Human Carbonic Anhydrases

VI. LEVELS OF ISOZYMES IN OLD AND YOUNG ERYTHROCYTES AND IN VARIOUS TISSUES*

(Received for publication, October 5, 1970)

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SUMMARY

The more acidic, minor isozymes of human carbonic anhydrase types B and C are present in higher concentrations in the older erythrocyte populations. The levels of all of these isozymes can be determined quantitatively by immunological and electrophoretic methods following their separation by electrofocusing. A wide variety of human tissues have been assayed for carbonic anhydrases. Significant levels of the C type were found in all tissues except cardiac muscle and testis. Carbonic anhydrases of the B type were found only in kidney medulla and gall bladder mucosa.

A rather large number of carbonic anhydrase isozymes have been isolated from human erythrocytes (1). These can be classified immunologically as either B or as C type isozymes (1), i.e. isozymes B, A, D, T, E + F, and O + P are of the B type, and C, G + H, and M + N are of the C type. All of these isozymes exist in vivo. They can be converted in vitro to more acidic isozymes (2), i.e. isozymes B to A, A to D, D to T, C to G + H, etc. The conversion of a given isozyme to the next more acidic form results from the loss of one amide group per molecule of enzyme (2). The physicochemical properties of the A and D isozymes generated in vitro were quite similar to the isozymes of the same electrophoretic mobilities isolated from erythrocytes (2). This suggested that the minor isozymes are generated in vitro from the B and C forms of these enzymes.

The levels of some of the minor isozymes of the B and C types of carbonic anhydrases have been determined in human erythrocytes, and it has been found that they are present in increased amounts in the older erythrocytes. Determination of the levels of the B and C type isozymes in various human tissues has shown that the C type is widely distributed but that the B isoenzyme is virtually absent except in kidney medulla and in gall bladder mucosa.

MATERIALS AND METHODS

Erythrocyte fractions of different density were separated by high speed centrifugation (68,000 × g for 2 hours at 4°C) with a Spinco model L centrifuge and a SW-39 rotor) by the method of Rigas and Koler (3) or by the relatively low speed centrifugation method (2 hours at 20°C and 3500 rpm with a Sorvall RC-3 type centrifuge) of O’Connell, Caruso, and Sass (4). Both of these groups of workers have shown that the upper layer of centrifuged erythrocytes of patients who had been given 55Fe a short time previously contained almost all of the radioactivity. This indicates that the most recently synthesized erythrocytes, i.e. the young erythrocytes, have a lower density than the older ones. The heparinized blood samples used were first centrifuged for a few minutes at 1000 rpm in the latter centrifuge to effect the separation of plasma and buffy coat.

Following density separation of the erythrocytes, the centrifuged samples were carefully cooled to 0°C and then frozen by immersion in a -15°C methanol bath. The frozen material in plastic centrifuge tubes was sectioned with a saw to provide samples representing the top and bottom quarter portions of the erythrocytes so separated. These samples containing the younger (top) and older (bottom) erythrocyte populations were lyed by the addition of an equal volume of water and subsequent repeated freezing and thawing of the material. The stroma of the samples in which gel filtration was later used to separate the hemoglobin was removed by centrifuging at 4°C for 2 hours at 68,000 × g. The hemoglobin contents of the lysates were determined by the methemoglobin cyanide method of Kampen and Zijlstra (5). Hemoglobin was removed by either the ethanol-chloroform fractionation procedure of Tsuchihashi (6) or by filtration of stroma-free hemolysates over columns of Sephadex G-75 as previously described (1) to provide crude fractions containing essentially all of the erythrocyte carbonic anhydrases. These fractions were concentrated in the usual manner by precipitation with saturated ammonium sulfate (1).

Tissue samples were obtained from autopsy cases that were relatively fresh. The tissues were cooled to 0°C in either 0.15 M NaCl or in pH 7.4 isotonic NaCl-borate buffer and then sliced to near 1-mm thickness. The tissue slices were then washed several times by decantation with 0.15 M NaCl at 0°C, blotted dry with filter paper, chopped into small pieces with a razor blade, and then dispersed at 0°C with a glass pestle type homogenizer. In initial experiments the tissue slices were chopped on filter paper, but this led to considerable loss of fluid and activity. In the more successful experiment, the washed tissue slices were chopped on a glass plate and all of the tissue and fluid was transferred to the homogenizer tube. Water had to be added to samples minced on filter paper; those cut on glass plates could be homogenized satisfactorily without addition of water.
mogenates were frozen (−70°) and thawed three times and then centrifuged at 0° for 30 min at 30,000×g. The supernatants were assayed for hemoglobin and for carbonic anhydrases B and C by the radial immunodiffusion method of Mancini, Carbonara, and Heremans (7). A hemolysate of the same individual was also subjected to these assays. The amounts of carbonic anhydrase B and C contributed by erythrocytes to each tissue homogenate could be then determined from the amount of hemoglobin found in the tissue extracts.

The carbonic anhydrase antibody preparations used in the radial diffusion assays were the same as those used in previous work (1, 8). An antibody preparation to human hemoglobin was prepared by immunization of rabbits to hemoglobin prepared by gel filtration techniques. It failed to react with carbonic anhydrases, cytocuprein, or catalase and gave a single zone of precipitation in radial immunodiffusion tests with hemolysates.

Electrofocusing experiments were carried out by the method of Svensson (9) with the LKB apparatus. Vertical starch gel electrophoresis experiments used the method of Smithies (10), and all experiments were conducted at pH 8.6 in a Tris-EDTA-borate buffer.

Nitrogen analyses were carried out by a micro-Kjeldahl procedure.

RESULTS

In a preliminary experiment, the crude carbonic anhydrase mixtures obtained from young and old erythrocytes that had been separated by low speed centrifugation were subjected to starch gel electrophoresis. An unstained portion of the gel was cut at the positions indicated by a stained section of a reference sample to contain the B, A, and D isozymes. That portion of the gel containing the B isozyme also contained the C types designated as G + H (1). The carbonic anhydrase activity of the extracts of the gel sections were assayed titrametrically in the manner previously described (1). The results shown in Table I suggest that the relative level of minor isozymes A and D are higher in the material from older erythrocytes. These results are somewhat qualitative since, with the enzymatic assay used, the purified C type isozymes are found to possess from near 5 to 7 times more activity than the B type (1) and, furthermore, the C type isozymes in crude extracts may be variably activated by various substances (8, 11, 12). This prompted further investigation by quantitative immunochemical methods to evaluate isozyme levels.

<table>
<thead>
<tr>
<th>CA</th>
<th>Total enzymatic activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Young cells</td>
</tr>
<tr>
<td>B and G + H</td>
<td>92.2</td>
</tr>
<tr>
<td>A</td>
<td>6.9</td>
</tr>
<tr>
<td>D</td>
<td>0.9</td>
</tr>
</tbody>
</table>

* CA, carbonic anhydrases.
* Carbonic anhydrases G + H which are C type isozymes contribute about 10% of the activity of this fraction.

Aliquots of the crude carbonic anhydrase mixtures from the young and the old erythrocyte populations, each equivalent to 2 g of hemoglobin, were subjected to electrofocusing. The protein levels of the 1-ml fractions collected in the experiments presented were determined by $E_{280\text{nm}}$ measurements with the extinction coefficients of the purified enzymes determined previously (2). The levels of the B and of the C type carbonic anhydrases in each fraction were assayed immunochemically. Results of an experiment for one blood erythrocyte sample are shown in Fig. 1, A and B. It is to be noted that in spite of the complexity of the system the results obtained for the two experiments, one with
DISTRIBUTION OF CARBONIC ANHYDRASE ISOZYMES IN OLD AND YOUNG ERYTHROCYTES

<table>
<thead>
<tr>
<th>Type and CA</th>
<th>Total isozymes recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment I</td>
</tr>
<tr>
<td></td>
<td>Young</td>
</tr>
<tr>
<td></td>
<td>%</td>
</tr>
<tr>
<td>B. T</td>
<td>–</td>
</tr>
<tr>
<td>D</td>
<td>0.30</td>
</tr>
<tr>
<td>A</td>
<td>5.9</td>
</tr>
<tr>
<td>B</td>
<td>92.7</td>
</tr>
<tr>
<td>Basic components</td>
<td>1.0</td>
</tr>
<tr>
<td>C. M + N</td>
<td>–</td>
</tr>
<tr>
<td>G + H</td>
<td>8.9</td>
</tr>
<tr>
<td>C</td>
<td>91.1</td>
</tr>
<tr>
<td>Basic components</td>
<td>4.6</td>
</tr>
<tr>
<td>Ratio of B type to C type</td>
<td>7.8</td>
</tr>
</tbody>
</table>

- CA, carbonic anhydrases.
- Blood from a single individual was used for both experiments.
- The Tsuchikashi procedure was used to remove hemoglobin after separation of young and old cells. The young and old erythrocytes used in Experiment I were separated by centrifugation for 2 hours at 4°C near 68,000 × g (3) and in Experiment II for 2 hours at 20°C near 2,500 × g (4). In Experiment I, each 1-ml fraction collected from the electrofocusing column was assayed immunochromatically. In Experiment II, these assays were performed on the fractions pooled according to the results of optical density measurements.
- The + indicates a positive reaction, but the protein was present at a level of less than 15 μg per ml and could not be determined quantitatively.
- The basic components are proteins with higher isoelectric points than carbonic anhydrase B and C which react with antibody to carbonic anhydrase B and C, respectively.

Activity measurements cannot be used to determine quantitatively the carbonic anhydrase isozymes in tissues. Several problems present themselves. All isozymes of the B or of the C type exhibit from 5 to 20 times more than those of the B type, depending on the assay method used. Furthermore, the activity of the C isozyme is augmented by the presence of extraneous substances (2). For these reasons, a more quantitative method, such as that based on immunochromological properties of these enzymes, is required for their determination.
mucosa for one individual was much higher than two other

B to C isozyme in human erythrocytes is near 7, and it is difficult

part by erythrocyte contamination. However, the ratio of the

kidney cortex protein is near the above level. The high level of

the B isozyme found by Wistrand and Rao may be explained in

human kidney cortex contained only the C isozyme. Wistrand

Rao (13). To explain the ratio of 65 that was reported by Wistrand and

anhydrases in various tissues. However, they are based on

results of 1.71 fig of carbonic anhydrase C per mg of extractable

cortex. The C isozyme constituted 4 pg of the total. Our

and C could be extracted from 1 mg of freeze-dried human kidney

proven to be ideal for this purpose. The ratios of the D, A, and

isozyme found in human erythrocytes as reported in Table II

are near 90:15:1. These data agree with the amounts of these

isozymes that were isolated from human erythrocytes in previous

investigations (1). The increased levels of the minor isozymes in the older erythro-

cyte populations is indicative of a slow loss in vivo of amide

groups from the C and B enzymes which results in the generation

of more acidic forms. The quantitative estimation of these minor isozymes might give an index of the relative average age of an erythrocyte population.

Many studies have been made on the distribution of carbonic anhydrases in various tissues. However, they are based on activity measurement and, for reasons indicated above, lack quantitative significance.

Wstrand and Rao (13) have determined the carbonic anhydrase levels of an extract of human kidney cortex by a hemagglutination technique. They report a ratio of B to C isozyme of near 65. This result contrasts sharply with our finding that

the B type isozymes were found only in kidney cortex and gall bladder mucosa. It is

of tissues will be required to determine the extent of the variations in levels of this enzyme in different individuals.

The immunochemical approach to the measurement of carbonic anhydrase in tissues would appear to be the only valid method available at present to determine quantitatively the level of the B and C isozymes. Adequate controls must be run to determine the erythrocyte contribution to the total carbonic anhydrase level of a tissue extract. It is, furthermore, to be emphasized that the results presented here relate only to the tissue carbonic anhydrases that are readily extractable.

The results reported for tissues in the present study are for B and C type isozymes and not specifically for carbonic anhydrases B and C. The quantitative determination of immunological reactivity with electrophoretic properties was established for the hemolysates but not for the other tissue extracts. Thus, it is conceivable that the tissues could possess isozymes in relatively high concentration which are present in erythrocytes in low levels.

Studies of the carbonic anhydrases of tissues are of particular interest in view of the finding that the B type isozymes were found only in kidney cortex and gall bladder mucosa. It is

known that these isozymes of erythrocytes are markedly depressed in thyrotoxicosis (14–16) but that the levels of the C isozymes are only slightly lowered. The question of whether the absence of carbonic anhydrase B in most human tissues is due to a normal action of thyroxine is raised. Preliminary studies of the erythrocyte carbonic anhydrases (17) of various animals have indicated considerable species variation but suggest that an animal model such as the rabbit would permit investigation of the effects of excess thyroxine, of the hypothyroid condi-

**Table III**

Levels of carbonic anhydrases B and C in various tissues of single human

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Protein</th>
<th>B</th>
<th>C</th>
<th>B/C</th>
<th>CA-B</th>
<th>CA-C</th>
<th>CA-B/CA-C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain white matter</td>
<td>28.5</td>
<td>1103</td>
<td>125.0</td>
<td>50</td>
<td>14.6</td>
<td>2.11</td>
<td>(0.016)</td>
</tr>
<tr>
<td>Brain gray matter</td>
<td>26.7</td>
<td>1613</td>
<td>20.3</td>
<td>34</td>
<td>21.3</td>
<td>3.08</td>
<td>(-0.036)</td>
</tr>
<tr>
<td>Thymus</td>
<td>79.5</td>
<td>2213</td>
<td>25.7</td>
<td>0</td>
<td>29.9</td>
<td>4.73</td>
<td>(-0.045)</td>
</tr>
<tr>
<td>Liver</td>
<td>81.6</td>
<td>5950</td>
<td>73.3</td>
<td>94</td>
<td>78.5</td>
<td>11.4</td>
<td>(-0.065)</td>
</tr>
<tr>
<td>Kidney cortex</td>
<td>48.9</td>
<td>14601</td>
<td>189</td>
<td>111</td>
<td>193</td>
<td>27.9</td>
<td>(-0.083)</td>
</tr>
<tr>
<td>Kidney medulla</td>
<td>21.1</td>
<td>8267</td>
<td>130</td>
<td>52</td>
<td>109</td>
<td>15.8</td>
<td>0.990</td>
</tr>
<tr>
<td>Gastric mucosa</td>
<td>17.6</td>
<td>9.7</td>
<td>10</td>
<td>30</td>
<td>12.1</td>
<td>1.75</td>
<td>(-0.119)</td>
</tr>
<tr>
<td>Gastric mucosa S-1p</td>
<td>59.1</td>
<td>2.72</td>
<td>49.3</td>
<td>329</td>
<td>39.7</td>
<td>5.8</td>
<td>(0.162)</td>
</tr>
<tr>
<td>Gastric mucosa S-2</td>
<td>83.4</td>
<td>1.83</td>
<td>38.7</td>
<td>117</td>
<td>22.3</td>
<td>3.5</td>
<td>(0.197)</td>
</tr>
<tr>
<td>Gall bladder S-3</td>
<td>57.1</td>
<td>1.47</td>
<td>128</td>
<td>113</td>
<td>22.5</td>
<td>3.6</td>
<td>1.85</td>
</tr>
<tr>
<td>Cardiac muscle</td>
<td>40.8</td>
<td>4100</td>
<td>61</td>
<td>0</td>
<td>54.1</td>
<td>7.83</td>
<td>(0.108)</td>
</tr>
<tr>
<td>Testis</td>
<td>34.4</td>
<td>406</td>
<td>0</td>
<td>0</td>
<td>5.36</td>
<td>7.75</td>
<td>(-0.154)</td>
</tr>
</tbody>
</table>

a CA, carbonic anhydrases.

b Values in parentheses are considered to be representative of assay fluctuations and the enzyme is probably essentially absent in the tissue.

c Hb, hemoglobin.

d CA-B, type B carbonic anhydrase.

e CA-C, type C carbonic anhydrase.

f The pyloric portion of the stomach was used as the source of mucosa.

Samples designated S are from individual surgery cases.
tion, and of other physiological states on the levels of these enzymes. Studies of carbonic anhydrases have thus projected themselves into a series of new areas which are attractive to pursue.

REFERENCES

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Human Carbonic Anhydrases: VI. LEVELS OF ISOZYMES IN OLD AND YOUNG ERYTHROCYTES AND IN VARIOUS TISSUES
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