

# ISOLATION OF HUMAN CARBONIC ANHYDRASE B AND C AND APOCARBONIC ANHYDRASE BY AFFINITY CHROMATOGRAPHY

by

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Human carbonic anhydrase B and C have been purified in high yields by affinity chromatography, using two different affinity gels, incorporating the potent benzenesulfonamide inhibitor, but coupled to Sepharose through different «spacer-arms». Although both affinity gels bind carbonic anhydrase B and C quantitatively, they exhibit different binding capacities. Sepharose-glycyl-L-tyrosine-azobenzenesulfonamide was used to separate the mixture of isoenzymes from the bulk hemoglobin in the hemolysate of human erythrocytes. This affinity gel had a capacity of 15 mg of enzyme pr. ml packed gel. The isoenzymes were eluted with thiocyanate an inhibitor of carbonic anhydrase.

The mixture of hemoglobin free isoenzymes was separated efficiently on a Sepharose-ethyl (p-carboxybenzenesulfonamide) carboxamide affinity gel by exploiting the difference in binding strengths for the B- and C- enzyme, respectively. Furthermore, the affinity gels could be used to separate the apoenzyme from the zinc enzyme.

## 1. INTRODUCTION

Purification of human carbonic anhydrase isoenzymes, carbonic anhydrase B and C, from human erythrocytes can be accomplished using DEAE-Sephadex chromatography to separate the carbonic anhydrase from the large amounts of hemoglobin and also to separate the isoenzymes from each other (2). However, large-scale preparation using this procedure requires large columns and is laborious and time consuming. Hence, searches for new procedu-

res based on affinity chromatography were investigated. Numerous studies have shown that aromatic and heteroaromatic sulfonamides are specific and strong inhibitors of carbonic anhydrase (9). Furthermore, the effect of pH on the binding constants, the differences in the binding constants for the two isoenzymes, the isoenzyme dependent inhibition by anions and the competition between anions and sulfonamides for the same binding sites are known in great details (9). These properties suggest

that sulfonamide inhibitors would provide excellent ligands for the synthesis of affinity gels for carbonic anhydrase, since both the conditions for ideal adsorption and elution of the individual isoenzymes theoretically are attainable. FALKBRING *et al.* (6) and WHITNEY (13) have previously described the isolation of carbonic anhydrase B and C by affinity chromatography, employing *p*-aminobenzenesulfonamide coupled to Sephadex and *p*-(aminoethyl) benzenesulfonamide coupled to Sepharose, respectively. However, these affinity gels exhibit a relatively low capacity for carbonic anhydrase, and hence these are not suitable for large-scale procedures.

The present paper describes the synthesis of two improved affinity gels which basically have the same ligand, benzenesulfonamide, as described by FALKBRING *et al.* (6) and WHITNEY (4), but differ in the nature of the »spacer-arm«. The Sepharose-Gly-Tyr-(*p*-azobenzenesulfonamide) affinity gel exhibits very high binding capacity for carbonic anhydrase, and is used to separate the mixture of isoenzymes from the bulk amount of hemoglobin. In addition, this affinity gel has the additional advantage of its red color, which enables visual estimation of its coupling efficiency, as well as the slow leakage of the ligand with time. Furthermore, due to its strong binding of carbonic anhydrase, it can be used in a batch-wise procedure. In contrast to this affinity gel another gel, Sepharose-ethyl(*p*-carboxybenzenesulfonamide) carboxamide, has low capacity, and can not be used in a batch-wise procedure. However, this affinity gel exhibits much stronger affinity for the C enzyme than the B enzyme. This specificity was exploited to separate a large amount of an isoenzyme mixture on a relative small column. After the present work was completed OSBORNE and TASHIAN (11) described the preparation of an affinity gel for carbonic anhydrase, in which (2,3 diaminophenyl) azobenzenesulfonamide was coupled to CM-

Sephadex, resulting in a gel with a capacity very similar to the azobenzenesulfonamide gel reported in the present paper.

Human carbonic anhydrase contains one gram atom of zinc per molecule, which is essential both for enzymatic activity and strong sulfonamide binding (4). Hence the affinity gels can also be employed to prepare metal-free apocarbonic anhydrases.

## 2. MATERIALS AND METHODS

Sepharose 4 B was obtained from Pharmacia, Uppsala, Sweden, Cyanogen bromide and *p*-carboxybenzenesulfonamide from Aldrich, Milwaukee, U. S. A. *p*-aminobenzenesulfonamide, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimid-HCl (EDC)<sup>1</sup> and glycyl-L-tyrosine from Sigma Chemical Company, St. Louis, U. S. A. and ethylene diamine from Merck, Darmstadt. Human erythrocytes were obtained through the courtesy of Dr. O. Drachmann at the Blodbank, Københavns Amts Sygehus. Two sulfonamide affinity gels, Sepharose-Gly-L-Tyr-azobenzenesulfonamide and Sepharose-ethyl(*p*-carboxybenzenesulfonamide) carboxamide, respectively, were synthesized and used as affinity columns for the isolation of human carbonic anhydrase B and C. Both the Sepharose-Gly-L-Tyr and Sepharose-ethylamine were prepared by the cyanogen bromide procedure, essentially as described by CUATRECASAS (5). Sepharose-Gly-Tyr was prepared from Sepharose 4 B freshly activated with cyanogen bromide (180 mg/ml packed Sepharose) by coupling with Gly-Tyr (5 mg/ml packed Sepharose) in 0.1 M-sodium bicarbonate, pH 9.5. The affinity gel was obtained by diazotization of *p*-aminobenzenesulfonamide and coupling of this compound to the Sepharose-Gly-Tyr. *p*-aminobenzenesulfonamide (0.3 g) was suspended in 120 ml of ice-cold 1 M-HCl, and to the suspension was added 0.9 g of sodium nitrite in 45 ml ice-cold water.

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The following abbreviations have been used: HCA, human carbonic anhydrase; HCAA, human carbonic anhydrase A; HCAB, human carbonic anhydrase B; HCAC, human carbonic anhydrase C; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimid-HCl; SA-Sepharose, Sepharose-Gly-L-Tyr-azobenzenesulfonamide; SE-Sepharose, Sepharose-ethyl (*p*-carboxybenzene-sulfonamide) carboxamide; Gly-L-Tyr, glycyl-L-tyrosine; Tris-sulfate, 2-amino-2(hydroxymethyl)-1,3-propanediol titrated to the desired pH with sulfuric acid.

After 10 minutes of reaction the diazotized benzenesulfonamide was poured into a 600 ml suspension, containing 300 ml packed Sepharose-Gly-Tyr in 0.2 M-sodium bicarbonate pH 8.8. The pH was adjusted to 9.5 with 1 M-NaOH and, after gentle stirring for 3 hours at room temperature, the coupled red Sepharose derivative (SA-Sepharose) was washed with 10 liters of water and 2 liters of 0.05 M-Tris-sulfate pH 7.6.

The Sepharose-ethylamine derivative was prepared by adding 115 ml of an aqueous solution containing 15 ml ethylene diamine, adjusted to pH 10, to 100 ml freshly activated packed Sepharose. The affinity gel was prepared by coupling p-carboxybenzenesulfonamide to the Sepharose ethylamine by the carbodiimide method. One hundred milliliters of wet packed Sepharose ethylamine was suspended in 400 ml dimethylformamide containing 1 g of p-carboxybenzenesulfonamide. EDC (5 g in 30 ml water pH 4.7) was added slowly, and the pH maintained at pH 4.7. After 20 hours of reaction at room temperature the affinity gel, Sepharose-ethyl(p-carboxybenzenesulfonamide) carboxamide (SE-Sepharose) was washed with dimethylformamide and a large volume of water.

For preparation of human carbonic anhydrase isoenzymes, the erythrocytes were washed twice with 0.9% NaCl and then hemolyzed by addition of an equal volume of water. The cell stroma was removed by centrifugation at 3000 g for 30 min. The lysate was first pumped through a column with the SA-Sepharose equilibrated with 0.05 M-Tris-sulfate pH 7.5. After washing with 0.05 M-Tris-sulfate-1M-sodium sulfate pH 7.5, the mixture of carbonic anhydrase isoenzymes was eluted with 0.2 M-potassium thiocyanate in 0.05 M-Tris-sulfate pH 6.5. The enzyme containing solution was concentrated to 20 mg/ml by ultrafiltration in an Amicon Ultrafiltration cell equipped with a UM-10 membrane. The protein was precipitated by addition of solid ammonium sulfate. After washing of the precipitate with saturated ammonium sulfate solution to remove the thiocyanate the precipitate was dissolved in a minimum volume of 0.05 M-Tris-sulfate pH 7.5 and dialyzed overnight against the same buffer.

This solution was passed through the SE-Sepharose gel, which by a simple displacement procedure allowed separation of the isoenzymes, as described in section 3.

Apocarbonic anhydrase was prepared as described by COLEMAN (3) by dialysis against 0.01 M-1,10 phenanthroline pH 5.5.

Carbonic anhydrase activity was assayed by following the hydrolysis of p-nitrophenylacetate at 348 nm according to the method of ARMSTRONG *et al.* (2). Protein concentrations were determined from the absorbance at 280 nm using  $A_{280\text{nm}}^{1\%} = 16.3$  and 18.7 for the B and C enzymes, respectively.

Amino acid analyses were performed on the Spinco model 120 amino acid analyzer. Polyacrylamide disc gel electrophoresis was performed in 7.5% gels at pH 9.2 (7). The electrophoresis was run at 4 mA per tube. The gels were stained with 1% amidoblack in 7% acetic acid and excess stain was removed by electrophoretic destaining.

### 3. RESULTS AND DISCUSSION

Both the SA-Sepharose and the SE-Sepharose gel adsorb human carbonic anhydrase (HCA) quantitatively from hemolysates of red blood cells. However, they differ in their binding capacity and ability to separate the isoenzymes. The capacities of the affinity gels were determined by passing the hemolysates over a small column of the respective affinity gel until the amount of carbonic anhydrase activity of the effluent was the same as in the starting material. After washing of the column to remove non-specifically bound protein, the carbonic anhydrase was eluted and the amount determined from the absorbance at 280 nm. The washing and elution procedures were as described in Figure 1. The capacity of the SA-Sepharose gel was approximately 15 mg of HCA per ml gel, which is about 5 times higher than for the affinity gel reported by WHITNEY (13) but equivalent to the capacity of the affinity gel described by OSBORNE and TASHIAN (11). In contrast, the SE-Sepharose gel exhibits only a capacity of 2 mg HCA per ml gel. These differences in binding efficiency for affinity gels with the same essential ligand structure, the

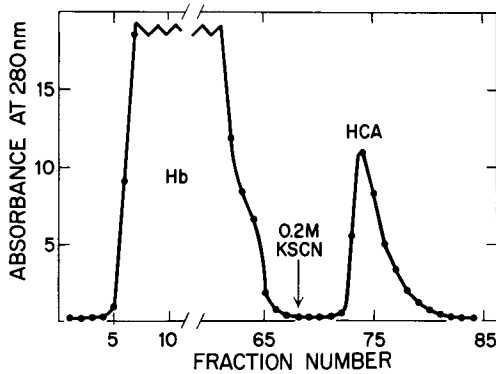


Figure 1. Purification of human carbonic anhydrase (HCA) from a hemolysate of erythrocytes on the SA-Sepharose gel. The column (1.5 cm diameter) contained 10 ml of affinity gel and was equilibrated with 0.05 M Tris-sulfate pH 7.5. The flow rate was 180 ml/hr and 4 ml fractions were collected. 50 ml of hemolysate was diluted with 150 ml water and passed through the column. The column was washed with 0.05 M Tris-sulfate -1M  $\text{Na}_2\text{SO}_4$  pH 7.5, and the enzymes eluted with 0.2 M KSCN-0.05 M Tris-sulfate pH 6.5 as indicated.

benzenesulfonamide, show that the nature of the »spacer-arm« can have strong influence on the performance of affinity gels, an effect which also has been observed in other systems (10). Furthermore, these observations suggest that not only a sufficient length of the »spacer-arm« to allow non-restricted binding is important in affinity chromatography, but also that the specific chemical structure of the »spacer-arm« can provide additional binding strength to the system and thereby enhance the efficiency of the affinity gel.

Figure 1 illustrates the isolation of HCA from 50 ml hemolysate of red blood cells with the SA-Sepharose affinity gel. HCA was adsorbed to the resin at pH 7.5. After extensive washing as described in section 2 to elute all of the hemoglobin and other nonspecific proteins, the HCA (90 mg) was eluted at pH 7.6 by  $\text{SCN}^-$ , which is a strong inhibitor of HCA and acts competitively with sulfonamides (9). Disc-gel electrophoresis (Figure 2, Gel 1) of the isolated HCA protein exhibited three bands, corresponding to the A, B and C isoenzymes, respectively. The amount of HCAA varied in the individual hemolysates, and it also depended on the age of the solution. However, when stored as an ammonium sulfate

precipitate, no change in the gel-electrophoresis pattern was observed. It has been suggested that HCAA is a deamidation product of HCAB (9).

For large-scale preparation of human carbonic anhydrase the SA-Sepharose derivative could be conveniently used in a batch-wise procedure. The SA-Sepharose gel, 300 ml in two liters of hemolysate, was stirred at room temperature and after 1 hour the carbonic anhydrase was quantitatively adsorbed. The gel was collected on a sintered glass-filter and washed with 4 liters of water followed by 1 liter of 0.05 M-Tris-sulfate, pH 7.5, 1 M- $\text{Na}_2\text{SO}_4$  to elute all the hemoglobin. The gel was then transferred to a 10 cm diameter column, and the HCA eluted with 1.0 liter of 0.2 M-KSCN in 0.05 M-Tris-sulfate, pH 6.5. After concentration to 100 ml by ultrafiltration, and precipitation of HCA with solid  $(\text{NH}_4)_2\text{SO}_4$ , about 4.5 g of a hemoglobin free mixture of the isoenzymes were obtained.

The binding of sulfonamides to both isoenzymes is pH-dependent, exhibiting weaker binding at low and high pH values (9). Furthermore, the pH-dependence of the binding constants is different for the two isoenzymes, and in addition HCAC generally binds sulfonamides stronger than the B enzyme (9). Anions show improved binding with decreasing pH, and ex-

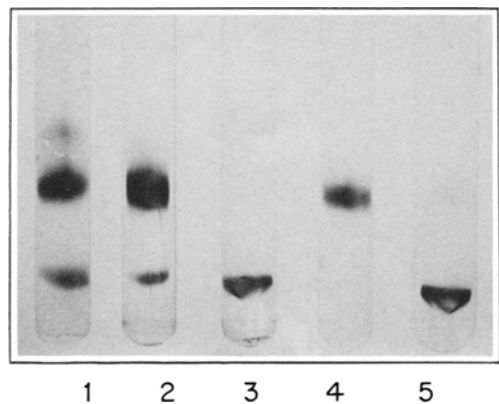


Figure 2. Polyacrylamide gels from the chromatographic peaks in Figure 1 and Figure 3. Gel 1 shows the isoenzymes isolated from the SA-Sepharose gel (Figure 1). Gel 2, 3, 4 and 5 represent the four chromatographic peaks I, II, III and IV, respectively in Figure 3A and B. For details see the text.

hibit at the same pH stronger binding to the B-enzyme than to the C-enzyme (9). Hence it should be possible to exploit these differences between the two isoenzymes to design elution condition specifically for each of them. However, several attempts to elute the isoenzymes selectively using several anions at various concentrations and pH's were unsuccessful. Chromatograms exhibiting two peaks could be obtained by eluting first with low concentration of  $\text{SCN}^-$ , followed by elution with stepwise increases in  $\text{SCN}^-$  concentration or by lowering the pH to improve  $\text{SCN}^-$  binding. However, these peaks always displayed long trailing edges, and analysis of the peaks by gel-electrophoresis indicated that they contained both isoenzymes in almost the same ratio as in the starting material. These findings indicate that either the binding constants for the immobilized sulfonamides are the same for the two isoenzymes or that the affinity gels contain a heterogeneous population of coupled sulfonamides with a wide range of binding constants resulting in enzyme-inhibitor complexes with overlapping stabilities for the two isoenzymes. In contrast to our results, FALKBRING *et al.* (6), WHITNEY (13) and OSBORNE and TASHIAN (11) using their respective benzenesulfonamide affinity gels could achieve separation of the isoenzymes by variation of anion concentration and pH. These facts further emphasize the involvement of the »spacer-arm« in the binding process.

Similarly to the SA-Sepharose column, HCA was also specifically adsorbed to the SE-Sepharose, and a hemoglobin free mixture of HCAB and HCAC could be isolated using the same elution conditions as described for SA-Sepharose. However, since its capacity was only 2 mg/ml gel it was not used for the separation of HCA from hemoglobin. However, it was noticed that the binding of the C enzyme to this gel was much stronger than for the B enzyme, and this phenomenon was used to separate the two isoenzymes as illustrated in Fig. 3 A and B. In this experiment 400 mg of HCA was passed through the SE-Sepharose column. When increasing amounts of the HCA mixture were applied on the column, the HCAC isoenzyme displaced the HCAB isoenzyme from the

binding sites, and when an excess of HCAC, as determined by the increase in specific activity in the eluate, had been applied to the column, all the binding sites were occupied with only the C-enzyme. After washing of the column with buffer, the HCAC was eluted with 0.2 M KSCN pH 6.5 in a yield of 30 mg. Disc gel-electrophoresis showed that the first peak mainly contained the B enzyme (360 mg) with a small amount of C enzyme, while the second peak contained pure C enzyme (Figure 2, gel 2 and 3). No traces of other protein bands were present. The small amount of C enzyme in the HCAB enzyme fraction was easily removed by rechromatography on the SE-Sepharose (Figure 3, B). The column was now unsaturated with respect to HCAC, although still saturated with HCAB. Hence the column captured all the HCAC passing through the column, and the breakthrough peak (III) contained pure HCAB (340 mg) while the second peak eluted with  $\text{SCN}^-$  now contained both isoenzymes, and in this experiment mainly HCAC (15 mg) as determined by disc gel electrophoresis (Figure 2, Gel 4 and 5, respectively). This procedure resulted in the isolation of homogeneous HCAB and HCAC isoenzymes by two affinity column runs on the SE-Sepharose.

In a large scale procedure 300 ml of SE-Sepharose was used to fractionate about 9 grams of a mixture of HCAB and HCAC, isolated by the SA-Sepharose, to homogeneous HCAB and HCAC isoenzymes. As a standard procedure, the affinity chromatography of the HCAC containing fractions were repeated until pure B enzyme was obtained. The HCAB and HCAC bound to the column corresponding to the second peak in Figure 3 B were not eluted but the column used directly with a new isoenzyme mixture from the SA-Sepharose column. In practical terms about 7.5 gram of HCAB and 1 g of HCAC were isolated from 9 grams of a mixture by two to three chromatographic runs, using about 300 ml of the SE-Sepharose.

The isolated pure isoenzymes were stored as ammonium sulfate precipitates at 4°. The amino acid composition (Table 1) of the HCAB and HCAC isoenzymes were in excellent agreement with the compositions calculated from their respective amino acid sequences (1, 8).

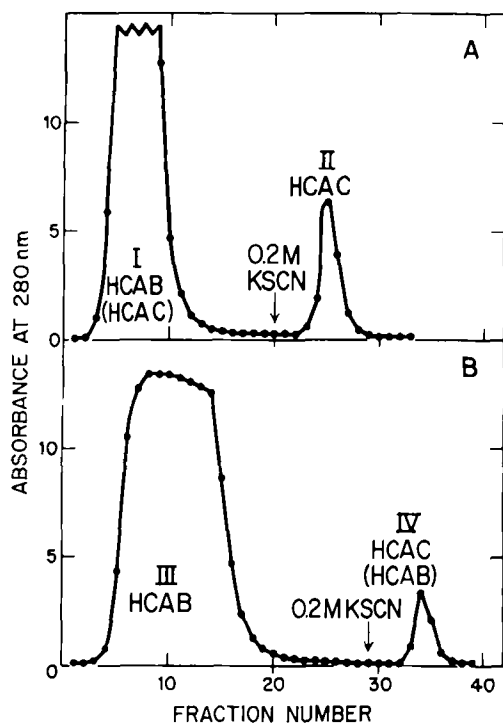


Figure 3. Separation of carbonic anhydrase B (HCAB) and C (HCAC) on the SE-Sephrose column. The column (1.5 cm in diameter) contained 15 ml of gel and was equilibrated with 0.05 M Tris-sulfate pH 7.5. The flow rate was 180 ml/hr and 4 ml fractions were collected. A: carbonic anhydrase (HCA) (400 mg in 6 ml 0.05 M Tris-sulfate pH 7.5) which had been purified as shown in Figure 1, was applied on the column. After washing with 0.05 M Tris-sulfate pH 7.5, the bound carbonic anhydrase was eluted with 0.2 M KSCN-0.05 M Tris-sulfate pH 6.5, as indicated.

B: Rechromatography of the pooled fractions from the break-through peak in A. The chromatogram was developed as in part A.

Furthermore, the turnover numbers for the hydrolysis of p-nitrophenyl acetate were 25 and 120  $\text{min}^{-1}$  for HCAB and HCAC at pH 7.5, respectively, which agree with the literature values for these two enzymes (12) with the assay procedure employed (2).

The binding of sulfonamides to apocarbonic anhydrase is much weaker than to native carbonic anhydrase (9), hence the affinity gel can be used to isolate metal free apocarbonic anhydrase. As shown in Figure 4 where a sample of HCAB, in which about 40% of the active site metal had been removed by dialysis against

1,10 phenanthroline, was passed over the column the apoenzyme passed unretarded through the SA-Sephrose, and as determined from the residual activity it contained less than 1% zinc. The column was initially made metal free by washing with 3 volumes of 0.1 N-HCl followed by glass-distilled water and metal-free dithiozone extracted 0.05 M-Tris-sulfate, pH 7.5. The same elution profile was obtained for the C-enzyme. Significantly, addition of stoichiometric amounts of  $\text{ZnSO}_4$  to the apoenzyme fully restored the activity. Preparation of apocarbonic anhydrase with very low residual zinc content by the standard dialysis procedure takes up to 2-3 weeks (3). With the present procedure this time can be reduced significantly. After dialysis for 2-3 days against 1,10 phenanthroline, where about 60% of the zinc is removed, followed by a dialysis against a metal-free 0.05 M-Tris-sulfate, pH 7.5 buffer, to remove 1,10 phenanthroline, the mixture of apo- and Zn-carbonic anhydrase was separated on the SA-Sephrose column as described above.

In general, the affinity gels described in the present paper should be useful for separation of all types of carbonic anhydrases with modified binding properties for sulfonamides e. g. the Cd

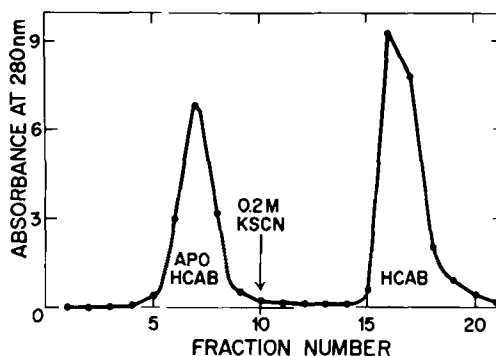


Figure 4. Separation of apocarbonic anhydrase B (Apo-HCAB) from the Zn-carbonic anhydrase (HCAB) on the SA-Sephrose column. The column (1.5 cm in diameter) contained 15 ml of the gel, and was equilibrated with 0.05 M Tris-sulfate pH 7.5. The flow rate was 150 ml/hr and 4 ml fractions were collected. 6 ml of a mixture of apo-HCAB (40%) and HCAB (60%) in 0.05 M Tris-sulfate pH 7.5 was passed through the column. After washing with the same buffer the HCAB was eluted with 0.2 M KSCN-0.05 M Tris-sulfate pH 6.5, as indicated.

**Table I****Amino acid composition of human carbonic anhydrase B and C<sup>1</sup>**

Amino acid	HCAB		HCAC	
	Experimental values (Gly=16)	From the Sequence (1)	Experimental values (Gly=22)	From the Sequence (8)
Lysine	18.3	18	25.8	24
Histidine	10.6	11	11.8	12
Arginine	7.0	7	7.2	7
Aspartic acid	31.1	31	30.3	29
Threonine <sup>2</sup>	14.2	14	12.7	12
Serine <sup>2</sup>	29.9	30	20.0	18
Glutamic acid	22.9	22	23.8	24
Proline	16.9	17	16.0	17
Glycine	16.0	16	22.0	22
Alanine	19.3	19	14.4	13
Valine <sup>3</sup>	17.1	17	17.4	17
Methionine	1.7	2	0.8	1
Isoleucine <sup>3</sup>	8.9	10	9.0	9
Leucine	20.5	20	26.8	26
Tyrosine	7.5	8	7.9	8
Phenylalanine	10.9	11	12.5	12

<sup>1</sup>) Samples of HCAB and HCAC were hydrolyzed in 6 N HCl at 110° for 22, 48 and 72 hours in evacuated sealed glass tubes.

<sup>2</sup>) The values for serine and threonine were corrected by extrapolating to zero time the values obtained at various times of hydrolysis.

<sup>3</sup>) The values of valine and isoleucine are the values obtained after 72 hours of hydrolysis.

enzyme can be separated from the Zn-enzyme (J. T. Johansen to be published). Finally, it should be mentioned that the Sepharose derivatives described have exhibited a very high degree of stability. They have been used repeatedly over one year without any losses of binding properties.

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