Biochemical Genetics, Vol. 17, Nos. 5/6, 1979

Chemical Characterization of a New Japanese Variant of Carbonic Anhydrase I, CA $I_{Nagasaki\,1}$ (76 Arg→Gln)

Kazuaki Goriki,[1,2] Richard E. Tashian,[1,3] Sharon K. Stroup,[1] Ya-Shiou L. Yu,[1] and Dag M. Henriksson[1,4]

Received 28 Aug. 1978—Final 6 Nov. 1978

A new inherited variant of carbonic anhydrase I (CA I), designated CA $I_{Nagasaki\,1}$ (CA $I_{NGS\,1}$), was discovered during a survey of hemolysates from 5852 individuals from the cities of Hiroshima and Nagasaki in Japan. Analysis of the amino acid composition of a tryptic peptide from the CA $I_{NGS\,1}$ variant indicated that a glutaminyl residue was substituted for an arginyl residue at position 76. Heat degradation studies showed that the CA $I_{NGS\,1}$ variant was less stable than normal CA I. The CO_2 hydrase and esterase activities of the normal and variant carbonic anhydrases I, as well as the relative amounts of the two enzymes in heterozygotes, were similar.

KEY WORDS: red cell carbonic anhydrase I; new Japanese variant; amino acid substitution; thermostability; CO_2 hydrase and esterase activities.

INTRODUCTION

Three isozymes of carbonic anhydrase, carbonic anhydrases I, II, and III (CA I, CA II, and CA III), are known to occur in humans (*cf.* Tashian, 1977; Carter

This work was supported in part by Contract E(11-1)-1552 with the Energy Research and Development Administration, Washington, D.C. (to J. V. Neel), and by U.S. Public Health Service Grant GM-24681.

[1] Department of Human Genetics, University of Michigan Medical School, Ann Arbor, Michigan 48109.
[2] Present address: Clinical Laboratories, Radiation Effects Research Foundation, Hiroshima, 730 Japan.
[3] Address correspondence to R.E.T.
[4] Present address: Department of Biochemistry, University of Göteborg, Göteborg, 5, Sweden.

0006-2928/79/0600-0449$03.00/0

et al., 1978). Our knowledge of the tissue distribution of these isozymes is still incomplete; however, CA I appears to be found mainly in erythrocytes and gastrointestinal mucosa, CA II in erythrocytes and a wide variety of tissues, and CA III in skeletal muscle. The CA I and CA II isozymes appear to be products of two closely linked genes in mammals (Carter, 1972; DeSimone *et al.*, 1973; Eicher *et al.*, 1976). The amino acid sequences of human CA I and CA II as well as their three-dimensional structures have been determined (Andersson *et al.*, 1972; Liljas *et al.*, 1972; Lin and Deutsch, 1973, 1974; Kannan *et al.*, 1975; Henderson *et al.*, 1976).

To date, 15 electrophoretic variants of CA I and three variants of CA II have been reported after screening of hemolysates from many different human populations (Moore *et al.*, 1971; Tashian and Carter, 1976; Blake and Kirk, 1978; Ghosh, 1978). Of these, the amino acid substitutions have been determined for five (and tentatively for two more) of the CA I variants and one of the CA II variants (Funakoshi and Deutsch, 1970; Lin and Deutsch, 1972; Tashian and Carter, 1976).

Here we report the amino acid substitution, comparative enzyme activities, and thermostability of a new variant of CA I discovered in a Japanese family from Nagasaki, Japan. This variant is designated CA $I_{Nagasaki\ 1}$ and abbreviated CA $I_{NGS\ 1}$. The CA $I_{NGS\ 1}$ designation is in keeping with the nomenclatural abbreviations recommended for protein variants by Ferrell *et al.* (1977). Previously, only one CA I variant, CA Ih Hiroshima (CA $I_{HIR\ 1}$), has been reported from the Japanese population (Ueda, 1974; Tanis *et al.*, 1976).

MATERIALS AND METHODS

Electrophoretic Techniques

The conditions for electrophoresis and staining used in this study were the same as those previously described (*cf.* Tashian and Carter, 1976). For the screening procedures, the esterase activities of red cell carbonic anhydrases after electrophoresis were detected using the fluorogenic substrates 4-methyl umbelliferyl acetate for CA I and fluorescein diacetate for CA II (Hopkinson *et al.*, 1974).

Purification of the Variant Carbonic Anhydrase

The normal and variant CA I isozymes were isolated from approximately 200 ml of whole blood pooled from the three individuals heterozygous for the CA $I_{NGS\ 1}$ variant. The two forms of CA I were initially separated by affinity chromatography on a sulfonamide-bound Sephadex column by the method of

Osborne and Tashian (1975). Further purification was achieved by rechromatography on a DEAE-Sephadex column (Armstrong *et al.*, 1966). The concentration of the carbonic anhydrases was determined using a molar absorption value at 280 nm of 4.9×10^4 liter $mole^{-1}$ cm^{-1}.

Peptide Analysis

Tryptic digestion and peptide mapping of the carbonic anhydrases were initially carried out as previously described (Tashian *et al.*,1966); however, in order to locate the altered tryptic peptide of the CA $I_{NGS\ 1}$ variant, tryptic digestion was carried out using a modification of an acid denaturation procedure (Henderson *et al.*, 1976). Twenty milligrams of CA I was first adjusted, with rapid stirring, to *p*H 3.0 with 0.1 N HCl and after about 10 min adjusted to *p*H 10.6 with 0.2 M NaOH. Trypsin (trypsin to CA I ratio of 2:100) was then added, and the mixture was incubated at 37 C for 16 hr. The *p*H was then adjusted to 5.0 with concentrated acetic acid, and after 2 hr the digest was centrifuged at 8000 rpm for 30 min. Separation of the tryptic peptides in the *p*H 5.0 insoluble fraction (containing the variant peptide) was carried out by dissolving the fraction in 5.0 M guanadine-HCl, *p*H 5.0, followed by chromatography on a Sephadex G-50 column equilibrated with the same buffer. The fractions containing the altered peptide were pooled, desalted, lyophilized, and dissolved in 10% acetic acid and rechromatographed on a Sephadex G-25 column equilibrated with 10% acetic acid.

One-dimensional electrophoretic separation of tryptic peptides was carried out on chromatography paper (Whatman 3 MM) in a pyridine-acetate buffer, *p*H 6.4, for 70 min at 2000 V. The peptides were eluted from the paper with 25% acetic acid.

Amino acid compositions of the eluted peptides were determined with an amino acid analyzer after hydrolysis for 21 hr in 6 N HCl at 100 C.

Thermostability Studies

Heat denaturation studies were performed on the normal and variant CA I isozymes by utilizing the azosulfonamide binding procedure of Osborne and Tashian (1974).

Enzyme Assays

The CO_2 hydrase activities of the carbonic anhydrases were measured by the method of Nyman (1963), which follows the rate of change in the ultraviolet absorbance of Veronal buffer at 276 nm.

The esterase activity toward *p*-nitrophenyl acetate was measured by the method of Armstrong *et al.* (1966).

RESULTS

The Survey

The CA $I_{NGS\,1}$ variant was discovered in a normal male member of a family from Nagasaki in an examination of blood samples from 5852 offspring of individuals exposed to atomic bomb radiation, and suitable controls, from the cities of Hiroshima (3267 individuals) and Nagasaki (2585 individuals). The variant was also found in the sister and father of the propositus. Since the variant was found in the father of the propositus, its presence is of course unrelated to the radiation history.

The electrophoretic pattern of CA $I_{NGS\,1}$ is shown in Fig. 1. The anodal migration of this variant suggests that it is the result of a single additional negative charge. The similar intensities of the protein stains of the normal and variant carbonic anhydrase I indicate that the levels are similar.

Isolation of the CA I Variant

After the initial separation of the normal and variant carbonic anhydrases I by affinity chromatography, the two forms were further purified on DEAE-Sephadex columns (Fig. 2). About 40 mg of normal CA I and 45 mg of CA $I_{NGS\,1}$ were separated from 200 ml of whole blood. The purity of the separated forms of CA I can be seen in the electrophoretic patterns in Fig. 3.

Peptide Analysis

The tryptic peptide patterns of normal CA I and the CA $I_{NGS\,1}$ variant are compared in Fig. 1. As can be seen, one of the peptides (arrow) present in normal CA I is absent in the variant. This peptide, designated T-2, was eluted from paper after electrophoresis and its amino acid composition showed that it represented residues 77–80 (Ser-Val-Leu-Lys) in the normal sequence (Tables I and II). The absence of this peptide in CA $I_{NGS\,1}$ suggests that Arg-76 has been substituted for an uncharged residue in the variant enzyme, and that the resultant tryptic peptide T-3 (positions 58–80) is located in the insoluble tryptic digest of CA $I_{NGS\,1}$.

In order to locate the insoluble T-3 peptide, the insoluble tryptic digest of CA $I_{NGS\,1}$ was dissolved in 5 M guanadine-HCl, *p*H 5.0, and the tryptic peptides were separated on a Sephadex G-50 column. The elution pattern is shown in Fig. 4. The fractions known to contain the insoluble peptide T-1 (positions

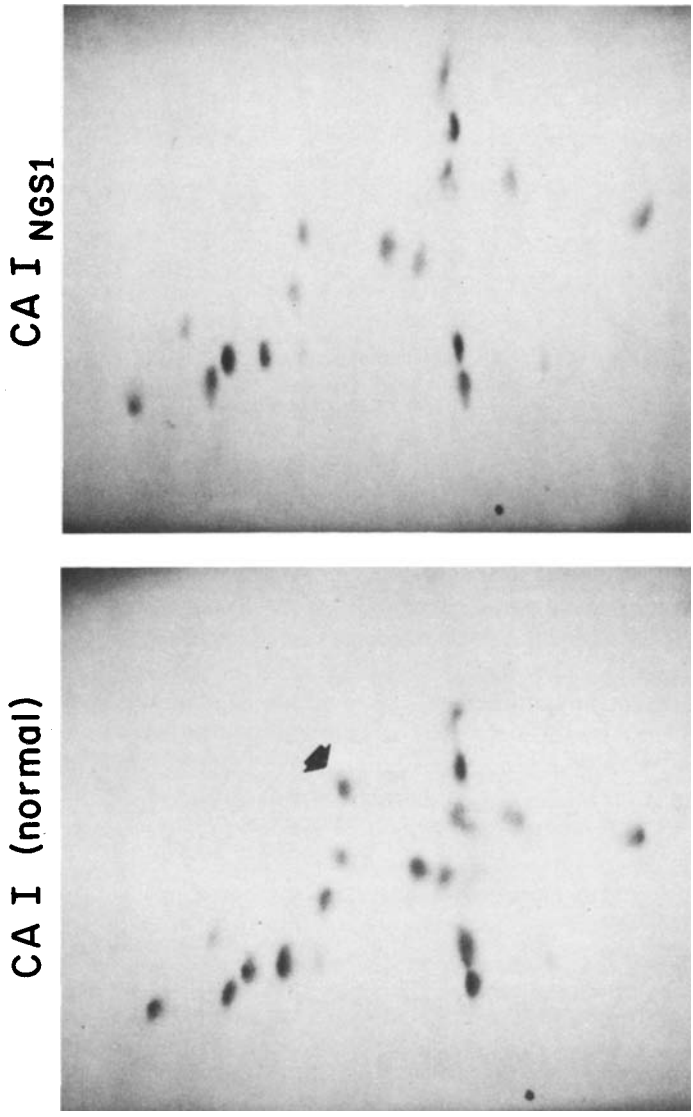


Fig. 1. Tryptic peptide patterns of normal CA I and CA $I_{NGS\ 1}$. The arrow indicates peptide T-2 (see Table I), which is missing in CA $I_{NGS\ 1}$.

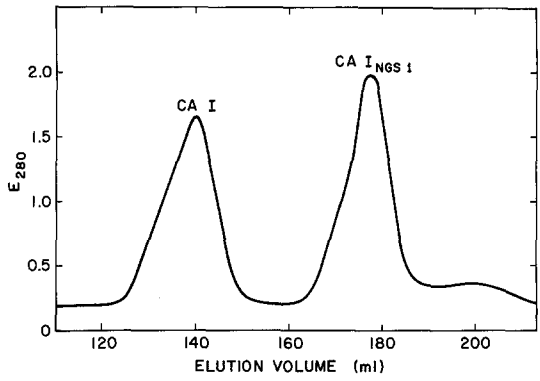


Fig. 2. Chromatography of normal CA I and $CAI_{NGS\ 1}$ on DEAE-Sephadex (1.5- by 90-cm column) eluted with 0–0.1 M NaCl gradient in 0.05 M tris-HCl, *p*H 8.7.

58–76) in normal CA I were pooled, desalted, lyophilized, dissolved in 10% acetic acid, and rechromatographed on a Sephadex G-25 column. The elution patterns from the normal and variant enzymes are shown in Fig. 5. The fractions containing the normal peptide T-1 from CA I and the variant peptide T-3 from CA $I_{NGS\ 1}$ were then lyophilized, resuspended in 10% acetic acid, and subjected to paper electrophoresis (see Materials and Methods). Since both peptides are negatively charged at *p*H 6.4, the anodally migrating bands were eluted from the paper with 25% acetic acid and hydrolyzed. The amino acid compositions of the two peptides are compared in Table II. As can be seen, the variant peptide contains one additional serine, valine, leucine, lysine, and glutamic acid, and no arginine. Since only one additional negative charge is indicated by the migration pattern of CA $I_{NGS\ 1}$ (Fig. 1), the most likely explanation is that a glutaminyl residue has substituted for an arginyl residue at position 76.

Thermostability

The CA $I_{NGS\ 1}$ variant was found to be less stable than normal CA I at 57 C, *p*H 8.0. The comparative thermostability curves are given in Fig. 6.

Enzyme Activities

The specific esterase and CO_2 hydrase activities of the purified normal and variant CA I isozymes were essentially the same. Both CA I and CA $I_{NGS\ 1}$ showed a value of 0.28 μmoles *p*-nitrophenol formed/min/mg toward *p*-nitro-

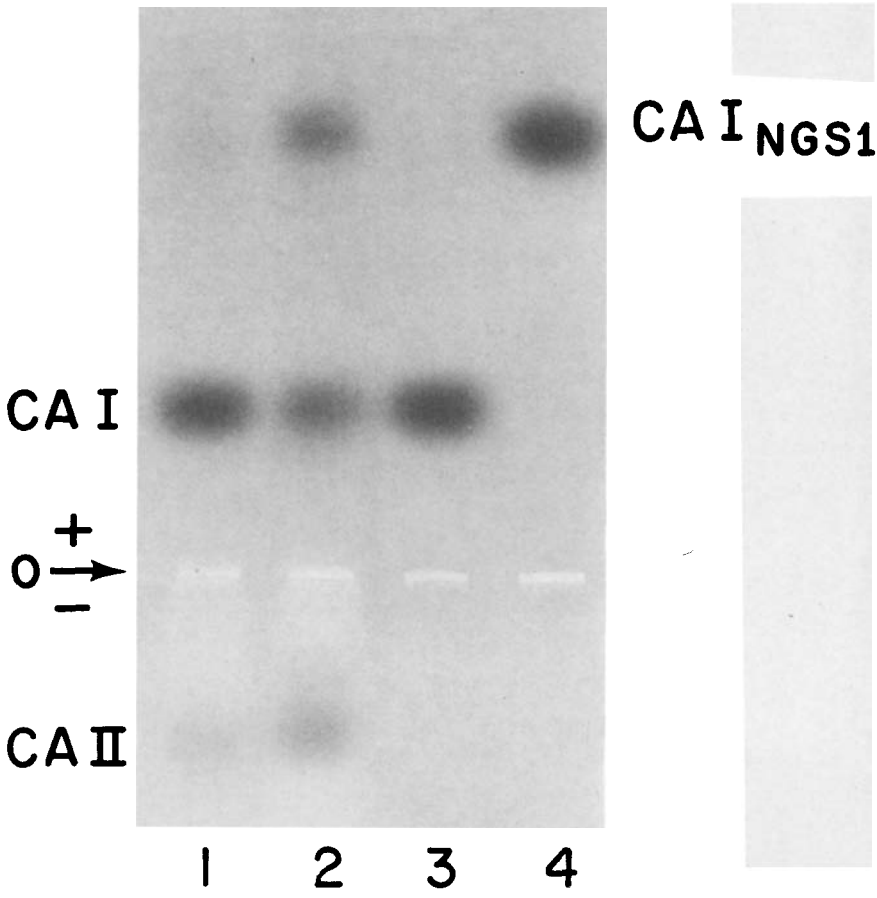


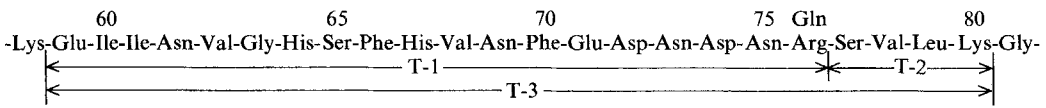
Fig. 3. Protein patterns (Nigrosin stained) of normal CA I and CA $I_{NGS\ 1}$ after vertical starch gel electrophoresis at 4 C for 18 hr at 8 V/cm, *p*H 8.6. Hemoglobin removed by extraction of hemolysates with a mixture of 40% ethanol and chloroform (1 : 0.5 v/v) at −10 C. 1, Normal CA I and CA II pattern; 2, CA $I_{NGS\ 1}$ /CA I heterozygote pattern; 3, purified CA I; 4, purified CA $I_{NGS\ 1}$. Purified normal and variant enzymes from chromatographic separation shown in Fig. 2.

phenyl acetate and values of, respectively, 4.8 and 4.9 units (ΔOD at 276 nm/sec)/mg toward CO_2.

DISCUSSION

The residue at position 76 has now been sequenced in the carbonic anhydrases I of orangutan, rhesus macaque, and ox (*cf.* Tashian, 1977). In these species

Table I. Portion of Human Carbonic Anhydrase I (CA I) Sequence Showing the Location of Tryptic Peptides T-1 and T-2 of Normal CA I, and Tryptic Peptide T-3 Produced by the Substitution of Gln for Arg at Position 76[a]



[a] CA I sequence from Andersson *et al.* (1972) and Lin and Deutsch (1973).

Table II. Comparison of the Molar Ratios of Amino Acid Residues in Tryptic Peptides T-1 and T-2 from CA I (Normal) and T-3 from CA $I_{NGS\ 1}$[a]

	CA I (normal)		CA $I_{NGS\ 1}$
Amino acid	T-1	T-2	T-3
Aspartic acid	6.36 (6)	—	6.07 (6)
Serine	0.99 (1)	0.53 (1)	1.87 (2)
Glutamic acid	2.25 (2)	—	2.81 (3)
Glycine	0.91 (1)	—	0.91 (1)
Valine	1.86 (2)	0.99 (1)	2.96 (3)
Isoleucine	1.87 (2)	—	2.31 (2)
Leucine	—	1.08 (1)	1.37 (1)
Phenylalanine	1.98 (2)	—	1.88 (2)
Histidine	1.98 (2)	—	1.75 (2)
Lysine	—	0.93 (1)	1.13 (1)
Arginine	0.99 (1)	—	—
Total residues	19	4	23

[a] Assumed number of residues is given in parentheses.

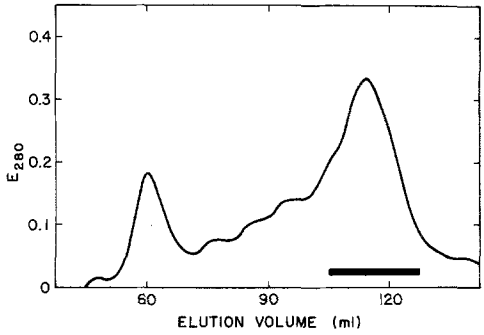


Fig. 4. Chromatography of *p*H 5.0 insoluble fraction of CA $I_{NGS\ 1}$ tryptic digest on Sephadex G-50 (0.9- by 120-cm column) eluted with 5.0 M guanadine-HCl. Bar indicates fractions containing peptide T-3; this region in the elution of normal CA I contains peptide T-1.

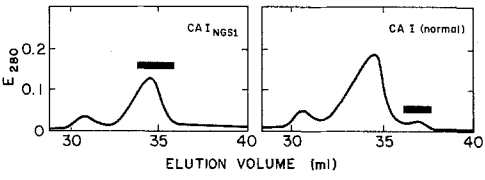


Fig. 5. Chromatography of pooled fractions (indicated by bar in Fig. 4) on Sephadex G-25 superfine (0.9- by 120-cm column) eluted with 10% acetic acid. Bars indicate fractions containing peptides T-3 from CA $I_{NGS\ 1}$ and T-1 from normal CA I. Peptide T-1 was isolated from the insoluble tryptic digest fraction of normal CA I which showed an elution pattern similar to that for CA $I_{NGS\ 1}$ in Fig. 4.

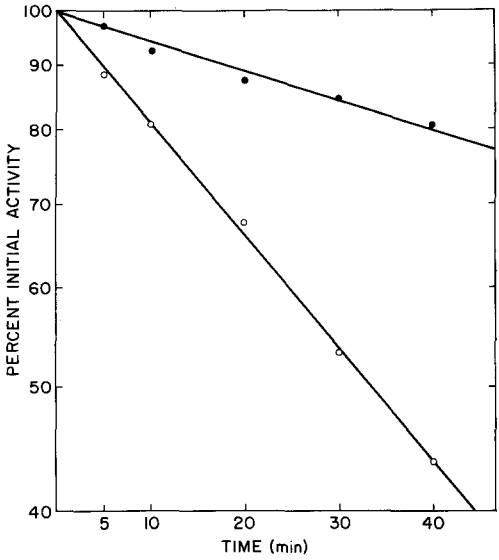


Fig. 6. Heat degradation of normal CA I (•) and CA $I_{NGS\ 1}$ (○) at 57 C in 10 mM Hepes buffer, *p*H 8.0.

the residue is arginine as in human CA I. The homologous residue in the carbonic anhydrases II of human, sheep, ox, and rabbit is lysine (*cf.* Tashian, 1977). In bovine CA III the homologous residue is arginine as in carbonic anhydrases I (Tashian, unpublished data). Thus a positive charge has been maintained at this position in all three carbonic anhydrase isozymes of mammals. However, except for the lower thermostability, the substitution of glutamine for arginine at this position does not seem to have affected either the activities or the quantitative levels of the variant enzyme. Based on the three-dimensional structures of human CA I and CA II (Kannan *et al.*, 1975; Liljas *et al.*, 1972), residue 76 is located on the outside of the molecule and is not a part of any secondary structure.

In an earlier survey of adults from Hiroshima (2604 individuals) and Nagasaki (1365 individuals) born prior to the atomic bombings, Ueda *et al.* (1977) found four unrelated individuals with the CA $I_{HIR\ 1}$ variant in the Hiroshima sample and none from the Nagasaki sample.

Of the 15 electrophoretic variants of CA I which have now been reported from various human populations, in only three (CA I 2-Australia, CA I 4-Australia, and CA I Parsi) have the frequencies of the variant alleles been found to be greater than 2% in some populations. Two of these, CA I

2-Australia and CA I 4-Australia (Blake and Kirk, 1978), were found in Australian Aborigines, and CA I Parsi was found in a Parsi population from Bombay, India (Ghosh, 1978).

CA $I_{NGS\ 1}$ and CA I 4-Australia are the only fast (anodally migrating) variants of CA I that have been reported. The electrophoretic patterns of these fast variants appear to be similar. As yet, however, the CA I 4-Australia variant has not been characterized chemically.

NOTE ADDED IN PROOF

A small amount (250 pmol) of the tryptic peptide T-3 from CA $I_{NGS\ 1}$ was kindly sequenced for us by Dr. Michael Hunkapiller (Division of Biology, California Institute of Technology). The sequence he obtained was the same as that given in Table I for T-3, thereby confirming the Arg→Gln substitution at position 76. In addition, this sequence shows that the Asp-Asn-Asp-Asn sequence for positions 72–75 is the correct one for human CA I originally reported by Lin and Deutsch (1973).

ACKNOWLEDGMENTS

We are grateful to Dr. James V. Neel for his helpful support during the course of this work. We also thank Dr. Howard B. Hamilton and Dr. Sadahisa Kawamoto (Radiation Effects Research Foundation Laboratory in Hiroshima and Nagasaki, respectively) for their help in collecting blood samples and Dr. Daniel Walz (Department of Physiology, Wayne State University School of Medicine, Detroit, Michigan) for his help in confirming the amino acid composition of the variant peptide.

REFERENCES

Andersson, B., Nyman, P. O., and Strid, L. (1972). Amino acid sequence of human erythrocyte carbonic anhydrase B. *Biochem. Biophys. Res. Commun.* **48:**670.

Armstrong, J. McD., Myers, D. V., Verpoorte, J. A., and Edsall, J. T. (1966). Purification and properties of human erythrocyte carbonic anhydrases. *J. Biol. Chem.* **241:**5137.

Blake, N. M., and Kirk, R. L. (1978). Widespread distribution of variant forms of carbonic anhydrase in Australian Aboriginals. *Med. J. Aust.* **1:**183.

Carter, N. D. (1972). Carbonic anhydrase isozymes in *Cavia porcellus, Cavia aperea* and their hybrids. *Comp. Biochem. Physiol.* **43B:**743.

Carter, N. D., Shiels, A., and Tashian, R. E. (1978). Carbonic anhydrase III isoenzyme from human and bovine muscle. *Biochem. Soc. Tr.* **6:**552.

DeSimone, J., Linde, M., and Tashian, R. E. (1973). Evidence for linkage of carbonic anhydrase isozyme genes in the pig-tailed macaque, *Macaca nemestrina*. *Nature New Biol.* **242:**55.

Eicher, E. M., Stern, R. H., Womack, J. E., Davisson, M. T., Roderick, T. H., and Reynolds, S. C. (1976). Evolution of mammalian carbonic anhydrase loci by tandem duplication: Close linkage of *Car-1* and *Car-2* to the centromere region of chromosome 3 of the mouse. *Biochem. Genet.* **14:**651.

Ferrell, R. E., Ueda, N., Satoh, C., Tanis, R. J., Neel, J. V., Hamilton, H. B., Inamizu, T., and Baba, K. (1977). The frequency in Japanese of genetic variants of 22 proteins. I. Albumin, ceruloplasmin, haptoglobin, and transferrin. *Ann. Hum. Genet.* **40**:407.

Funakoshi, S., and Deutsch, H. F. (1970). Human carbonic anhydrases. IV. properties of a mutant B type isozyme. *J. Biol. Chem.* **245**:4913.

Ghosh, A. K. (1978). The distribution of genetic variants of glyoxalase 1, esterase D, and carbonic anhydrase I and II in Indian populations. *Indian J. Phys. Anthropol. Hum. Genet.* (in press).

Henderson, L. E., Henriksson, D., and Nyman, P. O. (1976). Primary structure of human carbonic anhydrase C. *J. Biol. Chem.* **251**:5457.

Hopkinson, D. A., Coppock, J. S., Muhlemann, M. F., and Edwards, Y. H. (1974). The detection and differentiation of the products of the human carbonic anhydrase loci, CA I and CA II, using fluorogenic substrates. *Ann. Hum. Genet.* **38**:155.

Kannan, K. K., Notstrand, B., Fridborg, K., Lövgren, S., Ohlsson, A., and Petef, M. (1975). Crystal structure of human erythrocyte carbonic anhydrase B: Three-dimensional structure at a nominal 2.2 Å resolution. *Proc. Natl. Acad. Sci.* **72**:51.

Liljas, A., Kannan, K. K., Bergstén, P.-C., Warra, I., Fridborg, K., Strandberg, B., Carlbom, U., Järup, L., Lövgren, S., and Petef, M. (1972). Crystal structure of human carbonic anhydrase C. *Nature New Biol.* **235**:131.

Lin, K.-T. D., and Deutsch, H. F. (1972). Human carbonic anhydrases. VIII. Isolation and characterization of a polymorphic form of a C type isozyme. *J. Biol. Chem.* **247**:3761.

Lin, K.-T. D., and Deutsch, H. F. (1973). Human carbonic anhydrases. XI. The complete primary structure of carbonic anhydrase B. *J. Biol. Chem.* **248**:1885.

Lin, K.-T. D., and Deutsch, H. F. (1974). Human carbonic anhydrases. XII. The complete primary structure of the C isozyme. *J. Biol. Chem.* **249**:2329.

Moore, M. J., Funakoshi, S., and Deutsch, H. F. (1971). Human carbonic anhydrases. VII. A new C type isozyme in erythrocytes of American negroes. *Biochem. Genet.* **5**:497.

Nyman, P. O. (1963). A spectrophotometric method for the assay of carbonic anhydrase activity. *Acta. Chem. Scand.* **17**:429.

Osborne, W. R. A., and Tashian, R. E. (1974). Thermal inactivation studies of normal and variant human erythrocyte carbonic anhydrases by using a sulfonamide binding assay. *Biochem. J.* **141**:219.

Osborne, W. R. A., and Tashian, R. E. (1975). An improved method for the purification of carbonic anhydrase isozymes by affinity chromatography. *Anal. Biochem.* **64**:297.

Tanis, R. J., Osborne, W. R. A., Ueda, N., and Tashian, R. E. (1976). Biochemical characterization of the carbonic anhydrase variant CA Ih Hiroshima. *Hum. Genet.* **34**:29.

Tashian, R. E. (1977). Evolution and regulation of the carbonic anhydrase isozymes. In Rattazzi, M. C., Scandalios, J. G., and Whitt, G. S. (eds.), *Isozymes: Current Topics in Biological and Medical Research,* Vol. 2, Alan R. Liss, Inc., New York, pp. 21–62.

Tashian, R. E., and Carter, N. D. (1976). Biochemical genetics of carbonic anhydrase. In Harris, H., and Hirschhorn, K. (eds.), *Advances in Human Genetics,* Vol. 7, Plenum Press, New York, pp. 1–56.

Tashian, R. E., Riggs, S. K., and Yu, Y. S. (1966). Characterization of a mutant human erythrocyte carbonic anhydrase: Carbonic anhydrase Ic Guam. *Arch. Biochem. Biophys.* **117**:320.

Ueda, N. (1974). New Japanese variant of human erythrocyte carbonic anhydrase. *Jpn. J. Hum. Genet.* **19**:161.

Ueda, N., Satoh, C., Tanis, R. J. Ferrell, R. E., Kishimoto, S., Neel, J. V., Hamilton, H. D., and Baba, K. (1977). The frequency in Japanese of genetic variants of 22 proteins. II. Carbonic anhydrase I and II, lactate dehydrogenase, malate dehydrogenase, nucleoside phospholylase, triose phosphate isomerase, haemoglobin A and haemoglobin A_2. *Ann. Hum. Genet.* **41**:43.