Isolation and Characterization of a Distinct Type of Collagen from Bovine Fetal Membranes and Other Tissues†

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ABSTRACT: A new procedure has been developed to isolate a distinct bovine collagen fraction which contains αA and αB chains and resembles that reported by Burgeson et al. [Burgeson, R. E., El Adli, F. A., Kaitila, I. I., & Hollister, D. W. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2579–2583]. This fraction was obtained first from the bovine amnion and chorion, and the method was subsequently applied to the pepsin-solubilized collagens dissolved from cornea, tendon, skin, dura, nerve endoneurium, and cartilage. Electron microscopy reveals a single type of segment long-spacing crystallite, identical in staining characteristics with the "type VI" segments we previously distinguished among the collagens in bovine Descemet's membrane and heart valve. We were unable to separate the αA and αB chains on carboxymethylcellulose but could resolve them by hydroxylapatite chromatography. The ratio of the αA to αB chains was 1:1.78. Amino acid analysis demonstrated that the compositions of the αA and αB chains are distinguishable and similar to analyses published for the homologous chains from human collagen. No cysteine or disulfide linkages were detected. Analysis of alkaline hydrolysates of both chains for sugar residues indicated that 84% of the hydroxylysines in αB and 45% in αA are glycosylated. The native collagen is apparently resistant to mammalian collagenase. We tentatively conclude that the αA and αB chains are both present in a native $\alpha A(\alpha B)_2$ molecule because (1) when isolated from various tissues this collagen consistently contains the αA and αB chains in a ratio approximating 1:2, (2) only a single type of segment long-spacing aggregate could be detected by electron microscopy in these preparations, and (3) both chains are resistant to collagenase.

In the course of studies on age-related changes in collagen, we compared the pepsin-solubilized collagens from many collagen-rich tissues of the body. In many cases gel electrophoresis revealed a component migrating between the α1 chain and β_{11} components of type I collagen. Burgeson et al. (1976) demonstrated that from pepsin-treated human placental membranes a collagen fraction could be obtained which was enriched in a similar, slowly migrating α chain (αB); another chain, αA, that migrated only slightly slower than the type I α1 chain purified with αB. We reexamined some of our preparations and obtained fractions which, like Burgeson's, yielded on electrophoresis α chains migrating like αA and αB; other investigators have reported similar findings (Trelstad & Lawley, 1977; Brown et al., 1978; Rhodes & Miller, 1978; Bentz et al., 1978). Most investigators have selectively precipitated this collagen fraction with neutral 4 M sodium chloride but other types of collagen are recovered in this precipitate, and at present confusion regarding the ratios of the αA and αB chains [compare Brown et al. (1978) and Bentz et al. (1978)] may be attributed to problems of fractionating the mixture of collagen types. We report here an improved method of fractionation and some further criteria for identifying the collagen that contains the αA and αB chains. We also describe the separation of the αA and αB chains on hydroxylapatite columns. A preliminary description of these methods has been presented (Hong et al., 1978).

Electron microscopy of the SLS[1] precipitates from this collagen shows a banding pattern identical with a novel SLS type which we noted in pepsin-solubilized bovine heart valve and Descemet's membrane collagen and which we designated "type VI" (Davison & Cannon, 1977). We have used the terms "AB collagen" and "type VI" interchangeably (Davison et al., 1979). To avoid confusion we will here designate the characteristic SLS as type VI but we will refer to the collagen as AB collagen.

Materials and Methods

Bovine tissues were obtained from the abattoir within 30 min of slaughter, and the tissues were transported on ice to the laboratory. Fetuses were obtained in the uterus, and for these studies the membranes from fetuses ~30 cm in length were used. The amniotic and chorionic membranes were separated from the villi and teased apart. The membranes were washed free of blood and soaked in cold 0.01 M EDTA. The larger blood vessels were dissected from the membranes. The clean membranes were blotted dry, weighed, and then homogenized with a Waring blender in 0.1 M acetic acid at high speed for 1 min. Crystalline pepsin (Worthington Corp.) was added to the homogenate to 1% wet weight of the tissues. Limited proteolysis was carried out by agitating the mixture at 4 °C for 24 h. The digest was neutralized with 5 M sodium hydroxide to inactivate pepsin and stirred at 4 °C for 1 h. The digest was readjusted to pH 3.5 by glacial acetic acid and centrifuged at 60000*g* for 10 min, and collagen was precipitated from the supernatant by NaCl added to a final concentration of 1.0 M. This precipitate of pepsin-solubilized collagen was collected by filtration on Whatman 52 paper or by centrifugation and was redissolved in 0.05 M Tris-HCl buffer, pH 7.5, containing 0.1 mM PMSF, 1 mM *N*-ethylmaleimide, 1 mM benzamidine hydrochloride, 25 mM EDTA, and 0.5 M NaCl. The collagen in this neutral salt solution

† From the Department of Fine Structure Research, Boston Biomedical Research Institute, Boston, Massachusetts 02114. *Received May 9, 1979.* This project was supported by Grants NS11122, AG1120, and RR5711 from the National Institutes of Health.

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[1] Abbreviations used: SLS, segment long spacing; PMSF, phenylmethanesulfonyl fluoride; $NaDodSO_4$, sodium dodecyl sulfate.

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was then subjected to differential salt precipitation at 1.7, 2.5, and 4.0 M (Trelstad et al.,1976). The precipitate from 4.0 M salt was redissolved in 0.05 M acetic acid and dialyzed against the same acid solution. After removal of any insoluble materials by centrifugation, the clear supernatant was then dialyzed against 0.05 M sodium acetate, pH 4.8, at 4 °C. The precipitate that formed during dialysis was collected by centrifugation. For more complete purification the collagen in the precipitate was treated a second time with either the 2.4 M NaCl step or the pH 4.8 precipitation step. The product was redissolved in 0.05 M acetic acid. After exhaustive dialysis against the same acetic acid, the salt-free, acid solution was lyophilized (P 4.8 fraction).

Similar collagen fractions were prepared from the pepsin-solubilized tissue from bovine corneas, dura, nerve sheaths, tendon, fetal skin, and nasal and laryngeal cartilage. The corneas from 50 calf eyes were used for each experiment (Davison et al., 1979). The epineurium was stripped from the second and fifth cranial nerves, and the nerves were then transversely sliced, crushed, and extracted with neutral buffer and then with neutral 3 M guanidine hydrochloride to remove denaturant-soluble proteins (Davison and Jones, unpublished experiments). The residue was digested with pepsin to solubilize the collagen of the endoneurium and perineurium. The thinner sheets of the dura were selected for the preparation, and major blood vessels were dissected out before the tissue was finely sliced for pepsin treatment. The cartilages were cleaned as far as possible from superficial tissue and adjacent bone; they were then finely sliced, and the pepsin-soluble collagen was isolated by the procedure of Miller (1972). The skin preparation followed the method of Rubin et al. (1965). All of the washing and first extracting solutions contained enzyme inhibitors to minimize autolysis.

Hydroxylapatite Chromatography. A column (0.9 × 12 cm) was prepared with hydroxylapatite (DNA grade Bio-Gel HTP, Bio-Rad Laboratories) and equilibrated with 0.005 M sodium phosphate buffer, pH 6.3, containing deionized 4 M urea and 0.1 mM PMSF. The lyophilized pH 4.8 precipitate fraction was dissolved in the buffer, heated at 100 °C for 2 min, cooled to 20 °C, and then applied to the column. The column was eluted at 20 °C first with the same buffer and then with a linear salt gradient with 100 mL of buffer to 100 mL of buffer containing 0.5 M NaCl. The flow rate was 20 mL/h, and 3-mL fractions were collected for each tube. The absorbance at 230 nm of each tube was measured in a Gilford spectrophotometer.

Polyacrylamide Gel Electrophoresis. The collagen fractions were examined by discontinuous polyacrylamide slab gel electrophoresis in the presence of $NaDodSO_4$ with the buffer solutions of Laemmli (1970). The gel formulations and staining and destaining procedures were the same as those described before (Davison, 1978). A 6% running gel was used for intact collagen chains, and an 8–18% gradient was used for cyanogen bromide cleaved peptides.

Collagenase Digestion. The collagen samples were dissolved in 0.1 M $CaCl_2$ and 0.05 M Tris, pH 7.8. Trypsin-activated rabbit corneal collagenase (Davison & Berman, 1973) was added, and the samples were incubated at 20 °C for 3–10 days. The preparation was then dialyzed against 0.05 M acetic acid, and aliquots were denatured, neutralized, and applied in $NaDodSO_4$ to polyacrylamide gel slabs or were dialyzed against 0.4% ATP in 0.05 M acetic acid to produce SLS crystallites for electron microscopic examination.

Amino Acid Analyses. The amino acid compositions were determined following hydrolysis at 100 °C for 24 h in 6 M HCl under nitrogen. The acid hydrolysates of the isolated collagens were analyzed on a Durrum D-500 analyzer. No correction has been made for amino acid destruction during hydrolysis. Hydroxylysine glycosides were estimated from alkaline hydrolysates of collagens by the same analyzer used with Spiro's system (Spiro, 1969).

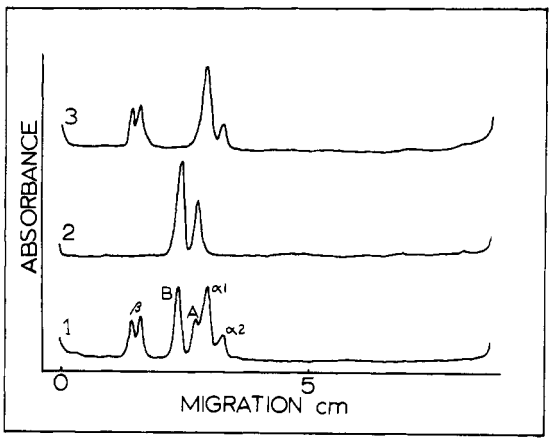

FIGURE 1: Densitometer traces showing component chains from fractions of pepsin-solubilized collagens of bovine amnion after separation by 6% polyacrylamide gel electrophoresis in $NaDodSO_4$: (1) 4.0 M NaCl precipitate in neutral salt buffer; (2) from sample 1, the P 4.8 fraction; (3) supernatant from 0.05 M sodium acetate, pH 4.8.

Cyanogen Bromide Cleavage. Cleavages of collagen α chains with cyanogen bromide were performed essentially according to the procedure described by Miller (1971). The weight ratio of cyanogen bromide to collagen was 5:1.

Electron Microscopy. SLS precipitates were prepared and stained as previously described (Davison & Cannon, 1977) and examined on a Philips EM 300 microscope.

Results

A limited pepsin digestion of bovine amnion or chorion membranes solubilized a mixture of different types of collagens. The collagens were fractionated by differential salt precipitation at neutral pH following the method of Trelstad et al. (1976). The AB type of collagen was precipitated by 4.0 M NaCl. The separation of the AB fraction from the other types of collagen was carried out by dialyzing the 4.0 M NaCl precipitate fraction first against 0.05 M acetic acid to dissolve it and then against 0.05 M sodium acetate, pH 4.8. Slab gel electrophoresis indicated that the fraction precipitating at pH 4.8 was relatively pure type VI, while the supernatant solution contained type I and perhaps other types of collagen (Figure 1). In a typical experiment 50% of the amnion membrane was recovered as pepsin-solubilized collagen, and from that (430 mg) we recovered 58, 34, and 6% in the 1.7, 2.4, and 4.0 M NaCl precipitates, respectively; further fractionation of the 4.0 M NaCl precipitate yielded 22 mg (5% of the solubilized collagen) of the AB collagen in the pH 4.8 precipitate.

Burgeson et al. (1976) showed that from the human fetal membrane collagens precipitated by 3.5 M NaCl the αA and αB chains could be separated by chromatography on carboxymethylcellulose under denaturing conditions. We attempted the same and variant procedures several times on the collagens obtained from bovine membranes, but the resolution of the αA and αB chains was very poor. However, the complete separation of αA chain from αB chain was achieved by chromatography on a hydroxylapatite column. When we applied the urea- and heat-denatured AB collagen to the hydroxylapatite column, the αA chain was not absorbed and passed directly through the column; a salt gradient was applied,

Table I: Amino Acid Compositions of Collagen α Chains

residue	residues/1000 residues										
	bovine							human			
	α1(I)[a]	α2(I)[a]	α1(II)[b]	α1(III)[c]	α1(IV)[d]	αA(VI)	αB(VI)	αA[e]	αB[e]	αA[f]	αB[f]
HO-Pro	92	88	96	121	140	92	92	111.5	111.9	111	108
Asp	42	49	48	46	51	44	50	51	50	51	51
Thr	16	18	23	16	20	28	20	26	19	25	19
Ser	34	35	26	47	37	34	21	31	26	33	24
Glu	76	74	93	68	79	86	97	84	91	86	99
Pro	138	117	121	107	65	96	129	97	118	104	120
Gly	319	328	326	348	328	362	361	319	322	330	336
Ala	126	98	103	95	37	60	41	52	46	62	46
Val	7	31	18	13	28	37	20	27	18	26	18
$^1/_2$-Cys				3	1						
Met	6	4	8	7	13	7	1	11	8	10	7
Ile	9	16	9	13	29	14	19	16	19	15	17
Leu	21	32	26	16	52	38	42	35	39	35	38
Tyr	3	2	1	2	2		1	1.8	2.1	0.7	1.2
Phe	13	14	13	8	29	10	18	14	12	11	12
HO-Lys	5	9	21	5	49	19	37	24	35	24	39
Lys	30	23	17	30	9	14	14	18	20	15	16
His	3	6	2	7	6	15	4	11	7.5	10	7
Arg	50	55	51	50	26	44	31	68	50	51	42
HO-Pro/(HO-Pro + Pro)	0.40	0.43	0.44	0.53	0.68	0.49	0.42	0.54	0.49	0.52	0.43
HO-Lys/(HO-Lys + Lys)	0.14	0.28	0.55	0.14	0.84	0.58	0.73	0.57	0.64	0.62	0.71

[a] Clark & Veis (1972). [b] Herbage et al. (1977). [c] Fujii & Kühn (1975). [d] Dehm & Kefalides (1978). [e] Burgeson et al. (1976). [f] Bentz et al. (1978).

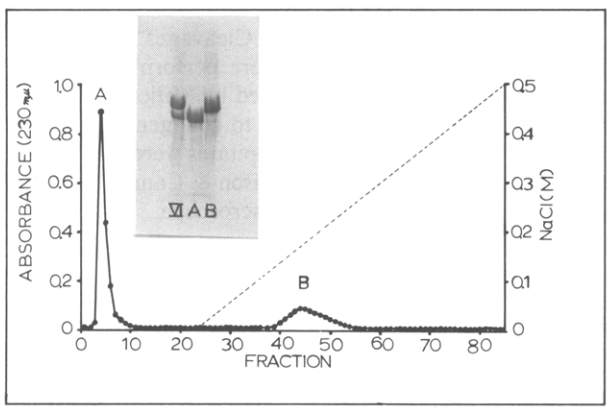

FIGURE 2: Hydroxylapatite chromatography of bovine type AB collagen. The bovine chorion collagen (12 mg) obtained after pH 4.8 precipitation was applied to a hydroxylapatite column as described under Materials and Methods. The elution positions of the αA and αB chains are indicated. Inset: 6% polyacrylamide gel electrophoresis in $NaDodSO_4$ of (VI) bovine chorion type AB collagen obtained after pH 4.8 precipitation, (A) αA chain from hydroxylapatite column chromatography, and (B) the αB chain from hydroxylapatite column chromatography.

and the αB chain was eluted at a concentration of ~0.15 M (Figure 2). Slab gel electrophoresis in $NaDodSO_4$ confirmed the resolution of the two chains (Figure 2, inset). The ratio of αB to αA chain was 1.78, and the total recovery of the collagen from the column was 95% based on the weight of lyophilized proteins from each peak.

The amino acid compositions of the αA and αB chains are shown in Table I. The content of hydroxylysine is high in both chains, but the two α chains differ substantially in the percentage of hydroxylation of lysyl residues (73% for αB and 58% for αA). The sum of the lysyl and hydroxylysyl residues in the αB chain (51) is 50% greater than in the αA chain. Similar findings with human AB collagen chains have been reported by Burgeson et al. (1976), Rhodes & Miller (1978), and Bentz et al. (1978). The percentage of hydroxylation of proline and the sum of hydroxyproline and proline are close to those of interstitial collagens. The low content in alanyl residues in both αA and αB chains is similar to the findings on basement membrane collagen (type IV). However, neither cysteine nor cystine was observed in either chain. Hydroxylysine glycoside analyses of the alkaline hydrolysates of both chains indicated that more than 84% of hydroxylysyl residues in αB but only 45% in αA are glycosylated. The amounts of glucosylgalactosylhydroxylysine and galactosylhydroxylysine are shown in Table II. The ratios of disaccharide to monosaccharide units in both αA and αB chains are lower than those in basement membrane collagen but higher than those in most interstitial collagens. Apart from the low values for methionyl residues, our analysis of the bovine chains shows a striking parallel with the analyses on human chains referenced above.

Table II: Hydroxylysine Glycosides of αA and αB Chains of Bovine Chorion AB Collagen

	residues/1000 residues	
	αA	αB
glucosylgalactosylhydroxylysine	6.9	26.8
galactosylhydroxylysine	1.4	4.3
unglycosylated hydroxylysine	10.6	5.8
total hydroxylysine	19	37
% glycosylation	43.9	84.2

The similarities in source, solubility characteristics, electrophoretic behavior of the α chains, and amino acid composition strongly suggest that we have isolated the bovine homologue of the human collagen fraction first described by Burgeson et al. (1976). We studied other characteristics of this collagen in order to permit its unambiguous identification in other tissue extracts.

Examination of SLS precipitates from collagen fractions rich in the AB type showed a segment with a banding pattern identical with the novel type we first noted in bovine Descemet's membrane and heart valve collagen (Davison & Cannon, 1977). Identical segments were also prepared from the AB-rich collagen fraction from human placental membranes (Figure 3). In negative stain (phosphotungstic acid)

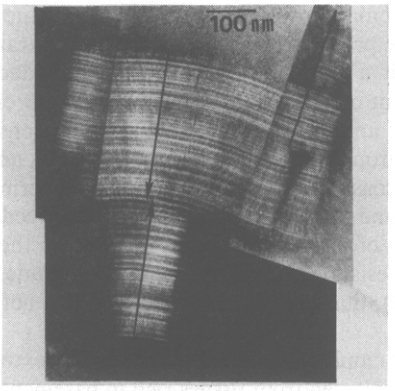

FIGURE 3: Electron micrographs of SLS crystallites from type AB collagen from (center) human amnion, (left) bovine amnion, and (right) pepsin-solubilized bovine tendon. The latter shows segments associating in a head-over-head, 40% overlap that appeared very often on the grids. The narrow amnion type VI segment (left) was from a collagenase digest, and it was selected because it shows a rare, fortuitous apposition with a three-quarter length fragment which is clearly different in banding pattern from the type VI; the AB collagen is not cleaved by the enzyme. We have not been able to identify the cleaved fragment from its banding; it does not match type I, II, III, or VII segments (Davison & Cannon, 1977). The 300-nm length of the human amnion segments is indicated by the arrows; the arrow head denotes the presumed carboxyl terminus of the collagen molecules. The crossbar (left) indicates the point of collagenase cleavage.

at pH 4.2, these segments were readily distinguishable from type I, II, or III collagens. The segments were commonly found in a 40% overlapping head-over-head association; they were never seen in the 30-nm overlapping head-to-tail associations commonly seen with type I SLS.

This bovine collagen fraction was resistant to digestion with mammalian collagenase. The digest of a mixture of native type I and AB collagen with rabbit corneal collagenase after agitation at 20 °C for 72 h–10 days was dialyzed against 0.05 M acetic acid and then lyophilized. Gel electrophoresis showed that the type I collagen was completely converted to one-quarter and three-quarter fragments after 5 days, while there was no indication of cleavage of the αA and αB chains (Figure 4). Electron microscopy revealed no cleaved type VI segments.

Samples of pure AB and type I collagens and a mixture of the two were cleaved by cyanogen bromide digestion, and the products were recovered by lyophilization and subjected to $NaDodSO_4$ gel electrophoresis on an 8–18% polyacrylamide gradient gel slab. The results were identical with those on corneal collagen fractions published elsewhere (Davison et al., 1979). The gel is illustrated in Figure 5; the figure also shows the peptides from the separated A and B chains.

Collagens from Other Tissue Sources. Gel electrophoresis of pepsin-solubilized extracts from various bovine tissues revealed a band in the αB position. To ascertain if the AB collagen was present in these extracts, they were fractionated and the products were examined and checked against the criteria for identification which we established above. In several cases the first step of fractionation was adjustment of a 0.05 M acetic acid–collagen solution to pH 4.8 with sodium hydroxide. The precipitate which formed was enriched in the AB collagen, and this precipitate was the starting material for the fractionation scheme already described.

We obtained pure AB or highly enriched samples from the following tissues: dura (1%); optic nerve endoneurium (1%); pepsin-solubilized extensor tendon (1%); cornea (8%); and fetal skin (8%). The percentages refer to the recovery relative to the yield of pepsin-solubilized collagen obtained from each tissue; the solubilized fraction was usually 75–95% of the tissue

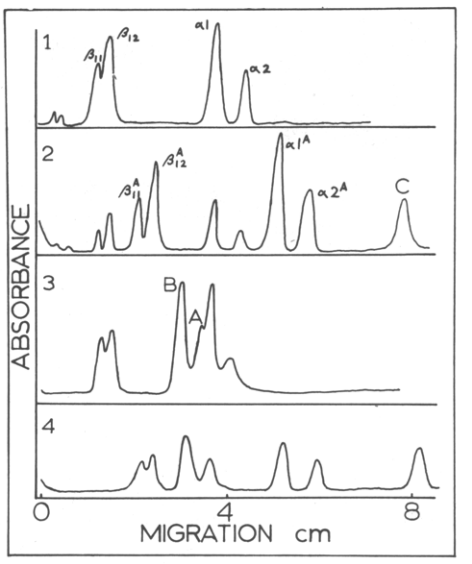

FIGURE 4: Densitometric profiles from untreated and collagenase-digested collagen fractions following $NaDodSO_4$–polyacrylamide gel electrophoresis. (1 and 2) Bovine type I collagen from tendon before and after digestion (the three-quarter length fragments of the β components and the α1 and α2 chains are designated with superscript A); (3 and 4) a mixture of type I and type AB collagen from bovine amnion before and after digestion. All the β, α1, and α2 chains have been cleaved by the fifth day of digestion at 20 °C (trace 4), but the αA and αB chains appear to be unchanged in amount and no fragments corresponding to three-quarters of their length are detectable. This evidence still allows the possibility that the αA and αB chains might be very slowly attacked or may be slowly cleaved into several chain lengths instead of the usual three-quarter and one-quarter pieces. Peak C is a component of the collagenase preparation.

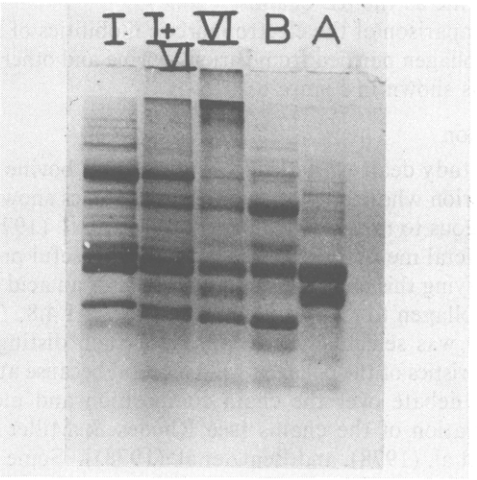

FIGURE 5: Photograph of a stained 8–18% polyacrylamide–$NaDodSO_4$ gel loaded with the cyanogen bromide cleavage products from (I) bovine tendon type I collagen, (VI) bovine amnion AB collagen, (I + VI) a mixture of the two preceding samples, and (B and A) the separated chains from bovine corneal type AB collagen. The identities of the cyanogen bromide peptides have been discussed previously (Davison, 1978; Davison et al., 1979).

collagen. In all of these cases the presence of AB collagen was confirmed by gel electrophoresis, by SLS identification, and by checking the resistance of the αA and αB chains to mammalian collagenase. While the cyanogen bromide cleaved peptides from this collagen are different from the type I collagen peptides, the major ones migrate sufficiently closely to make unambiguous analysis of the ratios of the two collagens difficult to achieve. Consequently, we found that the electrophoresis of the peptides was a poor criterion for identification unless the collagen samples were well purified.

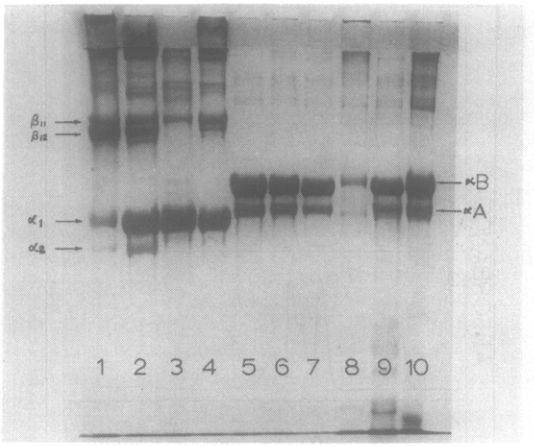

FIGURE 6: 5.5% polyacrylamide gel electrophoresis in $NaDodSO_4$ of collagens from different tissues: (1) acid-soluble rat tail tendon type I; (2) bovine chorion type I; (3) bovine cartilage type II; (4) bovine chorion type III; (5) bovine chorion type AB; (6) bovine amnion type AB; (7) bovine cornea type AB; (8) swine intestinal wall type AB; (9) chicken chorioallantoin type AB; (10) owl monkey sciatic nerve type AB. The somewhat lower mobility of the αB chain in sample 8 has been seen consistently, but this sample produced type VI SLS. The bands near the gel front (channels 8–10) are probably noncollagenous impurities.

We did not prove by these criteria the presence of this collagen in nasal cartilage. The P 4.8 fraction contained a collagen with a mobility similar to that of the αB chain as shown by gel electrophoresis and this collagen was collagenase resistant, but the αA chain was not resolved from the α1(II) chain and the type VI segments were not seen by electron microscopy. We have no analytical evidence that this α chain is the same as the αB chain.

A comparison of the electrophoretic mobilities of samples of AB collagen purified from various bovine and other species' tissues is shown in Figure 6.

Discussion

Our study dealt with a collagen type from bovine amnion and chorion whose analysis and characteristics show it to be homologous to that reported by Burgeson et al. (1976) from human fetal membranes. We found that a useful procedure for purifying this collagen was adjustment of an acid solution of the collagen to 0.05 M sodium acetate, pH 4.8. The AB collagen was selectively precipitated. Such distinguishing characteristics of the collagen will be useful because at present there is debate over the chain composition and molecular configuration of the chains [see Rhodes & Miller (1978), Brown et al. (1978), and Bentz et al. (1978)]. Some of these different results undoubtedly arise from mixtures of different collagen chains or cleaved chain fragments (Schwartz & Veis, 1978) in the preparations.

While we have consistently noted the resistance of bovine AB collagen to mammalian collagenase, we cannot claim on our present evidence that this resistance is absolute; the collagen may simply be very slowly attacked under the conditions selected. The same digestion conditions that cleave all type I molecules leave some cleaved and some intact type II molecules; under the same conditions no AB cleavage into three-quarter and one-quarter lengths was found and no loss of intact chains was detected, so this collagen is even more resistant than type II to this enzyme.

The αA and αB chains of human collagen were sucessfully separated by carboxymethylcellulose chromatography (Burgeson et al., 1976) and on phosphocellulose (Rhodes & Miller, 1978), but we were forced to resort to hydroxylapatite column chromatography to separate the bovine αA and αB chains. The procedure allowed complete separation of these chains with a high recovery, and it provided a reliable measurement of the weight ratio of αA to αB chains. Both gravimetric analysis and semiquantitative densitometry of stained electrophoresis gels suggest that the αA and αB chains are present consistently at a ratio of 1:2. Furthermore, because only one kind of SLS crystallite is produced from pure preparations of this collagen and because *both* the αA and αB chains are resistant to collagenase, we conclude, with Bentz et al. (1978), that AB collagen probably is a heteropolymer, $\alpha A(\alpha B)_2$.

Although emphasis has been placed on the extraction of the AB type of collagen from tissues rich in basement membranes (Burgeson et al., 1976; Trelstad & Lawley, 1977; Dehm & Kefalides, 1978; Chung et al., 1978), we, like Brown et al. (1978), have confirmed its presence in a variety of tissues and at levels which seem unlikely to relate to the amounts of basement membranes present. The fine structure of this collagen and its function remain to be determined.

Acknowledgments

We thank Randy Jones for her excellent technical assistance and Dr. Ben Peczon for performing the amino acid chromatography.

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