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# Synthesis of type II collagen *in vitro* by embryonic chick neural retina tissue

**(vitreous body/eye development/genetically distinct collagens)**

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**ABSTRACT** **Collagen produced by chick neural retina tissue *in vitro* has the properties of type II collagen. Isolated neural retina tissue from stage 29–32 chick embryos incorporated [$^{14}$C]glycine and [$^{3}$H]proline into collagen consisting of $\alpha$1 chains and a higher molecular weight species. The peptides produced by cyanogen bromide digestion of the collagen are identical to those from authentic type II collagen from cartilage in charge properties, as determined by carboxymethyl-cellulose chromatography, and size distribution, as determined by sodium dodecyl sulfate-acrylamide gel electrophoresis.**

Collagen and glycosaminoglycan produced by embryonic tissues are useful markers in development and may be active agents in tissue interactions leading to morphogenesis. In the early developing chick eye, where accumulation of vitreous materials provides the expansive pressure for the development of normal eye geometry (1, 2), the neural retina produces both the collagen (3) and the glycosaminoglycan (Smith and Newsome, in preparation) of the vitreous body.

Isolated neural retinas from early chick embryo incubated *in vitro* with appropriate isotopic precursor synthesize collagen (3) and glycosaminoglycan (Smith and Newsome, in preparation) with characteristics similar to those of molecules found in the vitreous body labeled *in ovo*. The vitreous glycosaminoglycans are almost exclusively chondroitin sulfates, while the collagen is predominantly an $(\alpha 1)_3$-type molecule, eluting from carboxymethyl (CM)-cellulose columns in the position of the $\alpha$1 type I chain of skin collagen and the $\alpha$1 type II chain of cartilage collagen. The other ocular tissues bordering the vitreous and the cells within the vitreous itself, in contrast, produce only small amounts of glycosaminoglycan and a collagen containing both $\alpha$1 and $\alpha$2 chains. Thus, it is reasonable to conclude that the neural retina secretes both the collagen and glycosaminoglycan components of the vitreous body during early development. The previously published data establish that neural retina collagen of early chick embryo is either type II (cartilage), or the $\alpha$1 type I trimer recently described by Mayne *et al.* (4), or an as yet undescribed separate gene product, but could not discriminate between these possibilities.

In the present report we have analyzed the collagen produced *in vitro* by stage 29–32 chick embryo neural retinas to determine the identity of the molecule.

## MATERIALS AND METHODS

**Production of Labeled Neural Retinal Collagen.** Neural retinas were dissected from the eyes of stage 29–32 (5) White Leghorn chick embryos and cultured overnight in Eagle's minimum essential medium supplemented with 10% fetal calf serum, 50 $\mu$g/ml of $\beta$-aminopropionitrile, 100 $\mu$g/ml of ascorbic acid, 50 $\mu$Ci/ml of [$^{14}$C]glycine (New England Nuclear; NEC-047H), and/or 50 $\mu$Ci/ml of [$^{3}$H]proline (New England Nuclear; NET-323). The inclusion of ascorbic acid promotes collagen synthesis and secretion, while the lathyrogen $\beta$-aminopropionitrile in the culture medium inhibits the formation of aldehyde-derived crosslinks, rendering types I and II collagen more extractable. After incubation, cultures plus medium were combined with unlabeled carrier collagen for extraction and purification of the collagens. The carrier collagens were either type I from chick skins or type II from chick sternae.

Cultures plus 1–10 mg of carrier were extracted for 48 hr in $\Gamma/2 = 0.4$ phosphate buffer, pH 7.6. Extraction and subsequent purification steps were at 4°. The extracts were centrifuged at 75,000 × *g* for 1 hr, and the collagen was precipitated by dialysis against 0.01 M $Na_2HPO_4$. The collagen precipitate was redissolved in 0.5 M acetic acid and reprecipitated by addition of solid NaCl to a final concentration of 10%.

After centrifugation, the pellets containing the collagens were redissolved in 0.1 M acetic acid and dialyzed against 0.1 M acetic acid before lyophilization and subsequent analysis.

**Collagenase Treatment.** Some samples were treated with protease-free collagenase (EC 3.4.24.3; Advanced Biofactures Form III) to verify their collagenous nature. Collagenase (125 units/mg of collagen) was added to collagen dissolved in 0.33

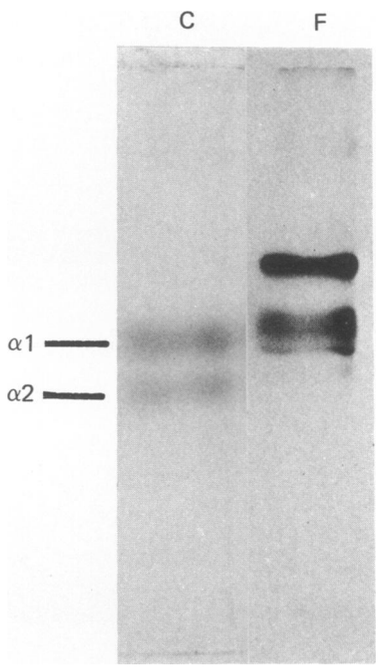



FIG. 1. **Fluorogram of purified whole collagen from neural retina cultures labeled with [$^{14}$C]glycine and [$^{3}$H]proline after separation on a 7.5% $NaDodSO_4$-polyacrylamide gel (F). Positions of the skin collagen carrier $\alpha$ chain are shown by the stained gel (C).**

Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; CM-cellulose, carboxymethyl-cellulose.

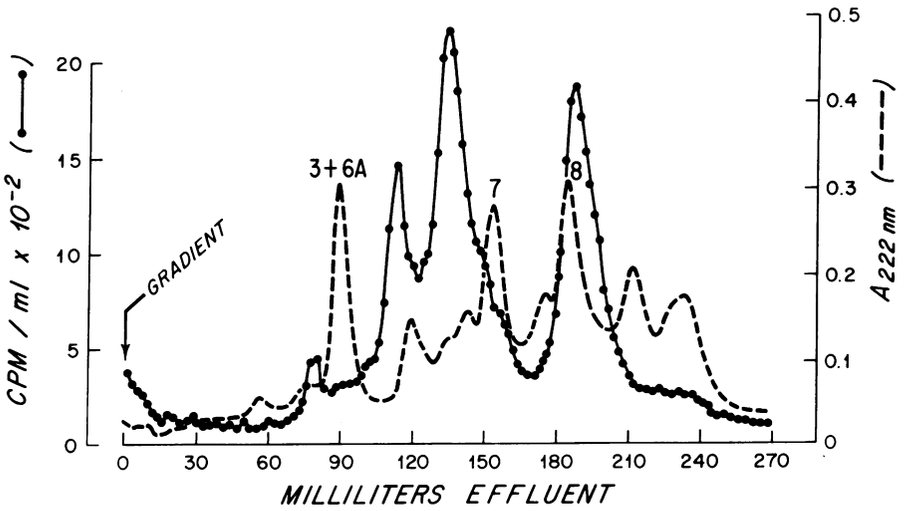



FIG. 2. CM-cellulose chromatogram of cyanogen bromide peptides (CB) of collagen from neural retina cultures (●—●) purified with type I carrier (- - -). The radioactive profile shows three large peaks, two eluting between carrier peaks CB3 + 6A and CB7, and the third near CB8. Very little material elutes with the two $\alpha 2$ peptide peaks that appear after $\alpha 1$-CB8. The peaks without numerical designation in the carrier profile between CB3 + 6A and CB7 are probably CB6B and CB4 + 5. The conditions for chromatography are given in the *text*.

M calcium acetate, 0.025 M Tris, pH 7.4, and the mixture was incubated at 37° for 5 hr. Phenylmethylsulfonyl fluoride (1 $\mu$M/ml) was added to suppress nonspecific protease activity. A control sample without enzyme was run in parallel. After treatment, the samples were dialyzed against 0.1 M acetic acid, lyophilized, and analyzed by sodium dodecyl sulfate ($NaDodSO_4$) gel electrophoresis (see below).

**Preparation of Cyanogen Bromide Peptides.** For preparation of cyanogen bromide peptides the labeled collagens plus carrier were dissolved in 1 ml of 70% formic acid, gassed with nitrogen, combined with cyanogen bromide (12 mg/ml), and incubated at 37° for 4 hr (6). The peptides were desalted on a column of Bio-Gel P-2 in 0.1 M acetic acid and lyophilized before further analysis. Alternatively, peptides were desalted on a similar column equilibrated with the starting buffer used in CM-cellulose analysis of the peptides (0.02 M formate/0.02 M NaCl, pH 3.8), and placed directly into the CM-cellulose column.

**CM-Cellulose Analysis of Cyanogen Bromide Peptides.** For CM-cellulose chromatographic analysis, 4–8 mg of peptides were applied to a 0.9 × 15 cm water-jacketed column of CM-cellulose equilibrated with 0.02 M formate/0.02 M NaCl, pH 3.8, and run at 42°. A linear gradient from 0 to 0.15 M NaCl was superimposed in a total gradient volume of 300 ml at a flow rate of 30 ml/hr (7, 8). The absorbance of the effluent was monitored at 222 nm, and 4-min fractions were collected. The identity of the peptides was confirmed by $NaDodSO_4$ gel electrophoresis with known standards, and the peptide nomenclature of Mayne *et al.* (4) and Lichtenstein *et al.* (7) was used. Radioactivity in the effluent fractions was measured by placing a 0.5-ml portion of each into 10 ml of Aquasol (New England Nuclear) in a Beckman liquid scintillation counter. Efficiency of the counter was 60% for $^{14}C$ and 40% for $^{3}H$. Recovery of collagen peptides that had been placed on the column was greater than 50%.

**Gel Electrophoresis and Fluorography.** The chain composition of the purified collagens was analyzed by electrophoresis in 7.5% $NaDodSO_4$-polyacrylamide gels using either a

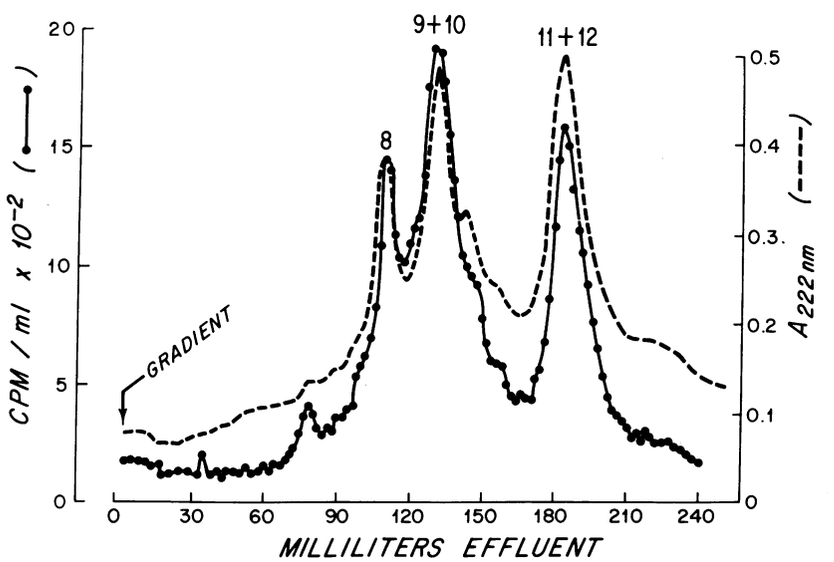
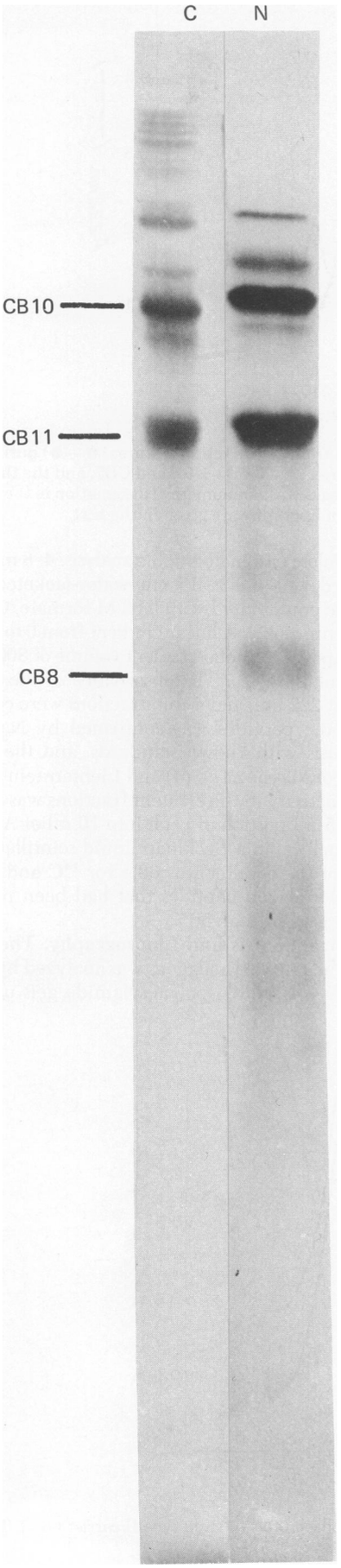



FIG. 3. CM-cellulose chromatogram of cyanogen bromide peptides of neural retina collagen (●—●) and type II carrier (- - -). The radioactive profile closely resembles that of the carrier. The details of chromatography are in the *text*.




Tris-glycine buffer system (8, 9) or a Tris-borate buffer system (10). Cyanogen bromide peptides were analyzed on 12.5% gels. All gels had bisacrylamide/acrylamide ratio of 0.8/30. The gels were prepared as slabs, 14 × 28 × 0.15 cm, topped with a 3% acrylamide stacking gel containing ten 1-cm wide sample wells. The bottoms of the sample wells were approximately 2 cm above the top of the separating gel. Samples contained 10–250 $\mu$g of protein in 50 $\mu$l of sample buffer. The slab was cooled with running tap water at 22 ± 2°, and electrophoresis was initiated under constant current conditions at 8 mA. After the samples had stacked and entered the separation gel, the current was raised to 16 mA and electrophoresis was continued overnight, until the bromphenol blue tracking dye had progressed 20–22 cm into the gel. The slab was then fixed for 4 hr in 30% methanol/10% acetic acid, stained in 0.25% Coomassie blue in 50% methanol/10% acetic acid for 1 hr, and destained in several changes of 10% methanol/10% acetic acid (11).

The radioactively labeled compounds were detected by fluorography (12). For fluorography, the water was removed from the previously stained and photographed gel by soaking in dimethyl sulfoxide, and the entire gel was infiltrated with 4 volumes of 20% 2,5-diphenyloxazole in dimethyl sulfoxide. It was then returned to water, causing the diphenyloxazole to precipitate within the gel, which was dried against Whatman no. 3 filter paper under reduced pressure. RP-Royal X-omat x-ray film presensitized by exposure to a flash of low intensity light (13) was pressed firmly against the gel and exposed at −70° for the time necessary to produce an image. Samples containing 50,000 dpm of $^{3}H$, or 20,000 dpm of $^{14}C$ were processed within 48 hr, but samples with 800 dpm of $^{14}C$ or 20,000 dpm of $^{3}H$ could also be detected by exposures of 1 or 2 weeks. The fluorographic image of the labeled collagen and the stained carrier bands in the dried gel were compared directly to ascertain the positions of labeled materials relative to known proteins.

## RESULTS

### Chain composition of extracted collagen

Fluorographic analysis of $NaDodSO_4$-polyacrylamide gels of denatured and reduced labeled collagen of embryonic neural retina revealed two bands of radioactivity, one of which comigrated with the $\alpha$1 chains of the carrier collagen and a slower band not represented in the carrier (Fig. 1). Only very small amounts of radioactive material migrated with $\alpha$2 chains of the carrier. Collagenase digestion completely eliminated the labeled material migrating with $\alpha$1 chains and that which migrated more slowly, confirming their collagenous nature. The slowly moving labeled band is as yet unidentified, but probably represents a higher molecular weight form of $\alpha$1 chain. These fractions probably represent the major collagen component synthesized under these conditions. No radioactive material with a sufficiently high specific activity for detection appeared trapped in the stacking gel, and pepsin extraction of the insoluble residues yielded about 10% additional collagen consisting of $\alpha$1 chains exclusively.

### CM-cellulose chromatography of cyanogen bromide peptides

Fig. 2 shows the absorbance profile of the cyanogen bromide

FIG. 4. Fluorogram of cyanogen bromide (CB) peptides from collagen prepared from neural retina (N) and cartilage (C) incubated with [$^{14}C$]glycine and [$^{3}H$]proline. The type II peptides from cartilage and the neural retina collagens yield an identical pattern, but the cartilage peptides consistently migrate ahead of those from the retinal collagen.

peptides of type I carrier from chick skin and the radioactive profile obtained from the neural retina collagen.

The radioactive profile for neural retina collagen has three major peaks, the first two eluting between the carrier peaks $\alpha 1$ (I)CB3 + 6a and $\alpha 1$(I)CB7, and the third eluting near $\alpha 1$(I)CB8 (Fig. 2). Note that no labeled material elutes in the region of the $\alpha 2$ peptides, which are the last two carrier peaks. Thus, the labeled peptides do not correspond to $\alpha 1$(I) peptides, demonstrating that this material is neither type I collagen nor the $\alpha 1$ trimer $[\alpha 1(I)]_3$.

Positive identification, by charge distribution, of this collagen with type II (the cartilage type) was made by comparison of the profiles of cyanogen bromide peptides of the labeled neural retina collagen cochromatographed on CM-cellulose with those of cartilage collagen (Fig. 3).

### Size distribution of the cyanogen bromide peptides

Further evidence for the identity of the newly synthesized collagen was obtained from the size distribution of radioactive cyanogen bromide peptides visualized by polyacrylamide gel electrophoresis and fluorography. The band pattern produced by the cyanogen bromide peptides of either type I or type II collagen is unique and distinctive. The distribution of cyanogen bromide peptides of neural retina collagen revealed by fluorography was nearly identical to the pattern from type II collagen (Fig. 4), although the neural retina peptides all migrated somewhat more slowly. No extra peptides not also found in type II preparations were seen.

## DISCUSSION

The collagen produced by the embryonic chick neural retina in culture is apparently identical to the type II collagen found in chick cartilage, as revealed by $\alpha$ chain composition and the size and charge distribution of the major peptides produced by cyanogen bromide cleavage. Thus, they are probably the same gene product.

Whether this molecule differs in more subtle ways in terms of terminal peptide sequences or post-translational changes from the collagen found in cartilage is not apparent from these data. We do not as yet know whether the higher molecular weight form of $\alpha$ chain seen in the neural retina cultures reflects another form of molecule, but the absence of any cyanogen bromide peptide not found in type II collagen suggests that the larger chains are incompletely processed procollagen. We are testing, by limited pepsin digestion, this possibility. This higher molecular weight material is not unique to neural retinas; we find similar chains in both type I and type II collagens made by corneal epithelium in culture (14). We noted slight differences in migration on $NaDodSO_4$ gels of cyanogen bromide peptides from cartilage collagen and the collagen produced by the neural retina. These might simply reflect amount of carrier collagen included in the samples, but alternatively could indicate minor differences in size or in $NaDodSO_4$ binding by the neural retina collagen.

Production of type II collagen is associated with cartilage and with tissues involved in embryonic induction of cartilage. It is characteristic of adult (15) and embryonic cartilages (16, 17), but is also synthesized by embryonic chick notochord (18, 19) and found in the notochord of the adult sturgeon (20) and in the adult mammalian notochordal derivative, the nucleus pulposus (21, 22). The type II collagen produced by the neural retina, in contrast, is probably deposited in the vitreous body as suggested by Newsome *et al.* (3). Confirmation that chick vitreous collagen is type II will require further analysis, but bovine vitreous collagen has many type II characteristics and is clearly related to cartilage collagen (23, 24).

The predominant glycosaminoglycan of the chick vitreous is chondroitin sulfate (Smith and Newsome, in preparation), and if the vitreous collagen is indeed type II, the vitreous of the chicken resembles cartilage in its chemical composition.

Despite the similarities in chemical composition between chick vitreous collagen and that from cartilage, the neural retina has no clear relationship to cartilage. It forms from an outpocketing of the embryonic brain and ultimately differentiates as a neurosensory epithelium. It contains no mesenchymally derived cells and is not directly in contact with any developing cartilages. The production of type II collagen by this tissue and our recent finding that isolated embryonic corneal epithelium synthesizes both type I and type II collagen (14) suggest that the production of type II collagen is more unbiquitous than previously suspected. Thus, in the embryo at least, type II collagen must have additional functions not related to cartilage.

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