Macaca mulata Carbonic Anhydrase. Crystallization and Physicochemical and Enzymatic Properties of Two Isozymes*

Thomas A. Duff† and Joseph E. Coleman

ABSTRACT: Two carbonic anhydrase isozymes, B and C, have been crystallized from the erythrocytes of *Macaca mulata*. They are homogeneous by ultracentrifugation, and the molecular weight of each isozyme is near 29,000 as determined by equilibrium and approach to equilibrium centrifugation and by calculation from the amino acid analysis. Free boundary and acrylamide gel electrophoresis detects minor faster moving components in both isozymes which may represent denatured enzyme. There are large differences in the amino acid compositions of the two isozymes but great similarities to those of the corresponding isozymes of human carbonic anhydrase. Both contain 1 g-atom of zinc/mole.

The ultraviolet optical rotatory dispersion indicates a similar three-dimensional structure for the two isozymes, but significant differences in detail. There are multiple Cotton effects in the region of the aromatic absorption bands and large troughs at 222–225 and 195 mμ. The C enzyme has threefold higher activity than the B enzyme catalyzing the hydration of CO_2. Both enzymes demonstrate metal ion dependent catalysis of the hydrolysis of *p*-nitrophenyl acetate, C > B. The enzymatic, spectral, and optical rotatory properties of a series of metallocarbonic anhydrases prepared from the B isozyme are described.

Carbonic anhydrase has been isolated in highly purified form from two mammalian sources, bovine and human erythrocytes. Human carbonic anhydrase exists as three isozymes: a major component, enzyme B, and two minor components, enzymes A and C (Lindskog, 1960; Nyman, 1961; Laurent *et al.*, 1962; Rickli *et al.*, 1964; Laurent *et al.*, 1965; Reynaud *et al.*, 1965). This pattern appears characteristic of individuals as well as pooled material (Rickli *et al.*, 1964). The bovine enzyme consists of two isozymes, A and B, similar to the human enzymes in molecular weights, general amino acid compositions, and a number of physicochemical properties (Lindskog, 1960; Nyman, 1961; Nyman and Lindskog, 1964). Two prominent features, however, distinguish the human from the bovine enzyme. The human B and C enzymes contain a single cysteine residue apparently absent in the bovine enzymes (Nyman and Lindskog, 1964; Rickli *et al.*, 1964). In addition, the human isozyme C has a specific activity some threefold higher than the B isozyme, while the bovine isozymes have similar specific activities (Gibbons and Edsall, 1964). Another preliminary characterization of primate carbonic anhydrases has been the comparison of the starch gel electrophoretic patterns for red cell hemolysates from a large number of baboons (Barnicot *et al.*, 1964). Two bands containing carbonic anhydrase activity were observed. Some individual animals had only the first of these bands, some only the second, some both. Recently, Tashian (1965) has carried out a much more extensive gel electrophoretic study of the hemolysates from 23 primate species. There were generally two isozymes of carbonic anhydrase identified, although one (corresponding to enzyme B in the nomenclature used in this paper) showed considerable quantitative and qualitative species variation.

The present paper reports the crystallization and physicochemical and enzymatic properties of two carbonic anhydrase isozymes, B and C, isolated from the erythrocytes of the common rhesus monkey, *Macaca mulata* (longtail variety). Molecular weights, zinc contents, amino acid compositions, and the ultracentrifugal, electrophoretic, and optical rotatory properties are strikingly similar to the human isozymes.

Materials and Methods

Reagents. Chemicals employed in the isolation procedure were reagent grade. All solutions used in connection with metal analyses and substitutions were made metal-free by extraction with dithizone in CCl_4 (Vallee and Gibson, 1948). Metal-free HCl was prepared from hydrogen chloride gas, and all glassware was acid cleaned (Thiers, 1957). Metal-free NaOH and bromothymol blue were prepared by passage over Chelex resin (Bio-Rad Laboratories). Spectrographically pure metals or their salts (Johnson Mattehey Co., Ltd.) were used to prepare the manganese, cobalt, nickel, copper, cadmium, and mercury carbonic anhydrases. Acetazolamide (2-acetylamino-1,3,4-thiadiazole-5-sulfonamide, Diamox) was kindly supplied by Dr. E. H. Dearborn of the Lederle Laboratories.

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† Present address: Western Reserve School of Medicine.

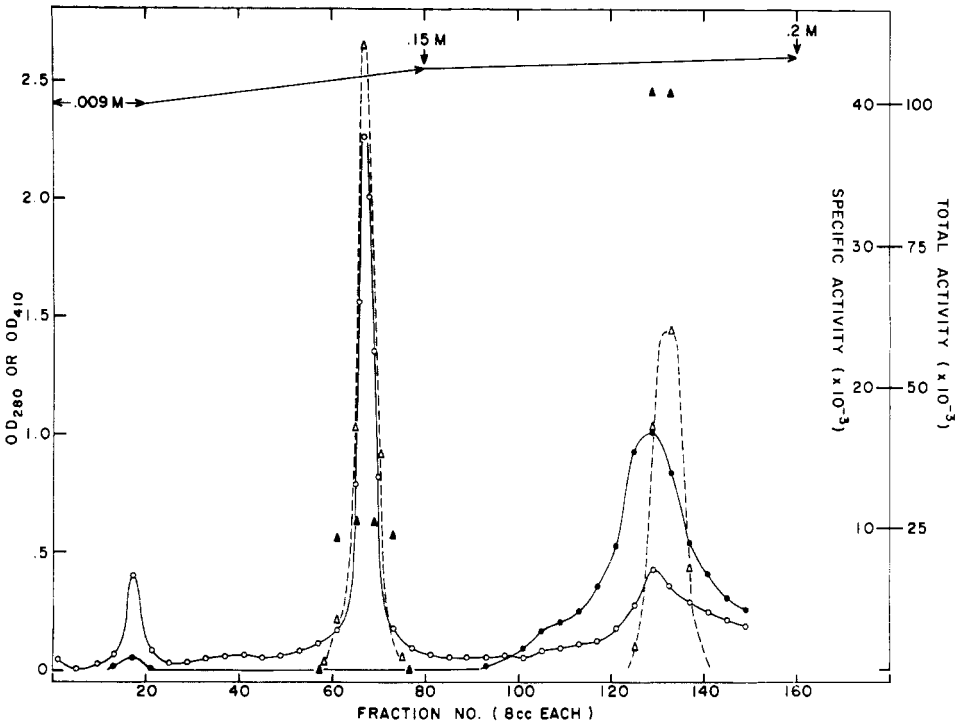

FIGURE 1: Chromatography of *M. mulata* carbonic anhydrases on hydroxylapatite. Column dimensions were 20 × 140 mm; 8-ml fractions were collected. Lyophilized powder from the initial Sephadex fractionation was dissolved in 10 ml of 0.009 M phosphate buffer, pH 6.8, 23°, and washed onto the column with 160 ml of the same buffer. The column was then eluted with two linear gradients of phosphate buffer, pH 6.8, from 0.009 to 0.15 M and from 0.15 to 0.2 M using the volumes indicated on the diagram. (○—○) optical density at 280 mμ; (●—●) optical density at 410 mμ; (△---△) total activity, U × milligrams of protein; (▲) specific activity, U. The enzyme emerging first from this column has been named B, that second, C.

Enzymatic Activities. HYDRATION OF CO_2 was measured by the procedure of Wilbur and Anderson (1948) (Rickli *et al.*, 1964; Coleman, 1965) employing bromothymol blue as indicator. Specific activity units, U, are defined by

$$U = \frac{10(T_b/T_e - 1)}{\text{mg of protein}}$$

where T_b is the time of the uncatalyzed reaction (color change of the indicator from blue to yellow-green) and T_e is the time for the enzyme-catalyzed reaction. Assays were performed at 4°.

ESTERASE ACTIVITY was measured using *p*-nitrophenyl acetate as the substrate (Tashian *et al.*, 1963) and following the optical density change at 400 mμ. The reaction cuvet contained 4.3×10^{-4} M *p*-nitrophenyl acetate in 0.025 M Tris, 5% acetonitrile, pH 7.5, 23°. Enzyme was added before the substrate and the reaction was started by the addition of the required amount of concentrated substrate dissolved in acetonitrile. The reaction mixture was read *vs.* a blank containing all components except the enzyme. Reaction rates were zero order and specific activities are expressed as micromoles of substrate hydrolyzed per minute per micromole of enzyme. An extinction coefficient of 1.82×10^4 M^{-1} cm^{-1} was determined for *p*-nitrophenol at 400 mμ, using the conditions of the reaction mixture.

ZINC ANALYSES were carried out by a dithizone procedure (Vallee and Gibson, 1948) and by atomic absorption spectroscopy (Fuwa and Vallee, 1963) using a Jarrell-Ash spectrometer.

AMINO ACID ANALYSES were performed by ion-exchange chromatography according to the method of Spackman *et al.* (1958). Hydrolyses were carried out for 22 hr in 6 N metal-free HCl at 110°. No corrections have been applied for losses of serine and threonine during hydrolysis. A Beckman amino acid analyzer, Model 120B, equipped with an Infotronics Model CRS-10A digital readout system, was employed. The instrument was modified by the incorporation of a 20-cm path length flow cell permitting tenfold greater sensitivity. Half-cystine was determined as cysteic acid after performic acid oxidation according to Moore (1963). Tryptophan was determined by the method of Goodwin and Morton (1946).

PROTEIN CONCENTRATIONS were determined from the optical density at 280 mμ. Molar absorptivities of 4.88 $\times 10^4$ M^{-1} cm^{-1} for enzyme B and 5.35 $\times 10^4$ M^{-1} cm^{-1} for enzyme C were determined by the trichloroacetic

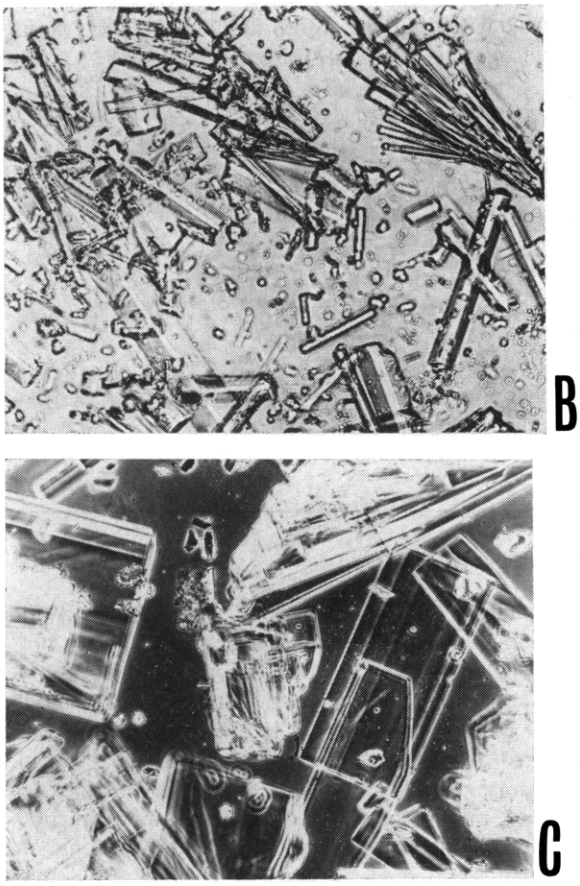

FIGURE 2: Crystals of *M. mulata* carbonic anhydrases B and C. Photomicrographs were taken under phase-contrast illumination at 100×, width of picture = 350 μ.

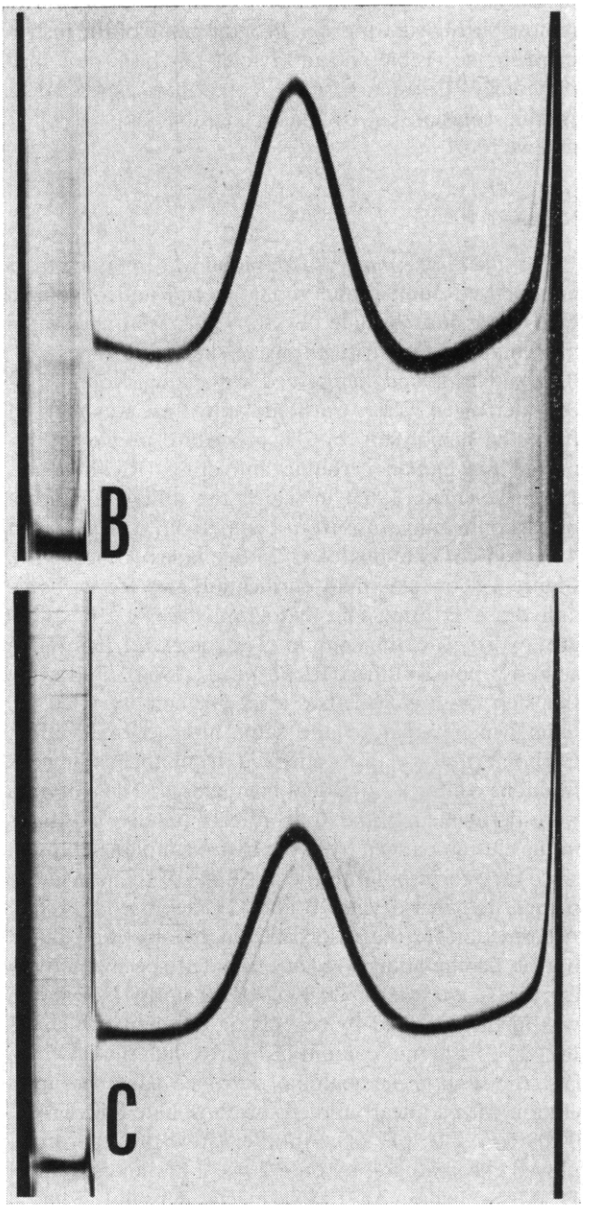

FIGURE 3: Sedimentation patterns of *M. mulata* carbonic anhydrases B and C. Conditions: 0.1 M phosphate buffer, pH 6.8, rotor speed, 59,780 rpm, 20°. B, 20.7 mg of protein/ml, 2 hr and 30 min after reaching full speed, bar angle = 55°. C, 21.6 mg of protein/ml, 2 hr and 14 min after reaching full speed, bar angle = 60°.

acid precipitation method of Hoch and Vallee (1953) assuming a molecular weight of 30,000 for both enzymes (*vide infra*).

Ultracentrifugation. Sedimentation velocity and equilibrium experiments were performed with a Spinco Model E analytical ultracentrifuge equipped with a phase plate as a schlieren diaphragm. Sedimentation coefficients were determined from experiments in which the rotor was operated at 59,780 rpm, 20°. Equilibrium and approach to equilibrium experiments were done at 15,220 rpm, 20°. Archibald experiments in which data at the cell bottom were used for calculating molecular weights employed silicone oil below the solution column. A partial specific volume of 0.74 was calculated from the amino acid composition of the enzyme and used in the calculation of $s_{20,w}$ and molecular weights.

Electrophoresis. ACRYLAMIDE GEL ELECTROPHORESIS was performed with a Canalco Model 6 disk electrophoresis apparatus. Gels were made up according to the directions supplied by the manufacturer. Two gels were employed, the first stacks at pH 5.0 (β-alanine–acetate buffer, pH 5.0) and runs at pH 4.3, the second gel stacks at pH 8.3 (Tris–glycine buffer, pH 8.3) and runs at pH 9.5. For one experiment the pH 4.3 gel and buffer were made up containing 8 M urea.

MOVING BOUNDARY electrophoresis was performed with a Spinco Model H electrophoresis and diffusion apparatus.

Absorption spectra were obtained with a Zeiss PMQII spectrophotometer or with a Perkin-Elmer Model 350 recording spectrophotometer equipped with a scale expander.

Optical rotatory dispersion (ORD) was measured with a Cary Model 60 recording spectropolarimeter, em-

ploying a water-jacked cell of 10-mm path length with removable quartz windows. Programming of the instrument in the visible and ultraviolet has been described previously (Coleman, 1965). Rotation is expressed as specific rotation $[\alpha]$ or molar rotation $[M] = [\alpha] \times$ mol wt/100.

Results

Enzyme Purification. Whole blood was obtained from anesthetized adult rhesus monkeys and anticoagulated with potassium oxalate. Erythrocytes were separated immediately by centrifugation, washed three times with 0.15 M NaCl, and hemolyzed with equal volumes of cold deionized water. Carbonic anhydrase was purified from the hemolysate by the procedure previously reported for human carbonic anhydrase (Rickli *et al.*, 1964; Coleman, 1965) in which the initial separation of carbonic anhydrase from hemoglobin was made on 4 × 150 cm columns of G-75 Sephadex (bead form). Figure 1 shows the final purification step for carbonic anhydrase B using chromatography on a 20 × 140 mm hydroxylapatite column (Tiselius *et al.*, 1956) with cellulose powder filler (Rickli *et al.*, 1964). Following this step the B enzyme had a specific activity of 10,500 units which remained the same after crystallization. Carbonic anhydrase C emerged from the column in fractions containing residual hemoglobin. The isozymes were designated B and C from their relative positions in the elution pattern from the hydroxylapatite column, since they correspond to the positions of the previously named human isozymes B and C (Rickli *et al.*, 1964). A correction for the absorbancy at 280 mμ contributed by the hemoglobin gave an estimated specific activity for the C enzyme of 30,000–40,000 units. Isozyme C was further purified by reapplying the active fractions to a 20 × 140 mm column of hydroxylapatite in which case the residual hemoglobin remained adsorbed upon elution with a linear gradient of phosphate buffer from 0.009 to 0.2 M, pH 6.8. An alternative procedure employed chromatography on a 20 × 140 mm column of DEAE-Sephadex A-50 (Pharmacia, Upsala). Elution of the latter column with a linear gradient of Tris buffer from 0.001 to 0.2 M, pH 8.8, eluted enzyme C as a narrow peak within the first 200 ml. This position was well before the position of any hemoglobin or of isozyme B when applied to this column.

Crystallization. Crystals of the B and C isozymes (Figure 2) were obtained from enzyme samples which had not undergone lyophilization at any point during the purification process. Enzyme solutions were concentrated by dialysis *vs.* Aquacide (Calbiochem) in powder form, and the final solutions, *ca.* 1% protein, dialyzed *vs.* 0.6 M ammonium sulfate–0.05 M Tris buffer, pH 8.5, 4°, to which solid ammonium sulfate was added periodically to raise the concentration by 0.6 M increments (Strandberg *et al.*, 1962). Isozyme B crystallized at 2.5 M ammonium sulfate while isozyme C crystallized best at 3.0 M ammonium sulfate.

Zinc Content and Specific Activity. Table I shows the zinc content and specific activity at the various steps during the purification procedure beginning with the hemolysate. There is a parallel 30-fold rise in both zinc content and specific activity using pure isozyme B as the referent. Within the accuracy of the methods, all of the zinc content of a red cell hemolysate is accounted for by the zinc contained in the carbonic anhydrase. Although isozyme C has a threefold greater activity, this is not reflected in the less pure fractions because of the small percentage of total carbonic anhydrase represented by isozyme C (Figure 1). Both purified enzymes have *ca.* 2200 μg of zinc/g of protein or 1 g-atom/mole assuming a molecular weight of 30,000 for both proteins. The specific activities shown are for the lyophilized material; however, the crystalline enzymes show the same specific activity within the error of the measurement.

TABLE I: Zinc Content and Specific Activity during Purification of *M. mulata* Carbonic Anhydrase.

Fraction	Zn (μg/g)	Sp Act. (U)
Hemolysate of washed erythrocytes	72	376
Pooled high act. fractions after initial Sephadex fractionation	1,000	4,620
Pooled high act. fractions after hydroxylapatite chromatography (enzyme B)	2,260	10,500
Pooled high act. fractions after rechromatography of enzyme C on DEAE-Sephadex (enzyme C)	2,220	32,000

Apo- and Metallocarbonic Anhydrases B. Dialysis of carbonic anhydrase B *vs.* a 500-fold volume excess of 5×10^{-3} M 1,10-phenanthroline in 0.04 M sodium acetate, pH 5.5, 4°, results in slow loss of both zinc and activity. After 20 days with one change of the dialysate, the preparation contained <0.04 g-atom of zinc/mole and had a specific activity of 150 units. Metallocarbonic anhydrases were prepared from this preparation as described previously (Coleman, 1965).

Amino Acid Compositions of Isozymes B and C are recorded in Table II. The total number of amino acid residues is very similar if not identical in the two isozymes. These particular analyses happen to give almost the same total number, 255 and 256, but the precision of the methods is at best ±1 residue for several of the amino acids.

Isozyme C contains six more lysyl, six less aspartyl, 12 less seryl, seven more glycyl, and five more leucyl residues than isozyme B. The contents of the other amino acids differ by 1–4 residues between the two isozymes. The amino acid composition for each isozyme is strikingly similar to the corresponding human isozymes as shown in Table II by representative data taken from the literature (Nyman and Lindskog, 1964; Gui-

TABLE II: Amino Acid Composition of Monkey Carbonic Anhydrases B and C. Comparison with the Amino Acid Compositions of the Human and Bovine Enzymes.

Amino Acid Residue	Monkey B		Human B	Bovine B	Monkey C		Human C
	Found	Nearest Integer	Guidotti and Spahr[a]	Nyman and Lindskog (1964)	Found	Nearest Integer	Nyman and Lindskog (1964)
Lysine	18.2	18	18	19	24.4	24	24
Histidine	9.1	9	11	11	11.6	12	12
Arginine	7.02	7	7	9	7.85	8	7
Aspartic acid	35.6	36	31	32	29.5	30	29
Threonine	13.0	13	13	15	11.4	11	13
Serine	30.0	30	27	16	18.1	18	19
Glutamic acid	21.8	22	22	24	25.6	26	27
Proline	17.2	17	15	20	15.7	16	17
Glycine	15.1	15	15	20	22.2	22	22
Alanine	15.7	16	17	17	11.7	12	13
Half-cystine	1.05	1	1	0	1.03	1	1
Valine	16.3	16	17	20	13.9	14	17
Methionine	1.08	1	2	3	1.95	2	1
Isoleucine	10.1	10	10	5	9.94	10	9
Leucine	19.0	19	19	26	23.5	24	25
Tyrosine	8.8	9	8	8	7.21	7	8
Phenylalanine	10.3	10	10	11	10.8	11	12
Tryptophan	7.4	7	6	7	6.8	7	7
Zn		1	1	1		1	1
Total residues		256	249	264		255	263

[a] Reported in Rickli *et al.*, 1964.

dotti and Spahr in Rickli *et al.*, 1964). Most remarkable is the maintenance of the characteristic differences in amino acid composition between isozymes B and C of the two species. The number of residues of a particular amino acid are the same or differ by one between the monkey and human enzymes in 11 out of the 18 types of residues in enzyme B and in 14 out of 18 in enzyme C. Such a comparison with the bovine B enzyme gives only five out of 18 (Table II).

Ultracentrifugal Properties. Figure 3 shows the sedimentation patterns of the B and C isozymes. The values of the sedimentation coefficients as a function of protein concentration are graphed in Figure 4. The protein concentrations are corrected for the dilution occurring in the sector-shaped cell and the concentrations shown on the abscissa of Figure 4 are the average values between the beginning and the end of each run (Schachman, 1957). An $s_{20,w}$ of 2.94 ± 0.01 S was determined for enzyme B by extrapolating to zero concentration. The slope, k, of the line calculated by the least mean squares method is -2.34×10^{-2} S ml mg^{-1}. Two single values of $s_{20,w}$ for the C isozyme fall very close to the same line.

Molecular Weight (M). Determination of the molecular weight of the B enzyme by the Archibald method, following the procedure of Schachman (1957), gave values of 29,800 ($M_{meniscus}$) and 28,400 (M_{bottom}). Individual runs were then extended to equilibrium and the molecular weight determined from the slope of $(1/r\ dn_c/dr)$ *vs.* n_c according to the methods of Van Holde and Baldwin (1958), giving a z-average molecular weight of 27,300. The molecular weight calculated from the amino acid analysis (Table II) is 28,300 for enzyme B. The z-average molecular weight for the C enzyme determined by the methods of Van Holde and Baldwin (1958) is 29,800 compared to 28,600 calculated from the amino acid analysis in Table II.

Thus both isozymes have similar molecular weights approaching 30,000, the value previously assigned to carbonic anhydrases from other mammalian sources (Lindskog, 1960; Nyman and Lindskog, 1964; Rickli *et al.*, 1964). The values obtained here by the different methods would seem to indicate a molecular weight nearer 29,000 for the monkey isozymes, although the deviation of the methods is still too great for a precise assignment.

Electrophoretic Properties of the B and C Isozymes. While isozymes B and C are homogeneous in the ultracentrifuge, free boundary electrophoresis detects two minor components in enzyme B, one slower moving and one faster moving, the latter separating only on longer runs (Figure 5). Isozyme C appears homo-

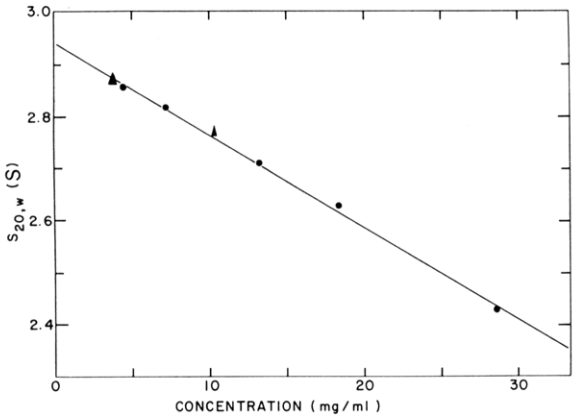

FIGURE 4: Concentration dependence of $s_{20,w}$ for isozymes B and C. (●) carbonic anhydrase B; (▲) carbonic anhydrase C. Concentrations are the average values between the beginning and end of the run determined by correcting for the sector-shaped cell.

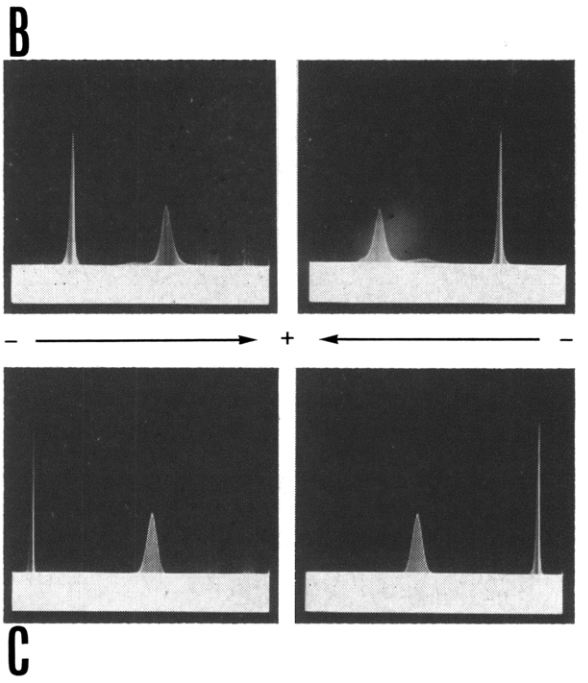

FIGURE 5: Free boundary electrophoresis of *M. mulata* carbonic anhydrases B and C. Conditions: 0.02 M sodium Veronal–0.08 M NaCl, pH 8.6, 1°, 15.2 v/cm, enzyme B = 11.7 mg/ml, enzyme C = 9.0 mg/ml. Descending boundaries are shown at left; B, 143 min of migration; C, 324 min of migration. Ascending boundaries are shown at right; B, 174 min of migration; C, 324 min of migration. Initial boundaries are shown in each picture. Mobilities are -2.28×10^{-5} cm^2 v^{-1} sec^{-1} and -1.29×10^{-5} cm^2 v^{-1} sec^{-1} for enzymes B and C, respectively.

geneous in the free boundary run with a mobility at pH 8.6 much smaller than isozyme B (Figure 5), implying a considerably higher isoelectric point, as expected from the amino acid analysis and in agreement with data on the human isozymes (Rickli *et al.*, 1964). The minor components of isozyme B vary between preparations, are almost absent in some, and may represent a small amount of denatured enzyme (*vide infra*). In contrast, acrylamide gel electrophoresis at pH 9.5 demonstrates second components for both isozymes (Figure 6). Under these conditions enzyme C has more of the minor component than B. At pH 4.3 both native isozymes move toward the cathode very slowly and barely enter the gel. In urea, guanidine hydrochloride, or at acid pH, the human isozymes have been shown to undergo marked conformational changes (Rickli *et al.*, 1964; Myers and Edsall, 1965; Coleman, 1965) accompanied by unmasking of a number of groups that can be protonated in the region pH 4–7, such that at pH 4.5 there are eight extra protons bound to the acid-denatured protein (Riddiford, 1965; Riddiford *et al.*, 1965). The monkey isozymes undergo very similar conformational changes under the same conditions. Hence, in an attempt to increase the rate of migration at pH 4.3, the gel electrophoresis was run in 8 M urea. This results in a very striking increase in mobility of both isozymes and at the same time they split into a number of electrophoretically separable components, four for isozyme B and five for isozyme C.

Spectra and Optical Rotatory Dispersion of Monkey Metallocarbonic Anhydrases. Figure 7 shows the ultraviolet ORD from 450 to 190 mμ for isozymes B and C. The general profiles of the curves for the two proteins are quite similar, but there are some significant differences in detail. Isozyme C shows moderately less negative rotation in the near ultraviolet, $[\alpha]_{450}$ −58° compared to $[\alpha]_{450}$ −110° for enzyme B. The first major trough for the B enzyme is located at 222 mμ, $[\alpha]_{222}$ −2400°, while that for the C isozyme occurs at 225 mμ and is not as deep, $[\alpha]_{225}$ −1800°. However, in the aromatic region the dispersion of enzyme C crosses that of enzyme B and falls to considerably more negative values of [α] before it crosses again at 226 mμ. Both enzymes have complex anomalous rotatory dispersion of relatively small amplitude in the region of the aromatic absorption bands from 250 to 300 mμ. Almost identical small Cotton effects are present at ~290 mμ, peaks at 292 mμ, troughs at 287 mμ, amplitudes of ~100°. These are followed by several small secondary peaks rising to a rather broad peak at 265 mμ. While this broad maximum with a peak at 265 mμ is present in both isozymes, it is considerably more developed in isozyme B, rising some 100° further from the minimum at 287 mμ.

In the far-ultraviolet region the isozymes show small positive rotation of a few hundred degrees in the region 202–210 mμ followed by troughs of large negative Cotton effects at 195 mμ, $[\alpha]_{195}$ −5200° and −4700° for enzymes B and C, respectively. Figure 8 shows the near ultraviolet absorption spectra of the solutions of enzymes B and C used to determine the detailed ORD of

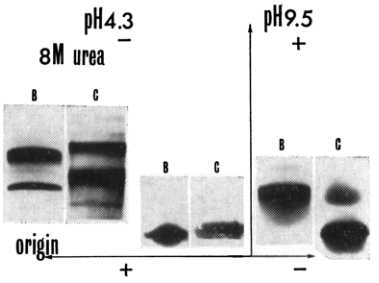

FIGURE 6: Acrylamide gel electrophoresis of *M. mulata* carbonic anhydrases B and C. Conditions were as outlined in methods.

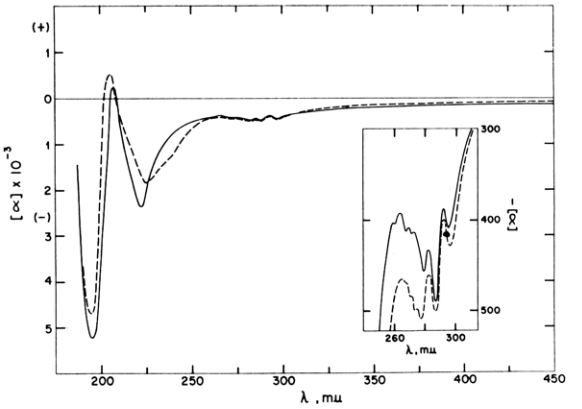

FIGURE 7: Ultraviolet ORD of *M. mulata* carbonic anhydrases B (——) and C (----). Concentrations of protein varied from 2×10^{-6} M for the far-ultraviolet determinations to 5×10^{-5} M for the near-ultraviolet determinations. Carbonic anhydrase was dissolved in 0.025 M Tris, pH 7.5, 27°. Insert: Detail of the aromatic regions. Protein concentrations were varied from 1×10^{-5} to 1×10^{-4} M in 0.025 M Tris, pH 7.5, 27°.

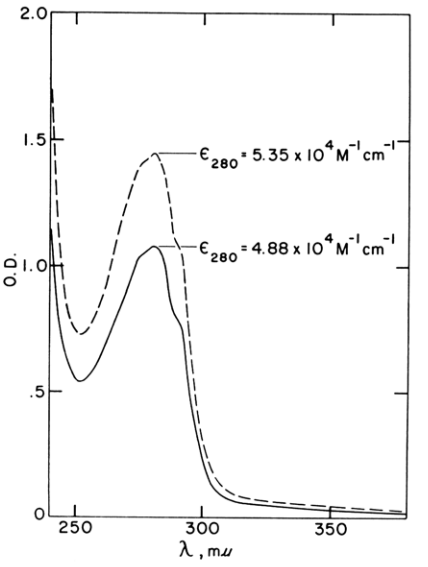

FIGURE 8: Near-ultraviolet absorption spectra of *M. mulata* carbonic anhydrases B (——) and C (----). Conditions: 0.025 M Tris, pH 7.5, 23°; B enzyme, 2.2×10^{-5} M; C enzyme, 2.7×10^{-5} M. Molar absorptivities given on the figure were determined by the method of Hoch and Vallee (1953).

the aromatic region shown in Figure 7. Except for the slightly higher extinction coefficient at 280 mμ shown by enzyme C, the isozymes have similar spectra with a peak at 280 mμ and a tryptophan shoulder at 290 mμ.

In the visible region of the spectrum, isozymes B (Figure 9) and C have plain dispersion curves. As in the case of the bovine and human enzymes (Lindskog and Malmström, 1962; Lindskog and Nyman, 1964), substitution of cobalt and copper for zinc induces characteristic visible absorption bands into the protein. Monkey cobalt carbonic anhydrase B has four maxima located at 520 mμ (ε 190), 550 (ε 300), 615 (ε 202), and 640 (ε 195), while copper carbonic anhydrase B has a broad maximum centered at 750 mμ (ε 100) (Figure 9). The band positions are almost identical with those observed in the human enzyme B, and as in the human enzyme the bands are optically inactive (Coleman, 1965).

Combination of sulfonamide and metal-binding inhibitors with either the cobalt or copper human (or bovine) enzymes has been shown to result in characteristic shifts of the visible absorption bands (Lindskog and Malmström, 1962; Lindskog, 1963; Lindskog and Nyman, 1964; Coleman, 1965). These spectral shifts in the human B enzymes are accompanied by the appearance of anomalous rotatory dispersion in the area of the copper and cobalt absorption bands (Coleman, 1965). Very similar phenomena occur on combination of sulfonamide and metal-binding inhibitors with the cobalt and copper monkey B enzymes as shown by two representative examples in Figure 9. The complex of a sulfonamide inhibitor, acetazolamide, with the monkey cobalt enzyme B has absorption maxima at 520 mμ (ε 315), 580 mμ (ε 540), and 605 mμ (ε 510). These bands show positive asymmetric anomalous rotatory dispersion with a peak at 592 mμ, a crossover point compared to the zinc enzyme at 555 mμ, and trough at 530 mμ. Total molar rotation is 4500°. The complex of a metal-binding inhibitor, CN^-, with the copper enzyme intensifies the absorption maximum and shifts it to 695 mμ (ε 125). Positive anomalous rotatory dispersion is induced centered at *ca.* 680 mμ with a peak at 790 mμ and a trough at 575 mμ. Total molar rotation approaches 6000°.

Enzymatic Activities of Monkey Metallocarbonic Anhydrases. Catalysis of the hydration of CO_2 by the two Zn(II) isozymes and Mn(II), Co(II), Ni(II), Cu(II), Cd(II), and Hg(II) carbonic anhydrases B is shown in Table III. Significant catalysis is limited to the two Zn(II) enzymes and Co(II) carbonic anhydrase B. The small activities shown by Mn(II) and Ni(II) are difficult

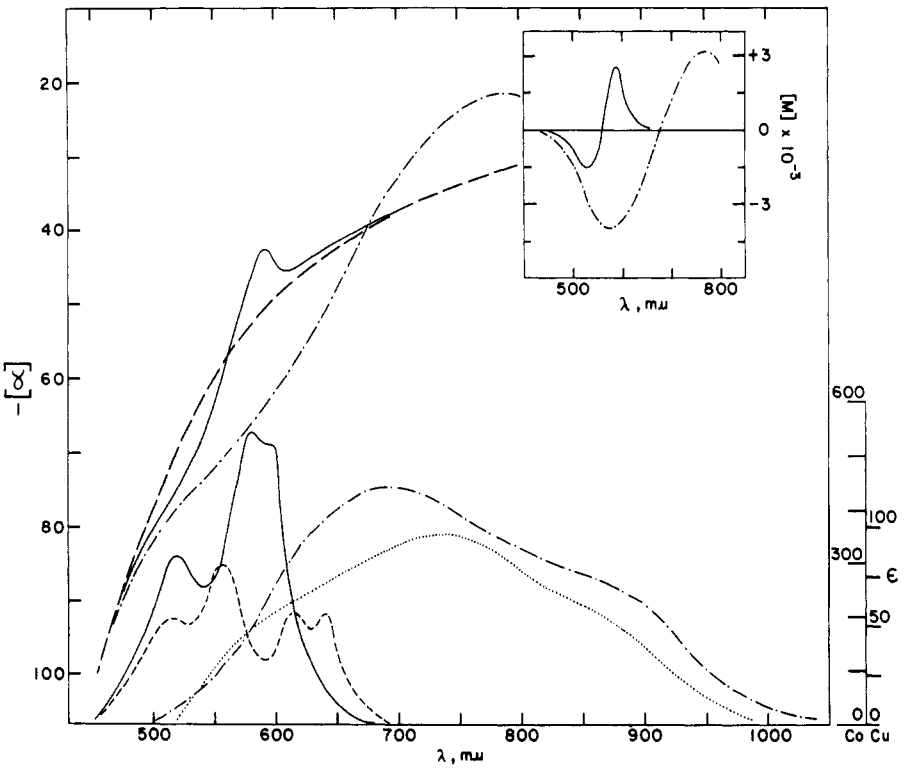

FIGURE 9: Visible ORD and spectra of Zn(II), Co(II), and Cu(II) carbonic anhydrases B and their inhibitor complexes. (-----) ORD of Zn(II), Co(II), and Cu(II) carbonic anhydrase B. (——) ORD of Co(II) carbonic anhydrase B plus acetazolamide. (— · —) ORD of Cu(II) carbonic anhydrase B plus CN^-. Insert: Difference ORD, (——) ($[M]_\lambda$ for Co(II) carbonic anhydrase B + acetazolamide) − ($[M]_\lambda$ for Co(II) carbonic anhydrase B). (— · —) ($[M]_\lambda$ for Cu(II) carbonic anhydrase B + CN^-) − ($[M]_\lambda$ for Cu(II) carbonic anhydrase B). Spectra: (-----) Co(II) carbonic anhydrase B; (——) Co(II) carbonic anhydrase B + acetazolamide; (. . . .) Cu(II) carbonic anhydrase B; (— · —) Cu(II) carbonic anhydrase B + CN^-. ϵ = molar absorptivity, M^{-1} cm^{-1}. Note separate scales for Co and Cu. Although the copper absorption bands are *ca.* one-fifth as intense as those of cobalt, rotatory power induced by inhibitor is greater. Zn(II) and Co(II) carbonic anhydrase were 3×10^{-4} M contained in 0.025 M Tris, pH 9.9, 27°. Cu(II) carbonic anhydrase was 8.4×10^{-4} M at pH 7.0; 0.025 M sodium acetate was added to hold Cu(II) in solution. Acetazolamide was added as a threefold molar excess, CN^- as a tenfold molar excess.

to evaluate because of possible zinc contamination in the assay mixture. The concentrated enzymes all contained <0.04 g-atom of zinc/mole. Several investigators have reported on the esterase activity shown by the other mammalian carbonic anhydrases (Tashian *et al.*, 1963; Malmström *et al.*, 1964; Pocker and Stone, 1965). The esterase activities (measured by the hydrolysis of *p*-nitrophenyl acetate) of the monkey enzymes are given in Table III. As in the hydration of CO_2, significant activity is confined to both Zn(II) isozymes and the Co(II) enzyme B. Again the low activities shown by the other first transition and IIB metallocarbonic anhydrases are difficult to evaluate, since their magnitudes approach the level expected to result from zinc contamination; however, they are significantly greater than the activity shown by the apoenzyme in the same assay mixture. The Tris complexes of the first transition metal ions show weak catalysis of the hydrolysis of *p*-nitrophenyl acetate, highest for Co(II). The rates given in Table III have been corrected for these blank values.

Enzyme C, while showing 130% of the esterase activity of enzyme B, does not approach the threefold greater activity it displays in the hydration of CO_2. On the other hand, Co(II) carbonic anhydrase B has a twofold greater esterase activity than the corresponding Zn(II) enzyme, while demonstrating <50% the activity of the Zn(II) enzyme when catalyzing the hydration of CO_2. Since the esterase reaction shows marked substrate inhibition, it should be emphasized that the esterase activities are compared at a substrate concentration of 4×10^{-3} M. Under the present assay conditions, there is a broad maximum between 4 and 8×10^{-3} M substrate followed by very marked inhibition at higher substrate concentrations.

Discussion

M. mulata erythrocyte carbonic anhydrase consists of at least two isozymes, a characteristic feature of all carbonic anhydrases isolated thus far from mammalian erythrocytes (Nyman, 1961; Laurent *et al.*, 1962;

TABLE III: Enzymatic Activities of the Metallocarbonic Anhydrases (*M. mulata*).

Enzyme	Hydration of CO_2[a] (U)	Esterase[b] (μmoles/min μmole of enzyme)
Zn(II) native B	10,500[c]	14.0
Zn(II) native C	32,000[c]	18.0
Apoenzyme B	150[d]	0.09
Mn(II) + apoenzyme B	640[d]	0.78
Co(II) + apoenzyme B	3,600[c]	33.0
Ni(II) + apoenzyme B	25[d]	0.38
Cu(II) + apoenzyme B	0[c]	0.71
Cd(II) + apoenzyme B	0[c]	0.26
Hg(II) + apoenzyme B	0[c]	0.21

[a] Determined by the colorimetric method of Wilbur and Anderson (1948) (see Methods). [b] Assayed by the hydrolysis of *p*-nitrophenyl acetate (see Methods); 2–6 $\times$ 10^{-6} M enzyme (0.06–0.18 mg) contained in 1.0 ml of reaction mixture. [c] 0.004–0.007 mg of enzyme contained in 3.0 ml of reaction mixture. [d] 0.015–0.018 mg of enzyme contained in 3.0 ml of reaction mixture.

Lindskog and Nyman, 1964; Rickli *et al.*, 1964). Both the enzymatic and physiochemical properties of the two rhesus monkey isozymes are remarkably similar to the corresponding carbonic anhydrase isozymes from human erythrocytes. Their chromatographic behavior (Figure 1), their molecular weights, their zinc contents (Table I), the presence of a single cysteine residue in both isozymes, the characteristic differences in amino acid composition between isozymes B and C (Table II), their spectra and ORD (Figure 7–9) make them almost indistinguishable from the human carbonic anhydrases. A functional similarity is also present in that the monkey enzymes have catalytic activities of the same magnitude as the human enzymes and show the rather unusual human characteristic that isozyme C has three times the catalytic activity of isozyme B (Table I) (Rickli *et al.*, 1964).

It would appear that the distinguishing features of the two isozymes of primate carbonic anhydrase evolved at a rather early stage in primate evolution and have remained essentially unchanged since. The differences in amino acid composition are far more striking between the isozymes of one species than they are between the same isozymes from different primate species. Only in comparing the primate enzyme B with the bovine enzyme B do the differences in amino acid composition approach those between the pairs of primate isozymes (Table II).

The rather considerable difference in amino acid composition between two isozymes containing the same number of residues necessitates differences in amino acid sequence. There must be at least 56 positions in the 255 residue sequence where substitutions occur between enzyme B and C (Table II). While there are moderate differences in three-dimensional structure of the two isozymes as indicated by the ultraviolet ORD (Figure 7), the differences in amino acid sequence must be accommodated in a structure which retains the chemical interactions leading to the distinctive carbonic anhydrase three-dimensional structure previously indicated by the ORD of the human isozymes (Myers and Edsall, 1965; Coleman, 1965). This includes the structure of the peptide backbone presumably contributing to the far-ultraviolet Cotton effects and the distinctive aromatic structure as reflected in the aromatic Cotton effects.

The moderate differences in three-dimensional structure between isozymes B and C indicated by the ultraviolet ORD presumably reflect their considerable differences in amino acid composition and consequent sequence. The ORD difference is most marked in the area of the first major trough which is 600° more negative in enzyme B, probably contributing to the more negative rotation of enzyme B in the near ultraviolet. The differences in the precise position of the first major trough in the two isozymes may be a reflection of differences in the environment of the aromatic chromophores, since the anomalous rotatory dispersion in the aromatic region is somewhat different in the two isozymes, especially in the 250–280-mμ region. This could easily influence the dispersion in the area between 225 and 250 mμ. There are two less tyrosines in enzyme C which does show a less prominent peak at 265 mμ. However, it cannot be assumed that all the aromatic residues contribute equally to the anomalous rotatory dispersion and the differences could well be reflections of structure rather than number. While the ORD of the monkey isozymes is very similar to that of the human isozymes, there is at least one minor difference in that the rotation of the monkey B enzyme is not quite as negative in the near-ultraviolet and visible regions of the spectrum, $[\alpha]_{450}$ $-110°$ rather than $-150°$ observed in the human enzyme. This is probably partially accounted for by the fact that the first major trough is 200° less negative in the monkey B enzyme than the human B enzyme (Coleman, 1965).

The presence of six more lysyl and six less aspartyl residues in isozyme C might be expected to make a considerable difference in electrophoretic properties between the two isozymes. In general the predicted behavior is borne out (Figure 5 and 6). At alkaline pH the faster migration of enzyme B toward the anode implies that it contains fewer positive and/or more negative charges than enzyme C. As expected, at pH 4.3 the relative rates of migration for isozymes B and C are reversed. A remarkable feature of the urea treatment of this enzyme is that the "denaturation" produces several electrophoretically distinct species (Figure 6). This may indicate that the conformational change induced by urea and characterized by striking ORD changes (Myers and Edsall, 1965; Coleman, 1965)

results in several relatively stable conformers and that equilibrium between them occurs slowly. Such conformational changes could induce charge differences by unmasking protonatable groups or in the case of the gel could alter the behavior of the protein toward the molecular sieving action. The existence of minor faster moving components at pH 9.5 could simply reflect a slightly different conformation of the molecule rather than a different primary structure.

Isozyme C consistently crystallizes as large plate-like crystals while isozyme B crystallizes as somewhat smaller and more regular polyhedra (Figure 2). It is not impossible that relatively minor differences in three-dimensional structure, especially the location of charged groups on the surface of the molecule, could influence gross crystal morphology by favoring or inhibiting growth along one or another crystal axis.

The structure and symmetry surrounding the metal ion is apparently the same in the monkey and human B enzymes, since the visible spectra and ORD of the cobalt and copper B enzymes are almost identical with those of the human enzyme and undergo the same changes on combination with sulfonamide and metal-binding inhibitors (Figure 9) (Coleman, 1965). On the basis of these spectral and ORD findings, a square-planar configuration for the metal-binding site has been postulated (Coleman, 1965). Like human metallocarbonic anhydrases, the two isozymes demonstrate activity not only in the hydration of CO_2, but in the hydrolysis of ester bonds as well (Table III) (Tashian *et al.*, 1963, 1964; Lindskog and Nyman, 1964; Malmström *et al.*, 1964). The esterase activities shown by the monkey enzymes under these conditions are somewhat higher than those reported for the human enzyme (Malmström *et al.*, 1964).

Although all the first transition and IIB metal ions apparently form complexes with the active site (Malmström *et al.*, 1964; Coleman, 1965), the electronic structures assumed by Co(II) and Zn(II) appear to be the only ones suitable for rapid catalysis of either type of reaction. The chemical reasons for this remain to be elucidated. The complexity of this reaction between protein, metal ion, and substrate is indicated by varying both the metal ion and the type of substrate. Substitution of Co(II) for Zn(II) has exactly opposite effects on the rate of hydration of CO_2 and of hydrolysis of the ester, a circumstance previously reported for the human enzyme (Malmström *et al.*, 1964).

Judging from the physicochemical and enzymatic characteristics of their carbonic anhydrases, the rhesus monkey does not appear to be very far removed from man on the scale of biochemical evolution. This is also indicated by the recent report of the amino acid composition of heart cytochrome *c* from this species (Rothfus and Smith, 1965) which is identical with that for human heart cytochrome *c* except for a single amino acid substitution.

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Organic Modifications of Metallocarboxypeptidases*

Joseph E. Coleman,† Pablo Pulido,‡ and Bert L. Vallee#

ABSTRACT: Both acetylation and iodination of tyrosyl residues and substitution of other metal ions for the native zinc atom alter the catalytic specificity of carboxypeptidase A. Both types of modification now have been employed jointly to explore further the intramolecular relationship between the catalytically essential metal atom and the functional tyrosines. The studies demonstrate that while the enzymatic consequences of such different chemical reactions are similar the mechanisms which bring them about are not. Acetylation or iodination of zinc carboxypeptidase abolishes peptidase activity through curtailment of the binding of synthetic dipeptide substrates such as carbobenzoxyglycyl-L-phenylalanine. Deacetylation of tyrosyl groups with hydroxylamine restores both binding and peptidase activity. However, both acetyl- or iodocarboxypeptidase bind the ester hippuryl-*dl*-β-phenyllactate.

The enzymatic consequences of metal substitution conjointly with acetylation or iodination of carboxypeptidase are not additive. The esterase activities of zinc, cadmium, cobalt, nickel, and manganese acetyl- and iodocarboxypeptidases are predominantly those characteristic of the respective metals. The peptidase activities, however, are those expected on acetylation or iodination of tyrosine. The esterase activity of mercury carboxypeptidase is comparable to that of the native zinc enzyme; but the mercury enzyme does not hydrolyze peptides even though these substrates are bound. On acetylation or iodination the esterase activity of mercury carboxypeptidase is abolished even though the ester substrate hippuryl-*dl*-β-phenyllactate continues to be bound. Further, under appropriate conditions, Zn^{2+} replaces mercury; the esterase activities then return to those of acetyl or iodo zinc carboxypeptidase indicating the distinctive but interdependent contribution both of the metal ion and of modification of amino acid residues to the enzymatic process.

A number of metallocarboxypeptidases have been prepared by replacing zinc with a series of other metal ions. When assayed under identical conditions these metalloenzymes differ from the native enzyme in specific activity and in some cases even in apparent substrate specificity. These replacement studies have presented a means to explore the role of the metal in the mechanism of action of carboxypeptidase and have also assisted in the delineation of the metal-binding site (Vallee *et al.*, 1960a,b; Coleman and Vallee, 1961; Vallee *et al.*, 1961). Acetylation and iodination of carboxypeptidase also has major functional consequences due to modification of two of the 19 tyrosyl residues of the enzyme (Riordan and Vallee, 1963; Simpson *et al.*, 1963; Simpson and Vallee, 1966).

A working hypothesis which invoked the operation of a minimal number of groups resulted in a scheme which attempted to attribute all functional changes to the modification of a single residue. The resultant model was proposed in order to generate and test alternate hypotheses concerning the possible mechanisms of action of the enzyme. The model neither assigned any role to the specificity-determining C-terminal carboxyl group of the substrate nor could it take into account possible effects of modification on substrate binding, since only minimal information on these

* From the Biophysics Research Laboratory, Department of Biological Chemistry, Harvard Medical School, and the Division of Medical Biology, Peter Bent Brigham Hospital, Boston, Massachusetts. *Received March 3, 1966.* This work was supported by grants-in-aid (HE-07297) from the National Institutes of Health of the Department of Health, Education and Welfare.

† Postdoctoral Fellow of the National Institutes of Health of the Department of Health, Education and Welfare. Present address: Department of Biochemistry, Yale University.

‡ Recipient, Lederle International Fellowship. Present address: Department of Physiology, Central University of Venezuela.

To whom reprint requests should be addressed.