Anterior Lens Capsule Collagens: Cyanogen Bromide Peptides of the C Chain†

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ABSTRACT: Collagen was extracted from bovine anterior lens capsule by limited proteolysis with pepsin for 40 h at 4 °C. After purification, the collagen preparation was denatured and fractionated by gel filtration and carboxymethylcellulose chromatography. Three polypeptide chains of approximately α-chain size designated C, C-1, and D are described. The apparent molecular weight estimated by gel filtration of the C and D chains is 95 000, while that of the C-1 chain is 110 000. However, the apparent molecular weight estimated by sodium dodecyl sulfate gel electrophoresis of C is 95 000, of D 75 000, and of C-1 140 000. The C and C-1 chains were cleaved with CNBr, and the resulting peptides were separated, purified, and characterized with respect to amino acid composition and apparent molecular weight. A total of 12 CNBr peptides was obtained from the digest of the C chain, which together accounted for the amino acid content of the intact chain. The CNBr cleavage of the C-1 chain yielded a total of 13 peptides, 12 of which were identical with those obtained from the C chain in amino acid composition and chromatographic properties. The one extra peptide, CB13, contained 183 residues of amino acid. These 13 peptides together account for the amino acid content of the C-1 chain. From these data, we conclude that the C and C-1 chains are derived from the same molecule and that the observed difference in the molecular weight might be attributable to different sites of scission by pepsin. The amino acid composition of the D chain differs significantly from that of the C or C-1 chain. Furthermore, the sodium dodecyl sulfate gel electrophoretic pattern of the CNBr digest of the D chain is substantially different from that of C or C-1. From these observations, we conclude that, contrary to earlier suggestions, bovine anterior lens capsule contains at least two separate collagen chains, C (or C-1) and D. Evidence is insufficient to decide whether the C and D chains are from the same or different molecules.

Basement membrane collagens with a distinct amino acid composition constitute a different family of collagens as compared with the interstitial collagens, types I, II, and III. They are characterized by the presence of higher contents of 3-hydroxyproline, hydroxylysine, leucine, and carbohydrates and low contents of alanine (Kefalides, 1973, 1975). Kefalides (1971) and recently Dehm & Kefalides (1978) reported an α-chain size collagenous component (type IV) from the pepsin digest of anterior lens capsule and proposed the chain composition $\alpha1(IV)_3$. Kidney glomeruli and descemet's membranes (Kefalides, 1971) were also reported to contain a single type (IV) of collagenous polypeptide chain. In contrast, Daniels & Chu (1975) isolated a heterogeneous mixture of collagenous fragments from bovine glomerular basement membrane. The work of Sato & Spiro (1976) and Freytag et al. (1976) also suggests the heterogeneity of collagenous fragments isolated without the use of proteases. Recently Burgeson et al. (1976), Chung et al. (1976), and Rhodes & Miller (1978) have isolated and characterized collagenous A and B chains from placenta, liver, skin, and the medial layer of vascular tissue. Similar A and B chains have also been reported by Bentz et al. (1978) from placenta and by Brown et al. (1978) from synovial membrane and gingiva. Although the low content of alanine and high content of hydroxylysine in the A and B chains resemble somewhat those typical for a basement membrane collagen, their origin from basement membranes remains to be documented.

This laboratory previously described two collagenous components of 110 000 and 50 000 molecular weights from bovine anterior lens capsule (Dixit, 1978). Schwartz & Veis (1978) have also shown in anterior lens capsule the presence of 115 000, 85 000, and 55 000 molecular weight components by $NaDodSO_4$[1]–polyacrylamide gel electrophoresis of reduced, pepsin-solubilized collagen. The present paper describes the isolation and characterization of three approximately α-chain size polypeptides, C, C-1, and D chains,[2] and the CNBr peptides of C and C-1 from bovine anterior lens capsule. The results show that the CNBr peptides of C and C-1 are identical except for the presence of an extra peptide in C-1, indicating that they are derived from the same molecule. The CNBr peptide pattern on $NaDodSO_4$–polyacrylamide gel electrophoresis of the D chain is different from that of C or C-1, indicating that D chain is most probably a structurally distinct chain.

Materials and Methods

Preparation of Collagen. Bovine eyes were obtained fresh from a local slaughter house and transported to the laboratory in an ice bucket. The anterior lens capsules were dissected, cleaned, and lyophilized as described previously (Dixit, 1978). The lyophilized capsules (1 g) were pulverized in a freezer mill (Spex Industries, Metuchen, NJ) in liquid nitrogen and suspended in 200 mL of 0.5 M acetic acid which contained a mixture of protease inhibitors (4 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, and 5 mM *N*-ethylmaleimide); the final pH was adjusted to 2.5 by the use of formic acid. Pepsin (100 mg) was added, and the mixture was shaken gently for 40 h at 4 °C. At the end of incubation, the supernates were collected by centrifugation (18000*g* for 1 h) and

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[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; CM, carboxymethyl.

[2] The nomenclature of C and D chains presented in this paper corresponds to that used by Kresina et al. (1978). The C and D chains have also been referred to as $\alpha1$(IV) and $\alpha2$(IV) chains, respectively, by Dr. Paul Bornstein.

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the collagens were precipitated by the addition of NaCl to a final concentration of 10%. The precipitate obtained after centrifugation was redissolved in 0.5 M acetic acid and reprecipitated with 10% NaCl. The precipitated collagens were collected by centrifugation and redissolved in 0.01 M Tris–1 M NaCl, pH 7.4, and the pH was adjusted to 7.4 by addition of cold 0.5 M NaOH. Solid NaCl was added to the clear solution to a concentration of 30%. The precipitate was collected by centrifugation, redissolved in 0.01 M Tris–0.1 M NaCl, pH 7.4, and dialyzed against 0.01 M Tris–0.02 M NaCl, pH 7.4, with several changes of the same buffer. The retentate was clarified by centrifugation and the clear supernatant was applied to a column of diethylaminoethylcellulose equilibrated with 0.01 M Tris–0.02 M NaCl, pH 7.4, and eluted with the same buffer. Fractions containing collagen eluting unretarded from the column were collected, and collagen was precipitated by the addition of NaCl to a final concentration of 30%. The precipitated collagens were harvested by centrifugation, dissolved in 0.5 M acetic acid, dialyzed vs. 0.1 M acetic acid, and lyophilized. All the experiments were performed at 4 °C. The mixture of protease inhibitors described above was incorporated into all solutions used during the purification except for the last step of dialysis vs. 0.1 M acetic acid.

Molecular Sieve Chromatography of Collagen Preparations. Lyophilized collagens were initially fractionated on a 4 × 50 cm column of 6% agarose A-5M (200–400 mesh, Bio-Rad Laboratories) with 0.01 M Tris–1 M $CaCl_2$, pH 7.4, essentially as described (Piez, 1968). The sample was dissolved in 6–8 mL of the same buffer by warming for 10–15 min at 45 °C and was applied to the column. The column was eluted with the same buffer at a flow rate of 55 mL/h. Column effluents were monitored continuously at 230 nm in a Gilford spectrophotometer equipped with a flow cell. The column was calibrated with unreduced human α1 type III, chick skin collagen β_{12}, and α1 chains. A drop of tritiated water was used to mark the column volume (Piez, 1968).

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. The polyacrylamide gel electrophoresis of various collagen fractions was performed on 7.5% slab gels in sodium dodecyl sulfate in the presence of mercaptoethanol as described (Laemmli, 1970). The gels were electrophoresed for 2 h at a constant current of 50 mA. The CNBr digests of C and D chains were electrophoresed for 5 h on 15% slab gels on a constant current of 25 mA. The gels were stained for 2–4 h in 0.1% Coomassie Brilliant Blue R-250 in 50% methanol and 7% acetic acid and destained in 10% methanol and 7% acetic acid until the background cleared.

Carboxymethylcellulose Chromatography. CM-cellulose chromatography of fractions obtained from molecular sieve chromatography on agarose A-5M column was performed on a 1 × 10 cm column of the resin equilibrated with 0.02 M sodium acetate–1 M urea, pH 4.8, at 44 °C. Samples were dissolved in 4–5 mL of starting buffer, denatured by heating at 40 °C for 10 min, and applied to the column. The column was eluted with a linear gradient of NaCl from 0 to 0.12 M over a total volume of 500 mL. Effluents were monitored continuously as above.

Reduction and Alkylation. The 220 000 molecular weight collagenous component (peak 2, Figure 1) was reduced with mercaptoethanol and carboxymethylated with iodoacetate essentially as described earlier (Crestfield et al., 1963). Fifty milligrams of the protein was dissolved in 36 mL of 0.25 M Tris–8 M urea, pH 8.5. Under a stream of N_2 atmosphere 0.2 mL of mercaptoethanol was added. Reduction was carried out with gentle stirring for 4 h at room temperature. At the end of incubation, a freshly prepared solution of iodoacetic acid (0.54 g dissolved in 2 mL of 1 M NaOH) was added with further stirring for 30 min in the dark. The reaction mixture was desalted on Bio-Gel P-2 (50–100 mesh, Bio-Rad Laboratories) immediately and lyophilized.

CNBr Cleavage. The purified collagen chains were cleaved with CNBr at 37 °C in 70% formic acid (Butler & Ponds, 1971; Miller et al., 1971; Volpin & Veis, 1971) for 4 h under an atmosphere of nitrogen as described originally by Bornstein & Piez (1966). An amount of CNBr equal to twice the weight of the protein was used. After 4 h the reaction was terminated by dilution of the reaction mixture with 10 volumes of cold distilled water and lyophilization.

Carboxymethylcellulose and Phosphocellulose Column Chromatography of CNBr Peptides. The initial fractionation of CNBr digests was performed on a CM-cellulose column (2.5 × 10 cm) equilibrated with 0.02 M sodium citrate, pH 3.8, at 44 °C. The samples were applied to the column in 8–10 mL of starting buffer, and the column was eluted with a linear gradient of NaCl from 0 to 0.12 M over a total volume of 1600 mL at a flow rate of 200 mL/h.

Small- and medium-size CNBr peptides were further purified on a phosphocellulose column (1 × 6 cm) using a linear gradient of NaCl from 0 to 0.3 M in 0.001 M sodium acetate, pH 3.6. Larger CNBr peptides were purified on a CM-cellulose column (1 × 6 cm) equilibrated with 0.02 M sodium acetate, pH 4.8, with a linear gradient of 0 to 0.16 M NaCl at 44 °C.

Effluents were monitored continuously at 230 nm in a Gilford spectrophotometer equipped with a flow cell. Desalting of the samples was performed on Bio-Gel P-2 (100–200 or 200–400 mesh) using 0.1 M acetic acid as the eluant.

Molecular Sieve Chromatography of CNBr Peptides. Various fractions obtained from ion-exchange chromatography were further purified on a Sephadex G-75S column (2 × 120 cm). The column was equilibrated with 0.04 sodium acetate, pH 4.8, and eluted at a flow rate of 15 mL/h. Smaller peptides were fractionated on Bio-Gel P-2 (200–400 mesh) on a 2 × 100 cm column using 0.1 M acetic acid as the eluant. Effluents were monitored continuously as described above. The samples were desalted on Bio-Gel P-2 as described previously.

Molecular Weight Determination. Molecular weights of small peptides were calculated from amino acid composition. The molecular weights of medium-size peptides were estimated by chromatography on a Sephadex G-75S column (2 × 120 cm). The chick skin CNBr peptides α1(I)-CB7, α1(I)-CB3, and α1(I)-CB2 mixed with tritiated water were used to calibrate the column. For determination of the molecular weights of large CNBr peptides, an agarose Bio-Gel A-1.5M (200–400 mesh, Bio-Rad Laboratories) column precalibrated with α2-CB(3–5), α2-CB3, α1(I)-CB7, and α1(I)-CB3 of chick skin collagen was used essentially as described by Piez (1968).

Amino Acid Analysis. Samples for amino acid analysis were prepared by hydrolysis in constant-boiling HCl at 108 °C for 24 h in an atmosphere of nitrogen. Analyses were performed on an automatic analyzer (Beckman 121) using a single-column method (Kang, 1972). Correction factors for the loss of labile amino acids (threonine, serine, methionine, and tyrosine) and the incomplete release of valine were used as determined by Piez et al. (1960). Hydroxylysine glycosides were determined by hydrolysis of samples in 2 N NaOH at 108 °C for 20 h. The hydrolyzed samples were diluted 10-fold with distilled water, adjusted to pH 2.0, and separated on an

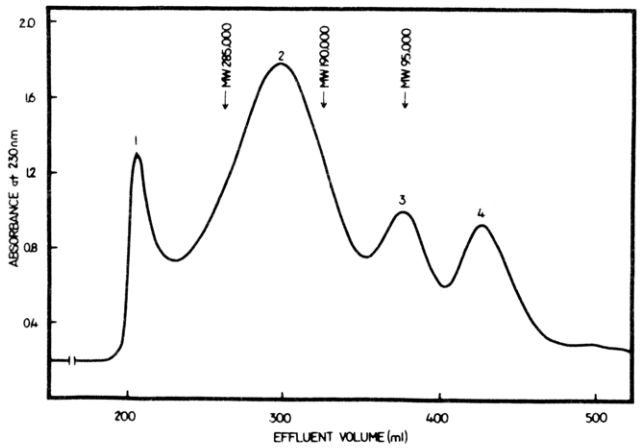

FIGURE 1: Molecular sieve chromatography of pepsin-extracted collagen (200 mg) from bovine anterior lens capsule on an agarose A-5M column (4 × 50 cm). The column was eluted with 0.01 M Tris–1 M $CaCl_2$, pH 7.4, at a flow rate of 55 mL/h. Fractions 2–4 were pooled separately, desalted on a column of Bio-Gel P-2 in 0.1 M acetic acid, and lyophilized. Uncorrected for any mechanical losses, 125, 20, and 20 mg of protein were obtained from fractions 2, 3, and 4, respectively.

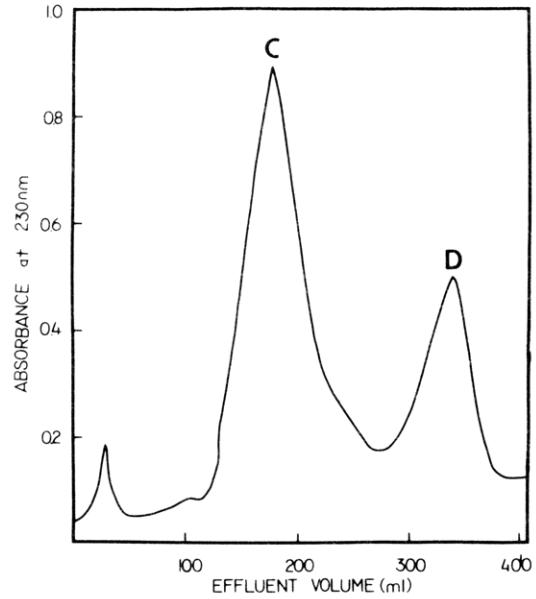

FIGURE 2: Chromatography of fraction 3 (25 mg) (Figure 1) on a carboxymethylcellulose column (1 × 10 cm) equilibrated with 0.02 M sodium acetate–1 M urea, pH 4.8, at 44 °C. The column was eluted at a flow rate of 100 mL/h with a linear gradient of NaCl from 0 to 0.12 M containing 1 M urea over a total volume of 500 mL. Uncorrected for any mechanical losses, 10 mg of C and 4 mg of D were obtained.

amino acid analyzer at pH 5.28 as described previously (Askenasi & Kefalides, 1972; Miller, 1972).

Results

Preparation of the C, D, and C-1 Chains. The initial fractionation of the collagen preparation was performed on an agarose A-5M column. A typical elution pattern is presented in Figure 1. The fractions 2–4 were pooled separately, desalted, and lyophilized. The lyophilized material from fraction 3 (Figure 1) was then subjected to CM-cellulose chromatography. A typical elution pattern is presented in Figure 2. Two major peaks were obtained, designated C and D. Molecular sieve chromatography on agarose A-5M of these chains yielded essentially homogeneous products. The $NaDodSO_4$–polyacrylamide gel electrophoresis patterns of fraction 3 (Figure 1) and the purified C and D chains are shown in Figure 3. It should be noted that the apparent molecular weight of the C and D chains estimated by gel filtration on agarose A-5M is 95 000 but that the apparent molecular weight estimated by $NaDodSO_4$–polyacrylamide gel electrophoresis of C and D is 95 000 and 75 000, respectively (Figure 3). The reason for the seemingly anomalous electrophoretic migration of the D chain is not clear. It is known that the α1(I) and α2 chains migrate with different mobility during $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 3) despite their identical molecular weight. The amino acid composition of the purified C and D chains is given in Table I.

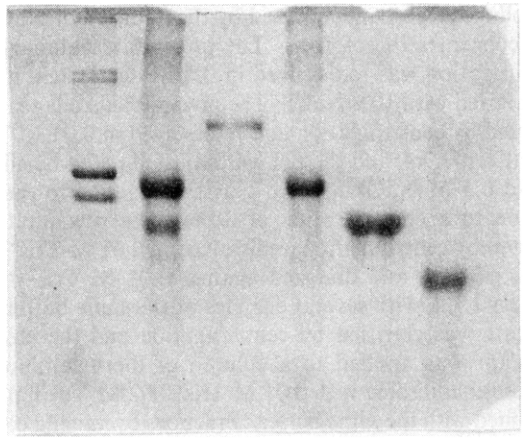

FIGURE 3: $NaDodSO_4$–polyacrylamide gel electrophoresis of (lane 1, from left) type I collagen of chick skin showing α1(I), α2, β_{11}, and β_{12}, (lane 2) fraction 3 from Figure 1, (lane 3) the C-1 chain prepared as described in the text, (lane 4) the C chain from Figure 2, (lane 5) the D chain from Figure 2, and (lane 6) material in fraction 4 (Figure 1) after purification by CM-cellulose chromatography. Gels (7.5%) were electrophoresed in the presence of mercaptoethanol for 2 h at a current of 50 mA.

Table I: Amino Acid Composition[a] of C and D Chains of Bovine Anterior Lens Capsule

	C-1[b]	C	D	fraction 4[c]
3-Hyp	3.2	3.8	6.3	2.8
4-Hyp	129	126	117	140
Asp	52	52	51	51
Thr	17	20	22	26
Ser	36	38	34	39
Glu	89	75	67	72
Pro	57	64	59	68
Gly	345	352	343	338
Ala	33	35	40	33
Val	18	26	27	28
1/2-Cys	2.9	-	2.2	-
Met	13	11	12	12
Ile	19	27	34	28
Leu	51	48	57	44
Tyr	3.0	2.3	2.1	1.9
Phe	29	28	34	25
Hyl	64	58	43	56
Lys	7.3	6.5	4.7	5.1
His	6.2	6.0	5.6	6.8
Arg	21	21	40	19
Glc-Gal-Hyl	52	48	34	ND[d]
Gal-Hyl	6	4.8	3.2	ND

[a] Residues calculated per 1000 amino acids. Values for residues present in number greater than 10 were rounded off to the nearest whole number. A dash indicates the presence of less than 0.2 residue. [b] Amino acid composition from Dixit (1978). [c] Fraction 4 of Figure 1 after further purification by CM-cellulose chromatography as described in the text. [d] ND, not determined.

The material obtained in fraction 2 (Figure 1) was reduced with mercaptoethanol in 8 M urea at room temperature,

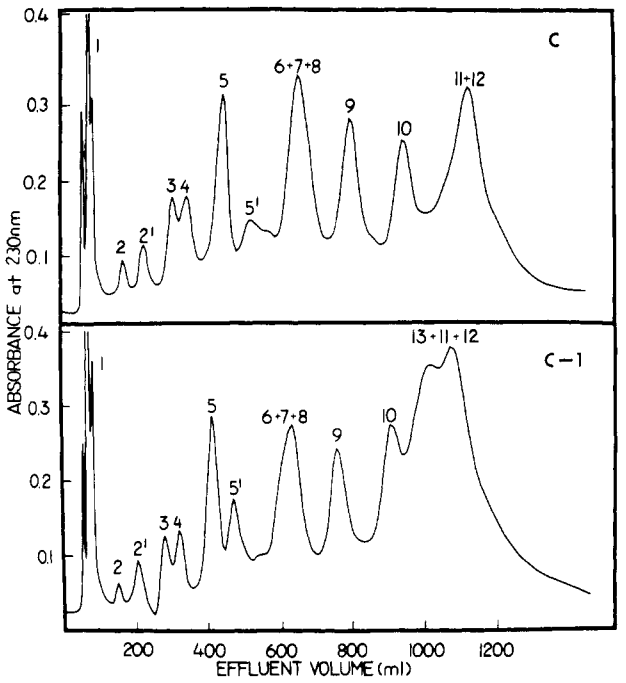

FIGURE 4: Elution profile of CNBr peptides of C (upper panel) and C-1 (lower panel) on a carboxymethylcellulose column (2.5 × 10 cm) equilibrated with 0.02 M sodium citrate, pH 3.8, at 44 °C. The column was eluted with a linear gradient of NaCl from 0 to 0.12 M NaCl over a total volume of 1000 mL at a flow rate of 200 mL/h.

carboxymethylated (see Materials and Methods), and subjected to molecular sieve chromatography on agarose A-5M followed by CM-cellulose chromatography. As documented in detail in a previous paper from our laboratory (Dixit, 1978), a polypeptide chain with an apparent molecular weight of 110 000 by gel filtration was obtained. Although this chain was left unnamed in that paper (Dixit, 1978), we now designate it C-1 because of its similarity to C (see below). Its $NaDodSO_4$–polyacrylamide gel electrophoresis pattern is shown in Figure 3. Its electrophoretic mobility is substantially slower than that of the α1(I) or C chain. Its apparent molecular weight calculated from $NaDodSO_4$–polyacrylamide gel electrophoresis is 140 000, but that estimated from its elution from agarose A-5M is 110 000 (Dixit, 1978). The amino acid composition of C-1 is given in Table I.

The material present in fraction 4 (Figure 1), after desalting, was further purified by CM-cellulose chromatography. A single homogeneous peak eluting near the region of α1(I) was obtained (not shown). Its purity was confirmed by $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 3), and the amino acid composition is presented in Table I. Present evidence is insufficient to decide its origin or relationship to the C and D chains.

Isolation and Characterization of the CNBr Peptides of the C Chain. The lyophilized CNBr digest of the C chain was initially chromatographed on a CM-cellulose column using 0.02 M sodium citrate, pH 3.8. The elution pattern is illustrated in Figure 4 (upper panel). Fractions 1 and 2 were lyophilized and desalted on Bio-Gel P-2 (200–400 mesh). Fraction 1 (Figure 4) was further chromatographed on a phosphocellulose column in 0.001 M sodium acetate, pH 3.6, when one major peak corresponding to CB1 was obtained (not shown). Under similar experimental conditions, fraction 2 (Figure 4) was resolved also into a major peak CB2 (not shown).

Fractions 3 and 4 (Figure 4) were pooled, desalted, and resolved into individual peptides, CB3 and -4 (Figure 5), on

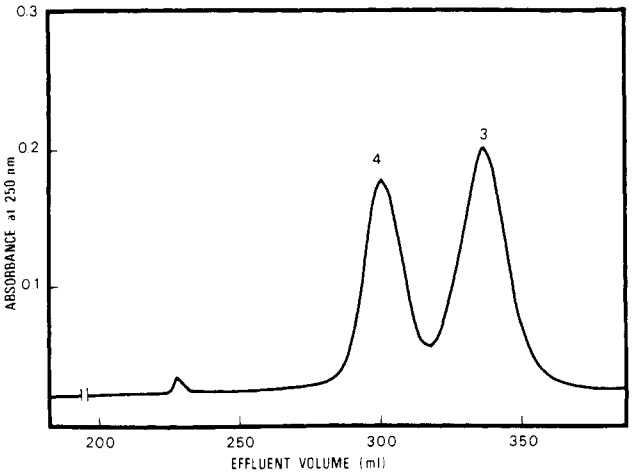

FIGURE 5: Molecular sieve chromatography of pooled fractions 3 and 4 (Figure 4, upper panel) on a column of Sephadex G-75S (2 × 120 cm). The column was equilibrated with 0.04 M sodium acetate, pH 4.8, and eluted at a flow rate of 15 mL/h.

a Sephadex G-75S column. The greater absorbance of CB3 as compared to CB4 (Figure 5) is possibly due to the presence of a residue of phenylalanine in CB3. Fraction 6 (Figure 4) contained three major peptides, CB6, -7, and -8, which were readily separated by rechromatography on Sephadex G-75S as shown in Figure 6. The unlabeled peak between fractions CB8 and -7 (Figure 6) was identified as CB9 by amino acid composition. Fractions 5, 9, and 10 (Figure 4) were also rechromatographed on Sephadex G-75S, and only one major peptide peak (CB5, -9, and -10, respectively) was obtained (not shown). The individual peptides CB3–CB10 were further rechromatographed on a phosphocellulose column at pH 3.6, and a homogeneous peak was obtained.

Fraction 2' (Figure 4) on phosphocellulose resolved into three minor components, possibly breakdown peptides, which were not investigated further. Fraction 5' was resolved into two minor components; one was identified as CB6 and the other as an uncleaved peptide consisting of CB3 and -7 by amino acid composition containing a residue of methionine (data not shown).

Fractions 11 and 12 (Figure 4) were resolved on a CM-cellulose column at pH 4.8. The CM-cellulose elution profile is depicted in Figure 7. The peptides CB11 and -12 were rechromatographed under identical conditions to achieve further purification. The relatively poor resolution of CB11 and -12 may perhaps be explained by the presence of high contents of carbohydrate linked to hydroxylysine residues.

The molecular weights of CB1 and -2 were calculated from amino acid composition. The molecular weights of all the other peptides were estimated by molecular sieve chromatography and are presented in Table II. The observed molecular weight on molecular sieve chromatography is higher when compared with the molecular weight calculated from amino acid composition. If the glycosylation of hydroxylysine residues is taken into account, however, the observed and calculated molecular weights agree with each other within experimental error.

The amino acid composition of the cyanogen bromide peptides obtained from the C chain is listed in Table II. The sum of the amino acid residues of the CNBr peptides accounts for, within experimental error, the observed amino acid composition of the C chain.

Isolation and Characterization of the CNBr Peptides of the C-1 Chain. The elution pattern of CNBr peptides obtained from the C-1 chain is illustrated in Figure 4 (lower panel). One extra peptide, CB13, was present in the CNBr digest of

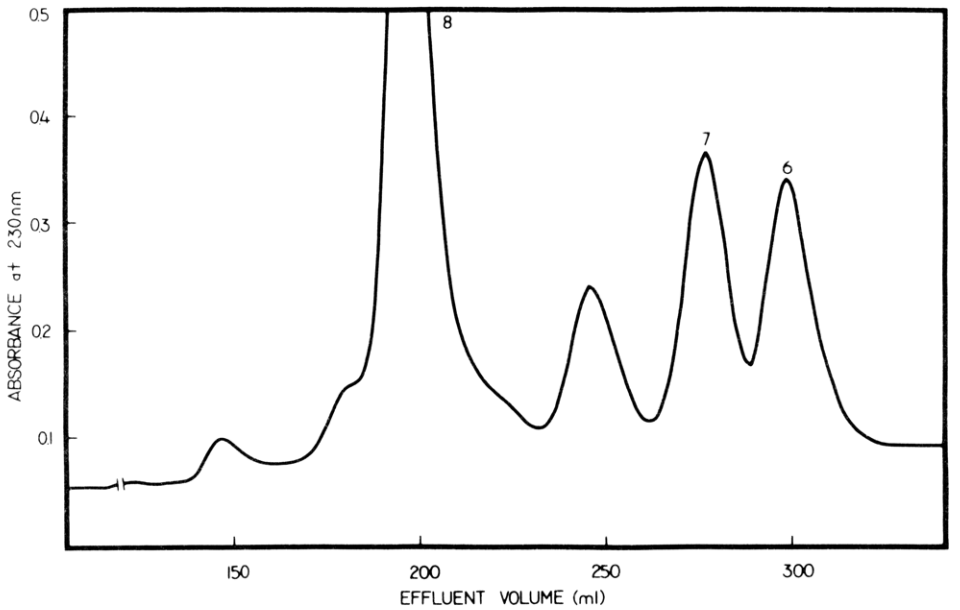

FIGURE 6: Molecular sieve chromatography of fractions 6 + 7 + 8 (Figure 4, upper panel) on Sephadex G-75S. Conditions were the same as for Figure 5.

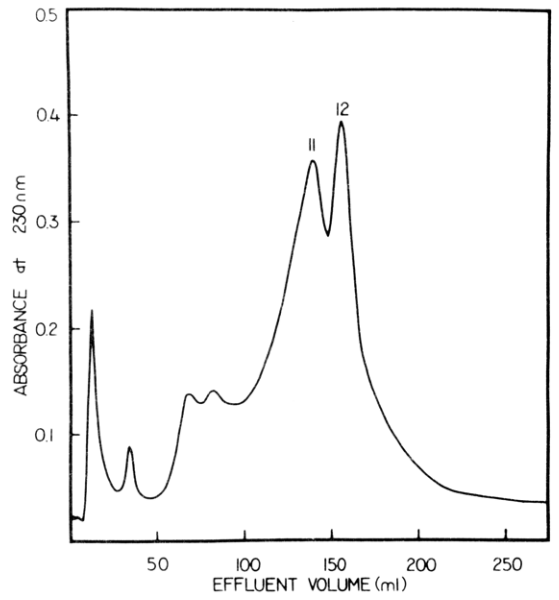

FIGURE 7: Carboxymethylcellulose chromatography of CB11 and -12. A column (1 × 6 cm) of carboxymethylcellulose was equilibrated with 0.02 M sodium acetate, pH 4.8, and after the application of the sample was eluted with a linear gradient of NaCl from 0 to 0.16 M at 44 °C.

C-1 that was not present in the CNBr digest of C chain. The fractions representing peptides 13, 11, and 12 were pooled, desalted, and lyophilized. The mixture was then fractionated on a column of CM-cellulose under identical conditions with those used for the separation of CB11 and -12. The peptide CB13 eluted unretarded from the column in the void volume, and the elution pattern of CB11 and -12 was indistinguishable from that given in Figure 7. The remainder of the fractions 1–10 (Figure 4, lower panel) was collected, purified, and characterized as described under C chain peptides. The amino acid compositions and molecular weights of the peptides CB1–CB12 were identical within experimental error with those of the cyanogen bromide peptides of the C chain. The amino acid composition of CB13 is presented in Table III.

CNBr Peptides of the D Chain. The CNBr digest of the purified D chain was analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis, and the resulting pattern was compared

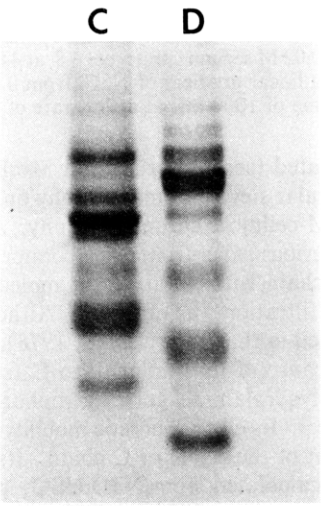

FIGURE 8: $NaDodSO_4$–polyacrylamide gel electrophoresis of CNBr digests of the purified C and D chains. Gels (15%) were electrophoresed for 5 h at a constant current of 25 mA.

with that obtained from the C chain. As shown in Figure 8, the patterns are different, suggesting that the D chain is a structurally different chain from the C chain.

Discussion

The work presented here describes the isolation of two distinct collagenous chains, C and D, from the pepsin digest of anterior lens capsule. Both chains are liberated by limited pepsin treatment of lens capsule and are eluted on an agarose A-5M column in the position of the $\alpha 1$(I) chain (Figure 1). The proteolytic cleavage by pepsin does not proceed to completion as higher molecular weight collagenous components were consistently present on molecular sieve chromatography (Figure 1). The use of protease inhibitors during extraction and purification of collagens probably helped to retard the proteolysis into small molecular weight components. The two chains, C and D, were readily separated by CM-cellulose chromatography (Figure 2). The amino acid compositions of the two chains are significantly different in their content of leucine, hydroxylysine, and arginine residues. The proportion of C/D chain was always found to be more than 2:1. As shown in Figure 8, their CNBr peptide patterns are also significantly

Table II: Amino Acid Composition[a] of CNBr Peptides from the C Chain of Bovine Anterior Lens Capsule

	1	2	3	4	5	6	7	8	9	10	11	12	total
3-Hyp	0	0	0	0	0	1 (0.7)	0	1 (1.1)	0	0	1 (0.8)	1 (0.9)	4
4-Hyp	1 (1.0)	2 (1.6)	4 (3.7)	6 (5.5)	6 (5.6)	6 (5.8)	8 (8.1)	12	10 (9.7)	12	19	23	109
Asp	1 (1.1)	1 (1.0)	1 (1.0)	1 (1.0)	2 (2.1)	1 (1.1)	4 (4.2)	4 (3.8)	4 (4.0)	4 (3.7)	8 (8.2)	9 (8.9)	40
Thr	0	1 (0.9)	0	2 (1.8)	1 (0.9)	1 (1.0)	1 (1.0)	2 (2.0)	2 (1.9)	1 (0.8)	2 (2.0)	2 (2.0)	15
Ser	0	1 (0.9)	1 (1.1)	2 (1.9)	0	0	4 (3.7)	3 (3.0)	2 (2.0)	6 (6.0)	8 (7.6)	7 (7.0)	34
Glu	0	0	2 (1.8)	5 (5.1)	3 (3.2)	5 (4.7)	3 (2.8)	10 (10.2)	7 (6.9)	7 (7.0)	16	17	75
Pro	0	1 (1.1)	2 (1.8)	2 (2.3)	3 (3.2)	5 (5.0)	2 (2.2)	10 (9.8)	4 (3.8)	4 (4.1)	12	14	59
Gly	2 (2.2)	3 (2.8)	8 (7.9)	12	13	13	20	39	26	31	63	71	301
Ala	0	0	0	0	1 (1.0)	1 (1.0)	2 (2.0)	3 (2.8)	4 (4.0)	2 (2.0)	6 (6.0)	7 (6.8)	26
Val	0	1 (0.9)	0	0	0	1 (0.9)	2 (1.8)	3 (3.0)	2 (1.7)	2 (1.8)	5 (4.7)	5 (5.2)	21
Ile	0	0	0	1 (1.0)	0	0	0	2 (2.1)	3 (2.7)	3 (2.8)	6 (6.0)	7 (6.8)	22
Leu	1 (1.0)	0	1 (1.0)	3 (3.2)	3 (2.8)	2 (2.0)	3 (2.7)	6 (6.1)	3 (3.1)	5 (5.0)	9 (9.2)	10	46
Tyr	0	0	0	0	0	0	0	0	0	1 (0.9)	0	0	1
Phe	0	1 (0.8)	1 (1.1)	0	1 (0.9)	0	2 (2.1)	5 (5.0)	1 (0.8)	3 (3.0)	5 (5.1)	6 (6.2)	25
Hyl	0	0	1 (1.2)	3 (2.7)	2 (1.8)	1 (1.1)	4 (3.8)	7 (6.6)	7 (6.6)	7 (6.7)	11	10	53
Lys	0	0	0	0	0	0	1 (1.1)	0 (0.2)	1 (0.7)	1 (1.3)	2 (1.6)	2 (1.7)	7
His	0	1 (0.8)	0	0	0	1 (0.8)	1 (1.0)	1 (0.8)	1 (0.8)	0	1 (0.9)	1 (1.1)	7
Arg	0	0	0	0	1 (1.0)	2 (1.9)	1 (1.1)	1 (1.0)	1 (1.1)	1 (1.1)	6 (6.0)	7 (7.2)	20
Hse	1 (1.0)	0	1 (0.8)	1 (0.8)	1 (1.0)	1 (0.9)	1 (0.9)	1 (0.8)	1 (0.8)	1 (0.8)	1 (0.8)	1 (0.9)	11
total	6	12	22	38	37	41	59	110	79	91	181	200	876
mol wt by amino acid analysis	604	1198	2115	3725	3576	4017	5515	10474	7624	9329	17310	19025	
mol wt by gel chromatography	604[b]	1198[b]	2850	4420	3800	4230	6820	13800	8400	12300	19200	20400	

[a] Residues per peptide to the nearest whole number. Actual values are given where less than 10 residues occur. [b] Molecular weight calculated by amino acid analysis.

Table III: Amino Acid Composition[a] of CB13 Obtained from CNBr Cleavage of the C-1 Chain

3-Hyp	1 (0.8)
4-Hyp	20
Asp	8 (8.2)
Thr	5 (4.7)
Ser	4 (3.7)
Glu	21
Pro	13
Gly	81
Ala	4 (4.2)
Val	2 (2.1)
1/2-Cys[b]	2 (1.7)
Ile	3 (3.0)
Leu	11
Tyr	2 (1.6)
Phe	5 (5.2)
Hyl	11
Lys	3 (2.6)
His	1 (0.9)
Arg	5 (5.0)
Hse	1 (0.8)
total	183
mol wt by amino acid analysis	18 184
mol wt by gel chromatography	19 800

[a] Residues per peptide to the nearest whole number. Actual values are given where less than 10 residues occur. [b] Calculated as (carboxymethyl)cystine.

different. The glycosylation of hydroxylysine to an extent of 91 and 86% was found in the C and D chains, respectively. The C chain described in this paper is very similar in amino acid composition to the three major fragments of apparent molecular weights of 140 000, 100 000, and 70 000 isolated from human placenta by Sage et al. (1979). Further, the D chain has an amino acid composition similar to that of the 70K-II fragment of 70 000 molecular weight as described by the same investigators (Sage et al., 1979).

The amino acid compositions and the molecular weights as estimated by gel filtration of the CNBr peptides obtained from the C chain of bovine anterior lens capsule are summarized in Table II. The data are in substantial agreement with the results reported earlier by Kefalides (1972) on the CNBr peptides of bovine anterior lens capsule with minor differences. The peptide CB2, a dodecapeptide, lacks a residue of homoserine and must be the COOH-terminal peptide, whereas peptide CB5 was reported earlier (Kefalides, 1972) as the COOH-terminal peptide. The peptides CB7, -8, -9, and -10 (Table II) correspond possibly to peptides 6, 9, 8, and 7, respectively, reported by Kefalides (1972) with some differences in amino acid composition. The peptides CB11 and CB12 (Table II) and CB13 (Table III) are similar to peptides 10, 11, and 12 of Kefalides (1972) with minor differences in amino acid composition. The sum of the total amino acid residues of 12 CNBr peptides obtained from the C chain is ~876 amino acid residues. The apparently larger molecular weight behavior of the C chain which eluted in the position of the $\alpha 1$(I) chain of 95 000 molecular weight is contributed by glycosylation of over 90% of the hydroxylysine residues in the C chain. These 876 amino acid residues, when summed up with the amino acid residues of peptide CB13 (Table III), constituted an α-size chain containing over 1050 amino acid residues, similar to other α chains of type I, II, and III collagens (Piez, 1976).

The C-1 chain has an apparent molecular weight of 110 000 (Dixit, 1978) by agarose A-5M gel filtration. The C-1 chain is obtained by reduction followed by carboxymethylation of the 220 000 molecular weight fraction (peak 2, Figure 1). The

fact that the C-1 chain is obtained only after reduction indicates that interchain disulfide bonds are present in the 220 000 molecular weight fraction. Further, since both the C and C-1 chains contain the COOH-terminal peptide, CB2 (Table II), the interchain disulfide bonds and the peptide CB13 are likely to be located at the NH_2-terminal end of the C-1 chain. This chain has been designated as C-1 because of its similarity to the C chain. The CNBr digestion of the C-1 chain resulted in the formation of 13 CNBr peptides. Twelve of these are identical within experimental error with the CNBr peptides of the C chain. There is an extra peptide, CB13, in C-1 (Table III) accounting for the higher molecular weight of the C-1 chain. Thus, we suggest that the C-1 and C chains are derived from the same parent molecule by pepsin cleavage at different loci. The values of molecular weight of C-1 estimated by gel filtration and $NaDodSO_4$–polyacrylamide gel electrophoresis do not agree with each other (110 000 and 140 000, respectively). On the basis of our characterization of C-1 and the CNBr peptides, we believe that the value of 110 000 obtained by gel filtration is more correct and that the larger value obtained by $NaDodSO_4$–polyacrylamide gel electrophoresis is due to the peculiar electrophoretic mobility of C-1 in that system in a manner analogous to the behavior of $\alpha 2$ relative to $\alpha 1$(I). Further studies are needed to define the precise pepsin cleavage site of the C-1 chain which results in the formation of the C chain.

The amino acid composition of the C chain presented in Table I is in agreement with that previously reported for the type IV chain (Kefalides, 1971; Dehm & Kefalides, 1978) from anterior lens capsule. The D chain is a new distinct chain not described earlier. We suspect that the D chain is more susceptible to pepsin cleavage as compared to the C chain. The best results were obtained when the proteolysis with pepsin was carried out at 4 °C for 40–48 h and a mixture of inhibitors was used during extraction and purification. The amino acid composition (Table I) of the C and D chains is characteristic of basement membrane collagens which are characterized by high contents of 3-hydroxyproline, hydroxylysine, and carbohydrates and low contents of alanine. As is evident from Table I, there are striking differences in the leucine, hydroxylysine, and arginine contents of the C and D chains.

From the present data, it is difficult to speculate on the molecular structure of the two C and D chains. The two chains may arise from two separate molecules with molecular structures of C_3 and D_3 or from the same molecule with a molecular structure of C_2D or D_2C.

Added in Proof

Since submission of this manuscript, we have found that the D chain is comprised of 75 000 and 15 000 molecular weight components held together by disulfide cross-links as reported earlier by Kresina & Miller (1979) in the D chain from human placenta. The behavior of the D chain on $NaDodSO_4$–polyacrylamide gel electrophoresis in the presence of mercaptoethanol is consistent with the observed mobility of the reduced D chain with the formation of the 75 000 component (Figure 3).

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