

Carbonic Anhydrases from Human Erythrocytes

PREPARATION AND PROPERTIES OF TWO ENZYMES*

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Apart from the hemoglobins, carbonic anhydrase (carbonate hydro-lyase, EC 4.2.1.1) represents the principal protein constituent of erythrocytes. Roughton (1, 2) has described its physiological role in the transport of carbon dioxide; Roughton and Clark (3) and Davis (4) have reviewed earlier work on its preparation and properties. Lindskog (5, 6) and Lindskog and Malmström (7, 8) have made recent important advances in the characterization of carbonic anhydrases from bovine erythrocytes. Until recently, however, there had been little work on the chemical characterization of this enzyme from human erythrocytes since its partial purification by Keilin and Mann (9) in 1940.

Since 1961, three different laboratories have reported evidence that carbonic anhydrase activity in human erythrocytes is associated with at least two, and perhaps three or more, distinct enzymes. Nyman (10) in Uppsala separated three active fractions by column electrophoresis. Two of us (11) separated two active fractions by hydroxylapatite chromatography with phosphate buffers. In Derrien's laboratory in Marseille, Laurent *et al.* (12, 13) have separated three components by chromatography on Amberlite CG-50 and have found them to contain carbonic anhydrase activity (14), one being much more active than the other two.

Of the three enzymes reported by Nyman (10) and by Laurent *et al.* (14), one has high specific activity but is present in relatively small amount; one has lower specific activity but is present in much larger amount; and the third is a minor component of low specific activity. We have observed only the first two of these and have previously designated them, respectively, as Fractions II and I (11). Through the kindness of Drs. P. O. Nyman and B. Malmström, who have exchanged materials and information with us, we have established with reasonable certainty the identity of our fractions with the two major fractions of Nyman (10). Here we describe their preparation and some of their properties, and at the beginning of our section entitled "Results"

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§ Predoctoral Fellow of the National Cancer Institute, National Institutes of Health, U. S. Public Health Service, 1959-1962. The sections of this paper dealing with the fractionation of the hemolysate on Sephadex G-75 and with the electrophoretic studies of the carbonic anhydrases are presented in more detail in portions of the Ph.D. thesis of Barbara H. Gibbons, Harvard University, 1963.

we set forth a new nomenclature to denote the different human carbonic anhydrases. This terminology represents the result of a joint agreement among workers in the different laboratories to achieve a common set of designations for these enzymes. A subsequent paper (15) describes the characterization of one of these enzymes by pH titrations and spectrophotometry. We have already presented preliminary reports of some of the work discussed here (11, 16) and have also carried out quantitative kinetic studies on the two carbonic anhydrases (17), which will be reported in detail later.

EXPERIMENTAL PROCEDURE

All chemicals employed were of reagent grade.

Carbonic anhydrase activity was generally assayed during fractionation by the colorimetric method of Wilbur and Anderson (18). The reaction mixture contained 2 ml of 0.025 M Veronal buffer, pH 8.2, and bromthymol blue, 1 mg per 100 ml, at 2°. Enzyme solution, 1 ml, of appropriate concentration is added, and 2 ml of a cold, saturated CO₂ solution are injected by means of a syringe into the Veronal buffer. The experimenter records the time from the moment of injection to the color change of the indicator from blue to greenish yellow. Activity units (*U*) are calculated according to the formula,

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

where *T_b* is the time of uncatalyzed reaction and *T_c*, the time of enzyme-catalyzed reaction. Conditions are generally so chosen that *T_b* is of the order of 90 to 100 seconds and *T_c*, of the order of 20 seconds. Although this assay is not suitable for quantitative determination of rate constants, the results proved reproducible and convenient for routine assay during fractionation. One of us (B. H. G.) has made quantitative kinetic measurements by a stopped flow method (17). The details of these studies will be reported later.

Measurements of pH were made on a Beckman 76 pH meter with expanded scale, or with a type 4 Radiometer pH meter.

Hydroxylapatite columns were prepared from CaCl₂ and Na₂HPO₄ as described by Tiselius, Hjertén, and Levin (19).

Zinc analysis was done by the diphenylthiocarbazone method of Vallee and Gibson (20) with strict attention to the purification of reagents and the removal of extraneous metallic ions from glassware and other containers (in this connection see References 21 and 22). We are indebted to Dr. B. L. Vallee for valuable advice and guidance in this work.

Moving boundary electrophoresis was done with schlieren optics in a Beckman/Spinco model H electrophoresis apparatus at 1°.

The buffers employed were: (a) acetic acid and sodium acetate, at pH 4 to 6; (b) imidazole and its hydrochloride, at pH 6.5 to 7.5; (c) sodium Veronate with added hydrochloric acid, at pH 8.0 to 8.6; (d) glycine and sodium glycinate, at pH values above 9. The ionic strength of the buffers was 0.020, except for the sodium glycinate buffer, for which it was 0.0027. We added sodium chloride to all buffers to bring the total ionic strength to 0.10. We measured the pH of the buffers at room temperature and then calculated the values for 1° by the data given by Miller and Golder (23), except for the imidazole buffer, for which we calculated the change of pH with temperature from the value for ΔH of 7.7 kcal per mole, which is given by Nozaki *et al.* (24). We measured conductance with an impedance bridge in a Shedlovsky cell (25) equilibrated to 0° in an ice water bath. The fact that the conductance value of the protein solution always agreed within 3% with that of the buffer with which it had been equilibrated shows that dialysis was adequate. The current was 13.0 ma in all electrophoretic runs, and it produced a potential drop of about 90 volts across the cell. We measured the positions of the boundaries on the photographs with a Gaertner microcomparator.

Starch gel electrophoresis was done according to the general procedure of Smithies (26); for further details of the procedure employed in this laboratory, see Waller and Harris (27). The starch was obtained from the Connaught Laboratories, Toronto, specially prepared for starch gel electrophoresis. Runs were generally carried out in a Tris buffer, pH 8.9, at 25° (0.075 M Tris-0.0125 M HCl). The protein samples were introduced into molded slots in the gel, and electrophoresis took place at 4° under a potential gradient of 10 to 12 volts cm^{-1} , generally for 22 to 24 hours. The temperature of the gel remained below 10°. The gels were stained for 1 to 5 minutes with saturated Amido black 10B (Eastman Kodak Company) in methanol-water-acetic acid (45:45:10 by volume) and then washed many times with the same solvent until the background was nearly white.

Sedimentation coefficients were determined on a Spinco model E analytical ultracentrifuge. The schlieren photographs were measured on a Gaertner microcomparator.

Amino end group determinations were made with 2,4-dinitrofluorobenzene by the method of Sanger (28, 29) as described by Fraenkel-Conrat, Harris, and Levy (30) and by Waller and Harris (27). Carboxyl end group determinations were made with carboxypeptidase, as described by Harris (p. 397 in Reference 30) and by the hydrazinolysis method of Akabori, Ohno, and Narita (31) as described by Niu and Fraenkel-Conrat (32). In one instance hydrazinolysis was done for 16 hours under the conditions described by Bradbury (33, 34).

Amino acid analyses were done by Dr. P. F. Spahr, and later by Dr. Guido Guidotti, on a Beckman/Spinco model 120 amino acid analyzer according to Spackman, Stein, and Moore (35). Tryptophan was determined by the method of Spies and Chambers (36). Most of the ultraviolet absorption measurements were done on a Zeiss PMQ II spectrophotometer, and some were done on a Cary 14 recording spectrophotometer that was made available to us by Professor George Wald. The measurements at very short wave lengths were made in a Beckman DK-2A spectrophotometer in the laboratory of Professor Paul Doty. Measurements of optical rotatory dispersion were also made on a Rudolph polarimeter in Professor Doty's laboratory.

Preparation of Hemolysate from Erythrocytes

We obtained human erythrocytes from the Protein Foundation Laboratories, Jamaica Plain, Massachusetts, through the kindness of Dr. R. B. Pennell. The blood donations had been collected in acid citrate-dextrose solution, and the plasma had been removed before we received the cells. We washed the erythrocytes twice with an equal volume of cold sodium chloride solution (0.15 M), centrifuging the cells for 10 minutes at 3000 r.p.m. in a refrigerated centrifuge (International Equipment Company, Boston) and discarding the supernatant liquid and the leukocyte layer after centrifuging. Addition of an equal volume of cold distilled water hemolyzes the washed erythrocytes. It is not necessary to centrifuge off the stroma since the subsequent precipitation of the hemoglobin removes it.

Separation of carbonic anhydrase from hemoglobin in the hemolysate presents a serious problem because of the great excess of the latter. In most preparations we have begun by denaturing the hemoglobin with ethanol and chloroform, according to Meldrum and Roughton (37). Since this is a rather drastic procedure that might modify the native structure of the carbonic anhydrase molecules, we have also employed on a small scale a much gentler method, in which carbonic anhydrase is separated from the hemoglobin on a column of Sephadex G-75.

Preparation of Crude Carbonic Anhydrase from Hemolysate by Ethanol-Chloroform Treatment

To 600 ml of cold hemolysate are added, with constant stirring, 125 ml of precooled 96% ethanol, followed by 155 ml of cold chloroform. The mixture is stirred vigorously until it becomes very viscous; it is then allowed to stand for 10 to 15 minutes in the cold at 0–2°. The denatured hemoglobin is centrifuged off, and the pale yellowish supernatant liquid ("chloroform extract") is filtered to remove suspended particles. To recover more carbonic anhydrase, the hemoglobin precipitate is taken up in approximately the same volume of cold 0.15 M sodium chloride, stirred directly in the centrifuge tubes until evenly suspended, and centrifuged again. The resulting supernatant solution (the "sodium chloride extract") is collected separately after filtration. This washing procedure commonly increases the yield of crude carbonic anhydrase by 80 to 100%. Even so, assays of enzyme activity in the original hemolysate and in the two extracts after the ethanol-chloroform treatment indicate that the recovery of activity is generally considerably less than 50%.

In the first preparations we dialyzed the enzyme-containing extracts extensively against distilled water and then lyophilized them. To reduce the large volumes involved, we employed dry Sephadex G-25 (Pharmacia, Uppsala, Sweden) according to the procedure described by Flodin, Gelotte, and Porath (38).

According to this procedure, 100 ml of packed red cells yield 150 to 200 mg of crude carbonic anhydrase.

Fractionation of Hemolysate on Sephadex G-75

It proved possible to achieve an almost complete separation of carbonic anhydrase from hemoglobin in the hemolysate by chromatography on Sephadex G-75 dextran gel (Pharmacia, Uppsala, Sweden). According to the manufacturer, this gel should exclude molecules with molecular weights of approximately 40,000 and higher. Theoretically, then, it should be capable of separating hemoglobin and carbonic anhydrase (39).

The ratio of hemoglobin to carbonic anhydrase is approxi-

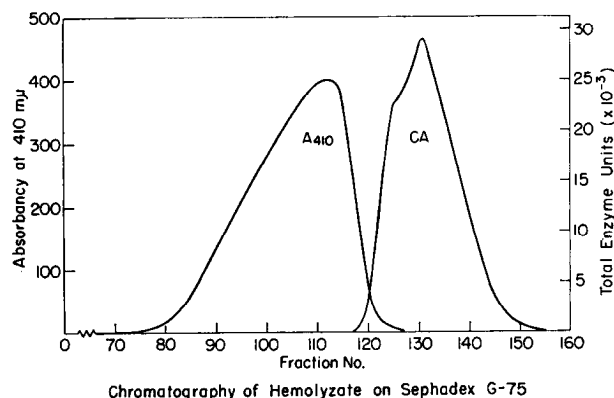


FIG. 1. Separation of carbonic anhydrase (CA) from hemoglobin in the human red cell hemolysate on Sephadex G-75. Note that the amount of protein under the hemoglobin peak (A_{410}) is actually more than 50 times as great as the amount of carbonic anhydrase.

mately 100:1 by weight. Separation in high yield and purity of the component present in smaller amount is difficult in such a system. Separation on Sephadex required careful attention to several experimental details.¹

Chromatography was performed at 4° on a column that measured 4 × 57 cm. Sodium phosphate buffer, pH 6.8, 0.2 M, was used to elute the proteins. Sephadex G-75, medium mesh, was sieved on a 100-mesh sieve to remove the coarser material, which was about half of the powder as supplied. Fines were removed by eight to ten decantations of an aqueous suspension of the gel. This step was essential to obtain high resolving power. The column was poured at room temperature according to the recommendations of Porath and Flodin (40). A wad of glass wool supported by glass beads at the bottom of the column helped to maintain a fairly even flow of buffer through the cross section of the gel. The use of a capillary of polyethylene to carry the effluent to the fraction collector reduced mixing below the end of the column. A disk of glass fiber filter paper (Hurlbut Paper Company) placed on the gel surface helped to achieve a sharp sample boundary. The flow rates were about 7 to 8 ml per hour; in fact, the solution would not flow much faster.

Samples of hemolysate (50 ml) were chromatographed satisfactorily on Sephadex G-75. To each sample were added 690 mg of $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ and 710 mg of anhydrous Na_2HPO_4 to bring the phosphate concentration roughly to 0.2 M, the same as that of the eluting buffer. Erythrocyte ghosts and a small amount of hemoglobin were removed by centrifugation at 10,000 r.p.m. for 10 minutes before the sample was applied to the column. Fractions of 3 ml each were collected.

A typical chromatogram is shown in Fig. 1. Hemoglobin concentration is expressed as the absorbance of the fractions at 410 μ , and carbonic anhydrase concentration, in terms of its activity in Wilbur-Anderson units according to Equation 1. It must be remembered that the hemoglobin peak includes 50 to 100 times as much protein as the carbonic anhydrase peak. The yield of carbonic anhydrase in enzyme units is usually 85 to 90% of the activity originally present in the hemolysate, so that the yield is much higher than by the chloroform-ethanol procedure. A small amount may be lost by adsorption onto the

¹ We are indebted to Drs. William H. Stein and Arthur Crestfield at the Rockefeller Institute for many helpful suggestions in the handling of Sephadex G-75.

glass paper or wool. At best, the resolution is such that about 1% of the hemoglobin overlaps the carbonic anhydrase peak. This means that about equal amounts by weight of the two proteins are present in the active peak.

The pooled fractions containing carbonic anhydrase activity were dialyzed against five 8-liter volumes of deionized water during 3 days and were lyophilized. The lyophilized powder weighed about 100 mg.

RESULTS

Naming of Observed Enzymes—Our findings deal with the preparation and properties of at least two distinct proteins with carbonic anhydrase activity. In previous reports (11, 16), we have referred to the first fraction emerging during hydroxylapatite chromatography as Fraction I, and to the second as Fraction II. Nyman (10) has separated three active fractions of human carbonic anhydrase by column electrophoresis and has designated them as Fractions II, III, and V. By comparison of data and samples of preparations with those obtained in the Uppsala laboratories, it has become clear to workers in both laboratories that Nyman's Fraction III corresponds to our Fraction I, which makes up most of the protein with carbonic anhydrase activity but is of relatively low specific activity. Nyman's Fraction V, with high specific activity but present in much smaller amount, corresponds to our Fraction II. Nyman's Fraction II is a minor component of low specific activity that we have not yet observed. The components that Laurent *et al.* (14) designate as X_2 , X_1 , and Y apparently correspond, respectively, to Nyman's II, III, and V.

In view of the multiple names in existence, it appears desirable to adopt a uniform terminology. We propose, by agreement with our colleagues in Uppsala and Marseille, to designate the minor component of low activity as carbonic anhydrase A, the major component of low specific activity as carbonic anhydrase B, and the component of high specific activity as carbonic anhydrase C. These would therefore correspond respectively with Nyman's Fractions II, III, and V; and enzymes B and C would correspond, respectively, with I and II of Rickli and Edsall (11).

In choosing this notation we have used capital letters rather than numbers to denote the three enzymes. Since Nyman (10) and Rickli and Edsall (11) used different sets of numerals to denote the enzymes, it seemed best to avoid the use of numerals altogether in the new notation. The order in which the components are designated by the letters A, B, and C was chosen to be consistent with the notation previously used by Lindskog (5) for the bovine carbonic anhydrases. Lindskog separated these enzymes on DEAE-cellulose at pH 8, designating the fraction that emerged first as B and the fraction emerging later as A. On zone electrophoresis at pH 8.2, component A moved with a higher (negative) mobility than B. Although we have not been able to obtain a satisfactory fractionation of the enzymes from human erythrocytes on DEAE-cellulose, Dr. Dirck V. Myers in this laboratory has recently developed a highly successful separation by chromatography on DEAE-Sephadex with Tris buffer at pH 8.9, employing a gradient with ionic strength of from 0.003 to 0.10. In this system, which will be described in detail in a subsequent paper, the fraction of high specific activity (C) emerges first; it is followed by the major fraction of low specific activity (B), and a little material of low activity is in a diffuse peak that emerges later. Likewise, in electrophoresis at pH 7, Nyman found his Fraction II (or A) to

have a higher negative mobility than III (or B), whereas Fraction V (or C) moved more slowly than either.

In order to make our notation for the human enzymes consistent with that employed by Lindskog (5) for the bovine enzymes, we give the first letter of the alphabet to the fraction that would presumably emerge last in chromatography on DEAE-Sephadex and that Nyman (10) has found to have the highest negative electrophoretic mobility at pH 7. This notation has the disadvantage that the letter A is used to designate a relatively minor component, the two major components being denoted as B and C. We believe, however, that this disadvantage is counterbalanced by the need for consistency in the notation used for the human and bovine enzymes. Table I summarizes the new notation and indicates its relation to the designations used by previous workers.

Separation of Carbonic Anhydrase B and C on Hydroxylapatite Columns

The columns, prepared by the method of Tiselius *et al.* (19), are packed with hydroxylapatite containing 1 g of Whatman standard cellulose per 5 ml of calcium phosphate suspension, in order to improve the flow rate. The column is then equilibrated with 0.009 M phosphate buffer, pH 6.8. The protein is dialyzed overnight against the same buffer. The amount of protein (in milligrams) to be chromatographed is taken as equivalent to the column volume in milliliters.

Chromatography of Crude Fraction from Chloroform-Ethanol Preparation

After removal of small amounts of insoluble material by centrifugation, the protein, dissolved in a small volume of 0.009 M phosphate buffer, pH 6.8, is placed on the column. The protein zone is displaced 2 to 3 cm down the column with 0.009 M phosphate buffer. The column is then washed with the same buffer. After passage of 1 hold-up volume of buffer, a small inactive peak emerges. In terms of absorption at 280 μ , it represents 10 to 12% of the applied protein. This fraction apparently contains some nucleotides, since it has an absorption maximum at 256 to 257 μ . (The ratio of the absorption at 280 μ to that at 260 μ is found to be 0.29.) We find this peak only in the "chloroform extract." There is little or none of it in the "sodium chloride extract."

We next apply a salt gradient from 0.009 to 0.06 M phosphate buffer, pH 6.8, the volume of buffer in the mixing chamber being equal to the volume of the column. This step yields another peak, which has carbonic anhydrase activity (carbonic anhydrase B). The specific activity is commonly near 10,000 units per mg of protein by the Wilbur and Anderson procedure (18).

This peak contains 65 to 70% of the protein in the crude fraction, expressed as integrated ultraviolet absorption at 280 μ .

We then employ a second salt gradient to increase the concentration of the phosphate buffer, pH 6.8, from 0.06 to 0.2 M. This elutes a second peak (carbonic anhydrase C) of specific activity close to 25,000 units per mg of protein. (Some of our later runs have yielded material with a specific activity of 40,000 to 60,000.) In terms of ultraviolet absorption at 280 μ , this peak contains 12 to 15% of the initial protein. Therefore the weight ratio of enzyme B to C is approximately 5:1.

Finally 0.4 M phosphate buffer elutes an additional small peak containing about 8% of the total protein. This peak is inactive and contains traces of denatured hemoglobin, which are always present in the crude fraction. We have previously published a figure showing typical results of this fractionation process (11). The over-all yields of protein originally present in the crude fraction are generally 95% or better, and the recovery of activity is almost 100%.

We carried out some experiments with only a single gradient from 0.009 to 0.2 M phosphate. This yielded the same elution pattern and the same protein distribution within the eluted fractions.

When either enzyme fraction, B or C, is rechromatographed on hydroxylapatite, it emerges from the column at the same position in the effluent as in the original chromatogram. This, combined with other evidence discussed below, appears to exclude the possibility that the two components are chromatographic artifacts. The two active peaks appear symmetrical, both in terms of the enzyme activity of the fractions separated and in terms of ultraviolet absorption.

Chromatography of Crude Carbonic Anhydrase Separated from Hemoglobin on Sephadex G-75

We have described in "Experimental Procedure" the separation of the mixed carbonic anhydrases from hemoglobin in the initial hemolysate. We dissolve the lyophilized protein from the carbonic anhydrase peak, shown as CA in Fig. 1, in phosphate buffer and chromatograph it on hydroxylapatite in exactly the same manner as the crude fraction derived from the chloroform-ethanol treatment. Fig. 2 shows the results. The two principal active peaks appear in the same positions, and with approximately the same relative specific activities and amounts of protein, as those obtained with the ethanol-chloroform extract. The residual hemoglobin, which slightly overlaps the carbonic anhydrase peak in Fig. 1, also shows some overlapping with enzyme C in the separation on hydroxylapatite. However, most of the hemoglobin emerges later. Some very small peaks, emerging in the early stages of elution in Fig. 2, may correspond to traces of other enzymes in the hemolysate. Most of these are probably denatured and removed by the alternative chloroform-ethanol treatment.

The fractionation on Sephadex G-75 represents an extremely gentle method of separating carbonic anhydrases from the hemoglobins, in contrast to the rather drastic chloroform-ethanol treatment. The data of Fig. 2 thus establish the conclusion that the two enzymes, B and C, already exist as such in the initial hemolysate and that neither is an artifact of the chloroform-ethanol treatment. One of us (B. H. G.) will later report the results of quantitative kinetic studies on enzymes B and C prepared by both procedures (see also Reference 17).

TABLE I
Notation of human carbonic anhydrases

| Proposed notation | Amount in hemolysate | Specific activity | Previous designations | | |
|--------------------------|----------------------|-------------------|-----------------------|------------------------|----------------------------|
| | | | Nyman (10) | Rickli and Edsall (11) | Laurent <i>et al.</i> (14) |
| Carbonic anhydrase A . . | Small | Low | II | | X ₂ |
| Carbonic anhydrase B . . | Large | Low | III | I | X ₁ |
| Carbonic anhydrase C . . | Small | High | V | II | Y |

As yet we have employed the fractionation of the initial hemolysate on Sephadex G-75 only for small scale preparations. The experimental results reported here refer almost entirely to material fractionated after the hemolysate had been treated by the chloroform-ethanol procedure.

Fractionation of Preparations Obtained from Individual Bloods

Most of the erythrocytes supplied by the Protein Foundation have been pooled samples from 10 to 20 or more donors. The question obviously arises whether the two different carbonic anhydrases, B and C, are determined by genetic factors differing from one individual to another. We have therefore analyzed 10 samples of crude carbonic anhydrase from different individuals, 1 by chromatography on hydroxylapatite and 9 by starch gel electrophoresis (see below). In every case the enzyme patterns were the same within experimental error. These limited findings of course leave open the possibility that some individuals may lack one or the other of the two principal carbonic anhydrases, or may contain others not yet observed in the pooled samples. However, our findings clearly exclude the hypothesis that enzyme B in the pooled samples arises only from one group of individuals and enzyme C only from others. We have not yet tested a sample of blood that lacked either enzyme or appeared to contain either in abnormally low or high concentration.

Crystallization of Enzymes B and C

Strandberg *et al.* (41) have crystallized carbonic anhydrase C (Nyman's Fraction V) from 2.5 M ammonium sulfate solution, after concentrating it by adsorption on sulfoethyl-Sephadex in dilute phosphate at pH 6 and eluting it with 0.1 M Na_2HPO_4 .² Dr. Direk Myers in this laboratory has obtained excellent crystals by their procedure with our preparations of enzyme C. He has also crystallized enzyme C from concentrated phosphate buffers. He and Dr. Lynn Riddiford have also obtained crystals from preparations of enzyme B in ammonium sulfate solutions, although these are smaller and less perfect than those of enzyme C. If crystals are to be obtained, it is important, as pointed out by Strandberg *et al.* (41), that the material should never have been lyophilized at any previous stage in the preparation. Further observations on the crystallized enzymes will be reported in later papers.

The solutions obtained by chromatography are quite dilute. To concentrate them without lyophilization, we have employed the osmotic technique of Hsiao and Putnam (42) and Squire, Starman, and Li (43). The solutions are placed in dialyzing bags with solid sucrose packed around them. The sucrose rapidly withdraws water from the protein solutions and thus concentrates them 6- to 10-fold. The sucrose that enters the protein solution is subsequently removed by dialysis against the ammonium sulfate solution employed for crystallization. Exposure of the enzymes to the sucrose solution has not resulted in any loss of enzyme activity or detectable change in physical properties.

² We should note a misprint in the paper of Strandberg *et al.* (41). The statement that crystallization was obtained from 4 M ammonium sulfate should be revised to read 2.5 M ammonium sulfate.

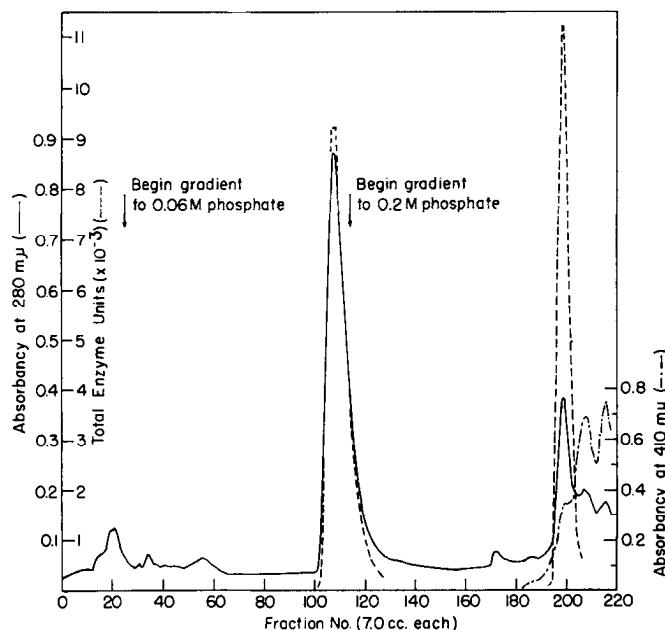


FIG. 2. Chromatography on hydroxylapatite of the total mixture of carbonic anhydrases separated on Sephadex G-75. The peak at Fractions 100 to 120 is enzyme B; the peak with a maximum near Fraction 200 is enzyme C. Absorbance at 410 μ measures the presence of hemoglobin.

Physical Properties of Enzymes B and C

Molecular Weights—We carried out determinations by the Archibald method of approach to sedimentation equilibrium (44). We studied enzyme B at pH 5.6 in 0.1 M acetate buffer, and at pH 6.0 in 0.1 M acetate buffer at a concentration of 9.5 mg per ml. We studied enzyme C in pH 6.0 phosphate buffer at 6.5 mg per ml. The centrifuge ran at 17,250 r.p.m. at 20°. We have taken the partial specific volumes of both enzymes as 0.733—a value determined on our preparation of enzyme B by Dr. Direk V. Myers and Mrs. Suchinta Mehta. The data on which this value is based will later be reported in detail; the determination of the weight of the anhydrous protein is the chief source of uncertainty.

The molecular weight so determined for carbonic anhydrase B was 26,500 at pH 5.6 and 26,700 at pH 6.0. Carbonic anhydrase C gave three values between 31,700 and 33,800, the average being 32,600.³ Reynaud *et al.* (45) have reported molecular weights of 29,100 to 29,600 for enzyme B (or X_1), and 31,600 to 32,200 for enzyme C, from sedimentation, diffusion, and partial specific volume studies. Nyman (10) earlier reported values of 34,000 for both enzymes from physical measurements, but Nyman and Lindskog (46) have recently calculated a value of 30,000 for both by finding what molecular weight gives the best fit to their amino acid analyses, so as to yield integral numbers of various amino acid residues from the data. Molecular weights may also be calculated from the amino acid analyses given below

³ In our first communication (11), we reported the molecular weight values as 28,000 for enzyme B and 34,000 for enzyme C. At that time we took \bar{v} (partial specific volume) as being 0.747 ml g^{-1} , a value which was derived from slightly less accurate density measurements than those we have since obtained and was also based on a slightly lower value for the specific absorption at 280 μ than the value we now employ (see below). The molecular weights given here involve the use of the value 0.733 for \bar{v} .

in Table IV, by summing the weights of all the residues, plus 1 atom of zinc, if it is assumed that no other constituents are present. The resulting values lie near 28,000 for enzyme B.

It is clear that some uncertainty still exists concerning the precise molecular weights. As a working basis for further calculations we adopt the value of 30,000 for both enzymes (46) since this gives a good fit to Riddiford's titration data (15).

Sedimentation Coefficients—We obtained values similar to those reported by Nyman (10); linear extrapolation of the data at five different concentrations gave a value for $s_{20, w}^0$ of 3.1 S for enzyme B and essentially identical values for enzyme C. In some later runs we have obtained values for both enzymes that were slightly lower—by about 0.15 S at any given concentration; in other physical properties, and in enzymatic activity, the later preparations appeared indistinguishable from the earlier. Nyman's limiting value for both enzymes is 3.0 S for $s_{20, w}^0$, and it thus lies between our own earlier and later values.

Reduced Viscosities—One of us (S. A. S. G.) has studied the viscosity of carbonic anhydrase B solutions in capillary viscometers, and Dr. Dirk V. Myers is now extending these studies with both enzyme B and enzyme C. The intrinsic viscosity of enzyme B appears to be less than 3 ml per g, and that of enzyme C is very similar. We defer reporting more precise values until Dr. Myers has completed his studies. The reduced viscosity of enzyme B increases greatly when the enzyme is dissolved in 8 M urea or when it undergoes a change of conformation in acid near pH 4 (see below). In the latter case there is marked aggregation of the protein, so that it is not clear how far the rise of viscosity may be due to aggregation, rather than to unfolding of individual molecules.

Zinc Content—Zinc analyses gave, for enzyme B, $0.208 \pm 0.006\%$ on one preparation (one anomalously low value of 0.170% was discarded, and four other measurements were averaged). A later measurement on another preparation in the laboratory of Dr. B. L. Vallee gave 0.188%. The value found for enzyme C was $0.187 \pm 0.009\%$. The recorded variations give the upper and lower limits of the analyses; the actual uncertainty in the measurements may be somewhat greater in view of the probable errors in the protein determinations and other factors.⁴ Our values agree with those of Nyman (10) and of Laurent *et al.* (14) within the limits of error. The data from all three laboratories show that 1 atom of zinc is present per molecule of enzyme in both proteins.

Ultraviolet Absorption—The specific absorption of the two enzymes at 280 $m\mu$ is important as a working standard for concentration measurements. Our determinations of total nitrogen and of total amino acids obtained on hydrolysis, for enzyme B are consistent with the latest figure reported by Nyman and Lindskog (46), which is 16.3 for absorbance at 280 $m\mu$ and 10 mg of protein per ml. Since our own dry weight determinations are not yet complete, we use this figure as a basis for the calculation; hence the molar absorptivity, ϵ_{280} , is 49,000 $M^{-1} cm^{-1}$ for enzyme B.⁵ For enzyme C we have used the earlier value of

Nyman (10) of 17.8 for A_{280} , rather than the newer figure (46) of 18.7. The latter is probably more accurate, but the difference is not significant for the measurements on enzyme C reported here.

Starch Gel Electrophoresis—The crude carbonic anhydrase, separated from the hemolysate by either the chloroform-ethanol or the Sephadex procedure, shows three principal bands with negative mobilities on starch gel electrophoresis in Tris buffer, pH 8.9, at 25°. The slowest moving band is identified, after chromatography on hydroxylapatite, as the principal component of carbonic anhydrase C; the next band, which moves about twice as rapidly as the first and is by far the most intense, is the principal component of carbonic anhydrase B. The most rapidly moving of the three bands is relatively small; after separation on hydroxylapatite it is found in enzyme B. If traces of hemoglobin are present in the crude fraction, as they generally are in material separated by the Sephadex G-75 procedure, they move in the starch gel considerably faster than any of the carbonic anhydrase components (Fig. 3).

The faster moving component in carbonic anhydrase B makes up about 15% of the total protein in B, as judged by ultraviolet absorption. It remains persistently with the main component on fractionation, and its relative amount is apparently unchanged after two or three crystallizations from ammonium sulfate solution. In one set of experiments we cut out separately the starch gel zones corresponding to the major and minor components of B and displaced the protein from each of the resulting pillars of starch gel into a small dialysis bag by further electrophoresis. The general method was a slight modification of the procedure of Gordon (47). Assay of the resulting protein solutions by the Wilbur-Anderson procedure showed that both the major and the minor bands separated on the starch gel had the same ratio of enzyme activity to ultraviolet absorption, within the limits of error ($\pm 5\%$).⁶

Starch gel electrophoresis of carbonic anhydrase C (Fig. 3) in pH 9 Tris buffer shows a major component of low negative mobility and 5 to 10% of a minor component with mobility virtually identical with that of enzyme B. We have not succeeded in separating this minor component by recrystallization or by further chromatography on hydroxylapatite; and we have not yet assayed it for enzyme activity separately from the major component.

Moving Boundary Electrophoresis—Tables II and III list the

lating the molar concentration of protein from the number of basic amino acids released on hydrolysis. This method of calculation, carried out by Dr. Guido Guidotti in conjunction with his amino acid analyses, gives an ϵ_{280} value of 46,000—a significantly lower figure than that given above. We have employed the value of 49,000, as given by Nyman and Lindskog, because of its consistency with their findings and with the titration data obtained by Riddiford (15) in this laboratory. The problem is obviously not finally resolved; it will be considered further in later reports from this laboratory by Drs. D. V. Myers and G. Guidotti.

⁶ It seemed possible that this minor component might represent carbonic anhydrase A (Fraction II of Nyman (10)). However, a sample of Nyman's enzyme B (his Fraction III), kindly provided by Dr. Nyman, showed a starch gel pattern virtually identical with that of our enzyme B, as separated by hydroxylapatite chromatography. The minor component of this enzyme, being so closely associated with it in fractionation and so nearly identical in enzymatic activity, may represent only a slight chemical modification of the major component. Nyman and Lindskog (46) report that enzymes A and B are virtually indistinguishable with respect to amino acid composition.

⁴ These values differ slightly from those reported in our earlier communication (11). The values given here are based on our latest revision of our calculation of the concentration of protein from its ultraviolet absorption as given in the present paper.

⁵ This value remains open to question. Since carbonic anhydrase B apparently contains 18 lysine, 11 histidine, and 7 arginine residues per molecule (see Table IV below) one may calculate a molar absorptivity value by doing an amino acid analysis on a hydrolysate from a solution with a known A_{280} value and calcu-

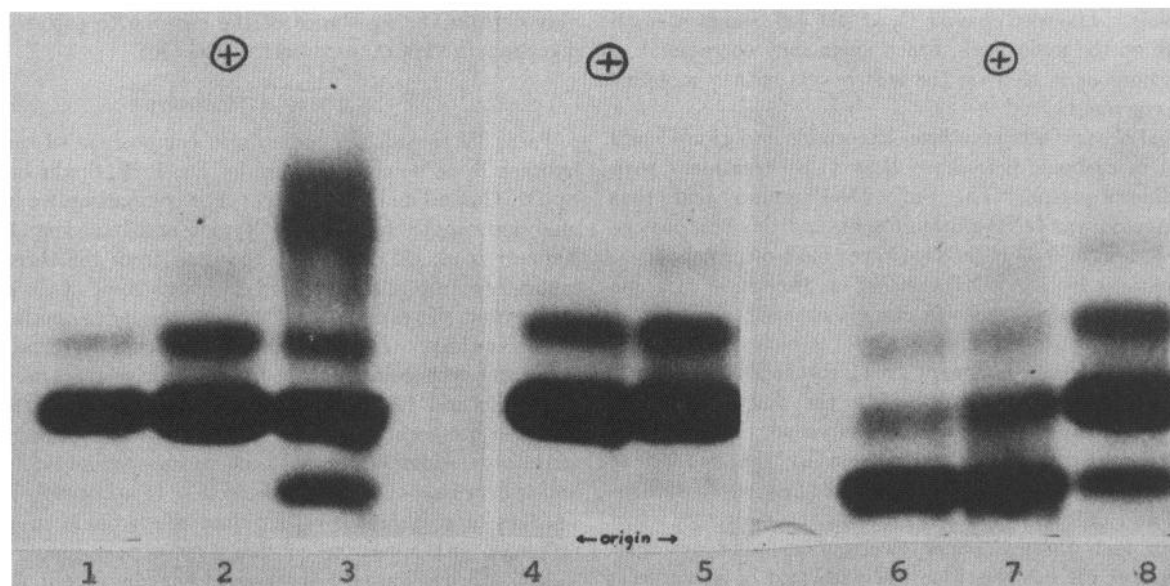


FIG. 3. Starch gel electrophoresis patterns, in Tris buffer, pH 8.9, of crude fraction from hemolysate and of carbonic anhydrases B and C. 1, enzyme B, 2 mg, from hemolysate separated on Sephadex G-75. 2, enzyme B, 3 mg of protein, from CHCl_3 -ethanol fractionation. 3, total crude carbonic anhydrase, 5 mg, from the original hemolysate after separation on Sephadex G-75. This is the material under the CA peak in Fig. 1. The most rapidly moving, rather diffuse band is chiefly hemoglobin. The other three principal bands, in order of decreasing mobility, are

the minor component of enzyme B, the major component of enzyme B, and the major component of enzyme C. Patterns 4 and 5 are enzyme C from chloroform-ethanol-treated hemolysates, 3 and 4 mg of protein, respectively. Patterns 6 and 7 are of enzyme C from chloroform-ethanol-treated hemolysate, two different preparations (2 to 2.5 mg of protein). These materials had been lyophilized and had not been crystallized. Pattern 8 is crude carbonic anhydrase, 4 mg, from chloroform-ethanol-treated hemolysate (compare with Pattern 3).

TABLE II

Mobility of carbonic anhydrase B

All buffers were at a total ionic strength of 0.1, of which 0.08 was contributed by sodium chloride (see "Experimental Procedure"). For a discussion of the two peaks at pH 4.04, see the text.

| pH at 1° | Buffer | Mobility |
|----------|-----------|--|
| | | $\text{cm}^2 \text{ sec}^{-1} \text{ volt}^{-1} \times 10^5$ |
| 4.04 | Acetate | +4.15 (major peak) |
| 4.04 | Acetate | +2.60 (minor peak) |
| 5.26 | Acetate | +0.40 |
| 5.55 | Acetate | +0.06 |
| 6.62 | Imidazole | -0.65 |
| 7.20 | Imidazole | -1.03 |
| 8.60 | Veronal | -1.72 |
| 9.67 | Glycine | -1.97 |

mobilities of enzymes B and C by the moving boundary technique in various buffers. The protein concentration in the various runs ranged from 2 to 4 mg per ml. Interpolation of the data in Tables II and III gives an isoelectric point of 5.7 ± 0.1 for enzyme B, and of 7.3 ± 0.1 for enzyme C. These pronounced differences in isoelectric point between the two enzymes are paralleled by differences in isoelectric point that have been determined by Dr. Lynn Riddiford and will be reported later.

Nyman (10) determined mobilities at pH 7.0 in phosphate buffer, 0.1 ionic strength; he reported -1.06×10^{-5} for enzyme B (his Fraction III) and -0.5×10^{-5} for enzyme C (his Fraction V). Both these values, especially that for enzyme C, are appreciably more negative than those found by interpolation from Tables II and III. The difference is in the direction to be

TABLE III

Mobility of carbonic anhydrase C

All buffers were at a total ionic strength of 0.1, of which 0.08 was contributed by sodium chloride (see "Experimental Procedure").

| pH at 1° | Buffer | Mobility |
|----------|-----------|--|
| | | $\text{cm}^2 \text{ sec}^{-1} \text{ volt}^{-1} \times 10^5$ |
| 5.52 | Acetate | +1.66 |
| 5.56 | Acetate | +1.65 |
| 6.55 | Imidazole | +0.57 |
| 6.62 | Imidazole | +0.51* |
| 7.26 | Imidazole | +0.04 |
| 8.12 | Veronal | -0.44 |
| 8.60 | Veronal | -1.01* |

* Runs on material prepared by E. E. R.; other runs on preparations made by B. H. G.

expected between measurements in imidazole and phosphate buffers, if there is some binding of the phosphate anions by the protein.

Below the isoelectric point of enzyme B a second component appears; it commonly comprises about 30% of the total protein in B. The major and minor peaks are completely separated after 225 minutes at pH 4. Since the enzyme undergoes drastic alterations in this pH range, as described in detail below, it seems probable that the appearance of the second peak is associated with these changes. The minor peak is detectable, but not prominent, at pH 5.3. Above the isoelectric point of enzyme B there is a small leading shoulder on the main peak, probably corresponding to the minor component observed in starch gel

electrophoresis. Likewise enzyme C, at pH 8.6, shows a small leading edge on the main peak, which again may correspond to the minor component seen on the starch gels with a mobility like that of enzyme B.

Amino End Group Determinations—No amino end group could be detected in carbonic anhydrase B or C by treatment with 2,4-dinitrofluorobenzene. The only DNP⁷-amino acid that could be detected was DNP-glutamic acid, and this was present only to the extent of 0.02 to 0.03 mole per mole of protein.⁸

Determinations by the DNP method on the crude carbonic anhydrase before hydroxylapatite chromatography showed the presence of DNP-glutamic acid, 0.8 to 1.0 mole per mole of protein. However, the component giving rise to DNP-glutamic acid on hydrolysis was dialyzable. In the dialysate it was present in equimolar amount with a component that could be titrated amperometrically with silver ion by the method of Benesch, Lardy, and Benesch (49). The mobility of the dinitrophenylated and unhydrolyzed substance in the dialysate, when subjected to high voltage paper electrophoresis in pyridine-acetate buffer at pH 6.5, was identical with that of a synthetic sample of DNP-glutathione used as a marker. Likewise the mobility of the untreated substance from the dialysate was the same as that of an authentic sample of glutathione. It therefore seems probable that the DNP-glutamic acid in the crude carbonic anhydrase arises from glutathione, of which traces may remain in the preparations of the separate enzymes, B and C.

Carboxyl-terminal End Group of Enzyme B—Hydrazinolysis (31, 32) yielded the most definite results. After periods of hydrazinolysis ranging from 5 to 16 hours and with 2,4-dinitrofluorobenzene treatment, DNP-phenylalanine was regularly identified, to the extent of 0.32 to 0.36 mole per mole of protein (uncorrected for losses). Other DNP-amino acids present did not exceed 0.06 mole per mole of protein (DNP-valine and alanine). DNP-glycine and serine were present in still smaller amounts.

In one experiment hydrazinolysis was carried out under the conditions described by Bradbury (33, 34); an uncorrected yield of 0.51 mole of DNP-phenylalanine per mole of protein was obtained, and there were only traces of other DNP-amino acids.

Treatment of enzyme B with carboxypeptidase A gave very low yields of free amino acids. At a weight ratio (carboxypeptidase to carbonic anhydrase) of 1:50, only traces of free amino acids appeared even after 24 hours of digestion at pH 8.5. At a weight ratio of 1:10, phenylalanine was detected (0.08 to 0.18 mole per mole of carbonic anhydrase), but there were traces of a number of other amino acids, perhaps produced by autodigestion of the carboxypeptidase.

We conclude from the hydrazinolysis experiments that phenylalanine is probably the COOH-terminal residue of enzyme B. We have obtained tentative indications from the study of a small peptide released during hydrazinolysis that a proline residue may immediately adjoin the COOH-terminal phenylalanine. This

⁷ The abbreviation used is: DNP, 2,4-dinitrophenyl.

⁸ We have also supplied samples of enzymes B and C to Dr. George R. Stark of the Rockefeller Institute, who has carried out amino end group determinations by the cyanate method (48). Although he detected minor amounts of end groups corresponding to several amino acids, all were present in amounts far smaller than 1 mole per mole of protein. These very careful studies, therefore, also lead to the conclusion that there is no free terminal amino group in carbonic anhydrase B or C.

may explain the resistance of the enzyme to carboxypeptidase digestion, in view of previous findings (50).

Amino Acid Analyses

Table IV records the amino acid composition of carbonic anhydrase B, as determined here by Dr. P. F. Spahr in 1961 and by Dr. Guido Guidotti in 1963. For comparison we include the analyses recently reported by Nyman and Lindskog (46) and by Laurent *et al.* (51). Clearly the data from the three different laboratories are in close accord, although some of the differences are beyond the experimental error of the determinations in any one laboratory. The findings make practically certain that the same protein is being studied by all the investigators.

Nyman and Lindskog (46) and Laurent *et al.* (51) have also reported amino acid analyses on carbonic anhydrase C, which contains considerably more lysine and less serine than enzyme B and differs in several other respects. It apparently contains 7 residues of tryptophan rather than the 6 found in enzyme B, although both appear to contain 8 tyrosine residues. We have as yet only preliminary analyses of enzyme C, but these are in general agreement with the findings of the other laboratories. A full report of the work of Drs. Guidotti and Spahr will be given later.

In another paper (11), we have discussed the earlier evidence that only 1 cysteine residue per molecule is present in enzyme B. We defer further discussion of the major question raised in that communication—the possible role of the cysteine sulfhydryl group in the binding of the zinc atom—until the completion of titration studies, which are now under way, on the zinc-free enzyme.

Acid Denaturation of Carbonic Anhydrase B

A sharp transition in the behavior of carbonic anhydrase B occurs near pH 4. From pH near 5 to 11 or above, the enzyme retains its activity for an indefinitely long period; its sedimentation coefficient remains near 3 S, and there is a single symmetrical peak. On exposure to pH below 4, enzyme activity is rapidly lost, and the sedimentation diagram becomes a broad, diffuse, rapidly moving peak with a mean value of s_{20} near 19 S. The breadth of the peak clearly indicates the presence of heterogeneous aggregates. Indeed the protein generally precipitates in this pH range, and its solubility is clearly much diminished. Below pH 2 it redissolves; on back-titration to pH 7, however, it remains relatively insoluble; it redissolves on further titration with alkali around pH 11. Studies with protein labeled with ⁶⁵Zn (11) indicate that the zinc is rapidly released in acid solution. We have not yet found conditions under which enzyme activity can be restored after acid denaturation. Titration studies (15) show that unmasking of a number of histidyl residues, which are unreactive in the native protein, accompanies the transition near pH 4.

Changes in ultraviolet absorption also appear. The *dashed curve* in Fig. 4 shows the characteristic difference spectrum between the native protein at pH 7.5 and the acid-denatured protein at pH 1.8, from 250 to 300 m μ . The spectrum shows two characteristic peaks, at approximately 285 and 292 m μ , the latter being the higher. The *solid line* in Fig. 4 shows measurements over a wider range of wave length in a Cary model 14 recording spectrophotometer. There is a peak at 236 m μ in the acid difference spectrum that is several times as high ($\Delta\epsilon \cong 30,000$) as either of the two peaks at longer wave length. Glazer and

TABLE IV

Amino acid analysis of carbonic anhydrase B

The first analytical data in our laboratory on carbonic anhydrase B were obtained by Dr. P. F. Spahr in 1961, on material separated on hydroxylapatite columns. Late in 1963, after this paper had originally been submitted, Dr. Guido Guidotti carried out further analyses on material separated by Dr. D. V. Myers on DEAE-Sephadex columns; on starch gel electrophoresis, this material shows only a single band, corresponding to the principal band shown for carbonic anhydrase B in Fig. 3. Except for tryptophan, for which only Dr. Spahr's value is available, the values listed in Column 2 are those of Guidotti; Spahr's data were essentially identical, except that he found 28 or 29 aspartic acid residues instead of 31 and 18 proline residues instead of 15. This difference may depend more on the exact analytical procedure than on a chemical difference in the protein samples analyzed.

Tryptophan was determined separately by the method of Spies and Chambers (36). Amide nitrogen, determined by the method of Stegemann (52), was 24 or 25 groups per molecule of enzyme, a value nearly identical with that found in the hydrolysates on the amino acid analyzer. Hydrolysis was carried out for 22 to 28 hours, for 48, and for 72 hours. The procedure was essentially that of Spahr and Edsall (53). The data in Column 2 are calculated on the assumption that the protein contains 18 lysine, 11 histidine, and 7 arginine residues. The data of Nyman and Lindskog (46) and of Laurent *et al.* (51) are expressed as moles of residues per 30,000 g of protein. The latter authors assumed a molecular weight of 29,350 g; we have therefore multiplied their reported values by the factor 3,000/2,935. This choice gives good numerical correspondence, to the nearest integer, among most of the results from the three laboratories. Note that the sum of the weights of the residues, plus 1 atom of Zn, is in all cases much closer to 28,000 than to 30,000. No constituent, other than amino acids and zinc, has yet been identified in carbonic anhydrase B; but the presence of other constituents has not been rigorously excluded. In view of the nearly quantitative recovery of nitrogen, such constituents, if present, are presumably not nitrogenous.

| Amino acid | Residues per mole | | | |
|---------------------------------|--------------------|-----------------|-------------------------|----------------------------|
| | Guidotti and Spahr | Nearest integer | Nyman and Lindskog (46) | Laurent <i>et al.</i> (51) |
| Lysine | 18.1 | 18 | 18.1 | 17.4 |
| Histidine | 11.0 | 11 | 11.1 | 11.4 |
| Arginine | 7.0 | 7 | 6.9 | 8.2 |
| Amide N | (25.4) | (25) | (26.5) | (29) |
| Aspartic acid | 30.5 | 31 | 31.3 | 30.8 |
| Glutamic acid | 21.7 | 22 | 23.8 | 22.4 |
| Serine | 26.6 | 27 | 30.3 | 29.5 |
| Threonine | 13.0 | 13 | 14.3 | 13.8 |
| Proline | 15.2 | 15 | 17.8 | 17.7 |
| Glycine | 15.1 | 15 | 16.0 | 15.3 |
| Alanine | 18.1 | 18 | 19.0 | 19.2 |
| Valine | 17.1 | 17 | 17.2 | 16.8 |
| Leucine | 18.9 | 19 | 20.0 | 20.5 |
| Isoleucine* | 9.45 | 10 | 9.7 | 8.4 |
| Cysteine† | 0.98 | 1 | 1.1 | 1.36 |
| Methionine | 1.91 | 2 | 2.0 | 2.02 |
| Phenylalanine | 10.3 | 10 | 11.0 | 11.75 |
| Tyrosine* | 7.4 | 8 | 8.1 | 8.7 |
| Tryptophan | 6.2 | 6 | 5.8 | 5.8 |
| Nitrogen recovered, % | 99.5 ± 2.5 | | 98.58 ± 2.13 | |

* Because the observed yields were expected to be slightly low

Smith (54) have observed a similar peak in the acid difference spectra of several other proteins.

Fig. 5 shows the pH dependence of the peaks at 285 and 292 mμ in the difference spectrum. The transition is extremely abrupt, with a midpoint near pH 3.5. The exact position of the curve depends somewhat on the time that has elapsed after the reference solution has been adjusted to acid pH, but the curves of Fig. 5 are representative of the difference spectra between neutral and acid solutions after the first hour or two. The data shown in Figs. 4 and 5 are not influenced by the increase of light scattering owing to the aggregation of the protein in the acid solutions. The fact that the observed value of Δε falls practically to zero above 300 mμ shows that the contribution of light scattering to the observed effects is very small and probably negligible. In any case, the sign of the observed Δε values is opposite to what would be expected from scattering alone; that is, the native protein absorbs more strongly than the acid-denatured protein, as in all other known cases.

Optical Rotatory Dispersion of Carbonic Anhydrase B—We have made optical rotatory dispersion measurements on enzyme B at wave lengths from 300 to 600 mμ in the native state, in acid solution at pH 1.9, in 8 M urea, and in 87% 2-chloroethanol. The chloroethanol solution was distinctly acid, owing probably to slight decomposition of the chloroethanol with liberation of HCl; the pH was approximately 2 after a 10-fold dilution with water.

Measurements in all the solutions gave approximately linear plots in terms of the Drude equation,

$$[\alpha]_{\lambda} = \frac{K}{\lambda^2 - \lambda_c^2} \tag{2}$$

on plotting λ²[α]λ as a function of [α]λ. The resulting values of λc are listed in Table V. The data are plotted in Fig. 6 in terms of the Moffitt-Yang equation (55),

$$[m']_{\lambda} = \left(\frac{3}{n^2 + 2} \right) \frac{W_m}{100} [\alpha]_{\lambda} = \frac{a_0 \lambda_0^2}{\lambda^2 - \lambda_0^2} + \frac{b_0 \lambda_0^4}{(\lambda^2 - \lambda_0^2)^2} \tag{3}$$

Here [α]λ is the specific rotation at wave length λ; n is the refractive index of the solvent at the same wave length; Wm is the mean residue weight of the protein—taken here as 115; and λ0 is assumed equal to 212 mμ. The values of a0 and b0 in the various solutions, calculated from Equation 3, are listed in Table V. The implications of these data for the structure of the protein are considered further in the discussion below.

Short Wave Length Ultraviolet Absorption of Native and Acid-denatured Carbonic Anhydrase B—Fig. 7 records the ultraviolet absorption, from 185 to 230 mμ, of the native protein at pH 6.35 and of the acid-denatured protein at pH 1.67. Acidification was carried out with phosphoric acid, since H3PO4 and the H2PO4- ion absorb much less strongly than the chloride ion in this range of wave length. The general procedure was that of Rosenheck and Doty (56). It is immediately apparent that the native protein absorbs more strongly than the acid-denatured protein at all wave lengths in this range. The peak near 190 mμ is due primarily to absorption by the peptide bonds; in simple polypeptides, such as poly-L-glutamic acid, the absorption of the peptide linkages is stronger when the molecule is in the random

for these amino acids, the nearest higher integer has been chosen as the most probable value.

† Determined as cysteic acid.

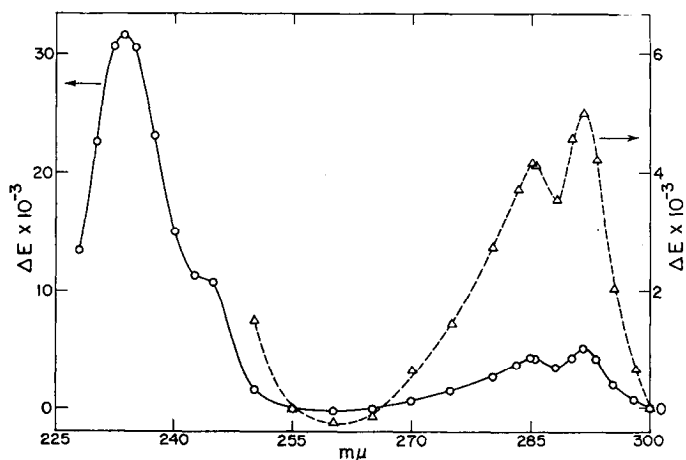


FIG. 4. Acid difference spectra of carbonic anhydrase B. The dashed line (right-hand ordinate scale) is from measurements made on the Zeiss spectrophotometer. The sample solution is native enzyme in phosphate buffer, pH 7.5, $\Gamma/2 = 0.15$. The reference solution is enzyme in HCl-KCl, pH 1.8, $\Gamma/2 = 0.15$. The solid line (left-hand ordinate scale) shows similar measurements made on another preparation over a wider range of wave lengths with the Cary model 14 spectrophotometer.

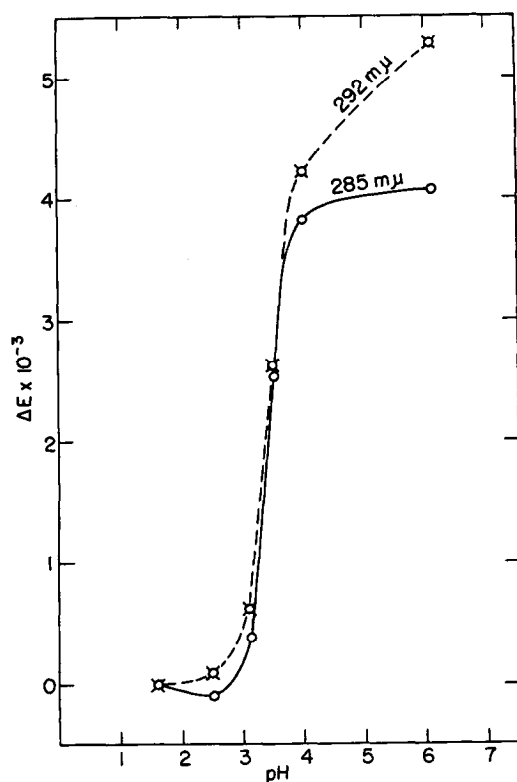


FIG. 5. Difference spectrum of carbonic anhydrase B as a function of pH at two wave lengths.

coil form than when it is in the form of an α -helix (56, 57). However, in a protein, there are also important contributions to the absorption, in this range of wave lengths, from the side chains of the aromatic amino acids, of methionine, and of histidine and from the carboxyl and amide groups of aspartic and glutamic acids and their amides. We have taken the values from Table IV for the content of these residues in carbonic anhydrase B

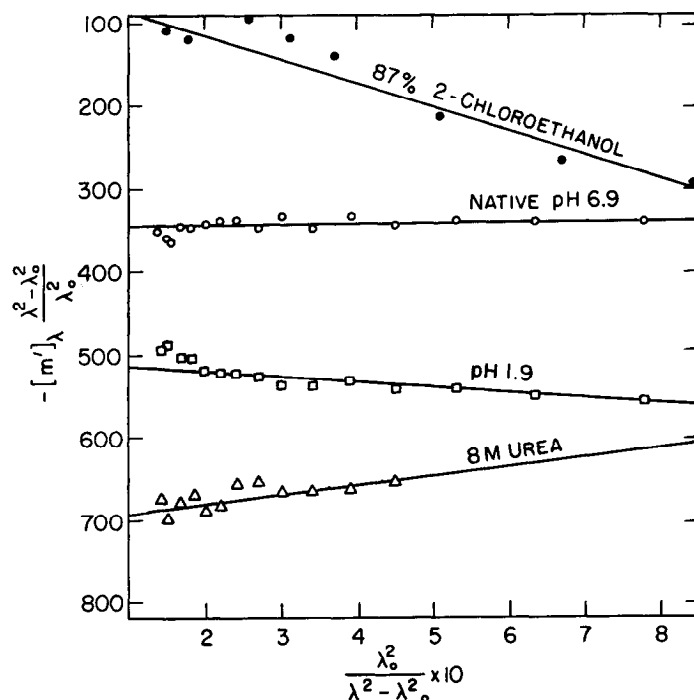


FIG. 6. Optical rotatory dispersion of carbonic anhydrase B plotted according to the Moffitt-Yang equation. The range of wave lengths covered by the measurements was from 313 to 600 μ . See Table V for a_0 and b_0 values.

TABLE V
Optical rotatory dispersion parameters for
carbonic anhydrase B

| Solvent | λ_c (Equation 2) $\mu\mu^*$ | a_0 (Equation 3) | b_0^* (Equation 3) |
|---------------------------------|--|--------------------|----------------------|
| Phosphate buffer, 0.1 M, pH 6.9 | 215 | -340° | -4.6° |
| Phosphoric acid, 0.1 M, pH 1.9 | 229 | -510° | -97° |
| 87% 2-chloroethanol, acid pH | 291 | -80° | -305° |
| 8 M urea, pH near 7 | 202 | -690° | $+44^\circ$ |

* The value of λ_0 in Equation 3 was taken as 212 $\mu\mu$ in calculating a_0 and b_0 in order to make our data comparable to most of those reported in the literature for other proteins.

and have calculated the contribution of the residues of each class to the absorption, making use of the values listed by Rosenheck and Doty. The horizontal lines marked 0 and 100, at 190, 197, and 205 $\mu\mu$ in Fig. 7 give the resulting values of molar absorption calculated for a completely helical (100%) and a completely unfolded molecule in the random coil form (0% helix). The experimental curve for the native molecule falls much closer to the latter curve than to the former. The curve for the acid-denatured protein is displaced downward, somewhat closer to the curve for 100% helix, but still considerably above it. The implications of these data are considered further in the discussion below.

Infrared Absorption of Carbonic Anhydrase B—We have made some preliminary observations of infrared absorption of enzyme B in deuterium oxide solution in the frequency region from 1300

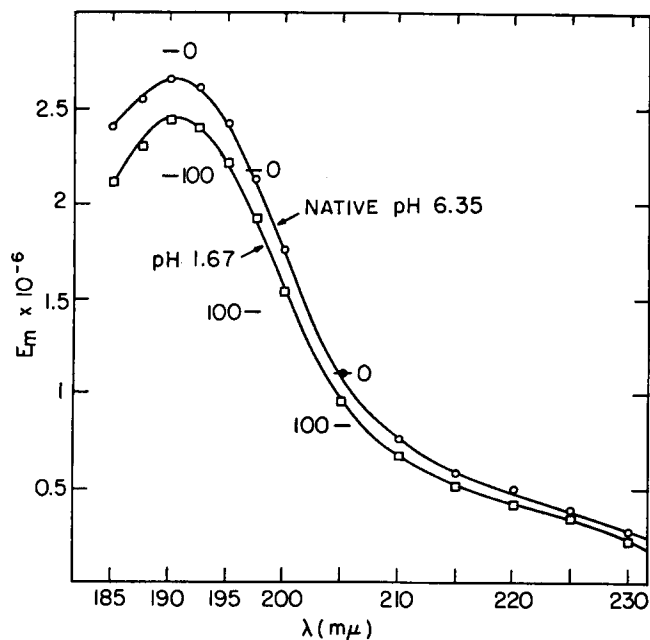


FIG. 7. Ultraviolet absorption of carbonic anhydrase B between 185 and 230 $m\mu$ for the native and for the acid-denatured enzyme. The scale marks 0 and 100 at 190, 197, and 205 $m\mu$ indicate the calculated values of molar absorptivity (E_m) for a random coil and a completely helical polypeptide chain of the amino acid composition of carbonic anhydrase B, calculated by the method of Rosenheck and Doty (56).

to 1800 cm^{-1} in order to determine the "hard-to-exchange amide hydrogen" by the method of Blout, de Loze, and Asadourian (58). Application of their method of analysis to our data led to the estimate that 20% of the peptide hydrogens in native enzyme B, and 30% in the acid-denatured enzyme, were difficult to exchange.

DISCUSSION

The presence of at least two carbonic anhydrases, of widely different specific activity, in human erythrocytes appears to be clearly established. The work of Nyman (10) and of Laurent *et al.* (14), in addition to our own studies, leaves little doubt of this conclusion. The work in the other two laboratories indicates the presence of a third minor component of relatively low activity (carbonic anhydrase A) that we have not yet identified. These different enzymes may of course be resolved into a larger number of distinct proteins by further research. Enzymes B and C, which we consider here, are strikingly similar in many respects. Both have molecular weights near 30,000, and each contains 1 atom of zinc per molecule of protein. They are very similar in their content of aromatic amino acids and of histidine, although they differ considerably in content of lysine and of several of the monoamino monocarboxylic acids. Their isoelectric points differ markedly, lying at pH 7.3 for enzyme C and at pH 5.7 for enzyme B. This difference might perhaps be expected from the higher lysine content of C, although its content of free dicarboxylic acids may also be somewhat higher (46, 51).

When two or more distinguishable isoenzymes are found in the same tissue, their specific activities are generally of the same order of magnitude. This appears to be true, for instance, of the bovine carbonic anhydrases studied by Lindskog (6); there are at least two major carbonic anhydrases, and perhaps some

minor ones, in bovine erythrocytes, but they do not differ widely in specific activity. The remarkable difference in activity between the human enzymes B and C has few parallels in previous experience. The difference is indeed even more pronounced when enzyme activity is assayed with higher precision by the stopped flow method (17);⁹ the maximal rate of turnover for enzyme C, in the hydration of CO_2 , at pH above 7 and 25°, is greater than 600,000 molecules of CO_2 hydrated per molecule of enzyme per second. The corresponding figure for enzyme B is close to 20,000. From these figures the activity ratio is about 30:1 and is thus of the same order of magnitude as the ratio of 20:1 reported by Laurent *et al.* (14) for the specific activities of components Y and X_1 from human erythrocytes. These components correspond presumably to our enzyme C and enzyme B, respectively. The ratio of specific activities, by the Wilbur-Anderson assay, is generally of the order of 3:1 but is sometimes as high as 5:1. Nyman's reported ratio (10) is similar to this. However, there are also marked differences (17) in the K_m values of enzymes C and B, and these tend to diminish the measured activity ratio at concentrations of substrate that are well below the saturation value. These apparent discrepancies, therefore, probably reflect only differences in the assay procedures. Since approximately 5 times as much of enzyme B as of C is present in human erythrocytes, both presumably contribute substantially to the catalysis of CO_2 hydration and HCO_3^- dehydration *in vivo*.

The presence of both these enzymes in the hemolysate in approximately the same proportions, whether the carbonic anhydrases are separated from the hemoglobins by the chloroform-ethanol procedure or by the far gentler gel filtration on Sephadex G-75, leaves little doubt that they are genuine components that are present in the original hemolysate and not artifacts of a particular method of preparation. Nyman (10) and Laurent *et al.* (14) have also drawn the same conclusion from studies on hemolysates fractionated by relatively gentle procedures. The genetic factors that control the production of the two enzymes appear to be present generally in the population, to judge from the very limited sample we have examined. None of the individual bloods we studied lacked either enzyme, B or C, nor were the proportions of the enzymes in any individual significantly different from those in pooled samples of blood.

Does the individual gain any physiological advantage from the presence in his blood of two enzymes differing so greatly in their K_m and specific activity values, yet both catalyzing the same reaction? Possibly he does, but we have not found any reason for believing this. Nor have we found evidence of any chemical procedure that would transform one enzyme into the other.

The value for $A_{280}^{1\%}$ of 16.3 ($\epsilon = 49,000$ for an assumed molecular weight of 30,000) for enzyme B appears compatible with the amino acid composition data in Table IV. If we assume that each tyrosyl residue makes a contribution of 1,340 to ϵ , and each tryptophan residue a contribution of 5,550 (see Wetlaufer (59)), the calculated residue contributions to ϵ are 44,000 for 8 tyrosines and 6 tryptophans. The value given above for enzyme B is 12% higher; the data for several other proteins, tabulated by Wetlaufer (p. 378), show that the observed value of ϵ is almost always higher than that calculated from the sum of the residue contributions, usually by 8 to 13%. This enhancement of absorption when the aromatic residues are enfolded in the structure of the

⁹ The full details of these studies are now being prepared for publication by one of us (B. H. G.).

native protein molecule is, of course, well known and theoretically is to be expected (60).

The two peaks at 285 and 292 $m\mu$ in the difference spectrum between native and acid-denatured enzyme B are characteristic of proteins with a high tryptophan content. The difference spectrum for enzyme B dissolved in concentrated urea solutions shows the characteristic "denaturation blue shift" (61), with peaks in positions virtually identical with those found by Chervenka (62) on chymotrypsinogen in urea solutions, measured against the native protein in water.¹⁰ Chymotrypsinogen contains 7 tryptophan and 4 tyrosine residues; carbonic anhydrase B contains 6 tryptophan and 8 tyrosine residues. In both cases, however, the far stronger absorption of the tryptophan residues tends to dominate the observed spectrum. The difference spectrum of free tryptophan between water and 7.5 M urea (63) shows well marked peaks near 283 and 292 $m\mu$. In all these cases, the peak at 292 is the higher of the two. The difference spectrum of tryptophan between neutral and acid solution, which arises from the ionization of the carboxyl group, shows peaks near 284 and 293 $m\mu$ (64). In this case the 293 peak is about 4 times as intense as that at 284. The peak at 284 is not apparent in the figures published by Donovan, Laskowski, and Scheraga (65), but these writers note in their discussion that it was observed at narrow slit widths.

It is of course quite likely that the tyrosyl residues also make some contribution to the observed difference spectra of carbonic anhydrase B, although the influence of the tryptophan residues is clearly predominant. Since we are now engaged in a much more detailed study of the difference spectra of enzymes B and C, we offer no further discussion on this point here.

The optical rotatory dispersion of native carbonic anhydrase B ($\lambda_c = 215 m\mu$, $b_0 = -5^\circ$) places it in the same general category with pepsin (66, 67), edestin, certain Bence-Jones proteins, α -conarachin, γ -globulin (68-70), and β -lactoglobulin (71), which are frequently referred to as "nonhelical." If indeed we apply the Moffitt-Yang equation (Equation 3) to proteins and accept the common assumption that a value for b_0 of -630° corresponds to a completely helical structure and a value of 0° represents the complete absence of helix, then there is virtually no α -helix in native carbonic anhydrase B,¹¹ or else right- and left-handed helices are present in equal amounts. Such inferences should be regarded with caution. It is, of course, true that peptides in the form of random coils commonly give values of b_0 near zero. A native protein such as carbonic anhydrase B or C, however, is far from being a random coil; it is an extremely compact and internally well ordered structure. The intrinsic viscosity of both enzyme B and enzyme C is certainly low (probably below 3 ml per g) and in the usual range for compact globular proteins (see for instance p. 692 in Reference 72). The molecular weights and sedimentation coefficients would also indicate that the molecules are compact and not far from spherical in shape. Their crystallizability indicates both internal and external order of the molecules in the crystal. The internal order may be, and probably is, of a very different sort from that found in an α -helix, but the

¹⁰ R. H. Stellwagen, L. M. Riddiford, and J. Dick, unpublished observations.

¹¹ Recent measurements by Dr. Dirck V. Myers in this laboratory give a value for b_0 of -50° for native carbonic anhydrase C near pH 7. By the same convention, this value would correspond to about 8% helix. The reader should remember that these calculations also involve the choice of λ_c as 212 $m\mu$ in the Moffitt-Yang equation.

component parts of the structure are not free to undergo constant transitions between a very large number of accessible conformations, as in a random coil. The data suggest that carbonic anhydrase B is "nonhelical," but this hypothesis is at present tentative. The short wave ultraviolet spectra of Fig. 7, however, give some support to it. The measured curves lie close to the values expected for the absorption of a peptide chain that has the form of a random coil and the amino acid composition of carbonic anhydrase B. The curves lie well above the calculated curve for a helical structure. These data, taken in conjunction with the optical rotatory dispersion measurements, tend to exclude the possibility that the b_0 value near zero might arise from the presence of equal amounts of right- and left-handed helix. Both kinds of helix would contribute equally to the measured ultraviolet absorption, but the helix content implied by this method is also near zero. The infrared measurements of "hard-to-exchange" amide hydrogen lead to similar conclusions.

By all these criteria, the acid-denatured protein appears more "helical" than the native protein. This is, of course, perfectly possible; when the denaturation process releases the constraints that hold the native molecule in its tightly coiled and compact form, considerable segments of the peptide chain may unfold and then refold into helical arrangements that were unattainable for the native molecule. Jirgensons (70) has recorded effects of detergents on several proteins— γ -globulins, Bence-Jones proteins, α -conarachin, edestin—that give b_0 values near 0° in the native state. Denaturation of these proteins with decyl or dodecyl sulfate shifts b_0 to negative values that are, in several cases, of the order of -120° . This phenomenon may be analogous to what we have observed in acid-denatured carbonic anhydrase B.

The rotatory dispersion data for enzyme B in acidic 87% 2-chloroethanol (Fig. 6 and Table V) would suggest that this relatively hydrophobic solvent strongly favors helix formation; the b_0 value of -305° would correspond to nearly 50% helix, according to the conventional interpretation. Similar effects of this solvent on many other proteins are, of course, well known (73).

SUMMARY

We have obtained two distinct enzymes with carbonic anhydrase activity from hemolysates of human erythrocytes by chromatography on hydroxylapatite columns. One, which we denote as carbonic anhydrase B, has low specific activity but is present in approximately 5 times the concentration of the other, carbonic anhydrase C, which has a specific activity several times as high as enzyme B. Starch gel electrophoresis reveals the presence of minor components in both enzymes; the minor component of enzyme B has essentially the same specific activity as the major component. These enzymes apparently correspond to carbonic anhydrases separated by Nyman (10) and by Laurent *et al.* (14) by quite different methods of fractionation. We have separated hemoglobins from carbonic anhydrases in the initial hemolysate either by denaturation of the hemoglobins with ethanol and chloroform or by gel filtration on Sephadex G-75. On further fractionation, enzymes B and C with essentially the same properties are obtained by either procedure; this fact minimizes the possibility that one or both of the components may be artifacts. Studies on ten individual bloods showed both components to be present in all cases and in nearly the same proportions.

Both enzymes have molecular weights near 30,000, and each

contains 1 atom of zinc per molecule. The isoelectric point of enzyme B is at pH 5.7; that of enzyme C, at pH 7.3. No free amino-terminal group was detected in either; there is evidence that the carboxyl-terminal group of enzyme B is phenylalanine. Amino acid composition data are reported for carbonic anhydrase B.

Studies of enzyme B in acid solutions show that it undergoes a sharp transition near pH 4, with loss of enzyme activity, loss of zinc, and increased tendency to aggregate. Between the native and the acid-denatured material there is a well marked difference spectrum with peaks at 285 and 292 $m\mu$ and a very intense peak at 236 $m\mu$.

Optical rotatory dispersion studies on native enzyme B give the Drude constant, λ_c , as 215 $m\mu$ and the Moffitt constant, b_0 , as -5° . These values suggest that a molecule of the protein may contain no or very little α -helix, although it is highly compact, as shown by its intrinsic viscosity and other properties. Short wave length ultraviolet absorption measurements also suggest a very low helix content. On acid denaturation, b_0 becomes more negative and the absorption near 190 $m\mu$ decreases; this finding may indicate that the acid-denatured protein is more helical than the native protein.

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**Carbonic Anhydrases from Human Erythrocytes: PREPARATION AND
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