

Collagens From the Skin and Cartilage of the Larval Salamander *Ambystoma tigrinum*

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ABSTRACT The major collagens of larval salamander (*Ambystoma tigrinum*) skin and cartilage were purified and characterized by CM-cellulose chromatography and amino acid analysis of the component α chains. The collagens are similar to type I (skin) and type II (cartilage) collagens of birds and mammals. The type I molecule has the chain composition $(\alpha 1)_2\alpha 2$, and the type II molecule is an $(\alpha 1)_3$ molecule. Both collagens have lower levels of proline hydroxylation than the collagens isolated from homoiotherms, and generally higher serine contents. The type II collagen molecule has about half of its lysine residues hydroxylated, as in cartilage collagens of other species. Cyanogen bromide digestion of the component α chains followed by CM-cellulose chromatography or SDS gel electrophoresis of the peptides confirms that each chain is a different gene product and allows identification of the collagens in this species. Similar collagens were isolated from newt (*Notophthalmus viridescens*) tissues by pepsin extraction and fractional salt precipitation. These data will allow more effective utilization of the regenerating salamander limb for studies of collagen metabolism during regeneration.

INTRODUCTION

Limb regeneration in urodele amphibians offers a model system for observing and manipulating developmental events, including the breakdown and removal of collagenous structures in the stump (Grillo et al., '68; Dresden and Gross, '70) and the synthesis and deposition of collagen in blastemal tissues and in differentiating regenerates (Mailman and Dresden, '76; Linsenmayer and Smith, '75, '76).

Collagen is the major fibrous component of connective tissues, and is a potential marker for differentiation in salamander limbs. Collagen molecules are composed of three polypeptide chains, called α chains, tightly coiled into a triple helix. The α chains have a molecular weight of 95,000 and many common compositional features, such as a glycine residue in every third position, and both hydroxyproline and hydroxylysine residues. However, in birds and mammals, several genetically distinct collagens have been described that differ in α chain composition (Miller, '76). Skin, bone, and tendons contain type I collagen, with two identical subunits called $\alpha 1(I)$ chains and a third, different chain called $\alpha 2$. It can be represented by the formula $[\alpha 1(I)]_2\alpha 2$. Type II collagen, $[\alpha 1(II)]_3$, is confined largely to cartilage. It is composed of three identical subunits called $\alpha 1(II)$, chains which are different from both $\alpha 1(I)$ chain and $\alpha 2$ chains in primary sequence. The third major interstitial collagen has three identical chains and is designated type III, or $[\alpha 1(III)]_3$. It is abundant in fetal skins and in internal organs. The $\alpha 1(III)$ chain is again different in primary structure from the other collagen α chains, and furthermore, the three chains within a type III molecule are linked in disulfide bonds.

In birds and mammals, skin contains type I and type III collagens, and may contain other types (Kefalides, '69; Burgeson et al., '76; Chung et al., '76), whereas cartilage contains mainly type II collagen (Miller and Matukas, '69; Miller, '76). In the larval salamander, skin and cartilage collagens have been characterized by the formula $[\alpha 1(I)]_2\alpha 2$. Type II collagen, $[\alpha 1(II)]_3$, is confined largely to cartilage. It is composed of three identical subunits called $\alpha 1(II)$, chains which are different from both $\alpha 1(I)$ chain and $\alpha 2$ chains in primary sequence. The third major interstitial collagen has three identical chains and is designated type III, or $[\alpha 1(III)]_3$. It is abundant in fetal skins and in internal organs. The $\alpha 1(III)$ chain is again different in primary structure from the other collagen α chains, and furthermore, the three chains within a type III molecule are linked in disulfide bonds.

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ized only by chain composition. We used radioactive collagens produced in vitro by explants of skin or cartilage to determine the α chain composition of the collagens produced by these tissues in larval *Ambystoma opacum*, *A. punctatum*, and *A. tigrinum* (Linsenmayer and Smith, '75, '76). Now, we have characterized chemical amounts of the major collagens of the salamander limb to allow more effective utilization of the regenerating limb system for the study of transitions in collagen types synthesized during differentiation of limb tissues, during blastema formation, and during experimentally induced scarring. We have confirmed that the major skin collagen in the larval salamander (*A. tigrinum*) is a type I-like molecule and that the major cartilage collagen is a type II-like molecule. The molecules can be identified by the behavior on gel electrophoresis and on CM-cellulose chromatography of the component α chains and the peptides derived from these preparations by cyanogen bromide cleavage.

MATERIALS AND METHODS

Preparation of collagens

Large (average wt = 8.2 g) larvae of *Ambystoma tigrinum* obtained from Michael Tolley, Donelson, Tennessee, were anaesthetized in a 1:1,000 solution of ethyl m-aminobenzoate methane sulfonate (Eastman), decapitated, and the skins and cartilages dissected free of other tissues and placed on ice. Subsequent manipulations were done at 4°C.

The skins were sequentially extracted in 0.4 ionic strength phosphate buffer, pH 7.6, and three changes of 0.5 M acetic acid. Each extract was clarified by centrifugation, and the collagen in the supernatant was purified as described previously (Gross, '58; Linsenmayer and Smith, '76). Collagens were precipitated from the neutral extracts by addition of 20% NaCl, then redissolved in 0.5 M acetic acid and precipitated by addition of 10% NaCl. Those extracted by acetic acid were first precipitated with 10% NaCl, then redissolved in 0.4 ionic strength phosphate buffer and precipitated with 20% NaCl. Finally, collagens from either extraction procedure were precipitated from potassium phosphate buffer by dialysis against 0.01 M Na₂HPO₄. These low ionic strength pellets were taken up in 0.1 M acetic acid, dialyzed, and lyophilized for further analysis. Collagen recovery was determined by hydroxyproline analysis (Woessner, '61).

The cartilages from the larval skeletons

were minced, extracted with 2 M MgCl₂, and suspended in 0.5 M acetic acid. Pepsin (20 mg/g wet tissue) was added to the suspension, the pH was adjusted to 2.5 with HCl, and the digestion allowed to proceed at 4°C for 72 h. Then, the suspension was neutralized to inactivate the pepsin and dialyzed into potassium phosphate buffer, pH 7.6, ionic strength, 0.4. The neutral solution was clarified by centrifugation, and the collagen in the supernatant was purified as described above for the skin extracts.

The pepsin digestion technique was also applied to tissues of the newt (*Notophthalmus viridescens*) and to *A. tigrinum* skin residues following acetic acid extraction. Some samples were fractionated by precipitation from neutral buffer (Trelstad et al., '76) by dropwise addition of 4.4 M NaCl in water to collagen solution in 0.4 ionic strength phosphate buffer. At 2.2 M NaCl/0.2 ionic strength phosphate buffer, type I precipitates and type II remains in solution.

Collagens from the skin of tadpoles of the bull frog (*Rana catesbiana*), from chicken tissues, from rat tissues, and from rabbit tissues were prepared by similar methods and used for comparison throughout the study.

Analyses of the collagens

The purified collagens and the cyanogen bromide peptides derived from the collagens were analyzed by CM-cellulose chromatography on jacketed columns maintained at 42°C. Collagen solutions were denatured by heating for 10 min at 52°C and applied to 0.9 × 4 cm columns of CM-cellulose equilibrated with 0.02 M acetate buffer, pH 4.8. The component polypeptide chains were eluted at a flow rate of 50 ml/h with a superimposed linear gradient of NaCl from 0 to 0.14 M over a volume of 200 ml, as described previously (Piez et al., '63; Linsenmayer and Smith, '75). The column effluent was continuously monitored at 228 nm and fractions of 4 ml were collected. All buffer solutions contained 1 M urea. Recoveries were determined on several samples by hydroxyproline analysis and were 40–50%.

Cyanogen bromide peptides were prepared in sealed tubes by dissolving the collagens in 70% formic acid at 10 mg/ml, gassing the solutions with nitrogen for 5 min, and adding 12 mg/ml cyanogen bromide. After 4 h at 37°C, the peptides were desalted on Biogel P2 columns equilibrated either with 0.02 M formate/0.02 M NaCl, pH 3.8, for chromatography of the peptides or with 0.1 M acetic acid for ly-

ophilization and gel electrophoresis.

The peptides were applied to a 0.9×12 cm column of CM-cellulose equilibrated with 0.02 M formate/0.02 M NaCl, pH 3.8, and eluted with a superimposed linear gradient of NaCl from 0 to 0.15 M over a total volume of 300 ml. The flow rate was 30 ml/h, and fractions of 5 ml were collected. Column effluents were monitored at 222 nm. For each collagen or α chain type, the chromatographic profile of the cyanogen bromide peptides was reproduced on 2–6 preparations without significant variation in pattern.

Some samples of collagen or collagen peptides were also separated by molecular sieve chromatography. Whole denatured collagens were chromatographed on Agarose A-5M in 1M CaCl_2 /0.05 M Tris, pH 7.5. Peptides were chromatographed on Sephacryl S-200 in the same buffer.

Pooled fractions containing the chromatographic peaks from either type of chromatography were dialyzed against dilute acetic acid and lyophilized for further analysis.

Amino acid analysis was performed on a Beckman 121 amino acid analyzer, using a single column method (Kang, '72). Samples were hydrolyzed under nitrogen in constant boiling HCl at 108°C for 24 h.

Both whole collagen preparations and cyanogen bromide peptides were analyzed by electrophoresis on SDS-polyacrylamide slab gels. The buffer systems used were either Tris-glycine (Laemmli, '70) or Tris-borate (Neville and Glossman, '74). Gel concentrations were 7.5% for α chains and 15% for peptides.

Gels $0.075 \text{ cm} \times 14 \text{ cm} \times 10 \text{ cm}$ were loaded and run overnight at 20 volts under constant voltage conditions. The gels were fixed in 30% methanol/10% acetic acid for four h, then stained with 2.5% Coomassie blue (Weber and Osborne, '69).

RESULTS

Type I collagen

More than 80% of the hydroxyproline containing proteins were consistently extracted from skin of *Ambystoma tigrinum* larvae with cold 0.4 ionic strength phosphate buffer, pH 7.6, followed by three changes of 0.5 M acetic acid. On SDS-polyacrylamide gel electrophoresis, the extracts contained materials that comigrated with standard collagen components from rabbits. Both neutral buffer and acid extracts contained apparent $\alpha 1$ chains, $\alpha 2$ chains, the covalently cross-linked dimers of

either two $\alpha 1$ chains ($\beta 1,1$) or one $\alpha 1$ and one $\alpha 2$ chain ($\beta 1,2$), as well as trimers (γ) (Fig. 1). Each of the extracts contained variable ratios of β and α components. On CM-cellulose chromatography, the salamander skin collagen again exhibits peaks corresponding to $\alpha 1$; $\beta 1,1$; $\beta 1,2$; and $\alpha 2$ components (Fig. 2). Materials recovered from these peaks comigrate with the appropriate known collagen components on SDS gel electrophoresis. The complexity of these chromatograms and the relatively poor separation between $\beta 1,2$ and $\alpha 2$ components preclude accurate assessment of $\alpha 1/\alpha 2$ ratios in these preparations. However, the triple helical portion of the native collagen molecule is resistant to limited pepsin digestion, and the covalent cross-links are located in short, non-helical regions at the ends of the molecule. Thus, digesting native collagen molecules in cold, dilute acetic acid with pepsin (4°C, 18 h) removes the nonhelical cross-link regions and converts the β and γ components to α chains (Fig. 2). The $\alpha 1/\alpha 2$ ratio determined from CM-

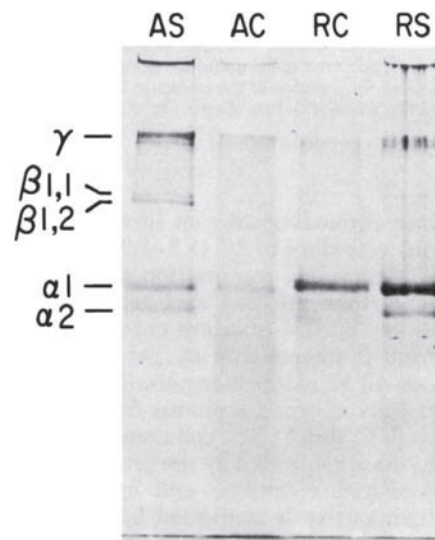


Fig. 1. SDS-polyacrylamide gel electropherogram of collagens from skin and cartilage of larval *Ambystoma tigrinum* plus reference collagens from rabbit tissues. Samples labeled AS and RS are from the skin of *Ambystoma tigrinum* and of the rabbit, respectively; AC and RC are from the cartilage of *A. tigrinum* and of the rabbit, respectively. The skin samples contain $\alpha 1$ and $\alpha 2$ chains, as well as the dimers composed of two $\alpha 1$ chains ($\beta 1,1$) or one $\alpha 1$ and one $\alpha 2$ chain ($\beta 1,2$). The cartilage samples contain largely $\alpha 1$ chains, although some samples have $\beta 1,1$ and α components.

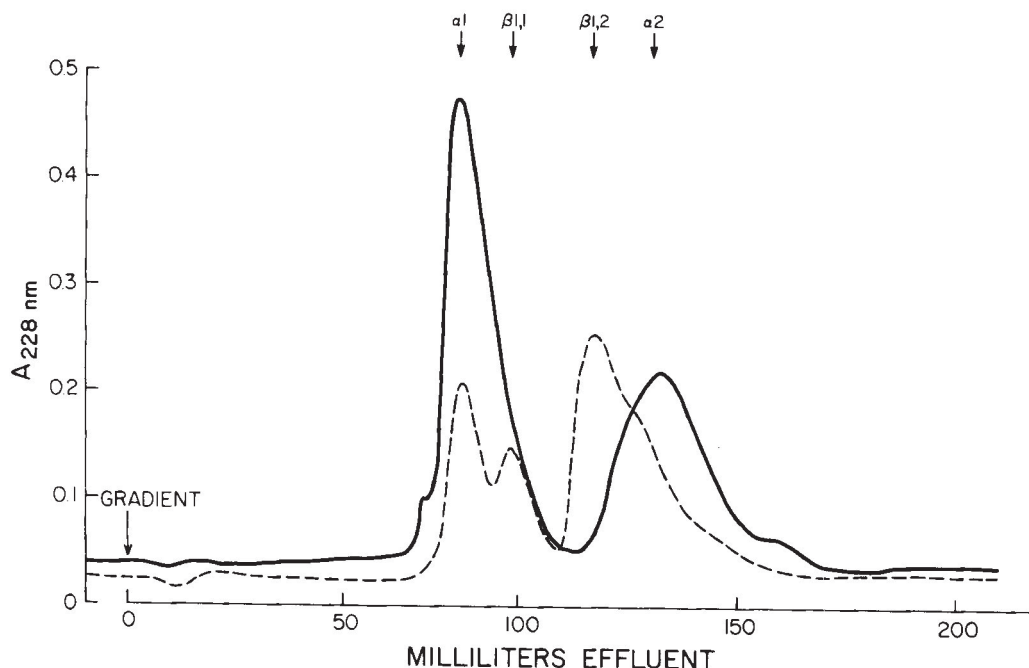


Fig. 2. Typical CM-cellulose chromatograms of collagen from skin of salamander larvae. The broken line is the chromatogram of the first extract with 0.5 M acetic acid. The peaks were identified as $\alpha 1$; $\beta 1,1$; $\beta 1,2$; and $\alpha 2$ by gel electrophoresis and amino acid analysis. The solid line is the profile of an aliquot of the acid extract after treatment with pepsin at 4°C for 18 h. Pepsin treatment under these conditions removes the nonhelical extension peptides from collagen, but does not attack the helical region of the molecule. Since the cross-links are located in the nonhelical ends, such treatment converts most of the β subunits to α chains. On this preparation, the resulting $\alpha 1/\alpha 2$ ratio was 1.9.

cellulose chromatograms on three such preparations was close to 2/1 (1.7–1.9) (Fig. 2).

The amino acid composition of $\alpha 1$ and $\alpha 2$ chains further purified and separated from each other by CM-cellulose chromatography, and from β components by gel filtration on Agarose A5 M, shows many similarities to the composition of type I α chains from birds and mammals (Table 1). The collagenous nature of the chains is confirmed by the presence of high levels of hydroxyproline and hydroxylysine, and their purity is confirmed by the absence of cystine and the presence of about one-third of the residues as glycine. The salamander α chains have lower levels of proline hydroxylation than are seen in the collagens of homeotherms. When compared to the $\alpha 1(I)$ chain of the chick, the $\alpha 1$ type I chain reveals a somewhat higher serine and lower alanine content. The $\alpha 2$ chain also shows a very high serine content. Both proline and lysine hydroxylation levels are lower in this chain than in the corresponding chain from chick skin.

Cyanogen bromide cleavage of the type I collagen yields peptides that can be partially resolved by CM-cellulose chromatography and on SDS gels. The $\alpha 1$ chain yields 5–7 peptides that can be resolved by chromatography (Fig. 3) and on SDS gel electrophoresis (Fig. 4). The $\alpha 2$ chain yields four poorly resolved peaks on CM-cellulose chromatography (Fig. 3).

Type II collagen

Cartilages from *Ambystoma* larvae, extracted with 2 M magnesium chloride to remove proteoglycan, then pepsinized, yield a collagen molecule similar to the type II collagen of birds and mammals. It contains only $\alpha 1$ chains as determined by CM-cellulose chromatography or by gel electrophoresis (Fig. 1). The amino acid composition (Table 1) reveals many similarities to the corresponding chains from other cartilaginous tissues. As previously noted for the skin collagen, the level of proline hydroxylation is lower than that seen in the

TABLE 1. Amino acid composition of collagen α chains from salamanders^a

	Ambystoma tigrinum $\alpha 1(I)$	Chicken ^b $\alpha 1(I)$	Ambystoma tigrinum $\alpha 2$	Chicken ^b $\alpha 2$	Ambystoma tigrinum $\alpha 1(II)$	Notophthalmus ^c viridescens $\alpha 1(II)$	Chicken ^b $\alpha 1(II)$	Sturgeon ^d $\alpha 1(II)$
3-Hydroxyproline	—	—	—	—	1	2	1	2
4-Hydroxyproline	81	99	78.5	94	86	86	97	88
Aspartic acid	39	46	54	52	52	50	50	46
Threonine	11	18	24	20	23	24	23	20
Serine	53	27	67	29	32	49	28	38
Glutamic acid	83	83	78	64	88	91	93	89
Proline	127	118	114	116	117	118	113	118
Glycine	353	336	331	340	344	325	336	338
Alanine	109	127	103	100	82	78	102	106
Cystine/2	0	0	0	0	0	0	0	0
Valine	11	16	20	31	16	22	23	14
Methionine	8	6	4	3	7	7	6	4
Isoleucine	6	7	8	18	9	10	9	8
Leucine	16	20	25	30	29	30	26	27
Tyrosine	—	2	3	3	2	4	1	1
Phenylalanine	13	13	13	14	14	10	12	12
Hydroxylysine	7	7	6	13	21	20	18	19
Lysine	29	25	24	17	21	12	17	16
Histidine	3	2	7	7	4	5	2	3
Arginine	50	47	44	49	53	51	43	51

^aValues are given as residues/1,000 and are the means of three determinations. The α chains were purified by chromatography on CM-cellulose and agarose A-5M.

^bChick α chain compositions are from Trelstad et al. ('72).

^cNewt $\alpha 1(II)$ collagen was isolated by fractional salt precipitation from pepsin extract of whole newt carcasses.

^dSturgeon type II α chain composition is from Miller and Matthews ('74).

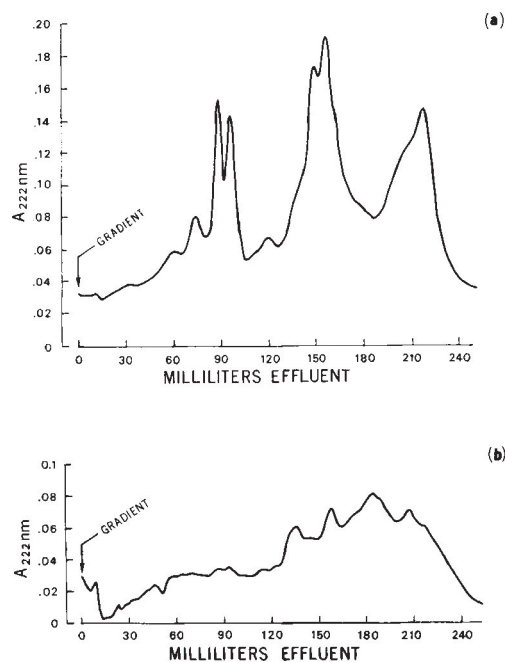


Fig. 3. Typical CM-cellulose chromatograms of cyanogen bromide peptides from (a) $\alpha 1$ and (b) $\alpha 2$ chains of salamander skin. The profiles are reproducible and characteristic for each chain. Because all of the $\alpha 2$ peptides elute before the last $\alpha 1$ peptide, it is advantageous to separate the α chains before cyanogen bromide digestion.

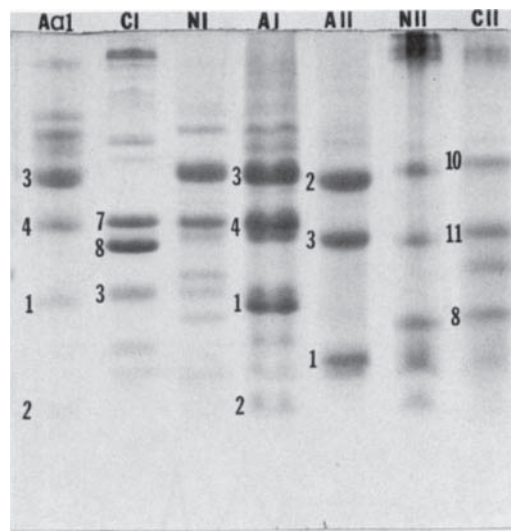


Fig. 4. SDS-polyacrylamide gel electropherogram of cyanogen bromide peptides of several amphibian collagens plus reference collagens from chickens. Samples labeled CII and CI are from chick $\alpha 1(II)$ and $\alpha 1(I)$ chains, respectively; AI and AII are from the homologous salamander collagens; NI and NII are from the homologous collagens of the newt; and A $\alpha 1$ is from an $\alpha 1$ chain from salamander skin collagen. The numbers to the left of CI and CII are the published designations of the chicken $\alpha 1(I)$ and $\alpha 1(II)$ peptides (Miller, '76). The numbers to the left of the salamander collagens correspond to the numbered peaks in Figure 5.

collagens of homoiotherms. However, the hydroxylysine content of the salamander type II is as high as or higher than the levels reported in birds and mammals. These high levels of lysine hydroxylation and very low alanine content distinguish this collagen from skin collagens.

Cyanogen bromide digestion of $\alpha(\text{II})$ followed by CM-cellulose chromatography yields a peptide map different from that produced by type I (Fig. 5), indicating that the collagen of the cartilage is a different gene product. The peptides resolved by gel electrophoresis (Fig. 4) are also unique. Direct digestion of the cartilage by cyanogen bromide yields a similar peptide map on either gels or CM-cellulose chromatography, confirming that type II collagen is the predominant collagen type in this tissue.

The newt, *Notophthalmus viridescens*, also has type II collagen (Fig. 4, Table 1) with properties similar to this collagen in larval salamanders, birds, and mammals. This molecular species was obtained through fractional salt precipitation of pepsin digests of newt tails and

limbs. Type I collagen is insoluble in 2.2 M NaCl/0.2 ionic strength phosphate, pH 7.6, whereas type II remains in solution.

DISCUSSION

Because type II collagen is the major collagen type produced by differentiated hyaline chondrocytes, it has been extremely useful both as a marker for differentiation during development (Linssenmayer et al., '73a, '73b; von der Mark et al., '76) and in the analysis of chondrocyte phenotypic expression in vitro (Mayne et al., '75, '76, '77; Benya et al., '78; Benya and Nimni, '79). Furthermore, the surprising observation that embryonic chick neural retina synthesizes type II collagen for export to the vitreous body (Smith et al., '76; Newsome et al., '76) and that the corneal epithelium also deposits both type I and II collagen in the primary corneal stroma (Linssenmayer et al., '77; von der Mark et al., '77) suggests wider distribution and additional, as yet unknown, functions for this collagen in development. Thus, characterization of the collagen of salamander skin and cartilage will facilitate similar studies in the amphibian developmental systems, particularly in regeneration.

We had previously reported that the collagen produced by cartilages incubated in vitro with ^3H -proline had the α chain composition $(\alpha_1)_3$, whereas the molecule produced by skin had the chain composition $(\alpha_1)_2\alpha_2$ (Linssenmayer and Smith, '75; '76). The existence of these chains has been confirmed with chemical amounts of collagen extracted from the tissues. Furthermore, the suggestion that each type of α chain is a product of different genes has been verified by amino acid analysis and cyanogen bromide peptide mapping. By analogy to the collagens of birds and mammals, we can now designate the skin molecule type I, or $[\alpha_1(\text{I})]_2\alpha_2$, and the cartilage molecule type II, or $[\alpha_1(\text{II})]_3$.

The type I molecule has a number of similarities to type I collagen from homoiotherms, including α_1 and α_2 chains as defined by their behavior on CM-cellulose chromatography and SDS gel electrophoresis, $\beta_1,1$ and $\beta_1,2$ components apparently cross-linked in nonhelical regions of the molecule, and the α_1/α_2 ratio of 2/1. The strongest evidence that the ratio of α_1/α_2 in type I collagen from *Ambystoma* larvae is 2/1 comes from our previous studies with radioactive collagens. In these experiments, β -aminopropionitrile was included in the medium to prevent cross-link formation. Thus, no β components formed and the CM-cellulose chromatograms were easily evaluated. Both skin preparations and the undifferentiated

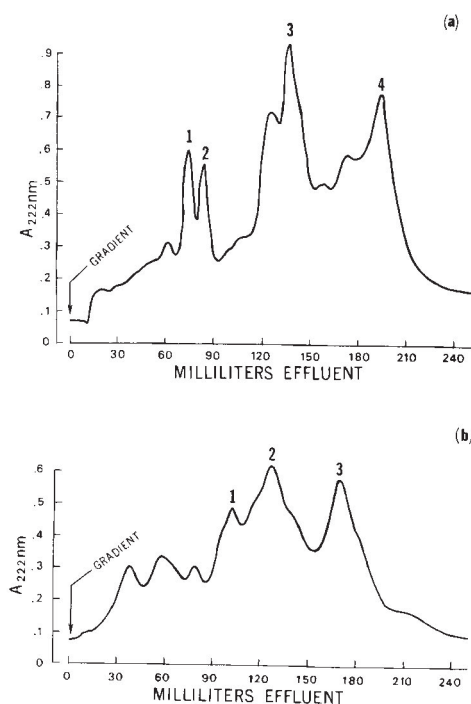


Fig. 5. Typical chromatographic profiles of (a) skin and (b) cartilage collagen from *A. tigrinum* larvae. The profiles are unique and reproducible. The major peptides from the numbered peaks are marked on Figure 4.

blastemal tissues reproducibly synthesized collagens with an $\alpha 1/\alpha 2$ ratio of 2/1. The ratios we obtained following pepsin digestion confirm the data from the studies in vitro.

The demonstration that the collagen found in the cartilage of larval urodeles is a product of different genes than the collagens of skin is of importance to both developmental studies and to phylogenetic considerations. During limb regeneration, chondrocytes are freed from the cartilage matrix and enter the blastema (Hay, '66). They lose their histological identity, proliferate as mesenchyme-like cells, then re-differentiate (Steen, '68). These changes that occur in vivo are similar in appearance to changes seen in chick and mammalian chondrocytes cultured in vitro. However, when we determined the chain composition of collagens produced by blastemal tissues derived in part from cartilage, we could detect only $(\alpha 1)_2\alpha 2$ molecules (Linsenmayer and Smith, '75, '76). In contrast, both chick and rabbit chondrocytes that lose chondrocyte morphology in vitro alter their collagen production from type II to include both type I, $[\alpha 1(I)]_2\alpha 2$, and a previously undetected collagen, the type I trimer, $[\alpha 1(I)]_3$ (Mayne et al., '75, '76, '77; Benya et al., '78; Benya and Nimni, '79). Investigation of the products of chondrocyte cultures derived from *Ambystoma* larvae should now allow verification of type I, type II, and type I trimer production. If chondrocytes that lose their histological identity in vitro are biochemically similar to those chondrocytes that lose their histological identity in vivo to enter the regeneration blastema, then these cultures should produce only $[\alpha 1(I)]_2\alpha 2$ collagen. If these cells produce $[\alpha 1(I)]_3$, then the loss of chondrocyte characteristics in vitro is a different process from the same loss in vivo.

At the phylogenetic level, salamander collagens have several features that distinguish them from the collagens of birds and mammals. Proline hydroxylation is uniformly lower than the levels seen in homoiotherms. Since the melting points that have been determined for newt collagen (Mailman et al., '74) are lower than the melting points found with mammalian collagens, the low level of proline hydroxylation is consistent with the proposition that the thermal stability of the collagen triple helix is proportional to hydroxyproline content (Ramachandran and Ramakrishnan, '76). As has been the case with other collagens from poikilotherms, lower hydroxylation of proline is accompanied by higher serine content (Rudall, '68; Pikkariinen and Kulonen, '69). Similar compositional features were also noted in the native collagen molecules ex-

tracted from newt (*Notophthalmus viridescens*) skin by dilute acetic acid (Mailman et al., '74) and from newt tails by limited pepsin digestion (Baum et al., '74).

The remaining differences between the amino acid content of collagens of the salamander and those of birds and mammals are slight. It is notable that lysine hydroxylation in the type II molecule is not lowered. Both the amphibian cartilage collagens studied here and the sturgeon cartilage and notochord type II molecules previously reported (Miller and Matthews, '73) have at least half of their lysine residues hydroxylated. This fact, and the conservation of the tissue distribution of type I and type II collagens in all vertebrates yet characterized, suggest functional advantages for these collagen molecules that are not yet fully understood.

Finally, although we have characterized the major collagens from skin and cartilage, other collagen types certainly exist. We have partially characterized two additional chains from salamander skin that have the electrophoretic mobility of the A and B chains thought to be associated with basement membranes in humans (Burgeson et al., '76; Chung et al., '76). The salamander chains also have high levels of hydroxylysine and measurable amounts of 3-hydroxyproline, features that are consistent with a basement membrane origin. We did not find any type III-like molecules in our extracts of skin, nor did we see any evidence of type III production in the radioactive collagens studied previously (Linsenmayer and Smith, '76). However, no systematic search of the 5% of the skin collagen that remains insoluble following pepsin extraction nor of the hydroxyproline containing molecules lost during purification has yet been made. This type of search has yielded evidence of at least three new chains in human cartilage (Burgeson and Hollister, '79) and was necessary to clearly identify type III collagen in chickens (Hermann and von der Mark, '77).

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