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## Structure of $\alpha 1$ -CB8, a Large Cyanogen Bromide Produced Fragment from the $\alpha 1$ Chain of Rat Collagen. The Nature of a Hydroxylamine-Sensitive Bond and Composition of Tryptic Peptides\*

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**ABSTRACT:** As a first step in the determination of the primary structure of an extended sequence in collagen, the 22 peptides resulting from tryptic digestion of rat collagen  $\alpha 1$ -CB8 were separated and their compositions determined. The peptides accounted for the entire cyanogen bromide produced fragment (mol wt 24,000) and no variant peptides were found. These observations provide additional support for the identity of the two  $\alpha 1$  chains in the rat collagen molecule. The task of peptide separation was facilitated by the finding that treatment of  $\alpha 1$ -CB8 with alkaline hydroxylamine yielded two fragments which could be separated and analyzed individually. Although hydroxylamine cleavage resulted in the formation of a new  $\text{NH}_2$ -terminal glycine, several lines of evidence indicated that hydroxylaminolysis of a peptide bond was not involved. The experimental data suggest that the hydroxylamine-sensitive bond exists as the cyclic imide,

anhydroaspartylglycine, which forms by cyclization of an asparaginyl (or aspartyl) side chain with the subsequent (glycyl) amide nitrogen in the polypeptide chain. Molecular models demonstrate that cyclization of an asparaginyl side chain is sterically hindered by the presence of a side chain on the subsequent amino acid. Collagen may therefore be particularly susceptible to hydroxylamine cleavage because of its high glycine content and the increased probability of asparaginyl-glycyl sequences. The elucidation of the nature of the hydroxylamine-sensitive bond in  $\alpha 1$ -CB8 provides an alternative explanation for many of the chemical findings previously believed to result from the existence of ester or other nonpeptide bonds in collagen. It is therefore probable that collagen chains are synthesized as single unbroken polypeptides, and that intrachain subunits linked by nonpeptide bonds do not exist in the protein.

In collagen, a fibrous protein, important intramolecular interactions appear to be limited to the hydrogen bonds which form between the polypeptide backbones of the three intertwined left-handed helical chains (Ramachandran, 1967). As a consequence of this structure, amino acid side chains extend radially from the central core of the molecule and are primarily involved in intermolecular interactions, as might

be expected in a protein which forms structural aggregates. However, because of the highly specific packing of molecules in the collagen fibril and the necessity for interactions with other macromolecules, the requirements for specificity in the amino acid sequence of collagen chains are at least as stringent as those in globular proteins. This specificity is reflected in the relative constancy of the structure of the helical regions of the collagen molecule in vertebrate species (Bornstein, 1968; Bornstein and Kang, 1970).

The determination of the amino acid sequence of a linear polypeptide such as collagen, combined with knowledge of the conformation of the peptide backbone, may therefore be expected to provide much of the information required to elucidate the relation of structure to function. As a step in this direction, and in order to ensure that any existing sequen-

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† Recipient of Research Career Development Award K4-AM-42582 from the U. S. Public Health Service.

tial patterns become apparent, a relatively long sequence of the  $\alpha 1$  chain of rat collagen was chosen for structural analysis. The CNBr-produced peptide  $\alpha 1$ -CB8 (mol wt 24,000) constitutes approximately one-quarter of the  $\alpha 1$  chain of rat collagen (Butler *et al.*, 1967) and is located in the middle of the  $\text{NH}_2$ -terminal-half of the chain (Piez *et al.*, 1968; Rauterberg and Kuhn, 1968). The fragment is some seven times as long as fragments  $\alpha 1$ -CB2 and  $\alpha 1$ -CB5 for which primary structures have been reported (Bornstein, 1967a; Butler, 1970) and its position in the chain makes it well suited to an examination of the proposal that nonpeptide bonds link subunits within  $\alpha$  chains (Gallop *et al.*, 1967). The determination of the primary structure of  $\alpha 1$ -CB8 would also clarify other features of the chemistry of collagen such as the pattern of hydroxylation of proline (Bornstein, 1967b) and lysine (Butler, 1968; Bornstein, 1969a), the pattern of glycosylation of hydroxylysine (Butler and Cunningham, 1966; Spiro, 1969), and the question of the existence of special carbonyl functions in collagen (Gallop *et al.*, 1968).

During the early phases of this work, it was found that nucleophilic attack with hydroxylamine (Blumenfeld and Gallop, 1962; Blumenfeld *et al.*, 1965) resulted in the specific cleavage of  $\alpha 1$ -CB8 into two fragments which could be readily separated by molecular sieve chromatography (Bornstein, 1969b). This limited nonenzymatic cleavage greatly facilitated the task of separating the enzymatically produced peptides of  $\alpha 1$ -CB8. The demonstration of a hydroxylamine-sensitive bond also led to efforts to elucidate the chemical nature of the linkage. This communication describes the fractionation and composition of the tryptic peptides of  $\alpha 1$ -CB8 and proposes a plausible mechanism by which hydroxylamine cleaves polypeptide chains.

## Materials and Methods

**Preparation of  $\alpha 1$ -CB8.** Fragment  $\alpha 1$ -CB8 was prepared from rat collagen by the procedure of Butler *et al.* (1967) with the following modifications. Sodium-containing rather than volatile buffers were employed for elution of  $\alpha$  chains from CM-cellulose and the resulting chains were then desalted on Sephadex G-25, coarse beads (Pharmacia) prior to CNBr cleavage. Preparations of  $\alpha 1$ -CB8 obtained by chromatography on CM-cellulose at pH 3.6 were exposed to a pH of 10.5 immediately prior to final purification by chromatography on CM-cellulose at pH 4.8. In this manner the chromatographic heterogeneity resulting from the equilibrium between homoserine and homoserine lactone was largely avoided. Both acid-extracted skin and tendon collagens from normal rats and salt-extracted skin and tendon collagens from lathyrus rats were used as sources of  $\alpha 1$ -CB8. The resulting fragments were indistinguishable by the techniques employed in these experiments. However, differences in the degree of hydroxylation of individual prolyl and lysyl residues may have been present in different batches of  $\alpha 1$ -CB8 (see Discussion).

**Hydroxylamine Cleavage.** Immediately prior to use a 2 M solution of hydroxylamine in 0.2 M  $\text{K}_2\text{CO}_3$ , pH 10.5, was prepared as follows.  $\text{NH}_2\text{OH} \cdot \text{HCl}$  (3.48 g, Eastman Organic Chemicals) was dissolved in 13 ml of  $\text{H}_2\text{O}$  in an ice bath and 2.5 ml of 12.5 N NaOH was added slowly with vigorous stirring; 5 ml of 1 M  $\text{K}_2\text{CO}_3$  was then added, the pH of the solution titrated to 10.5 with additional NaOH, and the

volume brought to 25 ml.  $\alpha 1$ -CB8 was dissolved in  $\text{H}_2\text{O}$  at a concentration of 5 mg/ml and denatured by warming to 40° for 10 min. An equal volume of cold 2 M  $\text{NH}_2\text{OH}$  was added to the peptide solution, mixed thoroughly, and incubated at 35° for 90 min. At the conclusion of the incubation the pH of the reaction mixture was reduced to 4 with 6 N HCl, and the peptide separated from the reagent and salts by chromatography at room temperature on Bio-Gel P2, 100–200 mesh (Bio-Rad Laboratories), equilibrated with 0.03 M ammonium propionate, pH 4.5.

**Gel Filtration on Agarose.** A  $1.8 \times 140$  cm column was packed with 8% agarose (Bio-Gel 1.5 M, 200–400 mesh, Bio-Rad Laboratories) equilibrated with 1 M  $\text{CaCl}_2$ –0.05 M Tris-HCl, pH 7.5, as described by Piez (1968). The sample was applied in 2 ml or less of the  $\text{CaCl}_2$  buffer and eluted at room temperature at a constant rate of 18 ml/hr using a Büchler Polystaltic pump. The optical density of the effluent was monitored at 230 m $\mu$  and at a constant temperature of 15° with a Gilford Model 2000 recording spectrophotometer equipped with a 10-mm light-path flow-through cell (0.125-ml volume) which was enclosed in a thermoregulated cell compartment. The agarose column was calibrated by applying a mixture of collagen chains and peptides, the molecular weights of each of which had been determined by sedimentation equilibrium (Butler *et al.*, 1967). Fragments were desalted by Bio-Gel P2 chromatography. The excluded volume of the column was determined in a separate experiment with Blue Dextran 2000 (Pharmacia).

**Hydroxamate Determinations.** Hydroxamate was detected colorimetrically after oxidation by iodine. The resulting nitrite was used to diazotize sulfanilic acid which then formed an azo dye with  $\alpha$ -naphthylamine (Bergmann and Segal, 1956). The procedure of Seifter *et al.* (1960) was followed except that volumes were scaled down to a total of 2 ml and the concentration of sodium thiosulfate was increased to 0.4 N. The final reaction mixture was allowed to stand for 60 min to permit complete and reproducible color development.

Optical density at 520 m $\mu$  was determined in a Gilford or Zeiss spectrophotometer in 1-ml cuvetts. In this fashion quantities of hydroxamate as low as 0.005  $\mu$ mole could be determined accurately and standard curves were linear to 0.05  $\mu$ mole. Equimolar quantities of sodium nitrite, hydroxylamine, and benzhydroxamic acid<sup>1</sup> yielded the same degree of color. The quantity of peptide was determined by amino acid analysis or hydroxyproline determination (Kivirikko *et al.*, 1967), or both.

**Enzymatic Hydrolyses.** Digestions with trypsin (L-1-tosylamido-2-phenylethyl chloromethyl ketone treated, Worthington) were performed in 0.2 M  $\text{NH}_4\text{HCO}_3$ , pH 7.8, containing  $1 \times 10^{-3}$  M  $\text{CaCl}_2$ . The enzyme (1% of the substrate by weight) was added as a 0.5% solution in  $1 \times 10^{-3}$  M HCl. Incubations were performed at room temperature for 4 hr and terminated by lyophilization. Digestion with purified collagenase (CLS-PA, Worthington) was performed in the same buffer at 37° for 24 hr. A drop of toluene was added to inhibit bacterial growth.

**Ion-Exchange Chromatography and Automated Analysis of Peptides.** Tryptic digests of  $\alpha 1$ -CB8,  $\alpha 1$ -CB8-HA1,<sup>2</sup> and

<sup>1</sup> Kindly provided by Dr. P. M. Gallop.

<sup>2</sup> HA is used to designate a hydroxylamine-produced fragment.

TABLE I: Composition of Gradients Used in Ion-Exchange Chromatography of Peptides.<sup>a</sup>

	Chamber (ml)								
	1	2	3	4	5	6	7	8	9
I. DC-1 and AG 50-X4 resins									
0.2 M Pyridine acetate, pH 3.1	60	60	40	13					
2.0 M Pyridine acetate, pH 5.1			20	47	60	27			
2.0 M Pyridine acetate, pH 6.5						33	60	60	60
II. AG 1-X2 resin									
3% Pyridine	50	50	25						
0.5 M Pyridine acetate, pH 6.0			25	50	50				
1.0 M Pyridine acetate, pH 6.0						50	50		
2.0 M Pyridine acetate, pH 5.1								50	50

<sup>a</sup> Molarities refer to concentration of pyridine. Pyridine was refluxed with 2 g of ninhydrin/l. for 4 hr and distilled prior to use.

$\alpha$ 1-CB8-HA2 were chromatographed with volatile buffers on DC-1 resin (Durrum Chemical Corp.). The cation-exchange polystyrene resin containing 6.5 to 9.0% divinyl benzene (nominal particle size, 10–22  $\mu$ ) was cycled in the usual way with strong base and acid. After equilibration in the H<sup>+</sup> form with the starting buffer, 0.2 M pyridine acetate, pH 3.1, the resin was packed in a jacketed column, 0.9 cm in diameter, to a height of 55 cm. The sample was dissolved in a small volume of starting buffer and eluted with a complex gradient of pyridine acetate buffers (Table I) delivered with a cylindrical glass Varigrad. Chromatography was performed at a flow rate of 36 ml/hr (Beckman Accuflo pump) at 55° under a back pressure of 90–100 psi. The entire effluent was collected in fractions of 3 ml in 3.5-ml plastic cups. The cups were transferred to a Sampler II (Technicon Corp.) and analyzed with the ninhydrin reagent in a modified Technicon peptide analyzer. Only the hydrolyzed peptide was reacted with ninhydrin and thus the second colorimeter and time delay coil of the conventional analyzer were not employed. Alkaline hydrolysis and reaction with ninhydrin were carried out in the same heating bath at 95°. A 2:1 ratio of water wash to sample (0.12 ml or 4% of each fraction) was used, permitting clearer visualization of individual fractions in the recorded trace of absorbance at 570 m $\mu$ . A standard solution of leucine was introduced into the system at the start of a run and interposed between every 40 cups to ensure an accurate correlation between the recorded trace and the sample fractions. Peptides identified in this fashion were lyophilized in preparation for amino acid analysis or other fractionation procedures.

Peaks eluting at or near the front of the gradient contained peptides which were too large or too acidic to be retarded by the resin. The fractions comprising these peaks were lyophilized and the peptide mixture chromatographed on Dowex 1-X2 (AG1-X2, 200–400 mesh, Bio-Rad Laboratories) using a complex gradient of pyridine acetate buffers (Table I). The conditions of chromatography were: column size, 55  $\times$  0.9 cm; temperature, 40°; and flow rate, 54 ml/hr. Fractions of 3 ml were again collected in sampler cups and monitored with ninhydrin as described above.

Those peaks obtained by Dowex 1-X2 chromatography which were judged by amino acid composition or paper

electrophoresis to contain mixtures of peptides were subjected to a third column chromatographic step using Dowex 50-X4 (AG50-X4, 200–400 mesh, Bio-Rad Laboratories). The conditions of chromatography and composition of the gradient were identical with those used in the initial chromatographic step with the DC-1 resin. Nevertheless, the different properties of the two resins permitted additional resolution.

**Paper Electrophoresis and Chromatography.** Electrophoresis was performed on Whatman No. 3MM paper at 2000 V for 90 min in a Gilson electrophorator (model D). The following buffers were employed: 1.24 M (in pyridine) pyridine acetate, pH 6.5; 0.04 M pyridine acetate, pH 3.6; 0.5 M acetic acid, pH 2.42; and 0.037 M pyridine formate, pH 2.1. Peptides were identified with the cadmium ninhydrin stain (Blackburn, 1965). Preparative electrophoresis was performed by streaking the sample at the origin to within 0.25 in. of the edge of the paper. Peptides were identified by cutting and staining 0.5-in. guide strips from both sides of the paper and were eluted with 1% acetic acid at 50°. Descending paper chromatography was performed on Whatman No. 3MM paper in pyridine-H<sub>2</sub>O (80:20).

**Acrylamide gel electrophoresis** was performed as described by Nagai *et al.* (1964) except that the concentration of acrylamide in the running gel was increased to 12.5%.

**NH<sub>2</sub>-terminal analysis and Edman degradation** were performed essentially as described recently (Bornstein, 1969a). Benzene rather than butyl acetate was used to extract diphenylthiourea and other by-products of the reaction with phenyl isothiocyanate. Acid hydrolysis of dansylated peptides was routinely limited to 4–6 hr (Gros and Labouesse, 1969).

Hydrazinolysis was performed using an Amberlite CG-50 catalyst (Braun and Schroeder, 1967). The  $\alpha$ - and  $\beta$ -hydrazides of aspartic acid were synthesized by hydrazinolysis of  $\alpha$ - and  $\beta$ -aspartyl benzyl esters (Fox Chemical Co.) and separated by electrophoresis at pH 2.42 (Narita and Ohta, 1959).

**Amino acid analyses** were performed on a Beckman 120 C analyzer modified for accelerated single column gradient elution (Miller and Piez, 1966; Bornstein, 1969a). Peptides were hydrolyzed *in vacuo* in doubly distilled constant-boiling point HCl at 110° for 24 hr. The following corrections were made for hydrolytic losses: threonine, 4.8%; serine, 12%;

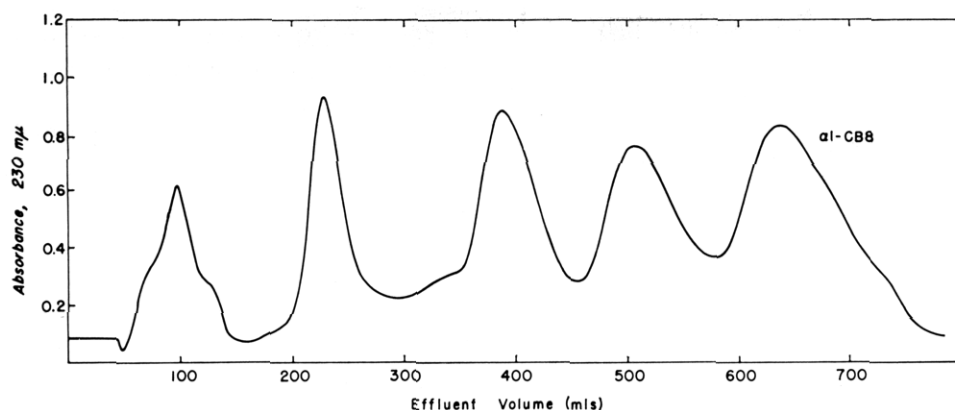


FIGURE 1: Elution pattern of peptides obtained by CNBr cleavage of the  $\alpha 1$  chain of rat collagen. Chromatography was performed on CM-cellulose at 40°. The sample (200 mg) was dissolved in 50 ml of starting buffer, 0.02 M sodium citrate, pH 3.6, containing 0.04 M NaCl, and eluted with a linear gradient of NaCl from 0.04 to 0.14 M over a volume of 1600 ml.

methionine, 14.3%; and tyrosine, 9%. A correction of 3.9% was applied for incomplete release of valine.

## Results

**Characterization of  $\alpha 1$ -CB8.** CNBr fragments from the  $\alpha 1$  chain of rat collagen were chromatographed on CM-cellulose at pH 3.6 (Figure 1) and the fractions comprising the effluent volume from 590 to 700 ml were pooled, desalted on Sephadex G-25, and lyophilized. The resulting material was rechromatographed on CM-cellulose at pH 4.8 (Figure 2). The rechromatographed peptide was essentially homogeneous by acrylamide gel electrophoresis (Figure 3, tube 1). The amino acid composition of  $\alpha 1$ -CB8 is listed in Table II. The

values are the same, within experimental error, as those previously reported by Butler *et al.* (1967).

**Cleavage of  $\alpha 1$ -CB8 with Hydroxylamine.** Because treatment of  $\alpha 1$ -CB8 with hydroxylamine yielded incomplete cleavage the factors which affected the extent of the reaction were investigated. An increase in temperature above 35° or an increase in the molarity of hydroxylamine or duration of the reaction beyond 90 min did not increase the degree of cleavage. The reaction was retarded below 30° and largely inhibited at 15°. The conditions which were eventually chosen (1 M hydroxylamine–0.2 M  $K_2CO_3$ , pH 10.5, 35°, 90 min) consistently resulted in approximately 50% cleavage of the starting material (Figures 3 and 4). Cleavage with 0.7 M hydroxylamine in 4 M LiCl at pH 9.5 for 7 hr (Volpin *et al.*, 1968) was equally, but no more, effective. Cleavage was appreciably reduced below pH 9.0. For reasons which are not

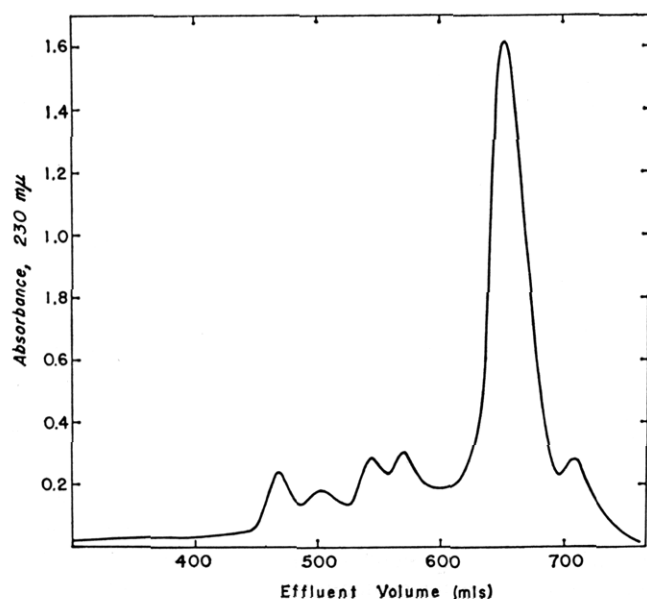


FIGURE 2: Elution pattern of  $\alpha 1$ -CB8 rechromatographed on CM-cellulose at pH 4.8. The sample (75 mg) was dissolved in 25 ml of starting buffer, 0.02 M sodium acetate, pH 4.8, and eluted with a linear gradient of NaCl from 0 to 0.1 M over a volume of 800 ml.

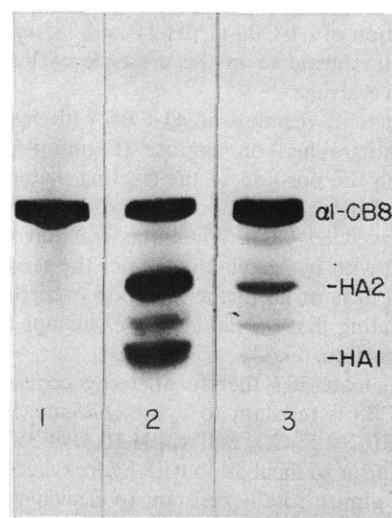


FIGURE 3: Acrylamide gel electrophoresis patterns of  $\alpha 1$ -CB8 before treatment (tube 1) and after cleavage with hydroxylamine (tube 2). Tube 3 represents the pattern obtained with a preparation of  $\alpha 1$ -CB8 which was resistant to hydroxylamine cleavage, after retreatment with hydroxylamine. Migration is toward the cathode from top to bottom.

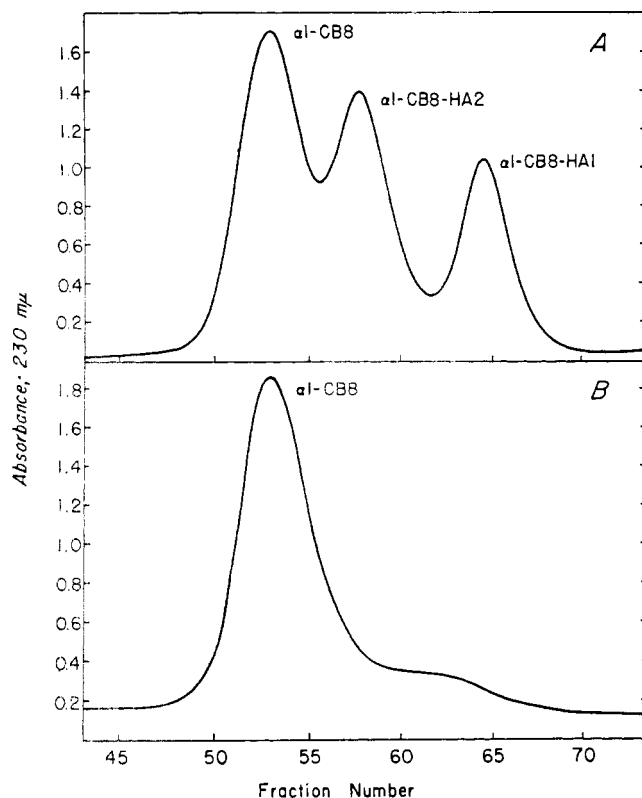


FIGURE 4: Agarose elution patterns. (A) Elution pattern of fragments obtained by cleavage of  $\alpha 1$ -CB8 with hydroxylamine. Chromatography performed on 8% agarose in 1 M  $\text{CaCl}_2$ -0.05 M Tris, pH 7.5. (B) Agarose elution pattern of alkali-pretreated  $\alpha 1$ -CB8 subsequently reacted with hydroxylamine.

understood, incubation in 1 M hydrazine at pH 10–11, although shown to be equally effective in the degradation of ichthyocol (Blumenfeld and Gallop, 1962) was far less effective than hydroxylamine in the cleavage of rat collagen  $\alpha 1$ -CB8. Incubation of  $\alpha 1$ -CB8 at pH 11 and 35° in the absence of nucleophilic agents or in the presence of 1 M piperidine resulted in no cleavage.

The products of reaction of  $\alpha 1$ -CB8 with hydroxylamine were chromatographed on agarose (Figure 4A). The first peak eluted in the position of the starting material, was unchanged in amino acid composition, and apparently represented uncleaved  $\alpha 1$ -CB8. When this fraction was isolated and retreated with hydroxylamine under the same conditions used initially, little or no further reaction occurred (Figure 3, tube 3) indicating that partial cleavage was not the result of incomplete reaction.

The starting material is therefore heterogeneous and roughly 50% of  $\alpha 1$ -CB8 is resistant to hydroxylamine. Pretreatment of  $\alpha 1$ -CB8 in 0.2 M  $\text{K}_2\text{CO}_3$  buffer, pH 10.5, at 35° for 90 min immediately prior to incubation with hydroxylamine rendered the fragment almost totally resistant to cleavage (Figure 4B). Pretreatment with 0.1 M HCl did not appreciably increase the degree of cleavage. The reasons for these findings are discussed below.

**Characterization of the Hydroxylamine Cleavage Products of  $\alpha 1$ -CB8.** In addition to the resistant fragment, two peaks,  $\alpha 1$ -CB8-HA1 and  $\alpha 1$ -CB8-HA2, were consistently observed

TABLE II: Amino Acid Composition and Molecular Weights of  $\alpha 1$ -CB8 and Its Hydroxylamine-Produced Fragments.<sup>a</sup>

	Residues/Peptide		
	$\alpha 1$ -CB8	$\alpha 1$ -CB8-HA1	$\alpha 1$ -CB8-HA2
4-Hydroxyproline	27	10	18
Aspartic acid	11	5 (4.9)	6 (6.3)
Threonine	5 (5.0)	2 (2.1)	3 (3.0)
Serine	10	2 (2.4)	8 (8.2)
Homoserine	1 (0.8)		1 (1.0)
Glutamic acid	20	7 (7.2)	13
Proline	30	11	18
Glycine	87	31	57
Alanine	36	16	20
Valine	5 (5.0)	2 (2.0)	3 (3.2)
Isoleucine	1 (1.0)		1 (0.9)
Leucine	4 (3.8)	1 (1.1)	3 (2.9)
Phenylalanine	3 (2.8)	1 (0.9)	2 (1.8)
Hydroxylysine	0.6	0.3	0.3
Lysine	8.6	1.9	7.0
Arginine	14	5 (5.3)	9 (8.8)
Total residues	263	95	169
Molecular weight <sup>b</sup>	23,600	8,400	15,200
Molecular weight <sup>c</sup>	24,000	8,500	14,500

<sup>a</sup> Values are the averages of three or more determinations. The actual values for amino acids present as less than ten residues are shown in parentheses. <sup>b</sup> Calculated from amino acid analysis. <sup>c</sup> Calculated by molecular-sieve chromatography.

TABLE III: Hydroxamate Contents of the Reaction Products of  $\alpha 1$ -CB8 Treated with Hydroxylamine.

Peptide	Hydroxamate/Peptide (equiv)
$\alpha 1$ -CB8 (untreated)	0
$\alpha 1$ -CB8 (resistant fraction)	0.6; 0.7
$\alpha 1$ -CB8-HA1	0.9; 1.1
$\alpha 1$ -CB8-HA2	0.3; 0.4
Gly-Ala-Asp-NHOH <sup>a</sup>	0.8; 0.9
Gly-Ala-Asp- $\alpha$ -NHOH	1.0; 0.9
Gly-Ala-Asp- $\beta$ -NHOH	0.5

<sup>a</sup> Low molecular weight fraction of a tryptic digest of HA1 containing both Gly-Ala-Asp and Gly-Pro-Arg. Value expressed is per equivalent of Gly-Ala-Asp.

by agarose chromatography of the hydroxylamine reaction mixture. Gel electrophoresis (Figure 3) also revealed two additional very minor components. These were not detected by molecular sieve chromatography and were not further investigated.

$\alpha 1$ -CB8-HA2 and  $\alpha 1$ -CB8-HA1 were rechromatographed on agarose to reduce the contamination with uncleaved

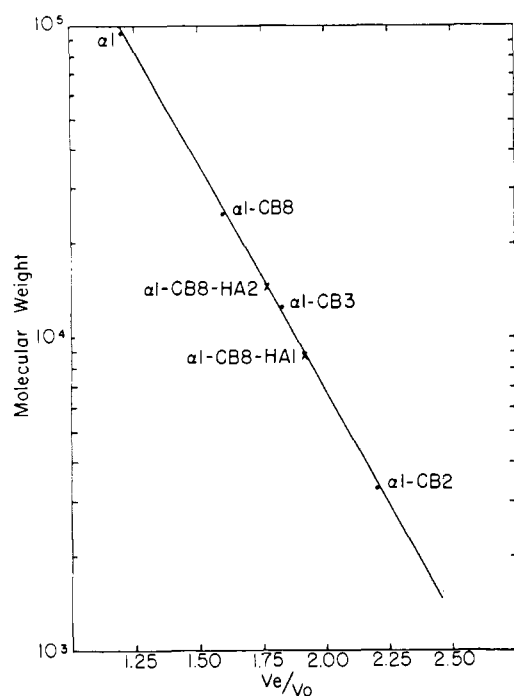


FIGURE 5: Semilogarithmic plot of molecular weight vs.  $V_e/V_0$ , the ratio of the observed elution volume to the totally excluded volume of the column. The agarose column was calibrated by plotting the observed values of  $V_e/V_0$  against the molecular weights of  $\alpha 1$ ,  $\alpha 1$ -CB8,  $\alpha 1$ -CB3, and  $\alpha 1$ -CB2 (determined by sedimentation equilibrium and amino acid analysis). The values of  $V_e/V_0$  for the hydroxylamine-produced fragments HA1 and HA2 are shown.

$\alpha 1$ -CB8 and HA2, respectively. The molecular weights of HA1 and HA2 were determined by molecular sieve chromatography on an agarose column calibrated with collagen chains and peptides of known molecular weights. The values of  $V_e/V_0$  for HA1 and HA2 yielded molecular weights of 8500 and 14,500, respectively (Figure 5 and Table II). The sum of the amino acid compositions of the two fragments is identical, within the experimental error of the analyses, with the amino acid composition of  $\alpha 1$ -CB8 (Table II) indicating that cleavage of a single bond produced HA1 and HA2. HA2 contains homoserine and therefore includes the COOH-terminal sequence of  $\alpha 1$ -CB8. Dansylation of HA2 indicated the presence of  $\text{NH}_2$ -terminal glycine which is apparently liberated as a result of hydroxylamine cleavage. Fragment HA1 contains 1 equiv of hydroxamate per peptide (Table III) and therefore contains the acyl moiety of the hydroxylamine-sensitive bond. However, both the resistant fraction of  $\alpha 1$ -CB8 and HA2 contain significant, although less than stoichiometric, quantities of hydroxamate (Table III).

**Composition of the Tryptic Peptides of  $\alpha 1$ -CB8-HA1.** The elution pattern, obtained by automated peptide analysis, of a tryptic digest of HA1 chromatographed on a cation-exchange resin (DC-1) is illustrated in Figure 6A. Five distinct peaks were obtained. Peptides T4, T6, T8, and T9 were homogeneous as judged by amino acid analysis (Table IV). However, peptides T4 and T8 each contained 2 equiv of arginine suggesting either the presence of arginyl bonds which were resistant to trypsin or the existence of a mixture of peptides. Attempts to fractionate T4 and T8 by paper electrophoresis

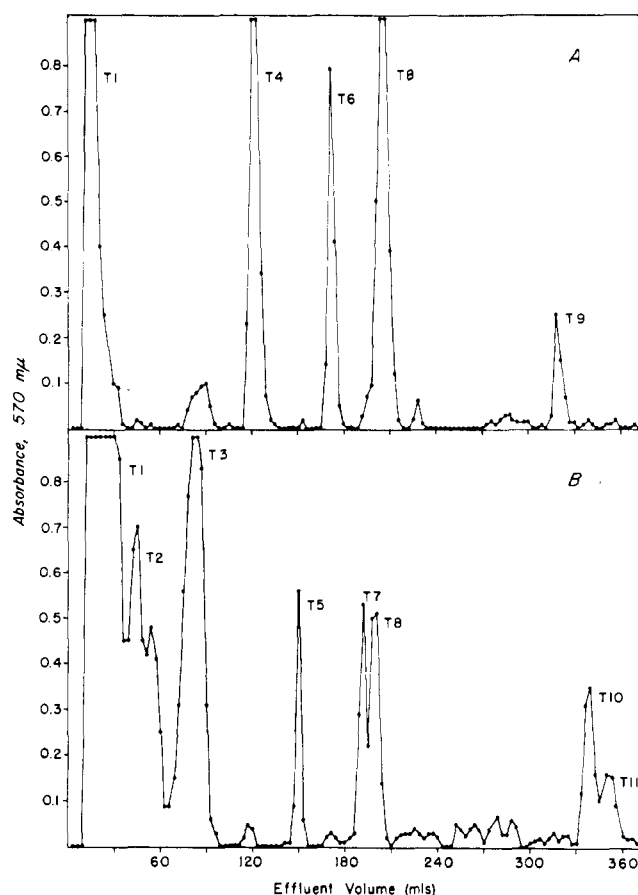


FIGURE 6: Fractionation of tryptic peptides of  $\alpha 1$ -CB8-HA1 (A) and  $\alpha 1$ -CB8-HA2 (B) by chromatography on the cation-exchange resin, DC-1. Individual fractions were analyzed with the ninhydrin reagent in an automated system. Fractions beyond 360 ml, effluent volume, yielded no significant absorbance above background.

at pH 3.5 and 6.5 or by paper chromatography in pyridine-water were unsuccessful. It is likely that the additional arginyl residues in these peptides are followed by imino acids. Lysyl-prolyl and lysyl-hydroxyprolyl bonds have been shown to be resistant to trypsin (Grimm and Grassmann, 1964).

The peak labeled T1 in Figure 6A was further separated into two peptides T13 and T14 by chromatography on Dowex 1-X2 (Figure 7A). The peak eluting at the breakthrough volume of the column contained nonpeptide ninhydrin-reactive material and small amounts of peptides derived from  $\alpha 1$ -CB8-HA2 (which contaminated the HA1 fragment to a small extent). Peptides T13 and T14 were essentially homogeneous by amino acid analysis (Table IV) and contained one lysyl residue (which was partially hydroxylated) per peptide. The uncorrected yields of the peptides listed in Table IV indicate the presence of these tryptic peptides in stoichiometric amounts in HA1. The somewhat lower yields of T13 and T14 were the result of the additional chromatographic step required in their purification.

All of the peptides obtained by ion-exchange chromatography contained arginine or lysine and none contained significant amounts of hydroxamate, indicating that the COOH-terminal sequence of HA1 had not been identified by these procedures. When a tryptic digest of HA1 was

TABLE IV: Amino Acid Composition of Tryptic Peptides of  $\alpha$ 1-CB8-HA1.<sup>a</sup>

	T4	T6	T8	T9	T13	T14	T19a	Total Tryptic Peptides	$\alpha$ 1-CB8-HA1 <sup>b</sup>
4-Hydroxyproline		0.9	1.9		3.3	3.6		9.7	10
Aspartic acid					2.1	2.1	1.0	5	5
Threonine					1.7			2	2
Serine	1.0		1.0		(0.2)			2	2
Glutamic acid	4.1	1.0			(0.2)	1.8		7	7
Proline	2.0		1.2	1.0	4.2	3.7		12.1	11
Glycine	5.9	2.0	4.0	1.0	10.2	9.3	1.0	33	31
Alanine	2.0		2.0		6.2	6.2	1.0	17	16
Valine	1.1				1.0			2	2
Leucine		1.0						1	1
Phenylalanine					1.0			1	1
Hydroxylysine					0.2	0.1		0.3	0.3
Lysine					0.8	0.9		1.7	1.9
Arginine	1.9	1.0	1.9	1.0				6	5
Yield, % <sup>c</sup>	39	47	46	40	29	36	48		

<sup>a</sup> The values are given as residues per peptide. Where no values are given a result of less than 0.2 residue was obtained. Residues in parentheses are fractional residues thought to be impurities. <sup>b</sup> Amino acid composition from Table II. <sup>c</sup> Uncorrected.

chromatographed, without prior fractionation, on Bio-Gel P2, the bulk of the tryptic peptides was separated from a hydroxamate-containing fraction which eluted in the totally included volume of the column (Bornstein, 1969b). Amino acid analysis of the latter fraction revealed the presence of two peptides (Gly,Pro,Arg) (T9) and (Gly,Ala,Asp) (T19a). The fraction contained 1 equiv of hydroxamate per equiv of T19a (Table III). These results indicate that tryptic cleavage of HA1 liberated a hydroxamate-containing tripeptide from the COOH-terminal sequence of the fragment which was not detected by ion-exchange chromatography. The further characterization of this tripeptide is described below.

The sum of the amino acids in the seven tryptic peptides listed in Table IV agrees well with the amino acid composition of HA1. Thus all of the sequence of HA1 is accounted for and overlapping sequences do not exist in these peptides.

*Composition of the Tryptic Peptides of  $\alpha$ 1-CB8-HA2.* The initial fractionation of a tryptic digest of HA2 is shown in Figure 6B. Peptides T5, T7, T8, T10, and T11 were homogeneous by amino acid analysis and each contained a single arginyl or lysyl residue (Table V). Peaks T1, T2, and T3 clearly contained mixtures of peptides and were subjected to further fractionation.

The elution pattern of peptides obtained by chromatography of peak T1 (Figure 6B) on Dowex 1-X2 is illustrated in Figure 7B. Peptides T13, T14, T16, and T17 were homogeneous by amino acid analyses (Table V). The composition of T15 was essentially identical with that of T17, but contained an additional arginyl residue. On the basis of the compositions and yields of peptides T11, T15, and T17, it was apparent that partial cleavage of a lysyl-arginyl bond in T15 resulted in the formation of peptides T11 (arginine) and T17. Peak T12 contained a mixture of peptides which was further fractionated by chromatography on Dowex 50-X4 (Figure 8A).

Separation of two peaks was obtained. Amino acid analysis of the first, HA2-T12, indicated the existence of a pure peptide (Table V). However, analysis of the second peak suggested a mixture. This mixture was eventually resolved into two peptides, HA2-T18 and HA2-T19b, by paper electrophoresis in pyridine formate at pH 2.1. The latter peptide, together with T19a, constitute the tryptic peptide in  $\alpha$ 1-CB8 which contains the hydroxylamine-sensitive bond (see below).

Rechromatography of peak T2 (Figure 6B) first on Dowex 1-X2 and then on Dowex 50-X4 (chromatograms not shown) yielded the tryptic peptides T20 and T21 and a mixture of peptides reflecting the contamination of T2 by T1. Peak T3 (Figure 6B) was also resolved by Dowex 1-X2 chromatography (not shown) into two peptides, T22 and T23.

The sum of the amino acid compositions of the 16 unique tryptic peptides derived from HA2 agrees, within experimental error, with the amino acid composition of the intact fragment (Table V). As in the case of HA1, 2 peptides (T18 and T23) contain more than one arginyl and/or lysyl residues. These peptides could not be further fractionated by a variety of electrophoretic and chromatographic procedures and presumably contain arginyl or lysyl residues followed by imino acids.

The yields of all of the tryptic peptides listed in Table V are consistent with the presence of these peptides in stoichiometric amounts in  $\alpha$ 1-CB8. The low yield of peptides T12, T18, and T19b (uncorrected for manipulative and analytical losses) resulted from the multiple steps required for their purification. Thus, peptides T18 and T19b were obtained only after five sequential steps, i.e., column chromatography on DC-1, Dowex 1-X2, and Dowex 50-X4 followed by paper electrophoresis at pH 3.5 and 2.1. The low yield of T11 resulted from partial release of arginine from a lysyl-arginyl sequence in T15.

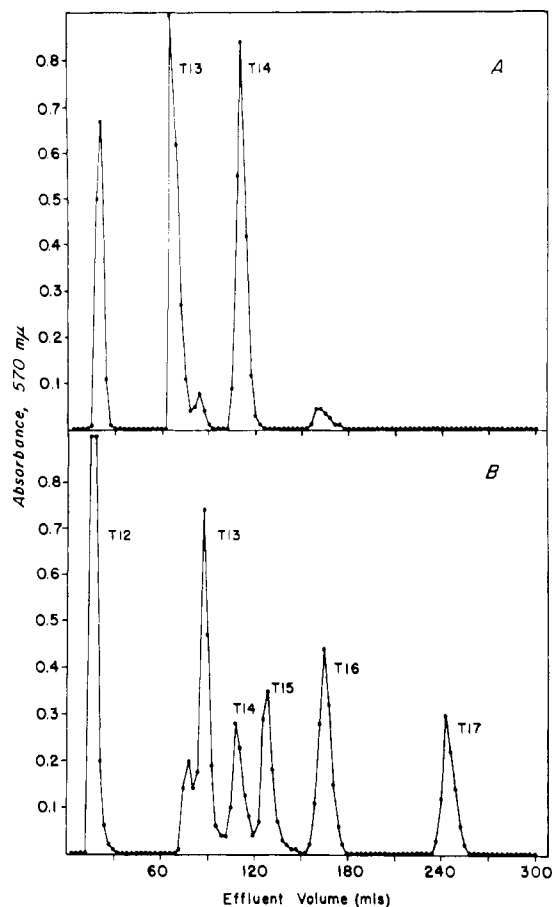


FIGURE 7: Rechromatography of  $\alpha 1$ -CB8-HA1-T1 (A) and  $\alpha 1$ -CB8-HA2-T1 (B) on Dowex 1-X2. The peak eluting at the start of chromatogram A contained nonpeptide material and minor contaminants. See Materials and Methods for further details.

Although the starting material, HA-2, contained 0.3 residue of hydroxylysine per peptide, hydroxylysine was not identified in any of the eight lysyl-containing peptides. The hydroxylysine at these positions may have been glycosylated (Butler and Cunningham, 1966; Spiro, 1969) thus perhaps changing the chromatographic positions of the peptides. However, assuming roughly equal hydroxylation of each of the lysyl residues, the quantity of hydroxylysine in the samples analyzed would have been too small to be detected with certainty (less than  $1 \times 10^{-3}$   $\mu$ mole).

The partial hydroxylation of lysine and proline contributed to the chromatographic heterogeneity of some of the larger tryptic peptides. This effect was particularly evident during paper chromatography and virtually excluded its use in the purification and analytical separation of complex mixtures of collagen-derived peptides.

**Location and Nature of the Hydroxylamine-Sensitive Bond in  $\alpha 1$ -CB8.** Initial efforts to locate the hydroxylamine-sensitive bond in  $\alpha 1$ -CB8 were devoted toward the separation and comparison of tryptic digests of  $\alpha 1$ -CB8, HA1, and HA2 by the paper fingerprint technique. These attempts were unsuccessful partly because of the large number of similar peptides present in the digests and because of the heterogeneity of the starting material. Consequently the tryptic peptides of the resistant fraction of  $\alpha 1$ -CB8 (Figure 4A) were separated

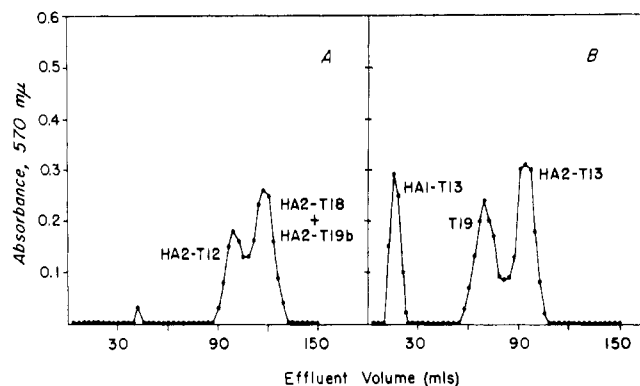


FIGURE 8: Chromatography on Dowex 50-X4: (A) peak  $\alpha 1$ -CB8-HA2-T12 (Figure 7B). (B) Rechromatography of a peptide fraction obtained by prior fractionation of a tryptic digest of intact  $\alpha 1$ -CB8 on DC-1 and Dowex 1-X2. See Materials and Methods for further details.

by the combination of column chromatographic and paper electrophoretic techniques described above. This task was even more complex than that required for the separation of the tryptic peptides from HA1 and HA2. However, the appropriate identification of the tryptic peptides from  $\alpha 1$ -CB8 was assisted by prior knowledge of the column peptide fingerprints of the two hydroxylamine-produced fragments.

Identification of the tryptic peptide containing the hydroxylamine-sensitive bond was also aided by the fortunate circumstance that the sole isoleucyl residue in  $\alpha 1$ -CB8 was present in this peptide. Peptides 19a and 19b were missing in digests of  $\alpha 1$ -CB8. In place of the tripeptide and the isoleucine-containing pentadecapeptide (Tables IV and V) an isoleucine-containing octadecapeptide was identified in tryptic digests of  $\alpha 1$ -CB8. The latter peptide chromatographed in the position of peak T13 from digests of HA2 (see Figure 7B). In the case of digests of  $\alpha 1$ -CB8 peak T13 therefore contained 3 peptides, HA1-T13, HA2-T13, and the isoleucine-containing octadecapeptide. These three peptides were adequately separated by rechromatography of  $\alpha 1$ -CB8-T13 on Dowex 50-X4 (Figure 8B). In contrast, peak T13 from digests of HA2 contained only peptide HA2-T13. The isoleucine-containing pentadecapeptide (T19b) from HA2 was located in peak T12. Its earlier elution from the Dowex 1-X2 column is in accord with the absence of the aspartyl residue.

The amino acid compositions of the octadecapeptide, T19, and of the two peptides T19a and T19b which are adjacent to the site of cleavage of hydroxylamine, are listed in Table VI. Peptide T19b contained a free  $\text{NH}_2$ -terminal glycine by dansylation. Hydroxylamine therefore cleaves an aspartyl (or asparaginyl) glycol bond or a derivative thereof in T19. The partial sequence of T19 is indicated in Figure 9. The initial sequence, glycine-alanine was obtained by Edman degradation of both T19 and T19a and aspartic acid was found in the third position in T19a. The phenylalanyl bond in T19 is resistant to chymotrypsin presumably because it exists in a Phe-Hyp sequence. A point of cleavage by collagenase is indicated. The products of collagenase digestion were isolated by paper electrophoresis at pH 3.6.

In order to further characterize the nature of the bond cleaved by hydroxylamine, the hydroxamate-containing low



TABLE V: Amino Acid Composition of Tryptic Peptides of  $\alpha 1$ -CB8-HA2.<sup>a</sup>

	T5	T7	T8	T10	T11	T12	T13	T14	T16	T17	T18	T19b	T20	T21	T22	T23	Total Tryptic Peptides	$\alpha 1$ -CB8- HA2 <sup>b</sup>
4-Hydroxyproline			1.0			1.1	2.1		2.8	2.0	2.3	2.7	1.8	0.9	1.6	2.8	21.1	18
Aspartic acid	1.1					1.1	1.1				1.0		2.1	1.0			6	6
Threonine	1.0					1.0	1.0				0.9						3	3
Serine		1.0				1.9	2.0		1.1				0.9		1.0	1.1	9	8
Homoserine								0.9									1	1
Glutamic acid		1.0				1.0		1.1	2.1	3.9	1.1		0.9			2.1	13	13
Proline		1.0				4.0	1.1		2.3	3.1	3.7			1.0	2.8		18.9	18
Glycine	2.1	2.0	2.9	1.0		4.9	5.0	2.1	5.0	7.1	6.9	5.4	3.9	3.8	4.1	6.2	62	57
Alanine	1.1	1.0	1.0			1.1	(0.2)	1.0		2.1	3.1	4.2	1.2	2.1	2.0	3.1	22	20
Valine								0.9		1.0				1.0			3	3
Isoleucine												1.0					1	1
Leucine						0.9			1.0							1.0	3	3
Phenylalanine												1.0		1.0			2	2
Hydroxylysine																		0.3
Lysine	0.9					0.9	0.9		0.9	0.9	1.8	0.9	1.0	0.9	0.9	1.0	8	7
Arginine		0.9	1.0	1.0	1.0				0.9							0.9	9	9
Yield, % <sup>c</sup>	34	61	62	56	21	20	47	45	53	44 <sup>d</sup>	6	7	38	40	34	36		

<sup>a</sup> The values are given as residues per peptide. Where no values are given a result of less than 0.2 residue was obtained. Residue in parenthesis is fractional residue thought to be impurities. <sup>b</sup> Amino acid composition from Table II. <sup>c</sup> Uncorrected. <sup>d</sup> Includes peptide T15 (Figure 9) which contains an additional arginyl residue. Partial cleavage of the lysyl-arginyl bond in T15 yields T11 (arginine) and T17.

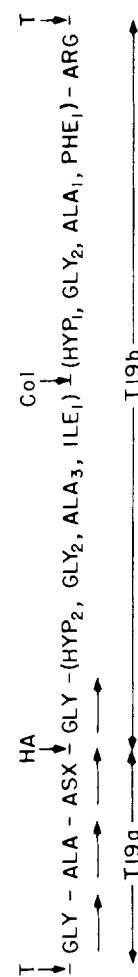


FIGURE 9: A partial amino acid sequence of peptide T19 in the vicinity of the hydroxylamine-sensitive bond. The vertical arrows indicate positions of cleavage by: T, trypsin; HA, hydroxylamine; and Col, collagenase. The horizontal arrows (→) indicate the extent of Edman degradation.

molecular weight fraction of a tryptic digest of HA2 was examined by high voltage electrophoresis. Amino acid analysis of this fraction was consistent with the presence of stoichiometric amounts of two tripeptides (Gly,Pro,Arg) (T9, Table IV) and (Gly-Ala-Asp) (T19a, Table IV). Hydroxamate analysis indicated the presence of roughly 1 equiv of hydroxamate, based on the content of (Gly-Ala-Asp) (Table III). However, paper electrophoresis of the mixture at pH 3.6 revealed 3 peptides. The most basic peptide was identified as (Gly,Pro,Arg) and contained no hydroxamate. The remaining two peptides were identical in amino acid composition (Gly,Ala,Asp) and both contained hydroxamate. Due to apparent lability of the hydroxamate group it has been difficult to measure the hydroxamate contents of the two forms of the tripeptide accurately. The more basic tripeptide, tentatively identified as the  $\alpha$ -hydroxamate, contained roughly twice as much hydroxamate as the more slowly migrating  $\beta$ -hydroxamate. Conceivably the more basic tripeptide represents the  $\alpha,\beta$ -dihydroxamate. It is also possible that the molar color yields of the  $\alpha$ - and  $\beta$ -hydroxamates differ with respect to the standards used. This could result from differences in the optimal pH for oxidation of hydroxamate to nitrite (Yashphe *et al.*, 1960). Studies are in progress to clarify this matter. In any event, the existence of two electrophoretic forms of the tripeptide (Gly-Ala-Asp), both containing hydroxamate, is established.

Hydrazinolysis of the tripeptide mixture and electrophoresis of the resulting hydrazides at pH 2.42 revealed the presence of both  $\alpha$ - and  $\beta$ -hydrazides of aspartic acid. However, this does not unequivocally demonstrate the prior existence of both  $\alpha$ - and  $\beta$ -hydroxamates since the  $\alpha$ - and  $\beta$ -hydrazides may be interconvertible during hydrazinolysis (Narita and Ohta, 1959).

## Discussion

The isolation, in pure form, of the tryptic peptides listed in Tables IV and V represents a major step in the determination of the primary structure of the cyanogen bromide peptide,  $\alpha$ 1-CB8, from rat collagen. Both its large size (mol wt 24,000) and the existence of repetitive or similar sequences significantly complicated the task of separating proteolytic digests of the fragment. On the other hand, the low content of amino acids such as isoleucine, phenylalanine, and threonine permitted the use of these amino acids as markers during the fractionation procedure. The 23 unique tryptic peptides separated from the two hydroxylamine-produced fragments were obtained in amounts consistent with their presence in stoichiometric amounts in the starting CNBr-produced peptide. These data, together with the absence of alternate peptides differing in amino acid composition, further support the view that the two  $\alpha$ 1 chains in the rat collagen molecule are identical in composition and sequence (Butler *et al.*, 1967; Miller *et al.*, 1967).

Until the order of the tryptic peptides and their internal structure is established detailed conclusions regarding the pattern of the amino acid sequence over extended stretches of the chain will be limited. Certain preliminary observations can nevertheless be made. Most of the tryptic peptides derived from  $\alpha$ 1-CB8 are composed of intact triplets, *i.e.*, the number of amino acids is a multiple of three. This indicates that the lysyl and arginyl residues in collagen are largely located in

TABLE VI: Amino Acid Composition of Peptides in the Vicinity of the Hydroxylamine-Sensitive Bond in  $\alpha$ 1-CB8.<sup>a</sup>

	T19	HA1-T19a	HA2-T19b
4-Hydroxyproline	2.9		2.7
Aspartic acid	1.0	1.0	
Glycine	6.1	1.0	5.4
Alanine	5.0	1.0	4.2
Isoleucine	1.1		1.0
Phenylalanine	0.9		1.0
Arginine	1.0		0.9

<sup>a</sup> The values are given as residues per peptide.

position Y in the repeating collagen sequence, Gly-X-Y-. This finding had been reported in another way by Grassmann *et al.* (1960) who noted that peptides resulting from tryptic digestion of calf skin collagen invariably contained NH<sub>2</sub>-terminal glycine. Peptides in which arginyl or lysyl bonds are resistant to trypsin may, however, contain these amino acids in position X followed by an imino acid in position Y. It will be of interest to determine the exact positions of charged amino acids and to compare these with the pattern of striations seen in electron micrographs of segment long-spacing crystallites of renatured  $\alpha$ 1-CB8 (Rauterberg and Kuhn, 1968). These striations are believed to reflect the presence of clusters of charged amino acids, primarily arginine and lysine, along the linear polypeptide chain.

The precise pattern of hydroxylation of proline in  $\alpha$ 1-CB8 cannot be established by these studies since both rat skin and tendon collagen chains were used to prepare  $\alpha$ 1-CB8. The collagens of these two tissues are hydroxylated to different extents as shown by the study of peptide  $\alpha$ 1-CB2 (Bornstein, 1967a,b). This investigation of  $\alpha$ 1-CB8 nevertheless indicates that the phenomenon of incomplete hydroxylation of individual prolyl residues is not limited to a sequence near the NH<sub>2</sub> terminus of the  $\alpha$  chain. Additional evidence for the incomplete hydroxylation of lysyl residues (Butler, 1968; Bornstein, 1969a) was also obtained. This partial hydroxylation is not completely random since 50% of the hydroxylysine in  $\alpha$ 1-CB8 is found at two of the ten lysyl residues in the fragment (HA1-T13 and HA1-T14).

The separation of the tryptic peptides of  $\alpha$ 1-CB8 was materially assisted by the ability to split the large cyanogen bromide peptide with hydroxylamine at a point roughly one-third from its NH<sub>2</sub> terminus, and to analyze the two hydroxylamine-produced peptides separately. Despite the appearance of a new NH<sub>2</sub>-terminal amino acid several lines of evidence indicate that cleavage did not result from hydroxylaminolysis of a peptide bond, but rather from scission of an intrachain cyclic imide.

(1) The starting material,  $\alpha$ 1-CB8, was heterogeneous in its susceptibility to hydroxylamine since only roughly 50% was cleaved by the nucleophile and the resistant fraction was not appreciably cleaved when retreated with hydroxylamine. In addition, the susceptible bond was stabilized by dilute alkali. Since the amino acid compositions of the resistant and susceptible fractions of  $\alpha$ 1-CB8 were not detectably different,

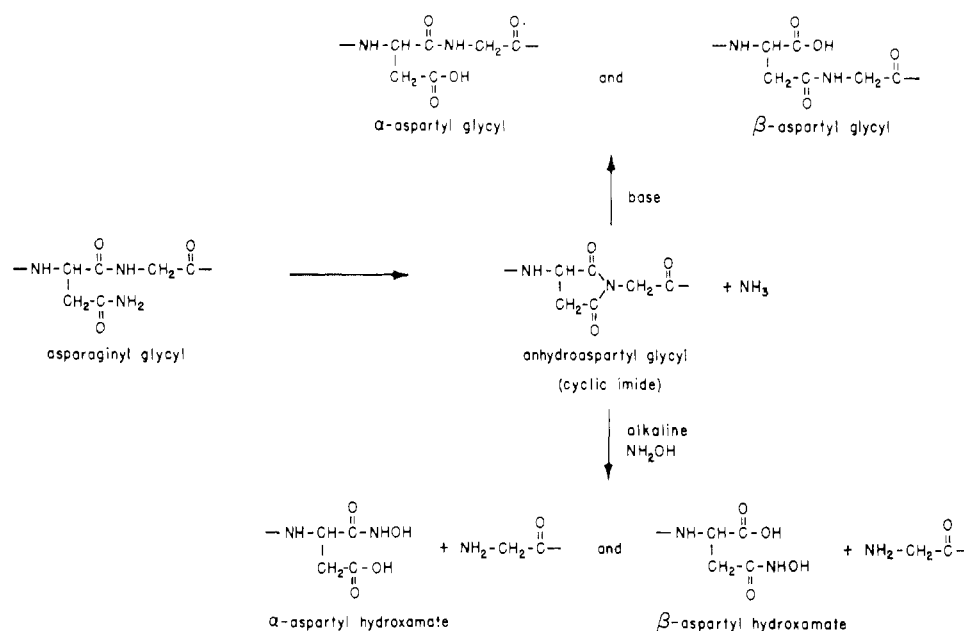


FIGURE 10: The proposed structure of the hydroxylamine-sensitive bond in  $\alpha$ 1-CB8. Cyclization of an asparaginyl- (or aspartyl-) glycylic bond gives rise to the cyclic imide, anhydroaspartyl-glycine. Cleavage with alkaline hydroxylamine yields a mixture of  $\alpha$ - and  $\beta$ -aspartyl hydroxamates and a new  $\text{NH}_2$ -terminal glycylic. The cyclic imide can also be saponified by base.

it was concluded that the heterogeneity in the starting material resulted from the existence of the potentially susceptible bond in two forms.

(2) The conversion of the bond from a susceptible into a resistant form was associated with the introduction of at least one additional negative charge in  $\alpha$ 1-CB8. This was demonstrated by the earlier elution of base-treated  $\alpha$ 1-CB8 from CM-cellulose. While the shift in chromatographic position of intact  $\alpha$ 1-CB8 may have been due in part to the conversion of homoserine lactone into homoserine, the effect was also seen with large chymotrypsin-produced fragments which contained the hydroxylamine-sensitive bond, but lacked the homoserine-containing  $\text{COOH}$ -terminal sequence (P. Bornstein, unpublished experiments).

(3) Although a new  $\text{NH}_2$ -terminal glycylic residue is liberated a result of hydroxylamine cleavage, examination of the acyl donor of the sensitive bond indicated that both the  $\alpha$ - and  $\beta$ -carboxyl groups of aspartic acid were involved in formation of the bond.

A scheme for the structure of the hydroxylamine-susceptible bond and its existence in interconvertible resistant and sensitive forms, which accounts for the above findings, is outlined in Figure 10. In this scheme an asparaginyl-glycyl (or possibly aspartyl-glycyl) sequence cyclizes to form the cyclic imide or anhydroaspartyl residue. This cyclic imide is readily saponified by mild base to yield a mixture of  $\alpha$ - and  $\beta$ -aspartyl-glycyl sequences. However, in the presence of alkaline hydroxylamine, nucleophilic attack and chain cleavage occur, resulting in a mixture of  $\alpha$ - and  $\beta$ -aspartyl hydroxamates and a new  $\text{NH}_2$ -terminal glycylic residue.

The precise manner in which chain cleavage occurs is not established. A mechanism which entails nucleophilic addition of hydroxylamine to first one and then a second carbonyl carbon of the anhydroaspartyl residue is illustrated in Figure 11. Compounds II and V are in equilib-

rium with their respective oximes III and VI. Rearrangement of the hydroxylamine adducts, II and V, gives rise to the mono- and dihydroxamates of aspartic acid (IV and VII, respectively). The formation of the dihydroxamate entails cleavage of the peptide chain and liberation of a new  $\text{NH}_2$ -terminal amino acid. The dihydroxamate is likely to be unstable, hydrolysis yielding a mixture of  $\alpha$ - and  $\beta$ -hydroxamates. Rearrangement of II can account for the incorporation of hydroxamate into the peptide without chain cleavage (see  $\alpha$ 1-CB8 resistant fraction, Table III). In this instance an  $\alpha$ -aspartyl hydroxamate and a  $\beta$ -aspartyl peptide bond are shown, but the reverse would result if nucleophilic addition occur at the  $\beta$ -carbonyl of the cyclic imide. Additional hydroxamate could be introduced into uncleaved  $\alpha$ 1-CB8 and HA2 by reaction of hydroxylamine with homoserine lactone, the cyclic ester of homoserine. Conceivably a low level of random hydroxylaminolysis of amide groups may also occur.

The heterogeneity of  $\alpha$ 1-CB8, as evidenced by the existence of hydroxylamine-sensitive and resistant forms of the peptide, can be explained in two ways. Since cleavage is performed at an alkaline pH, competition probably occurs between saponification of the cyclic imide (resulting from nucleophilic attack by hydroxide ion) to yield a mixture of  $\alpha$ - and  $\beta$ -aspartyl peptide bonds (Figure 10) and cleavage by hydroxylamine. Alternatively, rearrangement of II to IV (Figure 11) would result in a form of  $\alpha$ 1-CB8 which is less susceptible to further nucleophilic addition by hydroxylamine and to chain cleavage.

Although cleavage of cyclic imides by hydroxylamine has not been reported in detail before, the possibility of such cleavage is supported by the observations of Gallop *et al.* (1960). It was found that reaction of polyanhydroaspartic acid with hydroxylamine, under conditions very similar to those used in this work, resulted in extensive degradation of the polymer. An analogous reaction which involves the reac-

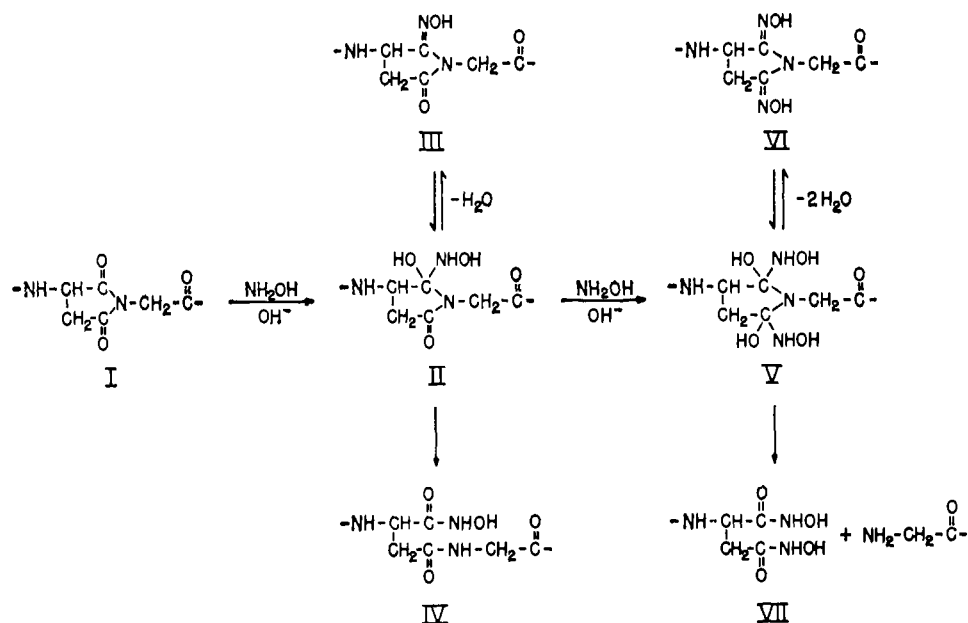


FIGURE 11: Proposed mechanism for the nucleophilic addition of hydroxylamine to the cyclic imide, anhydroaspartyl-glycine (I). Rearrangement of the hydroxylamine adduct (V) results in chain cleavage. See Discussion for further details.

tion of hydrazine with phthalylamino acids to form phthalyl hydrazide and a free amino group has been used extensively in peptide synthesis to remove the phthalimide-protecting group (Schroeder and Lübke, 1965).

Ample precedent exists for the remaining reactions described in Figure 10. The isomerization of aspartyl and asparaginyl peptides to form  $\alpha$ - and  $\beta$ -aspartyl peptides has been described repeatedly (John and Young, 1954; Bryant *et al.*, 1959; Haley *et al.*, 1966). This isomerization occurs by way of an imide intermediate, and base-catalyzed hydrolysis of the cyclic imide of peptidylaspartic acid occurs readily (Battersby and Robinson, 1955; Swallow and Abraham, 1958; Kovacs *et al.*, 1961). The hydrolysis of  $\beta$  esters of aspartyl peptides is also known to occur *via* an imide intermediate (Bernhard, 1958; Sondheimer and Holley, 1954). Imide formation is promoted by esterification of the  $\beta$ -carboxyl group of aspartic acid. In view of the chemical similarity between the ester and amide groups it is reasonable to assume that asparaginyl bonds would be more susceptible to imide formation and subsequent cleavage by hydroxylamine than aspartyl peptide bonds.

It is not known whether imide formation occurs *in vivo* in collagen and may therefore be of physiological importance or whether it represents an artifact in the preparation of the protein. The finding that intact collagen is readily cleaved by hydroxylamine (Blumenfeld and Gallop, 1962; Blumenfeld *et al.*, 1965) indicates that cyanogen bromide cleavage, and the subsequent procedures required for the preparation of  $\alpha 1$ -CB8, are not responsible for the formation of hydroxylamine-sensitive bonds. It is of interest that  $\beta$ -aspartyl glycine has been identified in the urine of normal individuals and that the quantity of the excreted compound can be increased by feeding a gelatin-rich diet (Haley *et al.*, 1966; Pisano *et al.*, 1966). Since the existence of cyclic imides in collagen could account for the appearance of  $\beta$ -aspartyl dipeptides (by saponification and subsequent metabolism of the protein)

imide formation may well occur under conditions other than those used during the laboratory preparation of the protein.

The cleavage, by hydroxylamine, of another cyanogen bromide peptide from the  $\alpha 1$  chain of rat collagen ( $\alpha 1$ -CB3) has recently been reported (Butler, 1969). In this work the reaction was performed with 0.7 M hydroxylamine, at pH 9.5 and 37°, for 7 or 24 hr. On the basis of the determination of the partial sequences of the relevant tryptic peptides and of the hydroxylamine-produced fragments, Butler concluded that an asparaginyl-glycyl peptide bond was cleaved by the nucleophile. However, the data presented do not exclude cleavage of a cyclic imide. Indeed, the finding that hydroxylamine cleaves an asparaginyl-glycyl bond in another region of collagen strongly supports the mechanism proposed in this work.

It has been reported that hydroxylaminolysis of tobacco mosaic virus coat protein under conditions sufficiently drastic to quantitatively convert amide into hydroxamate groups (3 M hydroxylamine, 60°, pH 6.9, 24 hr) also resulted in cleavage of an appreciable number of peptide bonds (Ramachandran and Narita, 1958). These bonds were almost exclusively of the X-Pro type in which the acyl donors were the carboxyl groups of a variety of amino acids. A low level of cleavage of peptide bonds with both hydroxylamine (Blumenfeld *et al.*, 1965) and hydrazine (de la Burde *et al.*, 1963) was also found during treatment of collagen under the milder conditions used in this work. The mechanism of chain cleavage in these instances therefore differs from that proposed for the degradation of  $\alpha 1$ -CB8.

The relatively unique susceptibility of collagen to specific cleavage with hydroxylamine may reflect the high glycine content and the increased probability of occurrence of asparaginyl-glycyl sequences in this protein. Cleavage of asparaginyl- (or aspartyl-) glycyl bonds may proceed preferentially under relatively mild conditions because the lack of a side chain on the glycyl residue reduces the possibility of steric

hindrance in the formation of the imide intermediate. This is readily demonstrable with space-filling molecular models. The addition of any side chain to the residue following asparagine seriously hinders the approach of the amide to the subsequent peptide nitrogen and seems likely to result in unacceptable contact distances, between this side chain and the oxygen of the  $\beta$ -carbonyl group, for most conformations of the polypeptide chain. Should hydroxylamine cleavage of proteins prove to be relatively specific for asparaginyl-glycyl sequences, it may be possible to develop the technique as a method for the nonenzymatic degradation of proteins. Additional complicating factors appear, however, to be operative since it is likely (although not proven) that other asparaginyl-glycyl sequences exist in  $\alpha$ 1-CB8, and yet cleavage occurs almost exclusively at one site.

A subunit model for collagen which proposes that each  $\alpha$  chain is composed of six lower molecular polypeptides linked by ester or special imide bonds (Gallop, 1966; Gallop *et al.*, 1967) is based largely on the interpretation of experiments which involved cleavage of collagen with hydroxylamine and hydrazine. In these experiments it was shown by Lossen rearrangement and lithium borohydride reduction that both  $\alpha$ - and  $\beta$ -aspartylcarboxyl groups participated in the formation of the nucleophile-sensitive bonds (Blumenfeld and Gallop, 1962). The products of the reaction of hydroxylamine with collagen were shown to be fragments which fell in well-defined weight classes (Blumenfeld *et al.*, 1965), a finding which was consistent with the existence of intrachain subunits.

It was originally proposed that the nucleophile-sensitive bonds were aspartyl esters, largely because a commensurate increase in free amino groups (as detected by the ninhydrin reaction) was not observed and because the hydroxylamine-sensitive bonds provided a plausible mechanism for the formation of interchain cross-links. However, it was recognized that the available data did not distinguish between ester and imide bonds (Blumenfeld and Gallop, 1962). The demonstration, in this work, that cyclic imides of aspartic acid exist in collagen and are cleaved by hydroxylamine reconciles virtually all of the chemical findings previously used to support the subunit hypothesis with the likelihood that collagen chains are synthesized as single unbroken polypeptides. There is, therefore, insufficient evidence to support the concept that intrachain subunits exist in collagen.

#### Acknowledgment

I am indebted to Dr. Y. Pocker for helpful discussions and to Felicia Arguelles and Nancy Rittall for skillful technical assistance.

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## Isolation of a Zinc $\alpha_2$ -Macroglobulin from Human Serum\*

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**ABSTRACT:** An  $\alpha_2$ -macroglobulin constitutes the principal zinc metalloprotein of human serum. The material, separated successively by DEAE-cellulose chromatography and gel filtration, accounts for 30 to 40% of the total zinc content of serum. It is homogeneous in the ultracentrifuge and exhibits a sedimentation constant consistent with that known for  $\alpha_2$ -macroglobulin. Disc gel and immuno electrophoresis show one major and two faint minor bands. Immunochemical properties, trypsin-protein esterase activity, and the failure

of soybean trypsin inhibitor to inactivate the trypsin-protein complex—under conditions where trypsin itself is inhibited—all serve to establish the identity of this zinc protein as  $\alpha_2$ -macroglobulin. During purification, trypsin-protein esterase activity of fractions increases in parallel with zinc content which varied from 320 to 770  $\mu\text{g}$  of zinc/g in a number of  $\alpha_2$ -macroglobulin preparations. A direct role for zinc in the esterase activity of the trypsin- $\alpha_2$ -macroglobulin complex is not known thus far.

Zinc in human serum is almost entirely bound to protein. Its concentration is maintained within well-defined limits in normal individuals and is depressed in several metabolic and neoplastic disorders (Vikbladh, 1951; Vallee, 1959; *Lancet*, 1968). During the past 15 years, the identification of a number of zinc metalloenzymes has clarified many general aspects of the metabolic role of zinc, but the zinc content of the known zinc enzymes accounts for very little of the element found in human serum (Himmelhoch *et al.*, 1966). Hence, the nature and significance of all the circulating zinc proteins in man has remained unknown.

Recently atomic absorption and spark emission spectroscopy have been employed to identify zinc and other metals in discrete protein fractions of human serum resolved by DEAE-cellulose chromatography (Himmelhoch *et al.*, 1966). The present communication extends these investigations and demonstrates that the zinc present in normal human serum is composed of two principal species: one fraction, which is firmly bound to  $\alpha_2$ -macroglobulin, and a second fraction, more loosely associated with albumin.

### Materials

Materials were chosen with a view toward minimizing metal contamination during experimental procedures, as de-

scribed previously (Himmelhoch *et al.*, 1966). Stock solutions of Tris (Trizma Base, Sigma Chemical Co.) were demineralized by passage through columns of Chelex 100 (Bio-Rad Laboratories) by the method of Himmelhoch *et al.* (1966), except that the resin was not prewashed with EDTA. Succinic acid (Fisher Scientific Co.) and HCl (Aristar, British Drug Houses, Ltd.) were found satisfactory for use without further demineralization.

**Chromatographic Media.** DEAE-cellulose (DE-32, Whatman Co.) was cycled as outlined previously (Peterson and Chiazze, 1962). After washing with 0.5 M HCl, it was brought to the pH and conductivity of the starting buffer and packed at 15 psi under nitrogen, without exposure to EDTA.

Sephadex G-100 (Pharmacia Fine Chemicals, Inc.) and agarose (Bio-Rad Laboratories, Agarose A 0.5 m) were washed with 0.1 M Tris-HCl, pH 8.0, until the zinc concentration of the effluent was comparable with that of the applied buffer, as verified by atomic absorption spectrometry (Fuwa and Vallee, 1963; Fuwa *et al.*, 1964; Iida *et al.*, 1967).

### Methods

Serum samples were obtained as previously noted (Himmelhoch *et al.*, 1966). Plasma was processed from material stored in commercially available plastic transfer packs (Fenwal Co.).

Prior to all chromatographic procedures the starting material was dialyzed against two 20-fold volumes of starting buffer at 5°. The buffer was changed at 18 hr, and then dialysis was stopped after 6 hr. Scanty precipitates were removed by centrifugation and discarded.

In each of four pilot studies, 10 ml of serum was chro-

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