II variants, the CA I patterns are not changed. This genetic evidence supports the already convincing chemical evidence[2 5] that these enzymes exist as monomers under the control of separate genetic loci.

Chemical Variation

Tryptic peptide patterns. In FIGURE 4, the tryptic peptide patterns of CA I are compared from three primate species: man, green monkey (an Old World monkey), spider monkey (a New World monkey), and the deer mouse. It can be seen that although there are many similarities between the patterns of man and the monkeys, they still differ noticeably. As might be expected, the mouse patterns bear little resemblance to the primate patterns. When the tryptic patterns of the other form of red cell carbonic anhydrase, CA II, are compared for the same four species (FIGURE 5), the patterns of man and the two monkeys show remarkably similar patterns, even though man and the spider

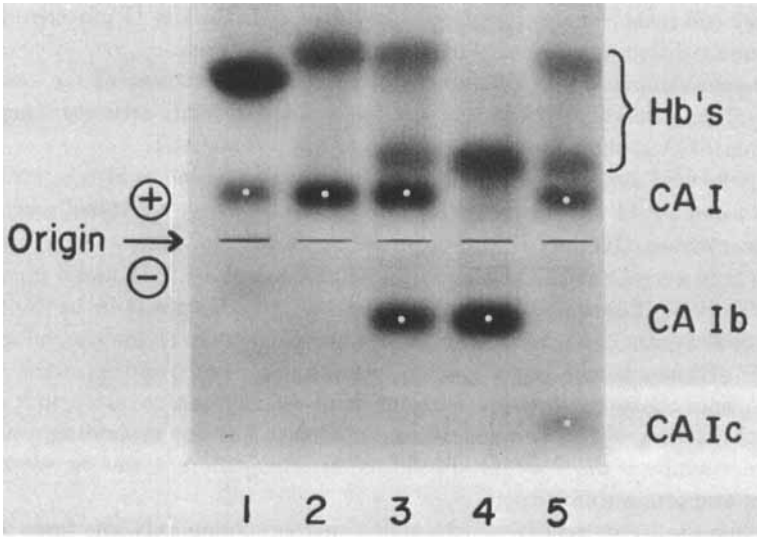

FIGURE 2. Electrophoretic patterns (esterase stain) of carbonic anhydrase I (CA I) variants in orangutan (*P. pygmaeus*). Human, normal pattern, 1; orangutan, normal pattern, 2; orangutan, heterozygous ab, 3; orangutan, homozygous b variant, 4; orangutan, heterozygous ac, 5. White dots indicate the carbonic anhydrases; all unmarked bands are normal and variant hemoglobins. Conditions for electrophoresis and staining same as in legend for FIGURE 1.

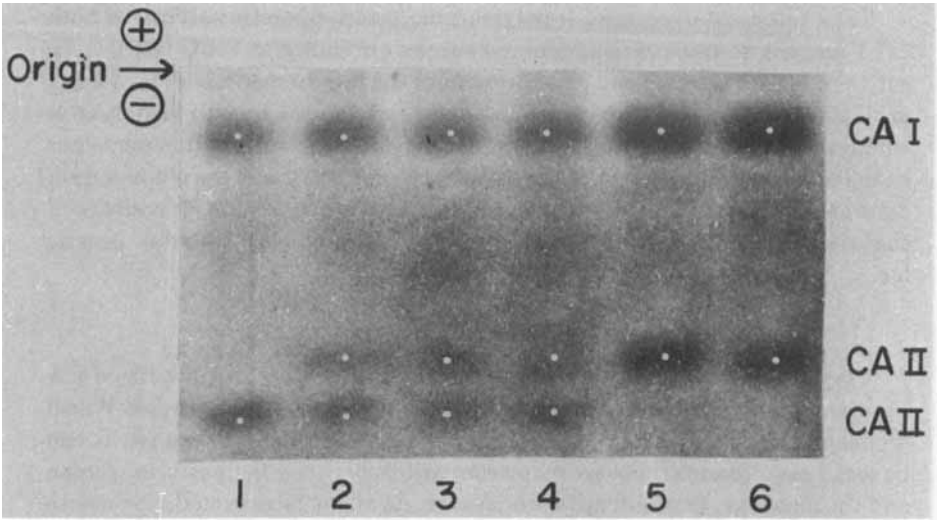

FIGURE 3. Electrophoretic patterns (protein stain) of red cell carbonic anhydrase I (CA II) variants in rhesus (*M. mulatta*) and cynomolgus (*M. irus*) macaques. Rhesus homozygous CA IIb pattern, 1; rhesus, heterozygous ab, 2,3; cynomolgus, heterozygou ab, 4; rhesus, homozygous CA IIa, 5; cynomolgus, homozygous CA IIa, 6. White dot indicate the carbonic anhydrases. Conditions for electrophoresis and staining same as i legend for FIGURE 1.

monkey are rather distantly related. As with CA I, the CA II pattern of th deer mouse differs considerably from the primate patterns.

These similarities and differences in the peptide patterns of the two en zymes from different species bring out the fact that certain structural aspect of primate CA II show less phylogenetic variation than CA I.

Amino acid compositions. In TABLE 2, the total amino acid composition of CA I and CA II are compared for man, green monkey, pig-tailed macaque and deer mouse. Overall, the compositions of the two enzymes are very similar which is in keeping with the hypothesis that CA I and CA II had a commo genetic origin. There are differences, however, which appear to be constan for each enzyme. This is especially noticeable when we examine the values fo serine, glycine, leucine, and lysine. These amino acid residues show goo interspecies constancy for each enzyme with no apparent overlap in value The similarities of the two mouse enzymes with the corresponding primat enzymes support the concept of the evolutionary homologies between th rodent and primate enzymes.

When the amino acid composition of a species having only one form of re cell carbonic anhydrase is examined (as Nyman and Lindskog[4] have done fo bovine carbonic anhydrase), the composition shows a higher degree of similar ity with CA II than with CA I of other species. This fact has recently bee supported by the findings of Nyman *et al.*[5] who showed that the sequence o the last 19–20 amino acid residues of bovine carbonic anhydrase more closel

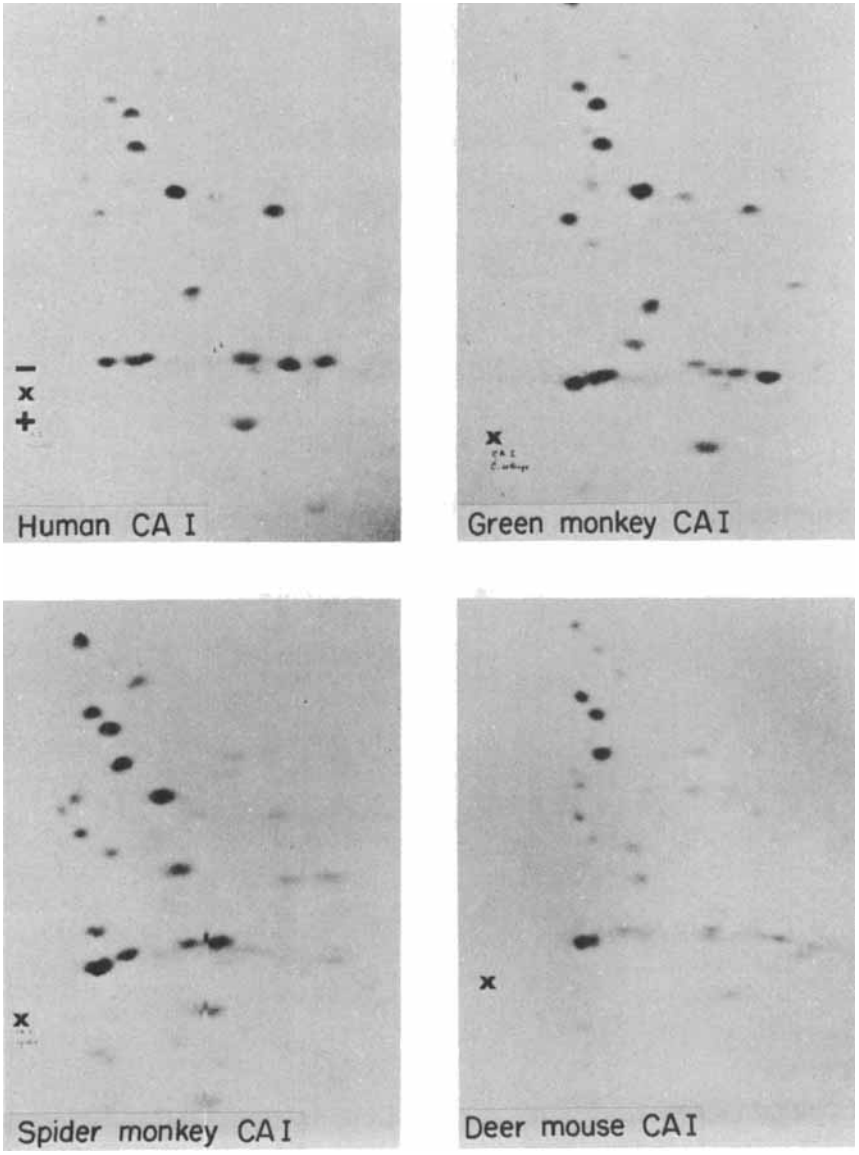

FIGURE 4. Tryptic peptide patterns of red cell carbonic anhydrase I (CA I) from man, green monkey (*C. aethiops*), spider monkey (*A. belzebuth*), and the deer mouse (*P. maniculatus*). See Reference 7 for details of methodology.

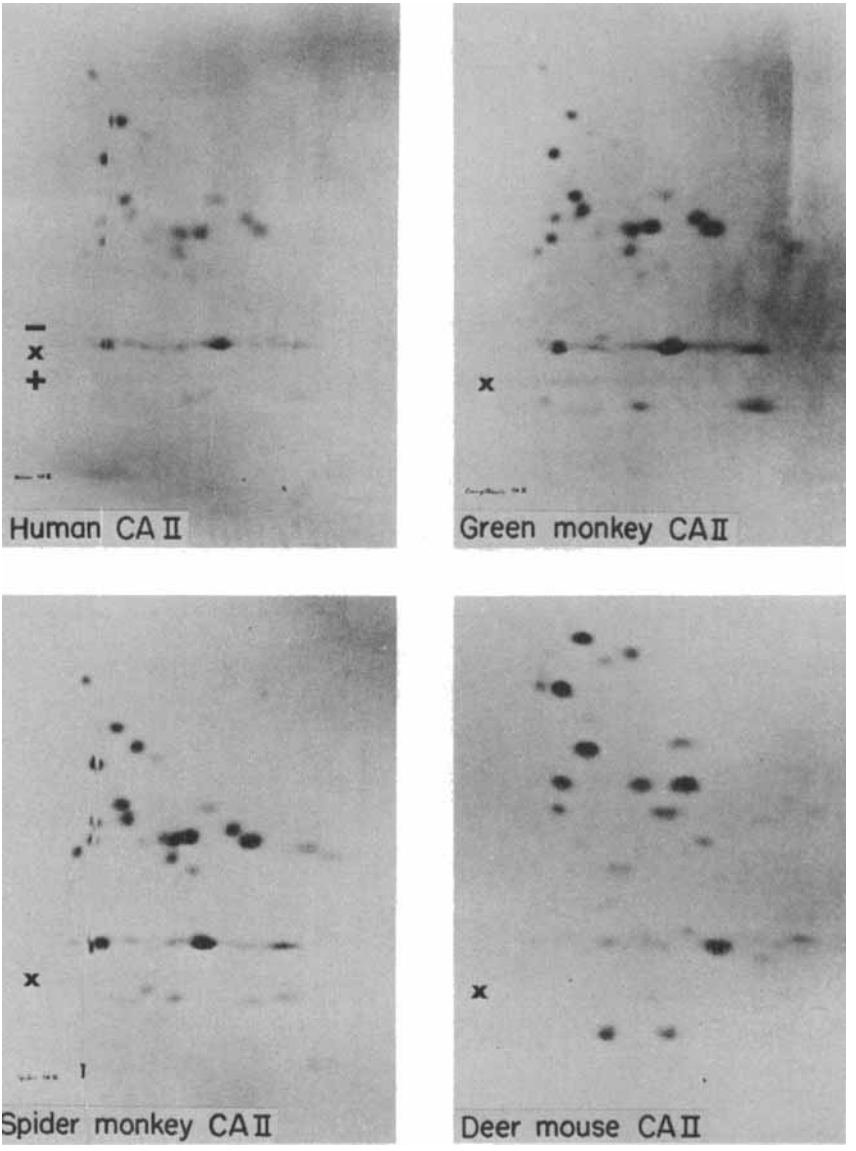

FIGURE 5. Tryptic peptide patterns of red cell carbonic anhydrase II (CA II) from man, green monkey (*C. aethiops*), spider monkey (*A. belzebuth*), and deer mouse (*P. maniculatus*). See Reference 7 for details of methodology.

TABLE 2
AMINO ACID COMPOSITION* OF ERYTHROCYTE CARBONIC ANHYDRASES FROM THREE PRIMATE SPECIES AND THE DEER MOUSE

	CA I				CA II			
	Man	Green monkey	Pig-tailed macaque	Deer mouse	Man	Green monkey	Pig-tailed macaque	Deer mouse
Aspartic acid	31	30	29	30	28	28	28	28
Threonine	13	13	11	13	10	11	10	15
Serine†	27	28	27	25	15	15	13	15
Glutamic acid	22	21	20	19	23	23	23	23
Proline	17	17	17	15	18	17	17	16
Glycine†	16	16	17	20	23	22	23	23
Alanine	19	16	16	19	13	13	12	17
Valine	17	17	16	16	17	15	15	12
Methionine	2	1	1	1	1	2	2	1
Isoleucine	9	9	10	11	9	9	9	9
Leucine†	20	20	19	20	25	24	23	24
Tyrosine	7	8	8	6	7	7	7	7
Phenylalanine	11	10	10	10	12	11	11	12
Lysine†	18	18	18	18	23	24	23	20
Histidine	11	10	9	11	11	10	11	10
Arginine	7	7	8	6	7	7	7	6

*Cysteine and tryptophan values have been omitted.
†Values underlined to emphasize dissimilarities between CA I and CA II.

resembles the sequence of human CA II than CA I. In addition, as will be shown later in this report, bovine carbonic anhydrase is also more similar to CA II with respect to its immunochemical properties and specific enzyme activities.

Enzyme activities. More evidence in support of the homologies of the carbonic anhydrase forms is shown in TABLE 3 where the esterase and hydrase

TABLE 3
ESTERASE AND HYDRASE ACTIVITIES OF ERYTHROCYTE CARBONIC ANHYDRASES IN DIFFERENT MAMMALIAN SPECIES

Species	Esterase Activity units*/mg		CO_2 Hydrase Activity units†/mg	
	CA I	CA II	CA I	CA II
Man *(Homo sapiens)*	0.044	0.029	7.9	27.2
Baboon *(Papio cynocephalus)*	0.094	0.025	10.0	28.2
Rhesus macaque *(Macaca mulatta)*	0.570	—	5.7	31.0
Pig-tailed macaque *(Macaca nemestrina)*	0.540	0.021	3.9	28.7
Green monkey *(Cercopithecus aethiops)*	0.066	0.020	7.0	30.5
Spider monkey *(Ateles belzebuth)*	0.200	0.020	3.4	25.3
Deer mouse *(Peromyscus maniculatus)*	0.048	0.020	7.1	23.0
Ox *(Bos taurus)*		0.010‡		17.2‡

*μ Moles β-naphthol formed/minute from β-naphthyl acetate.

†ΔOD of Veronal buffer at 276/mμ second. See Tashian *et al.*[7] for details of enzyme assays.

‡Values have been arbitrarily placed under CA II because of presumed homology. See text for explanation.

activities of the purified enzymes from several primate species, deer mouse, and ox are compared. Although considerable interspecies variability exists between the specific esterase activities of CA I, nevertheless, the overall activities show a fairly high degree of correspondence between the rodent and primate enzymes. The ratios between the CO_2 hydrase activities of CA I and CA II do not vary extensively in the species examined. These data also show that when interspecies comparisons are made between the activities of CA I and CA II, there is a greater tendency for variation in the activity of CA I than in CA II. A 13-fold range in esterase activity is seen for CA I, and only a 1.5-fold range for CA II. The higher variability of CA I is not as evident when the hydrase activities of the two forms are compared.

The activities of the bovine (ox) carbonic anhydrase, present as only one form,[4] seem to approach the values for CA II rather than CA I of the other species, a finding which is in keeping with the similarities noted above in amino acid compositions and amino acid sequences.

Immunochemical comparisons. Immunodiffusion studies of the carbonic anhydrases from different species, reacted against rabbit anti-human CA I and CA II sera, support the concepts, discussed above, of the relative structural constancy of CA II and the homologies between the primate and rodent enzymes.

The immunodiffusion patterns of purified CA I and CA II of man, pig-tailed macaque, green monkey, and deer mouse against rabbit anti-human CA I and CA II sera are shown in FIGURE 6. Although the CA I of the two monkey species gives a reaction of only partial identity with CA I of man, no spurring is detectable when purified CA II preparations from the three species are compared. This is consistent with the observations on the greater constancy of the peptide patterns of CA II (FIGURE 5) discussed earlier.

The immunodiffusion reactions of the *Peromyscus maniculatus* carbonic anhydrases (FIGURE 6) are of particular interest because both enzyme forms appear to cross-react with both anti-human CA I and CA II sera. The cross-reactivity of anti-CA I with *P. maniculatus* CA II has been shown to be due to the presence of a low level of *P. maniculatus* CA I (< 5%); however, the reaction of anti-CA II with *P. maniculatus* CA I is a true cross-reaction. Because similar cross-reactivities have been shown for house mouse and flying squirrel (TABLE 4), this may be a general pattern among rodents. This cross-reactivity of the different forms of rodent carbonic anhydrases is especially difficult to interpret in view of the facts that their tryptic peptide patterns are dissimilar (FIGURES 3 and 4), and that CA I and CA II of primate species tested to date do not show immunochemical cross-reactivity (*cf.* Micheli and Buzzi,[12] and TABLE 4). These immunochemical similarities of the rodent carbonic anhydrases may indicate that certain portions of the two molecules have not diverged as much as the homologous regions of the two primate enzymes.

In TABLE 4 are compared the immunodiffusion reactions against rabbit anti-CA I and anti-CA II sera of (1) the purified carbonic anhydrases, (2) hemolysates absorbed with anti-human CA I or CA II sera, or (3) unabsorbed hemolysates of various mammalian species. Examination of these data reveals several facts. First, it appears that four orders (Primates, Insectivora, Rodentia, and Carnivora) have species with two forms of carbonic anhydrase; obviously, the proposed gene duplication must have taken place before the divergence of these orders. Second, a clue as to which of the two carbonic anhydrases represents the evolutionally older form may be indicated by the fact that when only one form of carbonic anhydrase is present in a species (slow loris, shrew, ox, cat), it seems to correspond most often with CA II. Although the hemolysates of camel and opossum cross-reacted only with anti-

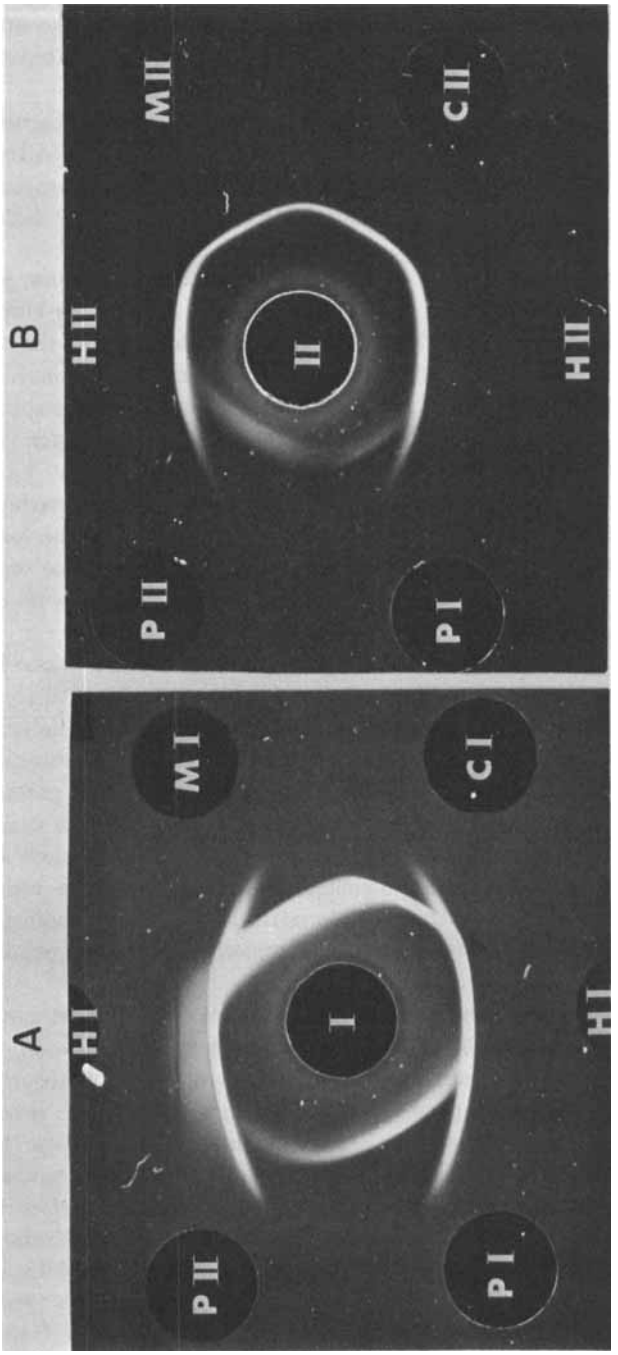

FIGURE 6. Immunodiffusion cross-reaction patterns between rabbit anti-human red cell CA I and CA II sera and purified red cell carbonic anhydrases from various species. A: center well—anti-human CA I; outer wells—human CA I (HI), pig-tailed macaque (*M. nemestrina*) CA I (MI), green monkey (*C. aethiops*) CA I (CI), and deer mouse (*P. maniculatus*) CA I (PI) and CA II (PII). B: center well—anti-human CA II; outer wells—human CA II (HII), pig-tailed macaque CA II (MII), green monkey CA II (CII), and deer mouse CA I (PI) and CA II (PII). See text for experimental details.

TABLE 4
IMMUNOCHEMICAL REACTIONS OF CARBONIC ANHYDRASES FROM DIFFERENT MAMMALIAN SPECIES

Species	Antigen Source*	Anti-Human CA I		Anti-Human CA II		Postulated Homologous Forms
Primates						
Man *(Homo)*	P	I (+)	II (-)	I (-)	II (+)	I, II
Pig-tailed macaque *(Macaca)*	P	I (+)	II (-)	I (-)	II (+)	I, II
Green monkey *(Cercopithecus)*	P	I (+)	II (-)	I (-)	II (+)	I, II
Galago *(Galago)*	H†	I (+)	II (?)	I (-)	II (+)	I, II
Slow loris *(Nycticebus)*	H	(-)		(+)		II
Angwantibo *(Arctocebus)*	H	(+)		(+)		I, II
Tree shrew *(Tupaia)*	H	(+)		(+)		I, II
Insectivora						
Elephant shrew *(Nasilio)*	H	(+)		(+)		I, II
Shrew *(Suncus)*	H	(-)		(+)		II
Rodentia						
Flying squirrel *(Glaucomys)*	H†	I (+)	II (+)	I (?)	II (?)	I, II
Deer mouse *(Peromyscus)*	P	I (+)	II (-)	I (+)	II (+)	I, II
House mouse *(Mus)*	P	I (+)	II (+)	I (+)	II (+)	I, II
Edentata						
Armadillo *(Tolypeutes)*	H	(-)		(-)		?
Carnivora						
Dog *(Canis)*	H†	I (+)	II (?)	I (?)	II (+)	I, II
Cat *(Felis)*	H	(-)		(+)		II
Artiodactyla						
Ox *(Bos)*	P	(-)		(+)		II
Camel *(Camelus)*	H	(+)		(-)		I
Marsupialia						
Opossum *(Didelphis)*	H	(+)		(-)		I

*P = purified enzymes; H = untreated hemolysates; H† = hemolysates absorbed with anti-CA I or anti-CA II.

human CA I serum, such cross-reactivity, where only a single form of carbonic anhydrase is present, may be misleading in view of the cross-reactions observed in rodents. Perhaps, when sequence data are available for these single forms, the homologies will become more evident. Third, it also appears that one or the other of the genes which control the synthesis of the two carbonic anhydrases can either be "turned on and off", or lost, so long as the other remains functional. In most cases, it appears that the cell can get along without CA I more readily than CA II. The fact that CA II has a much higher specific CO_2 hydrase activity than CA I (*cf.* Rickli *et al.*,[13] and TABLE 2) would seem to indicate that it may be more strongly selected for in this function than CA I. If this is true, then it would follow that the absence of CA I might not seriously upset the physiology of the red cell. This type of reasoning should be approached with caution, however, since at the present time we are quite ignorant of all of the physiological roles of these carbonic anhydrases.

Some evidence in support of the theory that CA II may be the older molecule comes from the work of Shimizu and Matsuura,[14] who isolated carbonic anhydrase from the red cells of a fish (yellow-fin tuna) and two species of mammals (blue-white dolphin and ox). The fish and mammals had only one form of the enzyme which, among other physicochemical features, showed the high specific hydrase activities characteristic of mammalian CA II rather than the relatively low specific CO_2 hydrase activities of CA I.

Summary

Two forms of erythrocyte carbonic anhydrase from a number of primate and other mammalian species were compared as to the extent of electrophoretic variability found in the enzymes, as well as several chemical parameters. On the basis of genetic variation, comparative tryptic peptide patterns, immunochemical cross-reactions, and variation in enzyme activities, carbonic anhydrase I appears to show greater variability than carbonic anhydrase II. In the limited number of species examined, wherever only one form of carbonic anhydrase is present, it usually appears to be homologous to form II of species which possess two forms of the enzyme. The possibility that carbonic anhydrase II represents the evolutionally older molecule is discussed.

Acknowledgments

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References

1. TASHIAN, R. E. 1965. Genetic variation and evolution of the carboxylic esterases and carbonic anhydrases of primate erythrocytes. Amer. J. Hum. Genet. **17:** 257-272.

2. ARMSTRONG, J. McD., D. V. MYERS, J. A. VERPOORTE & J. T. EDSALL. 1966. Purification and properties of human erythrocyte carbonic anhydrases. J. Biol. Chem. **241:** 5137–5149.
3. DUFF, T. A. & J. E. COLEMAN. 1966. *Macaca mulatta* carbonic anhydrase. Crystallization and physicochemical and enzymatic properties of two enzymes. Biochemistry **5:** 2009–2019.
4. NYMAN, P. O. & S. LINDSKOG. 1964. Amino acid composition of various forms of bovine and human erythrocyte carbonic anhydrase. Biochim. Biophys. Acta **85:** 141–151.
5. NYMAN, P. O., L. STRID & G. WESTERMARK. 1966. Carboxyl-terminal amino acid sequences of human and bovine erythrocyte carbonic anhydrase. Biochim. Biophys. Acta **122:** 554–556.
6. TASHIAN, R. E. & M. W. SHAW. 1962. Inheritance of an erythrocyte acetylesterase variant in man. Amer. J. Hum. Genet. **14:** 295–300.
7. TASHIAN, R. E., S. K. RIGGS & Y.-S. L. YU. 1966. Characterization of a mutant human erythrocyte carbonic anhydrase: Carbonic Anhydrase Ic Guam. The amino acid substitution and carboxylesterase and hydratase activities. Arch. Biochem. Biophys. **117:** 320–327.
8. NISWANDER, J. D., D. C. SHREFFLER & J. V. NEEL. 1964. Genetic studies of quantitative variation in a component of human saliva. Ann. Hum. Genet. **27:** 319–328.
9. SHAW, C. R., F. N. SYNER & R. E. TASHIAN. 1962. New genetically determined molecular form of erythrocyte esterase in man. Science **138:** 31–32.
10. TASHIAN, R. E., C. C. PLATO & T. B. SHOWS. 1963. Inherited variant of erythrocyte carbonic anhydrase in Micronesians from Guam and Saipan. Science **140:** 53–54.
11. SHOWS, T. B. 1966. The characterization of Carbonic Anhydrase Id Michigan, a human erythrocyte carbonic anhydrase variant. Ph.D. Dissertation. Univ. of Michigan. Ann Arbor, Mich.
12. MICHELI, A. & C. BUZZI. 1964. Electrophoretic and immuno-electrophoretic studies on the carbonic anhydrase of the human red blood cells. Biochim. Biophys. Acta **89:** 324–329.
13. RICKLI, E. E., S. A. S. GHAZENFAR, B. H. GIBBONS & J. T. EDSALL. 1964. Carbonic anhydrases from human erythrocytes. J. Biol. Chem. **239:** 1065–1078.
14. SHIMIZU, C. & F. MATSUURA. 1962. Some physico-chemical properties of crystalline carbonic anhydrase from erythrocytes of ox, blue-white dolphin and yellow-fin tuna. Bull. Japanese Soc. Scientific Fisheries **28:** 924–929.