Properties of Carbonic Anhydrase Isozymes Isolated from Porcine Erythrocytes*  

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SUMMARY

Two major components of carbonic anhydrase were purified from porcine red cells by column chromatography and electrophoretic techniques. Both forms behave as single components in sedimentation velocity experiments and during starch gel electrophoresis. The observed molecular weight of both forms was about $3 \times 10^6$. On the basis of their specific CO$_2$ hydrase activities and amino acid compositions, these two carbonic anhydrase isozymes were designated as high activity (carbonic anhydrase C) or low activity (carbonic anhydrase B) forms which appear to be homologous to the high and low activity carbonic anhydrases, respectively, of other mammals. When these pig B and C isozymes were compared with the red cell carbonic anhydrases of other ungulates (cattle and horse), several interesting features were observed. In contrast to the electrophoretic gel patterns of the horse B and C isozymes in which the C form is markedly more basic than the B form, the high activity C form of pig was observed to be more acidic than the low activity B form. The tryptic peptide map of bovine carbonic anhydrase appears to be more similar to that of porcine carbonic anhydrase C than to B, indicating that they are probably homologous proteins. Neoprotosil binding by the pig and horse B isozymes give rise to essentially identical spectra in the 425- to 600-nm region, whereas the C isozymes, from these two sources, generate quite different spectra.

Carbonic anhydrase is known to occur in mammalian red blood cells as either one or two molecular forms; the enzyme showing the highest specific activity in the reversible hydration of CO$_2$ is usually designated as carbonic anhydrase C or carbonic anhydrase II, and the low activity enzyme as carbonic anhydrase B or carbonic anhydrase I (for reviews see References 1-3). A number of other isozymes have been observed, but recent genetic and chemical studies indicate that these represent alternate forms of either the B or C molecules and are not the products of additional genetic loci (4-6). In several primate species, the two isozymes, carbonic anhydrase B and carbonic anhydrase C, have been shown to be under the control of different autosomal genes (see References 6 and 7).

Those mammalian species in which red cell carbonic anhydrases have been separated, and their activity or structure characterized to some extent, now include man and cattle (see 1-3); green monkey, pig-tailed macaque, baboon, spider monkey, deer mouse (7); rhesus macaque (8); dog (9); horse (10); guinea pig (11, 12); rat (13); and blue-white dolphin (14). With the exception of cattle, dog, and possibly dolphin, all of which appear to have one main high activity component, the erythrocytes of these species contained both the high and low activity isozymes. When both the high and low activity isozymes occur together in the same organism, all of the high activity forms reported to date appear to have higher isoelectric points than the low activity form.

The work reported here on the carbonic anhydrases of the domestic pig (Sus scrofa) appears to be yet another example of a species with erythrocytes containing two distinct forms of this enzyme which differ in specific activity and amino acid composition. On the basis of these differences, it was possible to classify them as a high activity form (carbonic anhydrase C) and a low activity form (carbonic anhydrase B). Additional physical and chemical studies were undertaken to characterize these enzymes in greater detail, and to compare them with the presumed evolutionarily homologous carbonic anhydrases of other mammalian species.

EXPERIMENTAL PROCEDURE

Enzyme Preparation—Porcine red blood cells were isolated from citrated whole blood obtained from Pentex Inc., Kankakee, Illinois. The cells were washed three times with 0.9% NaCl and then hemolyzed by suspending the packed cells in 1 volume of distilled water and storing the solution overnight at 4°C. Hemoglobin was removed by selective denaturation with ethanol and chloroform as previously described (15). After concentrating the supernatant liquid, the material was passed over a column of DEAE-cellulose equilibrated with 0.003 M phosphate buffer, pH 7.1, and elution was effected with this same buffer. The DEAE-cellulose column peak, containing the carbonic anhydrase activity, was further fractionated into two major forms by subsequent adsorption onto a DEAE-Sephadex column as described elsewhere (6, 16). The column was eluted with the equilibrating buffer, 0.05 M Tris-Cl, pH 8.7, to remove the B form of the enzyme. This step was followed with a 0 to 0.2 M NaCl gradient to elute the C form. All column fractions

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Electrophoresis was carried out at 4°C for 1.8 hours with a gradient buffer, pH 7.0, for 3 days, 0.42 ml of outer dialysis solution was contained and the material of interest was removed and re-electrofocused to further separate and purify the major and minor C forms of the enzyme. Subsequent studies involving the major C form were carried out using material prepared in this manner.

Protein Concentration—Protein concentration measurements were made on the basis of A_{280} values determined according to the method of Babul and Stellwagen (17). Centrifugation was carried out using the Spinco model E machine equilibrated to a temperature of 20°C and at a rotor speed of 10,000 rpm. The ultracentrifuge cell consisted of a standard double sector, synthetic boundary centerpiece and sapphire windows. After dialysis of the protein solution against 0.05 M sodium phosphate buffer, pH 7.0, for 8 days, 0.42 ml of outer dialysis solution was placed in one limb of the cell and 0.15 ml of the protein solution, after recording the optical density at 280 μM, in the other limb. The refractive increment (fringes per mg per ml) obtained in each case was converted to A_{280} using bovine α-chymotrypsin as the standard of refractive index comparison (17).

Starch Gel Electrophoresis—Starch gel electrophoresis was carried out as described in Reference 6. The gel was made up in 0.02 M borate-NaOH buffer, pH 8.6, and the bridge buffer consisted of 0.3 M borate-NaOH, pH 8.0, containing 0.03 M NaCl. Electrophoresis was carried out at 4°C for 18 hours with a gradient potential of 8.3 volts per cm. Protein staining was accomplished using 0.4% ninhydrin dissolved in water-methanol-acetic acid (5:5:1).

Peptide Mapping—The fingerprint methodology used in this work was essentially that described elsewhere (6, 18). After heat denaturation, the protein (2 to 2.5 mg) was degraded with a 1% trypsin solution (three times crystallized, Worthington) for 2 hours at 37°C. In the electrophoresis direction, pyridine-acetic acid-water (150:120:10) was used. The maps were developed by spraying with a 0.5% ninhydrin and ethanol solution and heating at 95°C for 5 min.

Amino Acid Composition—Amino acid analysis was carried out using Technicon equipment and methodology. Hydrolysis times of 24, 48, and 72 hours were employed to permit corrections for the destruction or slow release of certain amino acids (19). Duplicate samples were analysed for each of the time intervals. Hydrolyzates were prepared by heating 0.5- to 0.7-mg samples with 5.7 N HCl under reduced pressure. Cysteine content was determined by denaturing a known amount of enzyme with recrystallized 6 N guanidine hydrochloride and subsequently titrating the cysteine residue with 2-bromoaceticamido-4-nitrophenol (20). The molar absorptivity of this group bound to chymotrypsin was used to determine the amount of reagent bound to carbonic anhydrase. Tryptophan determinations were made by the method of Edelhoch (21) without modification. The amino acid composition data were used to calculate the partial specific volume of the two major forms of porcine carbonic anhydrase (22).

RESULTS

Enzyme Purification—The purification of porcine carbonic anhydrase was carried out using techniques which have been previously applied to isolation of this enzyme from other sources (1). Starting with a liter of whole blood, approximately a gram of protein was recovered after the chloroform and ethanol treatment. Subsequent purification with DEAE-cellulose reduced this to 700 mg of protein, the bulk of which was carbonic anhydrase. To separate the two major components from each other and from contaminating proteins, a DEAE-Sephadex column was employed in conjunction with a linear NaCl gradient. The gradient was found necessary to expedite elution of the C form of the enzyme from the column. As will be shown later, the C component isolated in this fashion required further purification. Shown in Fig. 1 is the elution pattern observed when the single peak obtained from DEAE-cellulose chromatography was fractionated on the DEAE-Sephadex column. The minor peak, centered at Fraction 40, contained no observable hydrase or esterase activity and therefore was discarded. Protein content of the B and C peaks generally were observed to be of a 3:1 ratio favoring the B isozyme. The designation of the two major peaks as the B and C components is based on the composition and activity results.
obtained. It has generally been agreed that the component showing low specific hydrase activity be designated the B form and the component with high hydrase activity be designated as the C form (see Reference 10). This convention was followed throughout this study.

Gel Electrophoresis Patterns—Shown in Fig. 2 are the starch gel patterns obtained after DEAE-Sephadex column purification of porcine carbonic anhydrase. As can be seen, the C form migrates more rapidly toward the anode than the B form. The migration rates did not appear to depend upon protein concentration. Wells 1, 2, and 3 represent the three peaks observed to separate on a DEAE-Sephadex column (Fig. 1). Well 1 contains the minor peak; no esterase or hydrase activity could be demonstrated on starch gels with this material. Well 2 contains the material comprising the second peak of Fig. 1. Only two protein bands were seen: a heavy band representing the B component and a very light band which migrates to a position just below the C form; the latter is not visible in Fig. 2. The middle peak (B) was considered homogeneous and no additional purification was undertaken. The material purifying the third peak is shown in Well 3. Although the third peak consists primarily of the C form and its minor component (C (+I)), there clearly are contaminating proteins present. To confirm that both major forms occurred in individual animals, a number of samples from different animals were screened using starch gel electrophoresis. In all cases it appeared that reproducible amounts of both the major forms occurred in each sample tested.

Electrofocusing was undertaken to further purify the third DEAE-Sephadex peak. The results obtained are shown in Fig. 3. Two major peaks and several minor peaks were observed when the material comprising the third peak (C) of Fig. 1 was fractionated on a pH gradient of 5 to 8. This is shown in the upper portion of Fig. 3. Because some contaminating material remained associated with the C component, it was necessary to remove that section of the pH gradient containing the C form and subject it to further electrofocusing. These results are...
TABLE I
Comparative physical properties of pig, horse, and bovine carbonic anhydrase B and C

<table>
<thead>
<tr>
<th></th>
<th>B form</th>
<th>C form</th>
<th></th>
<th>B form</th>
<th>C form</th>
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<tr>
<td></td>
<td>Pig</td>
<td>Horse</td>
<td>Pig</td>
<td>Horse</td>
<td>Bovine</td>
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<td>$D_{20,w}$ × 10^{-13}</td>
<td>3.2</td>
<td>2.7</td>
<td>2.9</td>
<td>2.7</td>
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<tr>
<td>$D_{20,w}$ × 10^{-14}</td>
<td>9.0</td>
<td>9.0</td>
<td>9.1</td>
<td></td>
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<td>Partial specific volume</td>
<td>0.73</td>
<td>0.73</td>
<td>0.73</td>
<td>0.73</td>
<td>0.73</td>
</tr>
<tr>
<td>$s/D$ molecular weight × 10^{-4}</td>
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<td>2.9</td>
<td>2.9</td>
<td>2.8</td>
<td>3.0</td>
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<tr>
<td>$A_{280}$</td>
<td>16.0</td>
<td>15.6</td>
<td>15.2</td>
<td>15.4</td>
<td></td>
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</tbody>
</table>

* Furth (10).
* Nyman and Lindskog (26).
* Based on amino acid composition.
* Determined according to Schachman (27).
* At a protein concentration of 7 mg per ml.

TABLE II
Amino acid composition of porcine, equine, and bovine carbonic anhydrases

Hydrolysis periods of 24, 48, and 72 hours were used to permit corrections for slow rate of release and amino acid destruction. The composition data for both forms of pig enzyme is based on an assumed molecular weight of 3 × 10^4. All values are given to the nearest integer. Cysteine and tryptophan were determined independently using spectrophotometric methods (see "Experimental Procedure.")

<table>
<thead>
<tr>
<th></th>
<th>Porcine B</th>
<th>Equine</th>
<th>Porcine C</th>
<th>Equine</th>
<th>Bovine</th>
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<tr>
<td>Found</td>
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<td>19</td>
<td>19.5</td>
<td>20</td>
<td>19</td>
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<tr>
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<td>22</td>
<td>19</td>
<td>19.5</td>
<td>20</td>
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<td>Lys.</td>
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<td>14</td>
<td>13.2</td>
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<td>12</td>
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<td>His.</td>
<td>5.6</td>
<td>6</td>
<td>5.8</td>
<td>9</td>
<td>9</td>
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<tr>
<td>Arg.</td>
<td>34.3</td>
<td>34</td>
<td>26.6</td>
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<td>27</td>
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<tr>
<td>Asp.</td>
<td>20.3</td>
<td>20</td>
<td>26.0</td>
<td>28</td>
<td>26</td>
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<tr>
<td>Glu.</td>
<td>9.9</td>
<td>10</td>
<td>12.5</td>
<td>13</td>
<td>12</td>
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<tr>
<td>Thr.</td>
<td>27.6</td>
<td>28</td>
<td>16.9</td>
<td>17</td>
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<tr>
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<td>17</td>
<td>16.9</td>
<td>17</td>
<td>16</td>
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<tr>
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<td>17.7</td>
<td>18</td>
<td>22.1</td>
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<tr>
<td>Gly.</td>
<td>18.6</td>
<td>19</td>
<td>12.5</td>
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<tr>
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<td>0.0</td>
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<td>Gly.</td>
<td>15.4</td>
<td>15</td>
<td>12.5</td>
<td>13</td>
<td>19</td>
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<tr>
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<td>1</td>
<td>2.8</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Ile.</td>
<td>14.5</td>
<td>15</td>
<td>10.3</td>
<td>10</td>
<td>7</td>
</tr>
<tr>
<td>Leu.</td>
<td>2.2</td>
<td>21</td>
<td>22.8</td>
<td>23</td>
<td>22</td>
</tr>
<tr>
<td>Tyr.</td>
<td>8.0</td>
<td>11</td>
<td>7.0</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Phe.</td>
<td>9.6</td>
<td>10</td>
<td>12.2</td>
<td>12</td>
<td>11</td>
</tr>
<tr>
<td>Try.</td>
<td>7.1</td>
<td>7</td>
<td>6.0</td>
<td>6</td>
<td>5</td>
</tr>
</tbody>
</table>

* Data taken from Furth (10).
* Data taken from Nyman and Lindskog (26).
* Determined by titration with 2-bromoacetamido-4-nitrophenol.
* Determined by spectrophotometric assay according to the procedure of Edelhoch (21).

shown in the lower half of Fig. 3 and in Wells 5 and 6 of Fig. 2. As can be seen in these two figures, the major and minor components are clearly separated from contaminating protein. Wall 4 of Fig. 2 contains material from the peak centered at Fraction 24, while Well 5 consists of the other peak seen at Fraction 42. All subsequent experiments were performed using C component prepared in this fashion.

Physical Properties—The analytical ultracentrifuge was utilized to ascertain the homogeneity of the purified B and C forms of the enzyme. Sedimentation velocity runs indicated a single component in each case. As shown in Table I, the B form had an $A_{280}$ value of 3.2 × 10^{-4}, while the C form had a corresponding $A_{280}$ value of 2.9 × 10^{-4} at a protein concentration of 8 mg per ml. The calculated $D_{20,w}$ values of the B and C components of porcine carbonic anhydrase were determined by the method of boundary spreading with the Spinco model E ultracentrifuge.

Using the data of Table I, the approximate $s/D$ molecular weights of the B and C forms were calculated. In both cases, the calculations indicated a molecular weight of about 30,000 which is in close agreement with values reported for other sources of this enzyme (10, 28).

The $A_{280}$ values reported in Table I are also in agreement with those reported previously for other sources of the enzyme (10, 25). The difference in $A_{280}$ values between the B and C forms of pig may reflect the higher tyrosine content of the B form. Because the amide nitrogen was not determined, it is not possible to calculate an exact molar absorptivity value from the amino acid data. However, the amino acid composition data can be used to calculate the partial specific volume of the two forms. Such calculations indicate a value of 0.73 cc per mg for both forms.

Amino Acid Composition—The amino acid compositions of the two purified forms of porcine carbonic anhydrase are shown in Table II. The average values were obtained on the basis of tyrosine content as determined according to the technique indicated under "Experimental Procedure." The methionine content in these data reflects inclusion of methionine sulfide oxide content only, since low levels of methionine sulfone are sometimes masked by the aspartic acid peak. An average value of 0.45 (range, 0.37 to 0.58) methionine residues was found for the B form; it was assumed that this probably represented 1 residue of this amino acid. Tryptophan and cysteine content were both determined spectrophotometrically, the former by the method of Edelhoch (21) and the latter by titration with 2-bromoacetamido-4-nitrophenol.

As in the case of bovine carbonic anhydrase B (26), both major forms of porcine carbonic anhydrase appear to have no cysteine. To confirm that there were no disulfide bridges involved, the enzyme was reduced with 2-mercaptoethanol in the presence of 6 m guanidine hydrochloride before titration.

Since the B component appeared to contain only a single methionine residue, it was of interest to determine if its position was homologous to the methionine residue found in the C-terminal portion of human and bovine carbonic anhydrases. Cyanogen bromide treatment and subsequent purification of a possible peptide fragment according to the methods of Sampath Kumar, Clegg, and Walsh (29) was undertaken. Previous unpublished results in this laboratory have indicated this method works well with human enzyme. However, when similar experiments were carried out with 1 μmole of the B component of porcine carbonic anhydrase, there was no indication of a peptide corresponding to the C-terminal fragment found in the human and bovine enzymes. It was therefore concluded that the methionine residue occurring in the B form does not correspond to that methionine residue known to be located 10 to 20 residues from the C-terminal end of these enzymes (30).
Neoprontosil Spectra—Fig. 4 shows the spectral envelopes of porcine carbonic anhydrase-azosulfonamide complexes. These complexes were formed by combining equal molar amounts of enzyme and the azosulfonamide compound, Neoprontosil. Shown in the figure are unbound compound, compound bound to the B and C forms of porcine carbonic anhydrase, and compound bound to the B form of equine carbonic anhydrase. The bathochromic and hypochromic shifts seen in the 500-nm region are indicative of differences surrounding the binding site of the zinc atom. Coleman (25) has suggested that such differences may be usefully employed to learn how closely related various forms of carbonic anhydrase are. The data in Fig. 4 indicate that the B and C forms of porcine carbonic anhydrase are quite different from each other, while the spectral properties of the B forms of equine and porcine enzyme are very similar. It was observed that both equine and porcine B forms shifted approximately 6 nm towards the red while the porcine C form shifted 10 nm. On the other hand, the porcine C form did not show the extensive hypochromic shift shown by both the porcine and equine B forms. Although not shown in Fig. 4, equine carbonic

The human enzyme data has been added for comparison purposes.

Table III

<table>
<thead>
<tr>
<th>Carbonic anhydrase</th>
<th>Esterase activities</th>
<th>CO-hydrazide activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>β-Naphthyl acetate</td>
<td>β-Nitrophenyl acetate</td>
</tr>
<tr>
<td>Porcine B</td>
<td>0.143</td>
<td>0.18</td>
</tr>
<tr>
<td>Human B</td>
<td>0.044</td>
<td>0.32</td>
</tr>
<tr>
<td>Porcine C</td>
<td>0.031</td>
<td>1.05</td>
</tr>
<tr>
<td>Human C</td>
<td>0.020</td>
<td>1.77</td>
</tr>
</tbody>
</table>

* 2-Hydroxy-5-nitro-α-toluene sulfonic acid sultone.
* Micromoles of β-naphthol formed per min.
* Micromoles of p-nitrophenol formed per min.
* Micromoles of nitrooxybenzylsulfonic acid formed per min.
* ΔO.D.270 per min.

Fig. 4. Spectral shifts induced in the Neoprontosil spectra by the binding of various carbonic anhydrases. The free Neoprontosil (5 × 10⁻⁶ M) was dissolved in 0.025 M Tris-HCl, pH 7.5. All enzyme solutions, made up in the Tris buffer, contained 5 × 10⁻⁶ M enzyme and 5 × 10⁻⁶ M Neoprontosil. Porcine carbonic anhydrase B (PCA B), ——; porcine carbonic anhydrase C (PCA C), ——; horse carbonic anhydrase B (HCA B), ——.

Fig. 5. Peptide maps of the tryptic digests of porcine carbonic anhydrases B (PCA B) and C (PCA C), and bovine carbonic anhydrase B (BCA B). Descending chromatography in pyridine-butanol-acetic acid-water (100:150:30:120) was followed by high voltage electrophoresis at pH 6.5 in pyridine-acetic acid-water (100:4:900). The fingerprints were developed using a ninhydrin spray.
anhydrate C, in the presence of equal molar amounts of Neoprontosil, generates a spectral envelope very similar to that of porcine and equine B components.

**Enzyme Activity**—It has been previously shown that the two isozymes of mammalian red cell carbonic anhydrase differ considerably in their specific activity regarding the CO₂ hydrase and esterase reactions (see References 1, 6–8, 10, 13). These differences, in terms of relative specific activities, appear to be emerging as follows for the following four substrates: enzyme C greater than enzyme B (CO₂, p-nitrophenyl acetate, 2-hydroxy-5-nitro-α-toluenic sulfonic acid sulphone); enzyme B greater than enzyme C (β-naphthyl acetate). The same pattern can be seen to hold for the porcine carbonic anhydrases as shown in Table III. Contrasting the human and porcine enzymes, it can be seen that both of the human forms are more reactive than the corresponding porcine forms in the hydrolysis of p-nitrophenyl acetate and 2-hydroxy-5-nitro-α-toluenic sulfonic acid sulphone.

Although kinetic data of the inhibition were not characterized, it was observed that the activities of both porcine forms were inhibited by 10⁻⁴M acetoazamidine, a compound known to be a potent, specific inhibitor of carbonic anhydrase.

**Peptide Mapping**—Further insight into similarities and differences between the two forms of porcine carbonic anhydrase was gained through the use of the fingerprint technique. Fig. 5, A and B, shows the peptide maps of the B and C forms obtained after digestion with trypsin followed by removal of a small amount of core material. Clearly, there are substantial differences between the two major forms of the enzyme. Such differences are also seen when other sources of the enzyme are compared in a similar fashion (7). However, there appear to be a number of similarities between the C form of porcine and the B form of bovine enzyme (Fig. 5, B and C). The latter is now considered to be evolutionarily homologous to other primate and ungulate C forms (30).

**Discussion**

The two major forms of porcine carbonic anhydrase studied in this work differed considerably in their ability to carry out the hydrase and esterase reactions. Furthermore, there is a clear dissimilarity regarding the total amount of each component. Typically, previous carbonic anhydrase systems that have contained two dissimilar major forms have exhibited a low and a small amount of core material. Clearly, there are substantial differences between the two major forms of the enzyme. Such differences are also seen when other sources of the enzyme are compared in a similar fashion (7). However, there appear to be a number of similarities between the C form of porcine and the B form of bovine enzyme (Fig. 5, B and C). The latter is now considered to be evolutionarily homologous to other primate and ungulate C forms (30).

The hypochromic shift is much less in the case of the C form, relative to the B form, indicating variation at the active site of these two isozymes. Coleman's (25) investigation of human and monkey carbonic anhydrases has also shown a similar effect; i.e. the B form has the larger hypochromic shift. Horse carbonic anhydrase B, also shown in Fig. 4, displays an analogous shift when azosulfonamide is added in equal molar amounts. As previously mentioned, the spectra of Neoprontosil bound to equine carbonic anhydrase C were also measured and were observed to be almost identical to equine carbonic anhydrase B in the 450- to 600-nm region. This would seem to indicate considerable similarity in the active site area, yet it has been previously shown that these two forms of equine carbonic anhydrase differ substantially in their ability to carry out both the hydrase and esterase reactions (10). It would thus appear that the hypocromatic shifts, seen when Neoprontosil is bound to the protein, are difficult to interpret as being generated specifically by the high and low activity forms of carbonic anhydrase.

Sufficient data are not available to attach much taxonomic

1 In the staining reaction, the β-naphthol-azo dye complex produces pink and the α-naphthol-azo dye complex produces gray (6). When equal amounts of the purified pig B and C enzymes are incubated (after starch gel electrophoresis) in an equal molar mixture of the α- and β-naphthyl acetate, the B form stains most intensely with the β-ester while the C form stains most strongly with the α-ester.

2 R. J. Tanis, unpublished data.
meaning to the findings on the three ungulate species studied to date; nevertheless, it is noteworthy that the electrophoretic pattern of the horse (order, Perissodactyla) carbonic anhydrases are not especially different from those of species in other mammalian orders, although its high activity form does appear to have the highest isoelectric point of any species examined to date (10). On the other hand, the pig, cattle (30), and sheep, which are in the order Artiodactyla, all have high activity forms which are characterized by low isoelectric points. In addition, the two ruminants (sheep and cattle) have only one major carbonic anhydrase which appears to be homologous to the high activity form of other species, whereas the pig has both the high and low activity forms. It will be particularly interesting to look at the red cell carbonic anhydrases of other ungulate species (domesticated as well as undomesticated) to see whether the patterns just described would be useful in classifying the various groups and subgroups of ungulates.

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