Human Carbonic Anhydrases

I. Isolation and Demonstration of Isozymes in Erythrocytes*

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SUMMARY

A combination of hydroxylapatite and diethylaminoethyl cellulose chromatographic fractionation procedures have been used to isolate a relatively large number of human erythrocyte carbonic anhydrases. Some of the crystalline minor isozymes isolated show two starch gel electrophoretic components, closely related in mobility. A combination of immunological, enzymatic, and 65Zn exchange reactions, in conjunction with starch gel electrophoretic separations, has permitted the demonstration of the presence in hemolysates of carbonic anhydrase isozymes with the same electrophoretic properties as the isolated isozymes.

Three human erythrocyte carbonic anhydrases appear to have been clearly delineated (1–4) and others have been recognized (1, 5, 6). Some investigators (1, 7) believe that only the B and C isozymes which have been obtained in crystalline form from both humans (3, 8) and monkeys (9) are native forms of these enzymes, the others being artifacts arising during isolation.

In our studies of nonhemoglobin human erythrocyte proteins we have observed the presence of a rather large number of carbonic anhydrase active proteins. Some of these have been isolated in crystalline form and some of their properties explored. A combination of enzymatic, immunochemical, and isotopic zinc exchange studies have indicated their presence in fresh hemolysates of human erythrocytes.

The various isozymes all appear to be immunologically related to either the B or C form. This paper presents a description of the methods used to isolate the various forms, and the results of experiments are presented that show that carbonic anhydrase active proteins with electrophoretic properties similar to those isolated are present in unfractionated hemolysates or in hemolysate fractions separated by very mild conditions.

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EXPERIMENTAL PROCEDURES

Enzyme Activity—This was determined potentiometrically by the method of Wilbur and Anderson (10). It is difficult to ascertain the yields of carbonic anhydrases from the starting hemolysate since the C isozymes have greater activity than the B ones. Thus the activity comparisons reported in such experiments are relative.

Chromatography—Hydroxylapatite was prepared according to the method of Tiselius, Hjerten, and Levin (11) and DEAE-cellulose by the procedure of Peterson and Sober (12) with Brown Company cellulose.

Isotope Exchange—A modification of the procedure of Lindskog and Malmström (13) was used. Solutions of the protein in 0.9-cm diameter Visking tubing were dialyzed against pH 5.5, 0.1 M/2 sodium acetate buffer containing 1 x 10^{-2} M 1,10-phenanthroline. The dialysate was exchanged after 24 hours and the sample was dialyzed for another 48-hour period. The dialysate was again discarded and the protein was dialyzed against 100 volumes of the pH 5.5, sodium acetate buffer to lower the 1,10-phenanthroline concentration to 1 x 10^{-4} M. The dialysate was then replaced with the same buffer containing carrier-free 65ZnCl₂ in an amount from 3 to 5 times the molar level of the carbonic anhydrases. The final concentration of the Zn was near 5 x 10^{-5} M; the 1,10-phenanthroline was 1 x 10^{-4} M. If small amounts of precipitate formed during the exchange reactions they were removed by centrifugation. Radioactivity was measured with a Packard model 3002 Tri-Carb scintillation spectrometer. When a purified isozyme was subjected to the zinc exchange reaction, the amount of radioactivity per mg of protein was determined.

Electrophoresis—Experiments were performed by the cellulose acetate zonal procedure with the Microzone apparatus of Beckman Instruments,1 on filter paper (Whatman No. 3MM) strips, and by the vertical starch gel electrophoresis method of Smithies (14). The latter experiments used 0.026 M/2, pH 8.6, Tris- Versene-borate and 0.005 M/2, pH 8.75, sodium borate buffers. Electrophoretic focusing experiments were carried out by the method of Svensson (15) with the LKB apparatus.

1 The authors are indebted to Miss Barbara Wallis of the University Hospital's Clinical Laboratory for performing these experiments.
Antiserum—Three times crystallized carbonic anhydrases B and C were used for the production of rabbit antiserum. The response to the C isozyme was relatively weak and only three of 15 animals developed adequate titers. The IgG portion of the antiserum was separated by ethanol fractionation procedures (16) and reconstituted into an isotonic 0.7% NaCl solution-borate buffer of pH 7.4. The antibody preparations to the B and C isozymes were absorbed with small amounts of the heterologous isozyme. These preparations were used in studies to determine the distribution of carbonic anhydrase in starch gel following electrophoresis. Small punch samples were taken over the entire length of the nonstained half of the starch gel and these were inserted into the antigen holes of Ouchterlony plates. The latter holes were prepared with the same punch used to remove the starch samples.

RESULTS

Preparation of Enzymes—Batch absorption of hemolysates with DEAE-cellulose suspensions similar to those used previously in the preparation of erythrocytpcin and catalase (17) could not be successful used, apparently because of the widely different charge properties of the different carbonic anhydrases (3). Fractionation of hemolysates used an initial step in which a modification of the Tsuchihashi method (18) was used to effect removal of the major portions of the hemoglobin.

Packed human erythrocytes previously washed three times by centrifugation with 3 volumes of 0.15 M NaCl at 3-4°C were lysed by the addition with stirring of 2 volumes of cold 0.15% saponin. Thirty minutes later 7 g of NaCl were added to each 800 ml of hemolysate. After cooling to 0°C in an ice bath, 170 ml of cold (0°C to -5°C) 95% ethanol were added slowly with good stirring. This was followed by the addition of 210 ml of cold (0°C to -5°C) chloroform. Fifteen minutes later the precipitated hemoglobin was removed by centrifugation at 0°C. The supernatant was filtered to remove suspended protein and then dialyzed to equilibrium against 0.15 M NaCl at 3-4°C. The time of contact of the protein with the ethanol-chloroform solution was minimized as far as possible. Dialysis bags containing the above supernatant were then placed in glass tubes containing sufficient solid (NH_4)_2SO_4 to bring the contents to saturation. The tubes were then gently shaken for about 24 hours in the cold and the precipitated protein in the dialysis sacks was separated by centrifugation, dissolved in pH 6.8, 0.009 M potassium phosphate, and then dialyzed against repeated changes of this buffer to effect removal of the ammonium sulfate. The dialyzed proteins in 6% solution, designated as "crude carbonic anhydrases," were applied to a column of hydroxylapatite and eluted by application of a linear salt gradient. The resolved fractions were further separated individually on DEAE-cellulose columns at pH 8 with ion exchange strengths lower than those used by Lindskog (7). A summary of the fractionations leading to the isolation of the various carbonic anhydrases is presented in Fig. 1. The naming of carbonic anhydrases A, B, and C, follows the nomenclature previously used (3). Additional carbonic anhydrase active components have been given alphabetical designations, usually as a function of our order of observation.

The erythrocytes were obtained from freshly drawn bloods which, because of undesirable serological activity, lipemia, etc., were not suitable for clinical use. We are grateful to the Badger Regional Blood Center of the American National Red Cross for making this material available to us.

Crystalization—The isolated proteins were crystallized by a modification of the method of Strandberg et al. (8). This was accomplished by dialyzing 0.5 to 1% solutions of the enzyme in 0.05 M, pH 8.5, Tris-HCl buffer against 1.75 M solutions of (NH_4)_2SO_4 in the same buffer, and then slowly increasing the concentration of this salt by periodic removal of small amounts of dialysate and replacement with an equal volume of the 3.5 M (NH_4)_2SO_4. The final salt concentration in which the various DEAE-cellulose fractions were obtained as beautifully birefringent suspensions of crystals ranged from 2.4 to 2.7 M for different preparations. Microscopically, the crystals most often appeared first as fine needles. Some of these underwent transitions later to thin, fragile plates. Photographs of some of the crystalline carbonic anhydrase preparations are shown in Fig. 2.

Electrophoresis Results—Starch gel electrophoretograms of these isozymes are presented in Fig. 3. The crystalline preparations of the minor protein fractions that we have designated as E + F, G + II, M + N, and O + P each showed two components on electrophoresis in starch gel. Their separation is not always readily observed because of the small differences in their electrophoretic mobilities and because the small amounts of these components available for study often barely permitted their detection. The two bands of G + H in Fig. 3 are clearly evident and similar banding patterns are seen for other binary mixtures when present in adequate concentration. It can be seen from Fig. 3 that the electrophoretic separation of the various isozymes is better in the relatively low 1/2 sodium borate buffer. In addition to the above named isozymes small amounts of other components with carbonic anhydrase activity have been noted.

The relative electrophoretic mobilities of the various carbonic anhydrases on filter paper, on cellulose acetate, and in starch gel near pH 8.6 are similar. This suggests that the electrophoretic mobility differences in starch gel are not due to polymers. The results of electrophoretic experiments on filter paper and on cellulose acetate of a mixture of carbonic anhydrase A, B, C, D, and T isozymes are shown in Fig. 4. The T isozyme is one not previously mentioned and is one that has not been prepared in crystalline form. It is readily apparent that these carbonic anhydrases resolve under conditions of free electrophoresis as well as in gels. Furthermore, known mixtures of these isozymes resolve readily in electofocusing experiments. The result for a mixture of the A, B, C, and D isozymes is shown in Fig. 5. Some asymmetry is noted for the A and D components. Thus, all of the experimental results indicate that the carbonic anhydrase isozymes of human erythrocytes have different electrophoretic properties and can be separated on the basis of this property.

The approximate relative enzymatic activities of the different fractions determined by the method of Wilbur and Anderson (10) and the average amounts of each component isolated from 1 liter of packed erythrocytes in the chromatographic experiments are presented in Table I. The yields of components and their relative activities are based on E = 100 values of 10.3 for the B, A, D, E + F, and O + P fractions and 18.7 for the C, G + II, and M + N fractions. The absorption coefficients are the average of values found for the isozymes of a given class (B or C) and are the same as those previously reported for the B and C components (19). It appears that the over-all recovery of the carbonic anhydrase activity is from 20 to 40% of the amount present in the hemolysate pools.
Fig. 1. Summary of carbonic anhydrase separations. All experiments except the initial hydroxylapatite chromatographic step (top graph) were carried out on DEAE-cellulose columns. The lettered components in parentheses are impurities to the main component (no parentheses). The left and right ordinates of the individual DEAE-cellulose chromatograms are E_{280} and r/2 values, respectively. The initial r/2 values of the buffers used in the diagrams illustrating the isolations of the different carbonic anhydrases are as follows: O + P, 0.0018, E + F and G + H, 0.0025, all others 0.006. All of the DEAE-cellulose subfractions of hydroxylapatite Fractions I, II, and III (second row) were initiated at 0.0025 r/2. All abscissa values are milliliters of effluent. The solid lines of each graph are the protein values; the dotted lines r/2.

Current studies in our laboratory1 have shown that the B isozyme can be converted into other isozymes and that all isozymes are immunologically related to either carbonic anhydrase B or C. Charrel, Laurent, and Darrien (20) have previously pointed out that components X1 and X2, i.e. B and A, are immunologically identical. Such results suggested that the many isozymes noted had been produced as a result of the isolation procedures employed. Various experiments were carried out to test this possibility.

1 Unpublished data from our laboratory.

When crystalline carbonic anhydrase D was refractionated over the hydroxylapatite and DEAE-cellulose chromatographic steps previously used in its isolation, none of the other B type isozymes were produced and the B enzyme had its original chromatographic properties and activity.

Neither reduction-alkylation nor sulphydryl exchange reactions (14) modified the relative starch gel electrophoretic properties of either carbonic anhydrase B or C. Treatment of a mixture 1% each in carbonic anhydrases B and C at 3-4°C for periods up to 48 hours, with an aqueous ethanol-chloroform solution of
the same composition as used in the Tsuchihashi (18) procedure, did not modify their starch gel electrophoretic properties.

The zinc exchange reaction likewise had no effect on the immunological or enzymatic activities or on the starch gel electrophoretic properties of the purified C, G + H, B, A, and D isozymes. Additional electrophoretic components were not generated when any of the isozymes was individually subjected to zinc exchange.

A mixture of equal amounts of isozymes C, B, A, and D was subjected to \(^{65}\text{Zn}\) exchange for 3 hours and for 7 days. A sample of each of these mixtures was then subjected to electrophoresis in starch gel and the radioactivities of 3.3-mm sections of the gel were determined. The result is presented in Fig. 6 and shows that the isozymes show marked differences in the rate and extent of labeling. The decrease in radioactivity with time noted for the A and D isozymes appears to be due to denaturation of these proteins. A previous report has indicated that the exchange rates of several bovine carbonic anhydrase isozymes are different (13). We have found that the C isozyme exchanges much more slowly than the B. Thus, while it is possible to show the presence of a given isozyme by radioactivity measurements, following starch gel electrophoretic separation of an isozyme mixture previously subjected to \(^{65}\text{Zn}\) exchange, such labeling cannot be used to determine a given component quantitatively.

Attempts to show the presence of various carbonic anhydrases in hemolysates utilized conditions in which denaturation of hemoglobin and ion exchange column chromatography procedures were not used. In a preliminary experiment, a pooled hemolysate was subjected to \(^{65}\text{Zn}\) exchange. A starch gel electrophoresis experiment was then performed on this material, with a mixture of unlabeled A, B, C, and D isozymes as a control. The control starch strip was stained with Amido black to locate the

![Fig. 2. Crystalline forms of various human erythrocyte carbonic anhydrase isozymes. C, \(\times 300\); all others, \(\times 600\).](image)

![Fig. 3. (left). Starch gel electrophoretograms of some carbonic anhydrase isozymes in pH 8.6 Tris-Versene-borate (upper) and in pH 8.75 sodium borate (lower) buffers.](image)

![Fig. 4 (right). Results of electrophoresis experiments on known mixtures of carbonic anhydrase isozymes. On Whatman No. 3MM filter paper in 0.1 \(\text{M} \) Tris-\(\text{HCl} \), pH 8.6, Tris-Versene-borate buffer (upper). On cellulose acetate strips in 0.005 \(\text{M} \) sodium borate buffer (lower). Sample 4 of this experiment is a normal human serum control (Versitol).](image)
TABLE I
Relative activities and approximate yields of carbonic anhydrase isozymes in pools of human hemolysates

<table>
<thead>
<tr>
<th>Isozymes</th>
<th>Relative activities</th>
<th>Approximate yields (mg/liter erythrocytes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E + F</td>
<td>5</td>
<td>&lt;1</td>
</tr>
<tr>
<td>C</td>
<td>100</td>
<td>160</td>
</tr>
<tr>
<td>O + P</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>G + H</td>
<td>100-180</td>
<td>5</td>
</tr>
<tr>
<td>D</td>
<td>15-20</td>
<td>1100</td>
</tr>
<tr>
<td>M + N</td>
<td>50</td>
<td>3</td>
</tr>
<tr>
<td>A</td>
<td>15</td>
<td>75</td>
</tr>
<tr>
<td>D</td>
<td>10-15</td>
<td>12</td>
</tr>
</tbody>
</table>

positions of the known isozymes. The starch strip containing hemolysate was sectioned at 3.3-mm intervals and counted for radioactivity. The result shown in Fig. 7 clearly shows that the unfractionated hemolysate contains $^{65}$Zn-labeled material with the electrophoretic mobilities of the A, B, C, and D isozymes. The other minor isozymes could not be detected in this experiment because of their low concentrations and inadequate resolution from the major components. Hemolysates aged for 3 weeks and for 7 months at 34°C prior to $^{65}$Zn exchange gave similar results.

Since the Tsuchihashi method for the removal of hemoglobin is a relatively rapid and easily applied method, an experiment to probe its effect on the distribution of the carbonic anhydrase isozymes was carried out. The hemoglobin from a portion of a hemolysate pool was removed by filtration over Sephadex G-75 and from a second portion by the Tsuchihashi procedure. The carbonic anhydrase active proteins were concentrated and subjected to $^{65}$Zn exchange. Aliquots of both preparations were then subjected to electrophoresis in starch gel and the radioactivity assays were again performed as previously described. The results obtained are shown in Fig. 8. It can be seen that the peak areas of radioactivity are in the positions of the A, B, C, and D isozymes. However, a good deal of radioactivity is also present in the regions characteristic of the electrophoretic migrations of the minor isozymes. A strongly labeled component with a greater anodic mobility than found for any of the carbonic anhydrases was shown to be erythrocytoperin on the basis of its reaction with a specific antibody preparation (21). Thus, this latter protein appears to undergo an exchange of its copper for $^{65}$Zn under the conditions used.

No significant difference in the electrophoretic distribution of $^{65}$Zn-labeled proteins derived from fractionation involving gel filtration or the Tsuchihashi method was noted. Thus, in agreement with Armstrong et al. (4), we find that treatment of hemol-

FIG. 5. Results for an electrofocusing experiment for a mixture of 5 mg each of carbonic anhydrases A, B, C, and D. One-milliliter fractions were collected.

FIG. 6. The radioactivity of sections of starch gel following electrophoresis of a mixture of 10 mg each of carbonic anhydrases C, B, A, and D subjected to the $^{65}$Zn exchange reaction for varying times.
ysates with ethanol-CHCl₃ does not introduce changes in the carbonic anhydrases that can be revealed by electrophoresis in starch gels.

Since the minor isozymes appear to be present in erythrocytes in low concentration, attempts to show their presence involved removal of the hemoglobin and concentration of the carbonic anhydrase active fraction by as mild conditions as possible. In one such study, the starting material was a hemolysate obtained from a pool of erythrocytes from 30 individuals from which the hemoglobin had been largely removed by precipitation at 2.3 M ammonium sulfate in pH 7.8, 0.05 M Tris buffer. The ammonium sulfate concentration of the supernatant was raised to 3.5 M and the precipitate containing the carbonic anhydrases was removed by centrifugation. It was dissolved in pH 7.4 phosphate-KCl buffer of 0.15 M and dialyzed against this buffer to remove the ammonium sulfate. The protein was then passed through a column of Sephadex G-75 to remove residual hemoglobin and the carbonic anhydrase active fraction was isolated and concentrated by pressure dialysis to a F₄0 cm = 283. This material was used for a series of studies. A portion of it was subjected to ⁶⁵Zn exchange. This fraction as well as aliquots of untreated protein was subjected to electrophoresis in starch gel. The gel was then sectioned at 3.3-mm intervals from its cathode to anode ends to provide 80 samples. The sections of the starch strip containing the sample which had been subjected to ⁶⁵Zn exchange reaction were individually counted for radioactivity. Another series of sections were individually placed in 4 ml of 0.1 ¹⁰⁻⁶ ¹⁰⁻⁴ ¹⁰⁻² ¹⁰ ¹⁰³ origin 3 6 9 12 CATHODE — STARCH GEL (cm) — ANODE

Fig. 7. The starch gel electrophoretic distribution of the radioactivity of whole hemolysate following ⁶⁵Zn labeling.

Fig. 8. The starch gel electrophoretic distribution of the radioactivity of hemolysate proteins from which the hemoglobin was removed by Sephadex G-75 filtration (○—○) and by the Tsuchihashi (18) method (□—□) before their labeling with ⁶⁵Zn.

I/2, pH 8.15, Veronal buffer and shaken for 24 hours at 3°. The starch gel piece was then removed and the extract was dialyzed against the same Veronal buffer. These solutions were then assayed for carbonic anhydrase activity. Another starch gel strip was used for immunological assay. A continuous series of 3-mm diameter samples were punched out of this portion of the gel and inserted into the holes of Ouchterlony (22) plates. These samples were then reacted against rabbit antibody to both carbonic anhydrase B and C. From the intensities and the positions of the precipitin bands formed, the relative amounts of antigen in the starch gel samples were graded from +1 to +5 by reference to a standard precipitin system prepared as follows. Small sections of fresh starch gel were placed in test tubes containing solutions of carbonic anhydrases B and C of various concentrations, and incubated for 24 hours to permit the enzyme to diffuse through the gel. The gel pieces were then removed and a punch sample was taken from each. These samples were placed in holes in Ouchterlony plates and allowed to react with specific antibody. The extent of the reactivity of these control starch gel samples containing different concentrations of a given isozyme were also graded from +1 to +5 to provide a scale to which unknown samples could be compared. The results of this experiment are shown in Fig. 9, which reveals that carbonic anhydrase active materials with electrophoretic mobilities over the range of the isozymes previously isolated are present in hemolysates from which the hemoglobin had been removed by precipitation with ammonium sulfate and gel filtration. Similar results were obtained in an experiment in which hemoglobin had been removed by gel filtration and only an immunological evaluation was made.
Fig. 9. The radioactivity ( ● — ● ), enzymatic ( ○ — ○ ), and immunological activities of hemoglobin-free components of human hemolysates separated by electrophoresis in starch gel.

In this experiment a control mixture of isozymes F + E, C, O + P, G + H, B, M + N, D, and T was used as the reference. It can be seen from Fig. 9 that there is good correspondence between the gel electrophoretic patterns of the carbonic anhydrases after Sephadex G-75 fractionation and the enzymatic and immunological activities in the starch gel. The responsible factor as previously indicated is the protein erythrocuprein.

Some pooled hemolysates are positive for E + F, others not. This may be due to the relatively rare occurrence of these isozymes in human erythrocytes. Some pools of 30 bloods may not encompass any individual of these isozyme types. These size pools always contained the other noted minor isozymes. We do not know, however, whether they are present in the erythrocytes of all individuals.

The isotope exchange reactions did not clearly show activity beyond the D component, apparently because of the low sensitivity of this method. The exchange of the copper in erythrocuprein by zinc, when such protein is present in the carbonic anhydrase-containing fraction separated by ammonium sulfate fractionation, complicated the results in the more anodic portion of the gel. The results obtained in the various experiments performed, however, show that the minor isozymes isolated are present in erythrocytes. Experiments with aged hemolysates indicate that these minor isozymes are more labile than the A, B, C, and D ones. It appears that rather than being generated in the fractionation conditions used some may be lost. At present there are insufficient data to discuss factors such as their lability and exact concentrations in erythrocytes or in hemolysates.

The hemoglobin fraction of the Sephadex G-75 column which elutes before the carbonic anhydrase active fraction was subjected to the 65Zn exchange reaction, and also to immunological and enzymatic assay. No carbonic anhydrase was detected but a low level of zinc binding was noted. The amount was too small to influence the results of the zinc exchange reactions for the C, B, A, and D components in the carbonic anhydrase fractions eluting from the Sephadex G-75 column or in those prepared with ammonium sulfate. However, when hemolysate is directly subjected to 65Zn exchange without any removal of hemoglobin the amount of the zinc binding is quite high. The responsible component (or components) has an electrophoretic mobility in the region of the G + H fraction. This necessitates that the hemoglobin removal procedure be used in zinc exchange experiments involving these isozymes.

**DISCUSSION**

The B and C isozymes which comprise the major erythrocyte carbonic anhydrases have been extensively studied. Nyman and Lindskog (19) and more recently Laurent et al. (23) have reported some of the properties of purified human carbonic anhydrase A but this component does not appear to have been crystallized. Their data indicate that it has an amino acid composition very similar to the B isozyme. Our experiments have led to the isolation of a relatively large number of isozymes from human erythrocytes and the presence of others are indicated. The combination of refractionation and of immunological, isotope exchange, and enzyme activity studies on electrophoretically resolved components indicate that the various isozymes are not artificial interconversion products of the B and C isozymes resulting from the fractionation conditions employed but are present in the original hemolysates.

All of the isozymes are immunologically related and have amino acid compositions similar to the B or the C form of this enzyme. The initial hydroxylapatite fractionation step effectively separates the B and C types and their further resolution on DEAE-cellulose can be effected. Direct application of DEAE-cellulose chromatography to the crude mixture of isozymes is possible but introduces many complications of separation. Refractionation of isolated isozymes over hydroxylapatite does not appear to modify their properties. Studies by Armstrong et al. (4) have indicated that carbonic anhydrase B isolated by a direct DEAE-Sephadex chromatographic procedure which avoided the Tsujihashi procedure gave a product which showed no significant deviation from the B isozyme prepared by the usual procedure. In agreement with these workers we find that the removal of hemoglobin by the Tsujihashi method does not influence the starch gel electrophoretic patterns of the carbonic anhydrase after 65Zn exchange. The electrophoretic mobilities of the B, C, A, and D isozymes isolated after procedures with ethanol-CHCl₃ are the same as those obtained when these isozymes are prepared by the procedure in which hemoglobin is removed by filtration over Sephadex G-75. The 65Zn-exchanged crude carbonic anhydrase fractions clearly show radioactivity in both the G + H and the O + P regions. The various studies which we performed did not indicate that detectable interconversions of the B and C isozymes into the minor component noted took place during fractionation or during zinc exchange reactions. Certain rather drastic condi-
tions such as aging at pH 11 to 12.5, however, do lead to inter-
conversions in vitro. 1

The minor isozymes which separate as mixtures of two com-
ponents closely related in starch gel electrophoretic properties
are difficult to detect in complex mixtures containing relatively
large amounts of the major isozymes. Their ready detection
and demonstration in the hemolysates of some individuals in
which they exist in relatively high concentrations will be re-
ported in future publications dealing with the properties of the
isozymes isolated to date. Studies of the distribution of the
minor isozymes in individuals will be necessary to define their
genetic implications. Our present data indicate that the minor
O + P fraction is quite similar electrophoretically to the so-
called “mutant” carbonic anhydrase previously described (5, 6).
This isozyme has been found to be a major carbonic anhydrase
isozyme in one of 54 individual hemolysates that we have ex-
amined to date.

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