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Isolation and Characterization of Basement Membrane Collagen from Human Placental Tissue. Evidence for the Presence of Two Genetically Distinct Collagen Chains†

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ABSTRACT: A unique collagen fraction containing basement membrane collagen molecules has been isolated from limited pepsin digests of placental tissue utilizing selective precipitation procedures and chromatography of the collagen in native form on CM-cellulose. Characterization of the denaturation products of this collagen has revealed the presence of two classes of components. The more acidic components (C′, C, and $50K_1$) represent, respectively, a modified form of the pro-C chain, a collagen chain virtually identical with the α-chain-sized component derived from limited pepsin digests of isolated basement membranes, and a mixture of smaller molecular weight components arising from proteolysis of either the pro-C or C chain. The more basic components (80K and $50K_2$) represent, respectively, the major portion of a genetically distinct chain, designated the D chain, and a mixture of proteolytic cleavage products of the latter chain. The C chain and the 80K component are readily distinguishable on the basis of chromatographic properties, compositional features, and their respective cyanogen bromide cleavage products. In addition, both chains appear to be derived from molecules originating in basement membrane structures since both components are observed in the denaturation products of collagen solubilized from isolated basement membranes. It is concluded from these data that basement membrane collagen molecules are comprised collectively of at least two genetically distinct collagen chains.

Basement membranes may be regarded as specialized extracellular matrices which serve to form the boundaries between various tissue compartments. In this role, the membranes may function as selective permeability barriers as well as supporting elements. They are, therefore, of critical importance in the maintenance of tissue integrity.

Early studies on the chemical composition of the larger and more readily isolated basement membranes such as the lens capsule, glomerular basement membrane, Reichert's membrane, and Descemet's membrane definitely established that such structures contain a high, albeit variable, proportion of molecules with collagenous sequences (Kefalides, 1973). However, the precise nature of the collagenous constituents and their molecular organization within basement membranes have not been well-defined and are the subject of some controversy. In this regard, studies on the pepsin-solubilized collagen from several basement membranes have led to the isolation of a single a1-like chain which exhibits a number of unique compositional features relative to other known collagen chains (Kefalides, 1971; Dehm & Kefalides, 1978). In addition, recent studies on basement membrane biosynthesis employing organ cultures of parietal yolk sac endoderm (Minor et al., 1976) and lens capsules (Heathcote et al., 1978) have shown that these tissues synthesize a high molecular weight collagenous protein comprised of three apparently identical procollagen-like chains, each of which exhibits an apparent molecular weight in the range of 160000–180000. Pulse chase experiments, however, indicated that the basement membrane procollagen species did not undergo a time-dependent conversion to a smaller molecular weight molecule on incorporation into the basement membrane structure. These results have led to the view that the collagenous constituents of basement membranes may be characterized as a homogeneous population of molecules (type IV collagen), each of which contains three identical chains (α1(IV) chains) and which are present in a form resembling procollagen molecules.

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Other investigators have observed a somewhat greater degree of heterogeneity in the collagenous components derived from basement membranes. Daniels & Chu (1975) reported the isolation of two α-chain-sized ($\sim$95 000) as well as two larger molecular weight ($\sim$140 000) collagenous components from pepsin digests of glomerular basement membrane. Extraction of a murine basement membrane tumor without addition of proteolytic enzymes to the extracting solvent has led to the isolation of a high molecular weight collagenous protein which on denaturation, reduction, and alkylation yielded two electrophoretically distinguishable components with molecular weights resembling those of pro-α chains (Timpl et al., 1978). In addition, an even greater degree of heterogeneity for the collagenous components of glomerular basement membrane has been reported by Hudson & Spiro (1972) and Sato & Spiro (1976) who examined the components extracted in detergent-containing solvents from reduced and alkylated membranes and noted the presence of several collagen-like polypeptides ranging in molecular weight from 25 000 to 220 000. Although the latter authors conceded the likelihood that at least some of the heterogeneity they observed in the collagenous components may have resulted from physiological degradation of collagenous molecules in situ, they as well as others (Daniels & Chu, 1975; Timpl et al., 1978) have generally concluded that their respective results do not support the concept that basement membrane collagen represents a homogeneous class of molecules comprised of identical chains.

Recent investigations on the collagens present in limited pepsin digests of several human tissues have resulted in the isolation and characterization of two new collagen chains, designated the A chain and the B chain (Burgeson et al., 1976; Chung et al., 1976; Rhodes & Miller, 1978). These chains have a molecular weight approximating that of collagen α chains and they each exhibit several compositional features resembling the α-chain-sized component recovered from pepsin digests of isolated basement membranes in previous studies (Kefalides, 1971, 1973). Molecules containing the A and B chains can be selectively precipitated from neutral-salt solvents at relatively high ionic strengths (Burgeson et al., 1976). Alternatively, these molecules may be selectively precipitated, purified, and recovered in good yield by employing differential salt fractionation procedures which are initiated directly on the acidic pepsin digest of a given tissue (Chung et al., 1976; Rhodes & Miller, 1978).

Utilizing and extending the latter procedures, we have now succeeded in isolating in native form a separate class of collagen molecules from limited pepsin digests of highly vascularized tissues such as the human placenta. On denaturation, or on denaturation plus reduction and alkylation, these molecules yield a complex mixture of components which can be resolved into two distinct components with a molecular weight in the range expected for collagen α chains plus smaller molecular weight polypeptide chains which appear to be proteolytic cleavage products of the two larger components. The two larger components are designated the C and D chains.[1] Several lines of evidence indicate that both chains are derived from molecules originating in basement membrane structures. The chains are readily distinguishable on the basis of individual compositional features and chromatographic properties, as well as their cyanogen bromide cleavage products. We report here the procedures useful in isolating this unique collagen fraction as well as the results of studies designed to elucidate the chemical properties of its constituent chains.

Materials and Methods

Preparation of Collagen. Collagen containing the C and D chains was isolated from minces of whole placental tissue by using essentially the same isolation and purification procedures as previously described for the preparation of collagen containing the A and B chains (Rhodes & Miller, 1978). In brief, clarified 24-h pepsin digests of the tissue were adjusted initially to 0.7 M NaCl. The precipitate formed at this step and consisting largely of type I and III collagens was removed by centrifugation and the NaCl concentration of the supernatant was subsequently adjusted to 1.2 M. The collagen precipitating at 1.2 M NaCl was redissolved in 1.0 M NaCl (0.05 M Tris,[2] pH 7.4) and reprecipitated by raising the NaCl concentration to 4.5 M. The latter precipitate was redissolved in an acidic solvent (0.1 M acetic acid) and reprecipitated by adjusting the NaCl concentration to 1.2 M. The precipitate obtained as a result of this procedure was redissolved in 0.1 M NaCl (0.05 M Tris, pH 7.4) and dialyzed vs. a large volume of 0.02 M NaCl containing 2.0 M urea (0.01 M Tris, pH 8.6). During the dialysis step, a precipitate consisting of collagens comprised of the A and B chains is formed (Rhodes & Miller, 1978). The supernatant solution, however, contains the molecules described in the present paper. A suspension of DEAE-cellulose (Whatman, microgranular, DE 32) particles equilibrated with 0.02 M NaCl containing 2.0 M urea (0.01 M Tris, pH 8.6) was added to this solution and incubated with shaking for 24 h. The particles were subsequently removed by centrifugation, and collagen was recovered from the supernatant solution following extensive dialysis vs. 0.5 M acetic acid and lyophilization of the retentate. The lyophilized material was used for all subsequent studies. All procedures described above were performed at 4 °C and all precipitations were performed from solutions containing collagen at a concentration of approximately 0.5 mg/mL. The salient features of these isolation and purification procedures are outlined in Figure 1.

CM-cellulose Chromatography (Nondenaturing Conditions). CM-cellulose chromatography of native collagen was accomplished following dissolution of 50-mg aliquots of the lyophilized material in 50 mL of 0.04 M (Na^+) sodium acetate (pH 4.8) containing 2.0 M urea (the starting buffer) at 4 °C. These samples were subsequently applied to a jacketed 2.5 × 10 cm column of CM-cellulose (Whatman, microgranular, CM 32) equilibrated with starting buffer and maintained at 8 °C by means of a circulating water bath. Chromatography was performed at a flow rate of 100 mL/h employing a linear gradient delivered to the column pump by a two-chamber constant-level device containing 500 mL of starting buffer in the mixing chamber and 500 mL of limit buffer (starting buffer containing 0.4 M NaCl) in the second chamber. The column effluents during this and all subsequent chromatographic procedures were monitored, recorded, and collected as previously described (Rhodes & Miller, 1978). Appropriate fractions of the CM-cellulose effluent obtained during chromatography under nondenaturing conditions were combined, desalted by dialysis vs. 0.5 M acetic acid, and lyophilized.

[1] In the absence of definitive information regarding the molecular organization of these chains, we have opted to follow a previous convention (Chung et al., 1976) and denote the chains by using letters of the alphabet.

[2] Abbreviations used: Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; CM, carboxymethyl; DEAE, diethylaminoethyl; CNBr, cyanogen bromide; $NaDodSO_4$, sodium dodecyl sulfate; Glc-Gal-Hyl, glucosylgalactosylhydroxylysine; Gal-Hyl, galactosylhydroxylysine; 3-Hyp and 4-Hyp, 3- and 4-hydroxyproline; Hyl, hydroxylysine; Hse, homoserine.

Molecular Sieve Chromatography. Initial resolution of the components obtained on denaturation of the collagen as well as molecular weight estimates for the components was achieved by chromatography of the denatured collagen on a calibrated 1.5 × 160 cm column of agarose beads (Bio-Gel A-5m, 200–400 mesh, Bio-Rad Laboratories) essentially as described by Chung et al. (1974). The column was calibrated for molecular weight estimates by use of the following collagenous standards: the trimeric form of α1(III) chains (285 000), β_{12} components from type I collagen (190 000), α1(I) chains (95 000), α2-CB3-5 (62 000), α1(II)-CB10 (32 000), α1-(II)-CB11 (25 000), and α1(II)-CB8 (13 000). In preparation for molecular sieve chromatography, samples of collagen were dissolved at approximately 5.0 mg/mL in 2.0 M guanidine hydrochloride (0.05 M Tris, pH 7.5) and denatured by warming to 42 °C for 30 min. Five-milliliter aliquots of these solutions were then applied to the column, which was equilibrated with the same solvent, and eluted at a flow rate of 12 mL/h. Appropriate fractions of the column effluent obtained during this procedure as well as all subsequent procedures involving chromatography of denatured components were combined and desalted on a 2.0 × 40 cm column of Bio-Gel P2 (100–200 mesh, Bio-Rad Laboratories) equilibrated with 0.1 M acetic acid.

Reduction and Alkylation of Denatured Components. Components recovered during agarose molecular sieve chromatography were redissolved in solutions of 5.0 M urea, pH 8.0 (pH adjusted by the addition of Tris-free base). Reduction was performed at 0.1 M 2-mercaptoethanol and alkylation was subsequently achieved on the addition of 0.2 M iodoacetic acid (sodium salt) under conditions described previously (Chung & Miller, 1974). Following reduction and alkylation, the reaction mixtures were applied directly to an agarose column (see above) in order to resolve and retrieve the reduction products.

CM-cellulose Chromatography (Denaturing Conditions). CM-cellulose chromatography of components recovered from agarose molecular sieve columns was performed on a jacketed 1.5 × 10 cm column of CM-cellulose (Whatman, microgranular, CM 32) under conditions essentially the same as those previously described (Miller, 1971). Samples of collagen components were dissolved at concentrations approaching 10 mg/mL in 0.02 M (Na^+) sodium acetate (pH 4.8) containing 1.0 M urea (the starting buffer) and applied to a column equilibrated with the same buffer. Chromatography was subsequently performed at 42 °C and at a flow rate of 150 mL/h by utilizing a linear salt gradient provided by 200 mL of starting buffer and 200 mL of limit buffer (starting buffer containing 0.12 M NaCl) in the respective chambers of a constant-level device.

Cleavage with CNBr. Purified components recovered from CM-cellulose chromatography under denaturing conditions were dissolved in 70% formic acid and cleaved with CNBr as described previously (Chung et al., 1974). In order to enhance cleavage at methionyl residues, however, the samples were first incubated in 0.2 M NH_4HCO_3, pH 7.0, containing 25% 2-mercaptoethanol for 22 h at 45 °C as described by Adelstein & Kuehl (1970) for the conversion of methionine sulfoxide to methionine. Samples pretreated in this fashion were recovered by desalting followed by lyophilization. The lyophilized material was then redissolved in 70% formic acid for CNBr cleavage.

Polyacrylamide Gel Electrophoresis. Polyacrylamide disc gel electrophoresis was performed in the presence of $NaDodSO_4$ essentially as described by Furthmayr & Timpl (1971). Samples of chains recovered during molecular sieve or ion-exchange chromatography as well as CNBr peptides derived from the chains were dissolved in 0.01 M sodium phosphate, pH 7.2, containing 5.0 M urea and 0.2% $NaDodSO_4$. The solutions were warmed at 45 °C for 30 min. For higher molecular weight components, 5–25-μg samples were electrophoresed in 5% acrylamide gels (0.5 × 60 mm) at 6 mA/gel by employing 0.1 M sodium phosphate, pH 7.2, containing 0.1% $NaDodSO_4$ in the electrophoresis chambers. Samples of CNBr peptides (75–250 μg) derived from these components were electrophoresed under identical conditions with the exception that 10% acrylamide gels were used. Electrophoresis was performed in all instances until the Bromophenol Blue tracking dye reached the end of the gel at approximately 5 h. The gels were subsequently stained for 4 h in a solution of 50% methanol, 5% acetic acid, and 0.25% Coomassie blue. Destaining was performed in a diffusion chamber in a solution containing 10% methanol and 10% acetic acid.

Amino Acid Analyses. Samples of collagen obtained during various stages of purification as well as purified components and CNBr peptides derived therefrom were hydrolyzed at 110 °C for 24 h in constant boiling 6.0 N HCl. Amino acid analyses were performed on the hydrolysates as previously described (Miller, 1972). The final calculations of amino acid content were corrected for destruction of threonine, serine, and tyrosine and incomplete release of valine as previously determined for collagen samples (Piez et al., 1960).

Analyses for Hydroxylysine Glycosides. For the determination of hydroxylysine glycoside (Glc-Gal-Hyl and Gal-Hyl) content, samples were hydrolyzed at 105 °C for 24 h in 2.0 N NaOH in sealed alkali-resistant tubes. The hydrolysates were subsequently cooled to room temperature, neutralized by the addition of 6.0 N HCl, and evaporated to dryness. Analyses were performed on an automatic amino acid analyzer (Miller, 1972) by employing only two buffers for the resolution of the more basic components of the hydrolysate as previously described (Chung et al., 1976).

Results

Isolation and Recovery of Collagens. As noted previously (Rhodes & Miller, 1978), limited pepsin proteolysis of minced placental tissue under the indicated conditions results in the solubilization of approximately 60% of the total tissue collagen. Of the solubilized collagen, some 6% can be recovered in the fraction containing collagen comprised of the A and B chains and we have observed that an equal proportion of the solubilized collagen is recovered in the fraction containing molecules comprised of the C and D chains (Figure 1). The estimates of recovery in the various fractions are based on hydroxyproline determinations following acid hydrolysis and amino acid analysis of aliquots of the indicated fractions. The collagen under investigation in the present communication, thus, represents approximately 4% of the total tissue collagen. This is a conservative estimate for the proportion of collagen containing the C and D chains in the total tissue collagen, however, since it may be assumed that some of this collagen was not solubilized during incubation with pepsin at 4 °C for 24 h. Moreover, we consistently observed that a portion of the collagen containing the C and D chains failed to remain in solution during fractionation of the initial pepsin digest at 0.7 M NaCl. The latter material, precipitating from the original digest along with type I and III collagens, could be recovered in the soluble fraction on redissolving the initial 0.7 M NaCl precipitate in 0.5 M acetic acid and repeating the 0.7 M NaCl precipitation step.

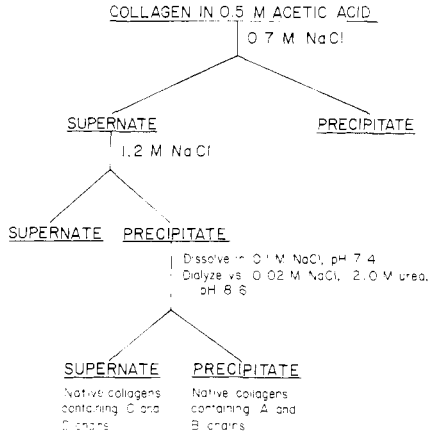

FIGURE 1: An outline of the isolation and purification procedures employed for collagen containing the C and D chains. On increasing the ionic strength of the pepsin digest of placental tissue to 0.7 M NaCl, 14% of the solubilized collagen remains in solution. Approximately 6% of the solubilized collagen is recovered in the fraction containing molecules comprised of the C and D chains, and an equal amount of collagen is recovered in molecules comprised of the A and B chains.

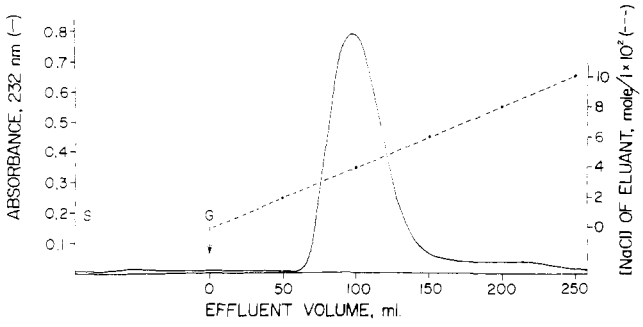

FIGURE 2: CM-cellulose chromatography of collagen containing the C and D chains under nondenaturing conditions. This illustration depicts the elution pattern of a 50-mg sample dissolved initially in 50 mL of starting buffer, 0.04 M (Na^+) sodium acetate, pH 4.8, containing 2.0 M urea. The sample was applied to the column which was maintained at 8 °C and eluted at a flow rate of 100 mL/h by means of a linear gradient described in the text. The letter S denotes the point of sample application, and G indicates initiation of gradient.

CM-cellulose Chromatography (Nondenaturing Conditions). When chromatographed in native form on CM-cellulose, collagen molecules comprised of the C and D chains were retained by the column and eluted early in the gradient as a single homogeneous peak (Figure 2). Approximately 95% of the collagen applied to the column is recovered routinely in the indicated peak. Under the conditions employed for chromatography, acidic noncollagenous contaminants of the collagen preparations are not retained by the column and are eluted during application of the sample to the column. In addition, native type I and III collagens as well as collagens comprised of the A and B chains are more strongly retained by the column and coelute at an effluent volume of 225 mL when the NaCl concentration of the eluant approaches a value of 0.09 mol/L. CM-cellulose chromatography of the collagen under nondenaturing conditions thus serves as a means of further purifying collagen preparations containing the C and D chains and provides a means of assaying their state of purity. As illustrated in Figure 2, use of the isolation and purification procedures described above allows the recovery of preparations which are virtually free of acidic noncollagenous proteins as well as other collagen types.

Molecular Sieve Chromatography. Figure 3A illustrates a representative molecular sieve elution pattern observed on

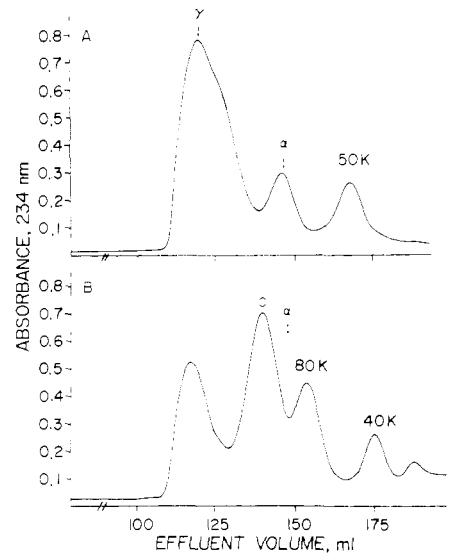

FIGURE 3: (A) Agarose molecular sieve chromatography (Bio-Gel A-5m) of collagen recovered from CM-cellulose under nondenaturing conditions (Figure 2). The chromatogram presented here represents the elution pattern of 25 mg of sample dissolved initially in 5 mL of 2.0 M guanidine hydrochloride (0.05 M Tris, pH 7.5). The sample was subsequently denatured by warming, applied to a 1.5 × 160 cm column, and eluted as described in the text. The elution positions of γ components (285 000 daltons) and α chains (95 000 daltons) are noted. The notation 50K is used for components exhibiting an apparent molecular weight of 50 000. (B) Agarose molecular sieve rechromatography of reduced and alkylated higher molecular weight components (effluent volume, 110–130 mL) recovered during the chromatographic procedures illustrated in Figure 3A. The chromatogram presented here represents the elution pattern of 20 mg of sample. Reduction and alkylation as well as elution of the sample were performed as described in the text. The elution position of α chains (95 000 daltons) is noted. The designations 80K and 40K indicate components exhibiting an apparent molecular weight of 80 000 and 40 000, respectively. Estimates of the proportion of collagen in each peak in the figure were made by planimetry. Fractions chosen for further study are α-chain-sized components (effluent volume, 135–155 mL) and 50K components (effluent volume, 160–175 mL) in Figure 3A; and higher molecular weight components (effluent volume, 110–125 mL), C′ and 80K components (effluent volume, 130–160 mL), and 40K components (effluent volume, 165–180 mL) in Figure 3B.

denaturation of the material recovered from CM-cellulose and rechromatography on Bio-Gel A-5m. In contrast to the apparent homogeneity of the material when chromatographed on CM-cellulose under nondenaturing conditions (Figure 2), the denaturation products were observed to be quite complex and to fall into three general molecular weight classes. These are higher molecular weight components eluting as a heterogeneous initial peak and comprising about 80% of the collagen applied to the column; components chromatographing in the region expected for α chains and accounting for 10% of the collagen; and smaller molecular weight components exhibiting an apparent molecular weight of 50 000 (50K) which account for the remainder of the sample.

As depicted in Figure 3B, reduction and alkylation of the higher molecular weight components (material eluting between 110 and 135 mL of effluent volume, Figure 3A) followed by rechromatography on Bio-Gel A-5m allow the resolution and recovery of several reduction products. Approximately 70% of the higher molecular weight components were routinely recovered as these reduction products. The latter are a component designated C′ and exhibiting an apparent molecular weight of 120 000; a component somewhat smaller than collagen α chains and exhibiting an apparent molecular weight of 80 000 (80K); and even smaller molecular weight com-

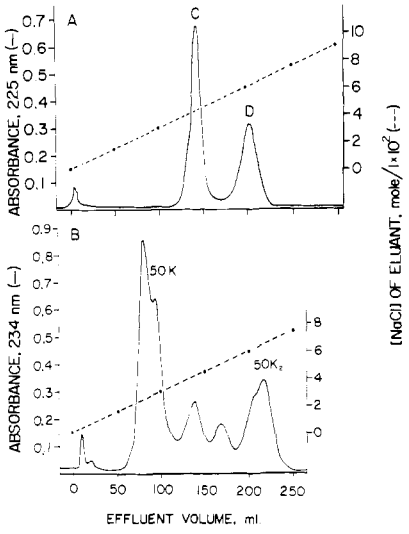

FIGURE 4: (A) CM-cellulose rechromatography of the α-chain-sized components recovered during agarose molecular sieve chromatography (Figure 3A). The chromatogram illustrated here represents the elution pattern of 8 mg of sample dissolved initially in 2 mL of starting buffer, 0.02 M (Na^+) sodium acetate, pH 4.8, containing 1.0 M urea. The sample was applied to a 1.5 × 10 cm column of CM-cellulose and elution was achieved as described in the text. (B) CM-cellulose rechromatography of the 50K material recovered during agarose molecular sieve chromatography (Figure 3A). This chromatogram represents the elution pattern of 25 mg of sample which was prepared for chromatography as described above for the sample chromatographed in Figure 4A.

ponents, the majority of which elute in a position corresponding to a molecular weight of 40 000 (40K). The higher molecular weight nonreducible components eluting prior to C′ were determined by amino acid analyses to represent collagenous components. However, rechromatography of these components on Bio-Gel A-15m under similar conditions revealed extreme heterogeneity with molecular weights ranging from well into the millions to approximately 300 000. No further attempts were made to characterize these presumably cross-linked components for the purposes of the present study.

CM-cellulose Chromatography (Denaturing Conditions). In order to more fully evaluate the nature of the components recovered during agarose molecular sieve chromatography, pooled samples of the various components were rechromatographed on columns of CM-cellulose under denaturing conditions. Rechromatography of the α-chain-sized components (see Figure 3A) in this fashion resulted in the resolution of two components, designated C and D, as illustrated in Figure 4A. In eight different preparations of the α-chain-sized components, the amount of the more acidic component, C, always exceeded the amount of D with ratios of C to D occurring in the range of 1.3:1 to 1.5:1.

Rechromatography of the 50K components (see Figure 3A) on CM-cellulose showed that this material is comprised of a much more heterogeneous collection of polypeptide fagments (Figure 4B). Nevertheless, the peptides were resolved by this procedure into two major fractions, $50K_1$ and $50K_2$. Similar to the results attained on rechromatography of the α-chain-sized components, the amount of the more acidic peptides recovered in the $50K_1$ fraction always exceeded the amount of the more basic $50K_2$ peptides. In this regard, the $50K_1$ and $50K_2$ peptides are present in ratios of 1.8:1 to 2.0:1 in various preparations.

CM-cellulose rechromatography of the C′ and 80K components (see Figure 3B) resulted in the complete resolution of these components as illustrated in Figure 5. As shown in

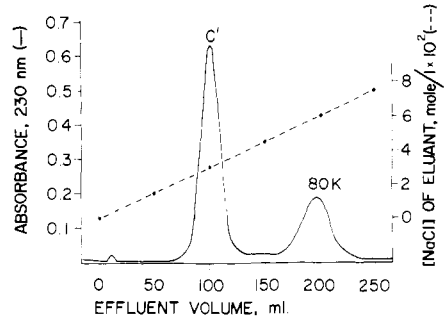

FIGURE 5: CM-cellulose rechromatography of the C′ and 80K components recovered during agarose molecular sieve chromatography (Figure 3B). This chromatogram represents the elution pattern of 15 mg of sample which was prepared for chromatography and eluted as described in the text.

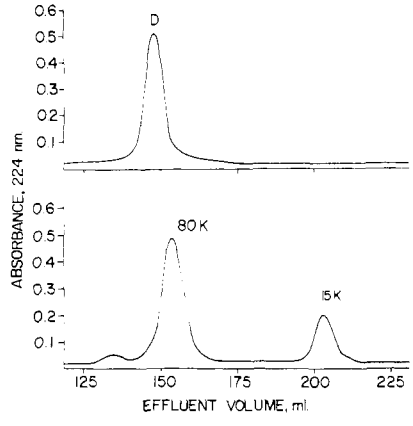

FIGURE 6: Agarose molecular sieve chromatography (Bio-Gel A-5m) of a 7-mg sample of the D component prior to (upper panel) and following (lower panel) reduction and alkylation. The notations 80K and 15K indicate polypeptides with apparent molecular weights of 80 000 and 15 000, respectively.

the figure, the more acidic component, C′, is the more prevalent chain in the mixture with ratios of C′ to 80K occurring in the range of 2.0:1 to 2.5:1 in various prepartions.

CM-cellulose rechromatography of the 40K components (see Figure 3B) revealed that this fraction was comprised of an extremely heterogeneous mixture of peptide fragments which were poorly resolved and showed no discernible tendency to be grouped into acidic and basic components (data not shown). These peptides were not examined further.

Recovery of material applied to CM-cellulose columns was in the range of 85–90% in all cases, and estimates of component ratios in the column effluents were made following planimetry measurements of the appropriate peaks.

Reduction and Alkylation of the D Component. In view of the similar ion-exchange properties of the D and 80K components (see Figures 4A and 5) as well as further data indicating a close similarity for these components with respect to compositional features (see Table I), samples of the D component were reduced and alkylated prior to rechromatography on Bio-Gel A-5m. As shown in Figure 6, reduction and alkylation of D resulted in its recovery as an 80K component plus a smaller molecular weight component designated 15K. The latter component proved to be a relatively basic collagenous peptide containing approximately 170 amino acid residues, but was not further examined in the present study. Reduction and alkylation of the other components recovered during CM-cellulose chromatography, i.e., C, $50K_1$, $50K_2$, C′, and 80K, did not alter their respective molecular sieve or ion-exchange properties.

Table I: Amino Acid Composition of the Components Recovered after CM-cellulose Chromatography[a]

amino acid	C′	C	50K$_1$	D	80K[b]	80K[c]	50K$_2$
3-Hyp	1	1	1	1	1	1	1
4-Hyp	118	122	116	107	110	110	107
Asp	45	45	45	50	49	50	52
Thr	20	19	20	28	30	29	29
Ser	36	38	39	30	30	30	30
Glu	88	78	76	64	65	65	66
Pro	88	85	88	73	73	73	72
Gly	324	334	333	328	324	322	322
Ala	30	30	30	47	47	46	48
1/2-cystine[d]	2	0	0	3	2	2	2
Val	32	33	31	25	27	27	28
Met	14	15	15	14	14	14	14
Ile	32	32	34	39	38	38	38
Leu	50	52	53	56	56	57	55
Tyr	8	5	6	6	7	7	7
Phe	28	27	26	36	36	36	36
Hyl	49	50	53	39	36	38	36
His	6	6	6	7	7	7	7
Lys	7	6	6	5	6	6	8
Arg	22	22	22	42	42	42	42
total	1000	1000	1000	1000	1000	1000	1000
Glc-Gal-Hyl	43	44	ND	ND	29	28	ND
Gal-Hyl	2	2	ND	ND	2	3	ND

[a] The values are presented as residues/1000 total residues and represent an average of values obtained in at least six different preparations of each component. The coefficient of variation for each amino acid in a given component was less than 3.0%. [b] Values for the 80K component recovered following reduction and alkylation of the D component (see Figure 6). [c] Values for the 80K component recovered following reduction and alkylation of higher molecular weight components (see Figures 3B and 5). [d] Calculated as the sum of half-cystine plus *S*-(carboxymethyl)cysteine.

Polyacrylamide Gel Electrophoresis of Components. NaDodSO$_4$–polyacrylamide gel electrophoresis in 5% gels of the components described above verified that each component had been isolated in a high state of purity and that the molecular weight estimates for each component based on agarose molecular sieve chromatography were essentially correct (data not shown). With respect to molecular weight estimates, the band for C′ migrated in the gels to a position suggesting a molecular weight of 140 000 as opposed to 120 000 indicated by molecular sieve chromatography. In addition, the somewhat diffuse bands for the 50K$_1$ and 50K$_2$ components suggested an average molecular weight of about 60 000 and 40 000 for these components, respectively. At the moment, the basis for the differences in molecular weight estimates for C′ and the 50K components when judged by molecular sieve chromatography and gel electrophoresis is not known.

Amino Acid and Hydroxylysine Glycoside Analyses. The amino acid analyses of the isolated components are presented in Table I. In general, these data substantiate the impressions gained in observing the ion-exchange properties of the individual components. In this regard, it is clear that the components fall into two general classes, i.e., the more acidic C′, C, and 50K$_1$ components and the more basic D, 80K, and 50K$_2$ components. With respect to the more acidic components, the C and 50K$_1$ components exhibit virtually identical compositional features. The amino acid composition of C′ is likewise quite similar to that of C and the 50K$_1$ components, but indicates the presence of some noncollagenous sequences which are relatively deficient in glycyl residues and relatively rich in glutamic acid (or glutamine), tyrosine, and cysteine residues. Given the properties and relative size of these components, these data strongly suggest that C′ represents a modified (somewhat truncated) form of the pro-C chain and that C represents the collagenous portion of the pro-C chain, while the 50K$_1$ components represent a somewhat heterogeneous collection of degradation products derived through proteolytic cleavage of the pro-C or C chain at a limited number of sites.

It is of interest to note that the C chain exhibits virtually all of the unique compositional features previously noted for the α-chain-sized component recovered following limited pepsin proteolysis of isolated basement membrane structures (Kefalides, 1971; Dehm & Kefalides, 1978). In this regard, the C chain is relatively rich in 4-hydroxyproline, the large hydrophobic amino acids, hydroxylysine, and the hydroxylysine glycosides, while it contains relatively low levels of alanine and arginine. Indeed, the amino acid composition of the C chain derived from human placental tissue shows a remarkable similarity to the α-chain-sized collagenous peptide recently isolated from bovine lens capsule (Dehm & Kefalides, 1978). The only striking difference between the chains derived from the indicated sources is that the lens capsule chain contains more 3- and 4-hydroxyproline and an approximately equivalent decrease in proline relative to the placental chain, suggesting a difference in the extent of prolyl hydroxylation for this particular chain in the two tissues. Moreover, the data of Dehm & Kefalides (1978) also indicated that the α-chain-sized lens capsule component was derived through proteolysis of a slightly larger component. It is likely that the latter component is equivalent to C′ which has been isolated in the present study.

The more basic components, D, 80K, and 50K$_2$, likewise exhibit very similar, but unique, compositional features. In this regard, it is clear that these components differ substantially from the more acidic components. This is most strikingly apparent in comparing their respective threonine–serine ratios as well as their respective contents of glutamic acid, alanine, phenylalanine, hydroxylsine, and arginine. These data also indicate that the 80K component obtained on reduction and alkylation of the D component (column 6, Table I) is identical with the 80K component recovered following reduction and alkylation of the high molecular weight components (column 7, Table I). We interpret these data to indicate that the 80K component represents a major portion of an additional genetically distinct collagen chain, designated the D chain, whereas the 50K$_2$ components represent a collection of smaller proteolytic cleavage products of this chain.

CNBr Cleavage Products. In order to more fully evaluate the relationship between C′, C, and 80K, samples of these polypeptide chains were cleaved with CNBr and the cleavage products were examined by NaDodSO$_4$–polyacrylamide gel electrophoresis in 10% gels. In initial experiments it was observed that the number of apparent cleavage products far exceeded the number of peptides expected for each chain and that many of the cleavage products ranged in molecular weight from 30 000 to 60 000. Moreover, amino acid analyses of the cleavage products revealed that in each case less than one-half the potential cleavage sites were actually cleaved as judged from the methionine and homoserine contents of the hydrolysates (Table II). These results suggested that failure to attain more complete cleavage might be attributed to the presence of appreciable quantities of oxidized methionyl residues in the indicated polypeptides. Accordingly, the samples were reduced in 0.2 M NH$_4$HCO$_3$ containing 25% 2-mercaptoethanol at pH 7.0 as described by Adelstein & Kuehl (1970) prior to cleavage with CNBr. As indicated in Table II, prior reduction of the samples resulted in much more efficient cleavage as judged by the conversion of methionine to homoserine which occurred to the extent of 90% or better in each polypeptide. The gel patterns of the peptides obtained under optimum cleavage conditions are shown in Figure 7.

Table II: Effect of Prior Reduction on the Conversion of Methionyl Residues to Homoserine during CNBr Cleavage

sample	Met content (residues/1000)	% conversion to Hse: CNBr	% conversion to Hse: reduction[a] plus CNBr
C′	14	43	93
C	15	35	91
80K	14	27	90

[a] Reduction was performed by a method adapted from Adelstein & Kuehl (1970) as described in the text.

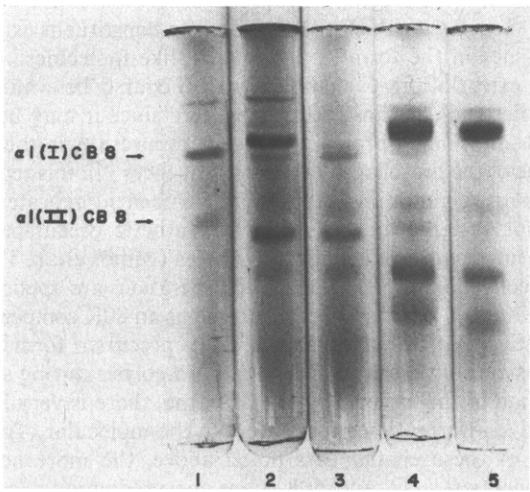

FIGURE 7: Polyacrylamide disc gel electrophoresis in $NaDodSO_4$. (Channel 1) Peptide standard showing the migration of α1(I)-CB8 (24000 daltons) and α1(II)-CB8 (13000 daltons). (Channel 2) CNBr cleavage products of C′. (Channel 3) CNBr cleavage products of C. (Channel 4) CNBr cleavage products of the 80K component derived as a reduction product of the D component (see Figure 6). (Channel 5) CNBr cleavage products of the 80K component obtained on reduction of higher molecular weight components (see Figures 3B and 5).

These patterns corroborate the proposed relationship between C′ and C in that the cleavage products of C′ (channel 2) correspond quite well with the cleavage products of C (channel 3), while the products derived from C′ contain an additional band corresponding to a fragment with a molecular weight of about 26000. In addition, the CNBr cleavage products of the 80K component (channels 4 and 5) migrate in different positions relative to those derived from C′ and C, indicating a different distribution of methionyl residues in the 80K polypeptide. These results, then, substantiate the conclusions based on the compositional data provided above and clearly indicate that the 80K component cannot originate as a smaller molecular weight fragment of C′ or C. Similar experiments were performed with samples of the $50K_1$ and $50K_2$ components. However, as expected, the CNBr cleavage products of these heterogeneous components migrated largely as very diffuse bands of low molecular weight.

Discussion

The studies presented here have demonstrated that it is possible to isolate a unique collagen fraction from limited pepsin digests of placental tissue. This has been accomplished essentially by selective precipitation of the other known collagens present in the pepsin digest and recovery of the collagen as the material soluble in a low ionic strength buffer at slightly alkaline pH (Figure 1). Use of the indicated isolation and purification procedures allows recovery of the collagen essentially free of contamination by noncollagenous proteins, type I collagen, type III collagen, and the collagens containing the A and B chains. In addition, current studies indicate that the isolation and purification procedures outlined here are applicable in studies designed to isolate the unique collagen fraction from other tissues as well.

The collagen isolated in this fashion chromatographs in native form on CM-cellulose in a manner suggesting the presence of a homogeneous population of molecules. Nevertheless, it yields on denaturation plus reduction and alkylation a complex mixture of denaturation products. The latter have been observed to be comprised of high molecular weight components and polypeptide chains with molecular weights in the range of collagen α chains, as well as smaller molecular weight components. The molecular weight distribution of these denaturation products thus closely resembles that observed in several previous studies on pepsin-solubilized collagen derived from isolated basement membranes such as the glomerular basement membrane (Daniels & Chu, 1975; Tryggvason & Kivirikko, 1978) and lens capsule (Dixit, 1978; Schwartz & Veis, 1978). A similar molecular weight distribution has also been observed for the denaturation products of a non-geling, basement-membrane-like collagen fraction isolated from the pepsin-solubilized collagen of a variety of tissues (Trelstad & Lawley, 1977).

In the present studies, isolation and characterization of the components approximating the size of collagen α chains as well as some of the smaller molecular weight components have provided considerable insight concerning the nature of these components and their interrelationships. As noted above, the most plausible interpretation of the data obtained for the more acidic components is that C′ represents a truncated form of the biosynthetic precursor of the C chain and that the $50K_1$ components represent proteolytic cleavage products of either the pro-C chain or C chain. This interpretation is also supported by our observations (not reported here) that reincubation of the isolated native collagen with pepsin at 17 °C for an additional 24 h results in augmentation in the proportion of all 50K components and concomitant reduction in the proportion of larger molecular weight components in the subsequent denaturation products. Similar observations with respect to the generation of smaller molecular weight components have been made on exposure of a preparation of murine tumor basement membrane collagen to pepsin (Timpl et al., 1978) as well as on reexposure of pepsin-solubilized lens capsule collagen to pepsin (Schwartz & Veis, 1978). Together, these observations indicate that the chains of basement membrane collagens, even when present in apparently native conformation, are unusually susceptible to the proteolytic activity of pepsin since similar cleavage products are not encountered in preparations of type I and III collagens (Chung & Miller, 1974; Chung et al, 1974), type II collagen (Miller, 1972), or collagens containing the A and B chains (Rhodes & Miller, 1978) when recovered following solubilization by limited pepsin proteolysis. These observations, however, do not rule out the possibility that at least some of the smaller molecular weight components recovered in the denaturation products of basement membrane collagens might originate as the result of proteolysis on the part of tissue proteases acting either prior to or during the extraction procedures.

The chemical properties of the C chain isolated in these studies likewise serve to identify it as a constituent of a basement membrane collagen. Indeed, as already pointed out above, the compositional features of the C chain correspond very well with those of an α-chain-sized component isolated from pepsin digests of lens capsule (Dehm & Kefalides, 1978). In addition, it has been shown that specific antibodies against

the placental C chain selectively stain basement membrane structures in several tissues (Gay & Miller, 1978; Gay et al., 1979). Of particular interest is the observation that the placental C chain is somewhat underhydroxylated with respect to proline relative to the chain derived from lens capsule. The decrease in prolyl hydroxylation is particularly striking for 3-hydroxyproline in that the placental C chain has only one of these residues while the lens capsule chain is reported to contain seven (Dehm & Kefalides, 1978). Should the placental chain be representative of the collagenous constituents in a variety of smaller basement membrane structures, it would appear that estimates of total basement membrane collagen in whole tissues based on 3-hydroxyproline to 4-hydroxyproline ratios might lead to significant underestimates of total basement membrane collagen content if it is assumed that all basement membrane chains exhibit relatively high contents of 3-hydroxyproline (Man & Adams, 1975; Man et al., 1978).

With respect to the more basic components isolated in the present study (D, 80K, and $50K_2$), the data are consistent with the view that the D component is the 80K component disulfide-linked to a smaller collagenous fragment, that the 80K component represents the major portion of an additional genetically distinct collagen chain (provisionally designated as the D chain), and that the $50K_2$ components are proteolytic cleavage products of the latter chain. In this regard, the compositional features of the 80K component as well as the nature of its CNBr cleavage products certainly indicate that this chain is not likely to be a derivative of either the C′ or C chains. Although the 80K component shows several compositional features which might be described as "basement membrane like" (a large complement of hydrophobic amino acids, hydroxylysine, and hydroxylysine glycosides and a relatively low alanine content), and although it is derived from the same collagen fraction from which the C chain is derived, these data in themselves are not sufficient to conclude that the 80K component or its parent chain originates in basement membrane structures since the starting material used in these studies is whole placental tissue. With respect to this point, however, some additional observations are particularly relevant. Thus, the $NaDodSO_4$–polyacrylamide gel patterns of reduced pepsin-solubilized glomerular basement membrane collagen published by Tryggvason & Kivirikko (1978) reveal a prominent band migrating in the position expected for an 80K component. Indeed, all of the components characterized in the present study (C′, C, D, 80K, $50K_1$, and $50K_2$) are present as the major bands observed by these authors. In addition, Dixit (1978) has isolated from pepsin digests of lens capsule a basic 50 000 molecular weight component with compositional features very similar to the $50K_2$ material described here. Moreover, further studies in our laboratory (S. Gay and E. J. Miller, manuscript in preparation) have shown that the most prominent components present in limited pepsin digests of bovine lens capsule are components homologous to the C′, C, and 80K components isolated from placental tissue in the present study.

It is of further interest to note that a smaller molecular weight basic component (designated CP45) previously isolated from the denaturation products of pepsin-treated collagen synthesized by smooth muscle cells (Mayne et al., 1978) exhibits many of the properties noted here for the $50K_2$ material. These comparisons strongly suggest that the component designated CP45 represents a proteolytic cleavage product of the parent D chain synthesized by the smooth muscle cells. It should also be pointed out that the 55 000 molecular weight components previously isolated from pepsin digests of vascular tissue (Chung et al., 1976) are not equivalent to the $50K_1$ or $50K_2$ components studied here. In this regard, the compositional features of the 55K components are much different than those observed for $50K_1$ and $50K_2$. It is likely that the former components represent cleavage products of additional, and as yet, unidentified collagen chains.

In summary, the data presented here support the notion that the collagenous molecules in several basement membrane structures are comprised collectively of two genetically distinct chains designated the C chain and the D chain. In view of the prevalence of the C′ component among the denaturation products of this collagen, the data also substantiate the concept that molecules in which the C chain is a constituent exist in the tissues in the form of procollagen-like molecules. The actual extent of pro-C chains cannot, of course, be evaluated in studies such as those performed here since it may be assumed that limited proteolysis with pepsin results in the loss of some of the procollagen extension sequences. In this regard, molecular weight estimates for the C′ component indicate that it is not as large as the initial biosynthetic precursors of basement membrane collagenous chains (Minor et al., 1976; Heathcote et al., 1978). Similar considerations are applicable to the D chain, which has been detected as an 80K component, suggestive that this chain as well as its precursor form may be considerably more susceptible to proteolysis during solubilization of the molecules. At this time, there is very little definitive information with respect to the molecular organization of these chains. As noted above, the more acidic components (C′, C, and $50K_1$) are the predominant denaturation products in the collagen prepared as described here. Indeed, these products are present in an approximately 2:1 ratio relative to the 80K and $50K_2$ components, suggesting the prevalence of molecules with the chain composition C_2D. Such conclusions must be regarded as highly tentative, however, in the absence of data concerning the different rates at which $[C]_3$ or $[D]_3$ molecules might be degraded during limited proteolysis.

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Achatina fulica Hemocyanin and Its Interactions with Imidazole, Potassium Cyanide, and Fluoride as Studied by Spectrophotometry and Nuclear Magnetic Resonance and Resonance Raman Spectrometry†

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ABSTRACT: Hemocyanin from Taiwan snails, *Achatina fulica*, has not been studied previously. We have used ultraviolet spectrophotometric, proton magnetic resonance, ^{19}F NMR, and resonance Raman methods to study the interaction of several small ligands with this hemocyanin species. Addition of imidazole (up to 0.5 M) and potassium cyanide (up to 0.001 M) to oxyhemocyanin causes a relatively rapid drop in absorbance at 345 nm, a primary reaction, followed by a much slower secondary process. The primary reaction between imidazole and hemocyanin takes about 2 h, while that between cyanide and hemocyanin takes only a fraction of a second. The ^1H NMR signal of 0.20 M imidazole (4,5-H) and the ^{19}F NMR signal of 0.20 M KF at pH 9.0 are broadened by oxyhemocyanin, and the extent of broadening is linearly dependent on hemocyanin concentration. Addition of KCN (up to 0.001 M) results in reduction of paramagnetic Cu(II) to diamagnetic Cu(I), with the copper still bound to the protein. Above 0.001 M, copper begins to be removed from the protein, forming aqueous cuprous–cyanide complexes. The rate constant of the primary reaction of KCN (0.0001–0.001 M) with hemocyanin decreases with pH increase in the pH range 8.5–9.5. The data suggest that the neutral HCN, or its kinetic equivalent, is the reactive species in the primary reaction. A resonance Raman spectrum of the oxyhemocyanin, by excitation with the 514.4-nm band, shows the O_2^{2-} vibration at 752 cm^{-1}. Literature values give 744 cm^{-1} in *Cancer magister* (arthropod) and 749 cm^{-1} in *Busycon canaliculatum* (mollusc) hemocyanins for the O_2^{2-} vibration. The position of the O_2^{2-} vibration together with our NMR and spectrophotometric results suggests that *A. fulica* and *B. canaliculatum* hemocyanins have similar structures and properties.

Hemocyanins are huge, oxygen-carrying proteins in the hemolymph of many molluscs and arthropods. It is known that one oxygen molecule binds to two copper ions in the protein, and much current effort is being directed toward determining the structure of the copper–oxygen complex and the nature(s) of the copper in oxyhemocyanin (Lontie & Vanquickenbourne, 1974; Freedman et al., 1976; Guo et al., 1978). Hemocyanin from Taiwan snails, *Achatina fulica*, has not been studied previously. In this paper we report studies on this hemocyanin species, by using ultraviolet spectrophotometry, nuclear magnetic resonance, and resonance Raman methods, and study the interaction between *A. fulica* hemocyanin and the following small ligands: imidazole, potassium cyanide, and fluoride.

Study of hemocyanin from snails in Taiwan has the advantage that the snails are plentiful in the immediate vicinity of the University Laboratory, so that collection of the hemolymph together with isolation and purification of hemocyanin from the hemolymph can be carried out in 1 day. This reduces greatly the complications which arise from aging of proteins.

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