VITREOUS BODY COLLAGEN

Evidence for a Dual Origin from the Neural Retina and Hyalocytes

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ABSTRACT

Two different cell types have been shown to synthesize embryonic chick vitreous collagen (vitrosin) at different stages of development. Identification of vitrosin was established by labeling the embryos *in ovo* with [^{3}H]proline at stages 23 and 28 and separating the extracted vitreous collagen α-chains by carboxymethylcellulose chromatography. The labeled collagen consisted predominately of $\alpha 1$ chains, indicating a molecule in the form of a trimer of identical chains designated $(\alpha 1)_3$. The molecular weight of the labeled chains measured approximately 95,000 daltons by molecular sieve chromatography, and contained 41% of their imino acid as 4-hydroxyproline. To establish which eye tissues synthesize vitrosin, the collagens produced in organ culture by the isolated neural retina, lens and vitreous body from stages 26–27, 29–30, and 40 were examined. At the two earlier stages, only the neural retina synthesized large quantities of $(\alpha 1)_3$ collagen whereas the lens and the cells within the vitreous body itself synthesized relatively small amounts of collagen characterized by an $\alpha 1{:}\alpha 2$ ratio of about 2:1. At stage 40, however, the cells of the vitreous body itself synthesized the greatest quantities of collagen, which now was predominantly an $(\alpha 1)_3$ type molecule. Stage 40 neural retina and lens synthesized lesser amounts of collagen with an $\alpha 1{:}\alpha 2$ ratio of 2 to 3:1. Chick vitrosin thus appears to be synthesized by the neural retina in early embryonic stages, whereas the major contribution derives from cells within the vitreous body in later development.

The normal spherical expansion of the avian eye during ocular morphogenesis appears to require the synthesis of the vitreous humor (4). The macromolecular constituents of this structure, largest of the ocular components, have been partially characterized in several adult organisms and shown to include a form of collagen(s) originally called vitrosin (8), and glycosaminoglycans (1). The source(s) of these extracellular matrix components has not been clearly established, although the likely tissues including lens, neural retina, pigmented epithelium, and hyalocytes (cells within

the vitreous itself) have been suggested (see review by Duke-Elder and Cook [6]).

The particular type of collagen within the vitreous has not been rigorously established, but two recent studies of vitrosin from adult organisms have indicated an α-chain distribution of the $(\alpha 1)_3$ type (21, 26). Swann et al. (21) have further suggested from amino acid analysis that this molecule is closely related to the Type II collagen, characteristic of cartilage.

To determine the tissue responsible for the production of vitrosin, we labeled *in ovo* the vitreous collagen synthesized at several stages of embryonic development, established its chain distribution, and subsequently analyzed the collagen synthesized during in vitro labeling of the separated, constituent eye tissues from correspondingly staged series of embryos.

MATERIALS AND METHODS

White Leghorn chick embryos were incubated at 38°C and staged according to Hamburger and Hamilton (9).

For *in ovo* labeling, embryonated eggs (either 4 or 6 days of incubation) were injected through the air sac with 20 μCi of [^{3}H]proline (NET-323) plus 20 μCi of [^{3}H]lysine (Schwarz-Mann Div. Becton, Dickinson & Co., Orangeburg, N. Y.) per embryo, and β-aminopropionitrile (βAPN) (12) (0.05 mg/embryo at 4 days and 0.4 mg/embryo at 6 days). The eggs were returned to the incubator for an additional 48 h, at which time the vitreous bodies were removed from the embryos by dissection and cleaned of any adhering foreign tissue.

The vitreous bodies, plus 5–10 mg of previously purified 3-wk old chick skin collagen for carrier, were extracted for 24 h at 4°C by gentle stirring in $\Gamma/2$ 0.4 PO_4 buffer, pH 7.6. The extract was clarified by centrifugation and the collagen in the supernate precipitated by dialysis against several changes of 0.01 M Na_2HPO_4. The collagen was dissolved in 0.5 M acetic acid and reprecipitated by the addition of solid NaCl to a concentration of 10%. This procedure was repeated, and the purified collagen was redissolved in 0.5 M acetic acid, then dialyzed exhaustively against dilute acetic acid.

The insoluble pellets remaining after the original extraction were dialyzed against 0.5 M acetic acid and digested with 0.1% pepsin at pH 2.5 for 24 h at 4°C, then clarified by centrifugation, neutralized to inactivate peptic activity, and the soluble collagens were precipitated, along with added carrier collagen, by dialysis against several changes of 0.01 M Na_2HPO_4. The collagen was dissolved in 0.5 M acetic acid and reprecipitated by the addition of NaCl to 20%. The precipitate was solubilized in 0.5 M acetic acid, dialyzed against dilute acid, and processed as described for the other collagen preparations. Recovery of incorporated proline was determined by measuring the radioactivity in the initial neutral extraction mixture and at each subsequent step in the purification, as well as determining the relative amounts of hydroxyproline present by split stream amino acid analysis, described below.

For in vitro labeling, the lens, neural retina, pecten oculi if present, and vitreous bodies were dissected from approximately one dozen eyes, the separated tissues pooled and incubated at 38°C overnight in 2.0 ml of Dulbecco-modified Eagle's medium supplemented with 25 μC/ml [^{3}H]proline, 100 μg/ml ascorbic acid and 50 μg/ml βAPN. The labeled cultures, both tissues and medium together, were extracted and purified along with added carrier collagen in the same manner as for the *in ovo*-labeled material.

Analysis of Synthesized Collagens

Carboxymethylcellulose (CM-cellulose) chromatography of the purified material was done according to Piez et al. (19), modified to use a starting buffer of 0.02 M Na acetate pH 4.8 containing 1 M urea. The radioactive collagen samples were dialyzed from dilute acetic acid against starting buffer for 1–2 h, denatured by heating at 50°C for 20 min then loaded onto 0.9 × 5 cm columns of CM-cellulose maintained at 42°C. The column was eluted with a superimposed linear gradient of 0–0.13 M NaCl (total gradient volume, 200 ml), and the effluent was continuously monitored at 228 or 230 nm with a Gilford recording spectrophotometer equipped with a flow cell. 5-min fractions were collected. An average of 64% of the loaded sample was eluted by the gradient as determined by measuring the amount of radioactivity loaded onto the column, the amount eluted by the gradient, and the amount eluted by washing the column with 50 ml of 1 M NaCl in 0.05 M NaOH. The CM-cellulose elution profile of the carrier collagen was highly reproducible from experiment to experiment.

Molecular weight was estimated by gel filtration on a 100 × 2.0 cm column of 8% Agarose (BioGel 1.5 M), equilibrated with 1 M $CaCl_2$ and 0.01 M Tris-HCl pH 7.5 (18).

The distribution of radioactivity between proline and 3- and 4-hydroxyproline was determined on the isolated α-chains after hydrolysis in constant boiling 6 N HCl at 108°C for 24 h under nitrogen. The amino acids were separated on a Jeolco model 5AH amino acid analyzer (10) from which a portion of the column effluent was diverted by stream splitting for radioactivity measurement.

In all analytical procedures, radioactivity was determined by mixing a portion of each effluent fraction (either 1.0 or 0.5 ml) with 10 ml of Aquasol (New England Nuclear Corp., Boston, Mass.), and counting in a Beckman liquid scintillation counter (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) with a tritium counting efficiency of 60%.

RESULTS

In ovo Labeling of Vitreous Collagen

In vitreouses from 6.5 to 8-day embryos, approximately 27% of the nondialyzable [^{3}H]proline was present as hydroxyproline, which indicates that approximately 50% of the labeled proline went into collagen. Neutral phosphate extraction and subsequent purification recovered 50% of the labeled collagen.

In several experiments, the insoluble pellet present after the original phosphate extraction was digested with pepsin, which degrades noncollagenous material and solubilizes additional collagen, presumably by removing the crosslinking peptides in the nonhelical terminal portions of the molecules (20, 16). The pepsin treatment recovered an additional 32% of the labeled collagen.

The α-chain composition of embryonic vitreous collagen labeled *in ovo* was determined by CM-cellulose chromatography. The chromatogram of labeled, neutral salt-extracted material deposited in the vitreous from stages 28 to 34 (days 6–8 of incubation) is shown in Fig. 1 and consists predominantly of a single, asymmetrical peak in the α1 chain region with only a small amount of material eluting in the region of the α2 chain (Fig. 1 and Table I). This indicates that the predominant collagen molecule in the vitreous consists of three α1 chains, $(\alpha 1)_3$. The labeled α1 chains eluted slightly earlier than the carrier α1 chains. The CM-cellulose chromatograms of the material purified from vitreous bodies labeled *in ovo* from stages 23 to 28 (4–6 days) were identical to those labeled during the stages 28–34 period except that the younger embryos incorporated a smaller amount of isotope into collagen. When analyzed by CM-cellulose chromatography, the radioactive profile of the pepsin-solubilized material had an α-chain distribution identical with that of the labeled collagen extracted in neutral phosphate, i.e. consisting almost entirely of α1 chains. Amino acid analysis of both neutral phosphate and pepsin-extracted α1 chains revealed that 41% of the incorporated proline was present as 4-transhydroxyproline; 3-hydroxyproline was either undetectable or present in amounts less than 1%.

The molecular weight of the *in ovo* labeled vitreous collagen α-chain as measured by molecular seive chromatography was approximately 95,000 daltons (Fig. 2).

Origin of Vitreous Collagen: In Vitro Labeling of Eye Tissues

As schematically diagrammed in Fig. 3, the anatomy of the eye suggests three likely possibilities for the source of the $(\alpha 1)_3$ vitreous collagen: (*a*) the cells of the vitreous itself; (*b*) the neural retina, which borders the vitreous body posteriorly; (*c*) the lens, which borders the vitreous body anteriorly.

We examined in vitro collagen synthesis by separated ocular tissues at three different stages of development: (*a*) stages 26–27, the earliest time at which we could obtain a readily dissectable organized vitreous gel; (*b*) stages 29–30, a period of rapid increase in size of the vitreous body, and (*c*) stage 40, a time when the rate of increase in size of the vitreous had markedly slowed. Tripli-

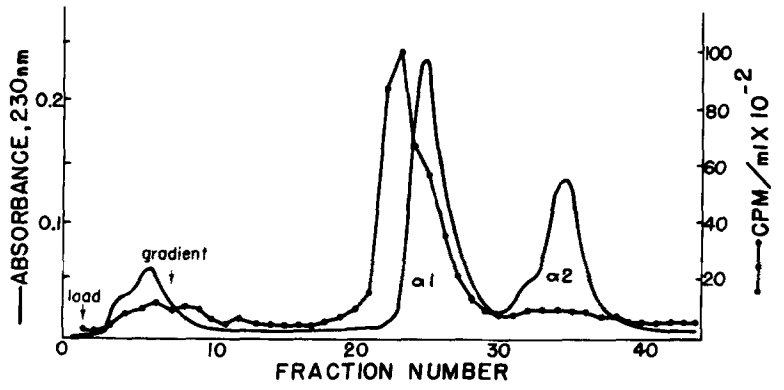

FIGURE 1 CM-cellulose chromatogram of the ^{3}H-proline-labeled, neutral salt-extractable collagen deposited *in ovo* within the vitreous body between stages 28 and 32 (dotted line) and skin collagen carrier from 3-wk old lathyritic chicks (solid line).

cate or quadruplicate experiments with replicate preparations in each were performed for each stage.

STAGE 29-30: Typical CM-cellulose chromatograms obtained from the purified radioactive collagens produced by the separated ocular tissues pooled from 12 stage 29-30 embryos are shown in Figs. 4-6. The neural retina at this stage produced 10-15 times more neutral salt-extractable collagen than either the lens or the vitreous body itself (Table II). The neural retinal collagen eluted from CM-cellulose columns predominately as a single peak in the $\alpha 1$ region, with only a small amount of material in the $\alpha 2$ position (Fig. 4). The $\alpha 1$ chains deposited in the vitreous bodies of stage 29-30 animals and those synthesized in vitro by stage 29-30 neural retinas eluted slightly ahead of the carrier $\alpha 1$, and each peak had a small shoulder that

TABLE I

$\alpha 1$:$\alpha 2$ Ratios of Neutral Phosphate and Pepsin‡ Extracts of Collagen Synthesized and Deposited In Ovo within the Vitreous Body (Vitreous Collagen)*

Stage	Material	$\alpha 1$:$\alpha 2$	Recovery
			%
24-29	Neutral extract	60:1	50
29-34	Neutral extract	130:1	
24-29	Pepsin extract	20:1	32
29-34	Pepsin extract	30:1	

* Ratios of total radioactivity minus estimated background in the $\alpha 1$ chain peak compared to the $\alpha 2$ chain peak from CM-cellulose chromatograms.

‡ Pepsin extracts of the insoluble pellet remaining after $\Gamma/2 = 0.4$ PO_4 extraction of whole, cleaned vitreous bodies.

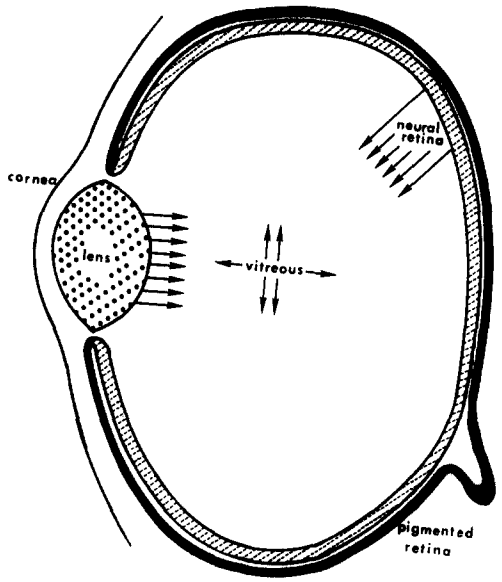

FIGURE 3 This schematic diagram of an embryonic chick eye emphasizes that on an anatomical basis the most likely sources of vitreous body collagen are the lens, neural retina, and cells of the vitreous itself.

may represent a somewhat heterogeneous composition (Figs. 4 and 6). These chromatograms were very similar to those of the vitreous body collagen isolated from the *in ovo* labeled embryos (Fig. 1), and the amino acid analysis (radioactivity) showed that about 41% of the incorporated proline was present as 4-transhydroxyproline, almost identical with that of bonafide *in ovo* labeled vitrosin.

The in vitro labeled lenses and vitreous bodies themselves produced less than 0.1 the amount of neutral salt-extractable collagen than was produced in vitro by the neural retinas from the same

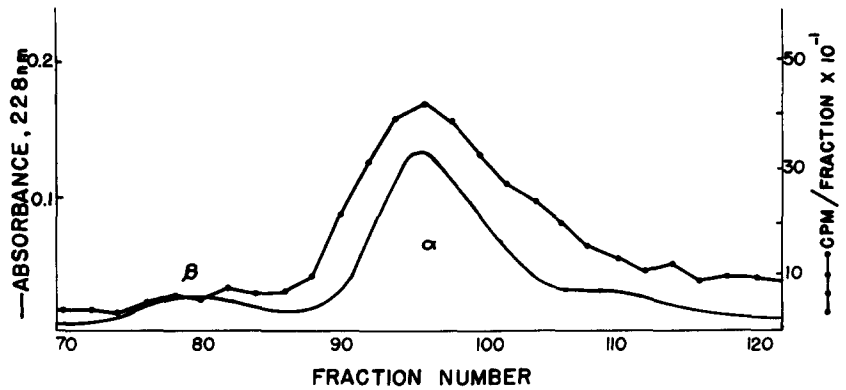

FIGURE 2 Elution pattern from a calibrated 8% Agarose column of the $\alpha 1$ peak (fractions 20 to 30) of Fig. 1. (Absorbance: solid line; radioactivity: dotted line).

eyes. CM-cellulose chromatograms of the collagens produced by these two tissues always showed considerable amounts of labeled material eluting in the $\alpha 2$ region seen in Figs. 5 (lens) and 6 (vitreous). In these chromatograms, the $\alpha 2$ chain peak was usually broad, and there was often a considerable amount of background radioactive material eluting in this latter $\alpha 2$ part of the chromatogram. It was possible, however, by direct assay of radioactivity in hydroxyproline to determine that the amounts of collagen eluting as $\alpha 2$ chains were clearly higher than that of the labeled collagen deposited *in ovo* in the vitreous body (Table II) and greater than that produced by neural retina. Thus, due to the small amount of collagen synthesized and the relatively considerable amounts of $\alpha 2$ chain present in the CM-cellulose chromatograms, it seems likely that neither the lens nor the cells of the vitreous body itself are responsible for synthesizing the major proportion of the collagen found in the vitreous body at this stage.

TABLE II

*Relative Synthesis In Vitro of α1 and α2 Collagen Chains**

Stage	Tissue	Total α1 counts (× 10^{-2})	Total α2 counts (× 10^{-2})	α1:α2
26	Lens	4.7	2.0	2.4:1
	Vitreous Body	3.5	1.8	2:1
	Neural Retina	40.0	4.0	10:1
29	Lens	4.5	1.9	2.4:1
	Vitreous Body	1.0	0.45	2.2:1
	Neural Retina	83.0	0.3	277:1
30	Lens	35.0	19.0	1.8:1
	Vitreous Body	29.0	15.0	1.9:1
	Neural Retina	220.0	1.5	147:1
40	Lens	15.0	10.0	1.5:1
	Vitreous without Pecten	61.0	1.0	61:1
	Pecten	200.0	10.0	20:1
	Neural Retina	55.0	15.0	3.7:1

* Total radioactivity (counts per minute) minus estimated background in the α1 chain and α2 chain peaks from CM-cellulose chromatograms. Ocular tissues from the same group of eyes at various stages of development. Results were consistent in either duplicate or triplicate experiments.

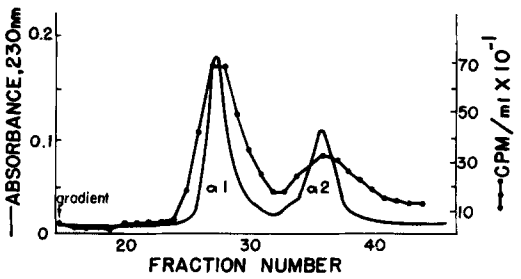

FIGURE 5 CM-cellulose chromatogram of the radioactively labeled collagen synthesized in organ culture by the vitreous bodies from the same stage 29-30 eyes that were the source of the neural retinas in Fig. 4 (dotted line) and skin collagen carrier (solid line).

STAGE 26-27: From the earliest time at which we could obtain a readily dissectible vitreous gel (stage 26-27), the results were largely the same as were found for the stage 29-30 embryos. Representative CM-cellulose chromatograms of the purified radioactive material synthesized in vitro by the separated lenses, vitreous bodies and neural retinas from one dozen stage 26 embryos are shown in Fig. 7. At this earlier stage, the lens and vitreous body produced less than 0.05 the amount of neutral salt-extractable collagen made

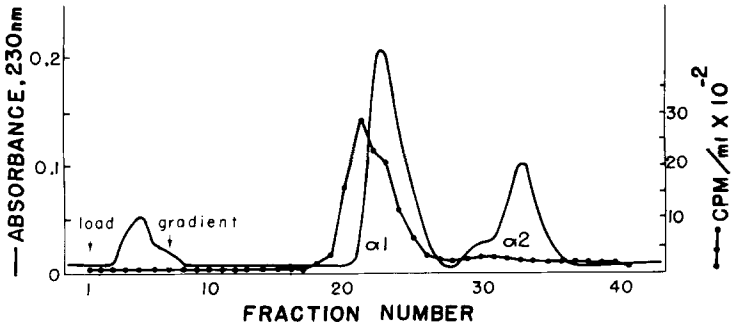

FIGURE 4 CM-cellulose chromatogram of the radioactively labeled collagen synthesized in organ culture by stage 29-30 neural retinas (dotted line) and skin collagen carrier (solid line).

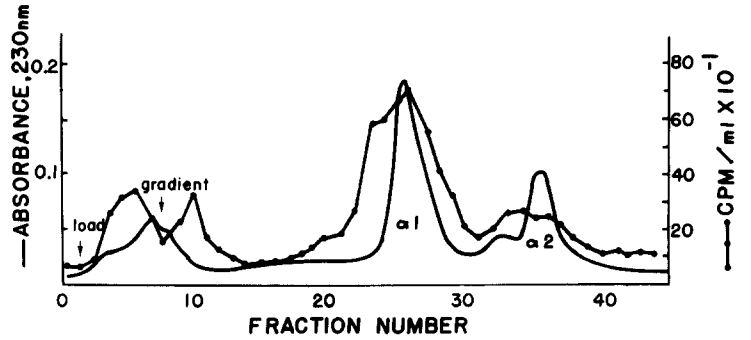

FIGURE 6 CM-cellulose chromatogram of the radioactively labeled collagen synthesized in organ culture by the lenses from the same stage 29-30 eyes that provided the neural retinas and vitreous bodies of Fig. 4 and 5 (dotted line) and skin carrier collagen (solid line).

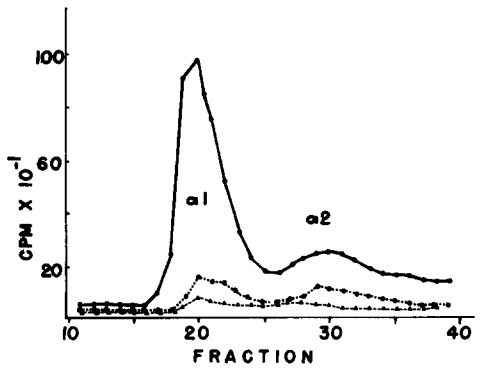

FIGURE 7 CM-cellulose chromatograms of radioactively labeled collagen synthesized in organ cultures by the neural retinas (solid line), vitreous bodies (dotted line) and lenses (dashes) from the same stage 26-27 eyes.

by the neural retina, an even greater differential than had been found for the stage 29-30 tissues. In fact, the amounts of collagen produced by both the cells of the vitreous body itself and the lens were so small that it was difficult to establish an accurate $\alpha 1{:}\alpha 2$ ratio (cpm in $\alpha 1$ chain region $<2\times$ background; see Fig. 7), although the chromatographic pattern suggests the presence of both $\alpha 1$ and $\alpha 2$ chains. The stage 26 retina at this early stage was already producing large amounts of collagen which, from the relative amounts of radioactivity in the $\alpha 1$ and $\alpha 2$ chain peaks (Fig. 7), is predominately an $(\alpha 1)_3$ type molecule ($\alpha 1{:}\alpha 2$ = 11). It should be noted, however, that the amount of radioactive material in the $\alpha 2$ chain region of the chromatogram was somewhat greater than was seen in the chromatograms of neural retina cultures from the stage 29-30 and also stage 40 embryos (compare Figs. 7 and 8; also Table II).

STAGE 40: Eye tissues removed from 14-day, stage-40, embryos were cultured and their products analyzed at a time when the growth of the vitreous had markedly slowed. At this stage, we noted a significant shift in the tissue synthesizing vitrosin. Representative CM-cellulose chromatograms of the purified collagen synthesized in vitro by the separated pooled tissues from two dozen stage-40 eyes are depicted in Fig. 8. In contrast to the situation at earlier stages, it now appears that the major source of $(\alpha 1)_3$ collagen is the vitreous itself ($\alpha 1/\alpha 2$ = 20) (Table II). If the pecten oculi (in avian eyes, the pleated lamina of vascular and pigmented tissue which lies ventrally in the eye surrounded by the vitreous gel) was removed, the cells remaining within the vitreous, presumably only the hyalocytes, synthesized a collagen almost exclusively consisting of $\alpha 1$ chains

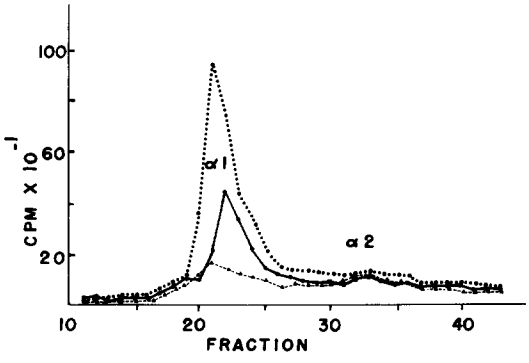

FIGURE 8 CM-cellulose chromatograms of radioactively labeled collagen synthesized in vitro by the neural retinas (solid line), vitreous bodies (dotted line), and lenses (dashes) from the same stage-40 eyes.

(Table II). The neural retina still synthesized collagen, but the $\alpha 1$ to $\alpha 2$ ratio had become 3 to 1 and the total amount synthesized was only about 0.2 that synthesized by the vitreous body (Fig. 8 and Table II). The lenses from the same eyes synthesized a relatively small amount of neutral extractable collagen with an $\alpha 1$ to $\alpha 2$ ratio of 1.5 to 2.

DISCUSSION

These *in ovo* and in vitro labeling experiments confirm earlier observations that vitrosin consists principally of $\alpha 1$ chains (21, 26) and further suggest that there may be several stage-specific and tissue-specific origins for this collagen—first the neural retina, and subsequently the vitreous body hyalocytes. The vitreous collagen $\alpha 1$ chains chromatograph on CM-cellulose in a position slightly ahead of the carrier $\alpha 1$ type I chains. The explanation of this finding is unknown. However, the chromatographic position of the vitreous $\alpha 1$ chains is also similar to that of the $\alpha 1$ type II chain from cartilage and the $\alpha 1$ type IV chain from basement membranes. It is not in the position of the type III chain which elutes slightly before $\alpha 2$ (2, 7, 23). No naturally occurring collagen species with $[\alpha_1(I)]_3$ composition has yet been described, although Mayne et al. (15) have reported the appearance of an $[\alpha 1(I)]_3$, type I trimer, collagen in cultures of chick chondrocytes following treatment with 5-bromo-2′-deoxyuridine. Thus, our data would initially suggest that the vitreous collagen is either type II, type IV, the type I trimer, or another genetically distinct collagen type.

The percent of radioactive proline residues converted to 4-hydroxyproline (41%), and the very small amount or absence of 3-hydroxyproline in the labeled vitreous $\alpha 1$ chain, are consistent with the hydroxyproline content in type II (24), but substantially less than the 55 to 60% 4-hydroxyproline and 19% 3-hydroxyproline found in type IV (11, 23), suggesting a closer identity with $\alpha 1$ type II. Neither Swann et al. (21) nor Trelstad and Kang (26) excluded the possibility that their vitreous collagens might represent either another genetically unique species of α-chain or a mixture of $\alpha 1$ chains, since a small amount of $\alpha 2$ was present. This rigorous identification of the $(\alpha 1)_3$ molecule present in the vitreous body has not yet been accomplished by criteria such as CNBr peptide maps.

Since we could recover, by combined neutral and pepsin extraction, over 80% of the collagen from *in ovo*-labeled vitreouses, it seems unlikely that we would have missed a significant component of vitreous collagen. The fact that the chromatographic profiles of the pepsin-solubilized collagen from vitreous residues were quite similar to those obtained from the initial neutral extracts indicates that the neutral soluble collagen is probably representative of the total vitreous collagen. This is important since type III and IV collagens which are solubilized by pepsin (23) can be overlooked in lathyritic tissues.

Our data demonstrate collagen synthesis by the embryonic chick neural retina, a tissue not usually associated with the production of connective tissue macromolecules. It is not surprising that this neuroepithelium should be synthesizing collagen, however, since other embryonic epithelia including the notochord (13), spinal cord (3, 25), anterior corneal epithelium (22, 5), and retinal pigmented epithelium (17) have all recently been shown to produce collagen. It seems, from our results, that the earliest (stage 26) neural retina tested was producing a mixture of at least two types of collagen, a predominant one of an $(\alpha 1)_3$ composition and a minor component with an $(\alpha 1)_2\alpha 2$ composition. Older neural retinas (stages 29 and 30) produced collagen with a much reduced amount of $\alpha 2$-containing species in comparison to the amount of $(\alpha 1)_3$.

Over the entire rapid growth period of the eye, the neural retina in vitro produced at least 10-fold more collagen than did the vitreous body or lens. In the light of other studies on lenses from mature animals (11), we might have expected to find a predominance of $\alpha 1$ chain from the type IV collagen deposited within the embryonic lens capsule. However, either our extraction procedure or the embryonic stages used could account for this discrepancy. Other work has shown that the chick embryo retinal pigmented epithelium produces an $(\alpha 1)_2\alpha 2$ type collagen.[1] Thus, this eye tissue also produces a collagen but one unlike that found in the vitreous.

Although we cannot entirely eliminate some contribution from the lens and vitreous body cells, using the criteria of the relative amount of collagen produced, the α-chain distribution and degree of hydroxylation of proline, we conclude that the neural retina is the chief source of vitreous body

[1] Newsome, D. A., and R. L. Trelstad. Unpublished observations.

collagen during early vitreous development. In a relative sense, the neural retina slows down its production of collagen some time between stages 34 and 40; concomitantly, the vitreous cells themselves appear to assume the synthesis of this protein during later development.

An intriguing question raised by this study is whether the $(\alpha 1)_3$ collagen produced later by the vitreous cells is the same as that made earlier by the neural retina. More discriminating criteria are required. In other developing organs such as the chick limb, a transition in the different types of collagens has been clearly documented (14). What we have described here for the vitreous may be a developmental transition in the tissue of origin, but perhaps for the same type of collagen. It is also possible that the vitreous cells, hyalocytes, whose origin has not been clearly established, represent a population of cells that have emigrated from the neural retina. The presence of varying amounts of $\alpha 2$ chains, in some preparations probably representing a type I collagen, may reflect cell heterogeneity and shifting populations with progressive stages of development.

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