# SYNTHESIS OF TYPE III COLLAGEN BY EMBRYONIC CHICK SKIN

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Summary. Analysis of cyanogen bromide peptides from the $\alpha_1$ chains synthesized by lathyritic embryonic chick skin during short-term tissue culture revealed significant levels of both Types I and III collagen prior to thirteen days of development, but mainly Type I collagen thereafter. The elevated ratio of $\alpha_1$ to $\alpha_2$ polypeptide chain synthesis during the period of maximal Type III production supports the proposed $[\alpha_1(III)]_3$ molecular structure for Type III collagen. Maximal synthesis of Type III collagen occurs during a period when collagen production is apparently necessary for normal dermal and hence normal ectodermal development.

## Introduction

Initial studies to characterize skin collagen revealed only Type I collagen which contains two $\alpha_1(I)$ chains for each $\alpha_2$ chain -- $[\alpha_1(I)]_2\alpha_2$ (1,2,3,4,5). More recently, CNBr peptides from insoluble bovine (6,7) and human skin (8) collagen were found which differed from $\alpha_1(I)$ CNBr peptides principally in the $\alpha_1$CB3 and $\alpha_1$CB4,5 peptides. As these CNBr peptides also differed from $\alpha_1(II)$ CNBr peptides from cartilage, it represents a third genetically distinct $\alpha_1$ chain and was referred to as $\alpha_1(III)$. Miller *et al.* (8) proposed on the basis of the relative amounts of $\alpha_1$ and $\alpha_2$ CNBr peptides that Type III collagen contained only $\alpha_1(III)$ chains -- $[\alpha_1(III)]_3$ .

We sought to determine if chick skin also contained Type III collagen and whether it was produced preferentially during any specific time of embryonic development. Analysis of lathyritic chick skin collagen labelled

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with [$^3$H]-proline during short term organ culture demonstrated synthesis of Type III collagen. This Type III collagen synthesis by chick skin was largely restricted from 7 to 12 days of embryonic development, during which time it represented as much as 60% of total skin collagen synthesis. The elevated ratio of $\alpha_1$ to $\alpha_2$ chains synthesized during the period of maximal Type III collagen synthesis supports the proposed $[\alpha_1(III)]_3$ structure for Type III collagen.

## Methods and Materials

Sterilly excised back and belly embryonic chick skin of various ages was maintained in F-10 medium plus 10% horse serum (GIBCO), 64 µg/ml β-amino-proprionitrile (BAPN, Aldrich), and 150 µg/ml sodium ascorbate (Sigma) in 5% $CO_2$, 95% air at 37°C. After an hour preincubation, 200 µc/ml of [2,3-$^3$H] L- proline (New England Nuclear Corp., 50 ci/mm) was added. The skin was removed after 18 hours incubation, washed twice with saline, and extracted for three days in 1 M NaCl (pH 7.0) at 4°C. All labelled collagen was salt soluble and no detectable collagen was found in the medium.

Salt soluble collagen together with 20 mg lathyritic chick collagen carrier was dialyzed against 0.03 M sodium acetate buffer (pH 4.8) in 1.3 M urea and then chromatographed on a 0.9 x 10 cm carboxymethyl cellulose column (42°C) at a flow rate of 5 ml/minute. Collagen was eluted using a 0 to 0.1 M NaCl gradient (250 ml total volume) in 0.03 M sodium acetate buffer (pH 4.8) and 1.3 M urea. The elution positions of $\alpha_1$ and $\alpha_2$ chains from the carrier collagen were determined by monitoring at OD 230 mµ. Radioactivity was measured from an aliquot of each tube and $\alpha_1$ and $\alpha_2$ chain fractions pooled for further analysis.

The $\alpha_1$ and $\alpha_2$ polypeptide chains were cleaved separately with cyanogen bromide (CNBr) together with 30 mg chick skin collagen in 70% formic acid according to the procedure of Miller *et al.* (8). Peptides, following desalting on a Bio-Gel P-2 column, were chromatographed on a 0.9 x 10 cm CM-52

(Whatman) column at 42°C and eluted with a gradient of 0.015 to 0.15 M NaCl (500 ml total volume) in 0.02 M sodium citrate (pH 3.6) which accentuated separation of $\alpha_1$CB3(I) and $\alpha_1$CB3(III) (from insoluble chick skin collagen). Radioactivity was measured using an aliquot of each fraction. The elution position of labelled $\alpha_1$CB3(III) was determined by co-chromatography of soluble and insoluble skin collagen CNBr peptides.

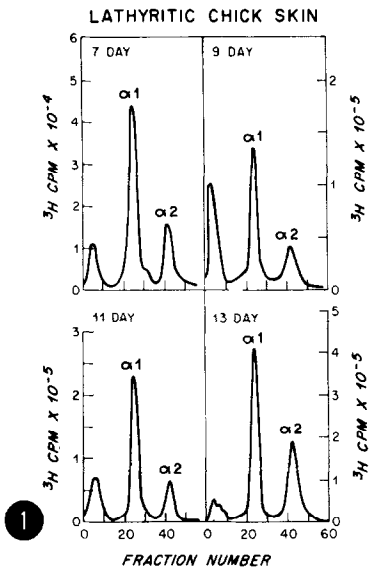
The $\alpha_1$ chains were chromatographed on DEAE-cellulose (Whatman, DE-52) according to the procedure of Trelstad et al. (9) except that all solutions contained 1.3 M urea. Soluble chick skin $\alpha_1$ chains were used as standard $\alpha_1$(I) chains and pepsin solubilized chick sternum $\alpha_1$ chains were used as standard $\alpha_1$(II) chains.

The $\alpha_1$ and $\alpha_2$ chains were also identified by electrophoresis on 4.5% acrylamide gels in 0.1% SDS according to the procedure of Weber and Osborn (10) except that freshly filtered urea (6 M) was added. Gels were sliced into 1 mm fractions with a Gilson Gel fractionator and radioactivity measured after dissolution in Protosol (New England Nuclear Corp.).

## Results

Lathyritic chick skin showed a pronounced age dependency in the ratio of $\alpha_1$ to $\alpha_2$ polypeptide chain synthesis as noted by CM cellulose chromatography (fig. 1). The embryonic chick skin characteristically produced the following ratios: 2.7 $\alpha_1$ chains per $\alpha_2$ chain at seven days, 3.7 $\alpha_1$ per $\alpha_2$ for 9 to 12 days, and 2.0 to 2.2 $\alpha_1$ per $\alpha_2$ for skin 13 days or older (fig. 2). The CM-cellulose isolated $\alpha$ chains were of the same size as normal $\alpha$ chains and were collagenase sensitive (fig. 3). Attempts to separate $\alpha_1$(I) and $\alpha_1$(III) chains by chromatography on DEAE cellulose according to the procedure of Trelstad et al. (9) for separation of $\alpha_1$(I) and $\alpha_1$(III) chains were unsuccessful as both types co-eluted (not shown).

Analysis of CNBr peptides from $\alpha_1$ chains revealed that 11-day embryonic chick skin made both Type I and Type III collagen. This was readily evident



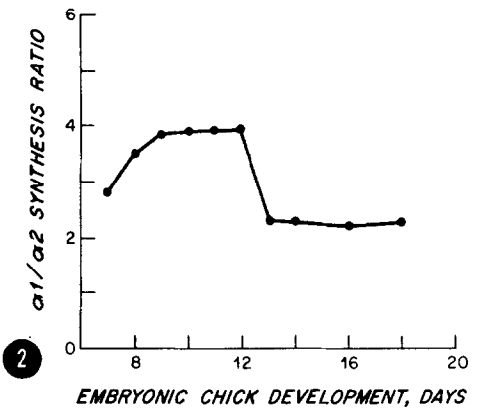




Figure 1. Salt soluble lathyritic collagen isolated from 7 to 18 day embryonic chick skin labelled with [$^3$H]-proline was chromatographed on CM cellulose at 42°C as described in Methods and Materials. The elution of labelled proteins was compared to the elution of carrier chick skin collagen $\alpha_1$ and $\alpha_2$ polypeptide chains, which are indicated as $\alpha_1$ and $\alpha_2$ respectively. Four representative chromatographs are presented here.

Figure 2. The ratio of $\alpha_1$ to $\alpha_2$ polypeptide chains synthesized by chick skin from 7 to 18 days of embryonic development.

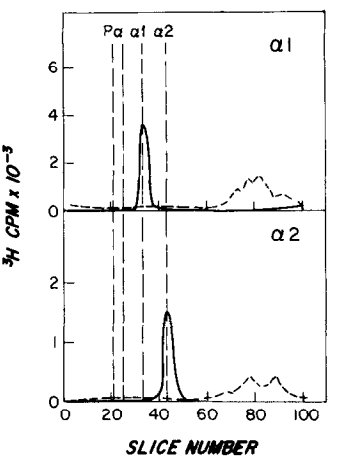



Figure 3. The $\alpha_1$ and $\alpha_2$ polypeptide chains isolated by CM-cellulose chromatography were electrophoresed on 4.5% acrylamide gels in 0.1% SDS and 6 M urea at 8 ma/gel for 6 hours according to the procedure of Weber and Osborn (10). Half of each sample was first digested in 5 mg of bacterial collagenase (3 hours, 37°C, 0.05 M $Ca^{+2}$, pH 7.0) prior to preparation for electrophoresis. Gels were sliced into 1 mm fractions and radioactivity measured. The co-electrophoresis of [$^{14}$C]-L-proline labelled embryonic chick calvaria procollagen (Pα, 125,000 daltons) and α chains (α, 95,000 daltons) were used to calibrate the gels.

after identification of labelled $\alpha_1$CB3(I) and $\alpha_1$CB3(III) by chromatography on CM cellulose at pH 3.6 (fig. 4). The CNBr peptides from 18-day skin $\alpha_1$ chains contained labelled $\alpha_1$CB3(I) but not $\alpha_1$CB3(III) (fig. 4).

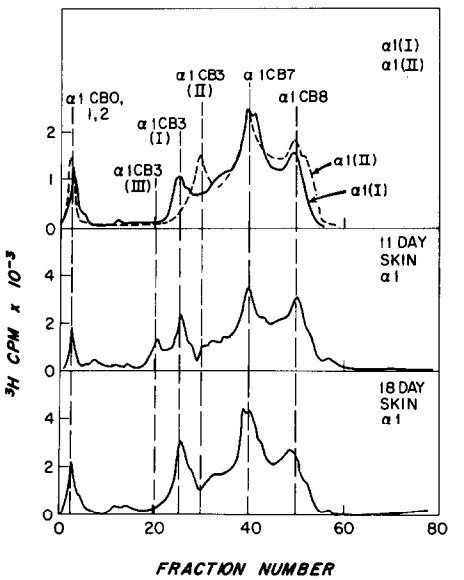



Figure 4. Labelled $\alpha_1$ polypeptide chains isolated by CM-cellulose chromatography of 11 and 18 day embryonic chick skin were digested with CNBr in 70% formic acid. The peptides were identified by chromatography on CM cellulose as described in Methods and Materials. The chromatography of isolated labelled $\alpha_1$(I) (solid line) and $\alpha_1$(II) (dashed line) CNBr peptides are also shown in the upper panel, 11-day skin $\alpha_1$CNBr peptides in the middle panel, and 18-day skin $\alpha_1$CNBr peptides in the lower panel. The elution position of different peptides are indicated by vertical dashed lines. The elution position of $\alpha_1$CB3(III) was determined by chromatography of CNBr peptides of insoluble chick skin collagen.

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Both $\alpha_1$CB3(I) and $\alpha_1$CB3(III) were the correct size ($\sim$ 13,000 daltons) and chromatographed on phospho-cellulose and CM cellulose at pH 4.8 as expected for $\alpha_1$CB3 peptides. As indicated in fig. 4, the CNBr peptide from $\alpha_1$(II) chains analogous to $\alpha_1$CB3(I) and $\alpha_1$CB3(III) peptides (referred to as $\alpha_1$CB3(II) for reasons of simplicity) elutes later than $\alpha_1$CB3(I) and hence is readily distinguishable from $\alpha_1$CB3(I) and $\alpha_1$CB3(III). Thus skin makes only Types I and III collagen. Formic acid alone, while causing slight degradation of $\alpha$ chains, failed to produce $\alpha_1$CB3(I), $\alpha_1$CB3(III), or any other recognizable

CNBr peptides eluting in this region. The CNBr peptides of $\alpha_2$ chains ($\alpha_2$CB1, $\alpha_2$CB2, $\alpha_2$CB3, $\alpha_2$CB4, $\alpha_2$CB5) from embryonic chick skin were homologous to those from embryonic chick tendon, calvaria, or cartilage (not shown).

## Discussion

Embryonic chick skin synthesizes Type III collagen at 11 days of development but not at 18 days. The elevated ratio of $\alpha_1$ to $\alpha_2$ polypeptide chain synthesis by embryonic chick skin from 7 through 12 days of development agrees with the proposed $[\alpha_1(III)]_3$ molecular structure for Type III collagen (8) as compared to $[\alpha_1(I)]_2\alpha_2(1)$ for Type I collagen. Assuming that the proposed Type III structure is correct, the relative synthesis of Types I and III collagen by embryonic chick skin of different ages can be estimated by the ratio of $\alpha_1$ to $\alpha_2$ chains (fig. 5). Thus, embryonic chick skin produces 60% Type I

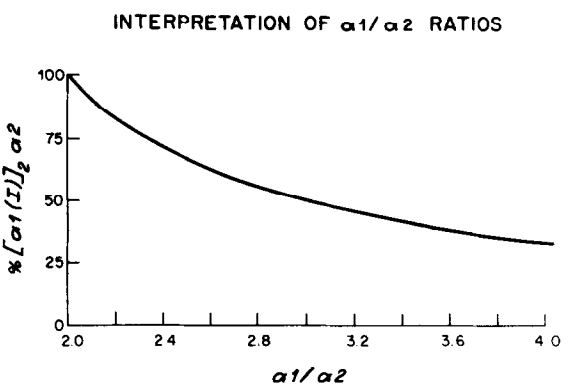


Figure 5. The ratio of $\alpha_1$ to $\alpha_2$ polypeptide chain synthesis was interpreted as synthesis of Type I, $[\alpha_1(I)]_2\alpha_2$ or Type III collagen, assuming that Type III collagen consists of only $\alpha_1$(III) chains -- $[\alpha_1(III)]_3$. This interpretation was possible since synthesis of other types of collagen were not detected. Percent of total collagen represented by Type I collagen $[\alpha_1(I)]_2\alpha_2$, at a particular $\alpha_1$ to $\alpha_2$ ratio, is plotted against that $\alpha_1$ to $\alpha_2$ ratio. Percent of Type III collagen synthesis represented by a particular $\alpha_1$ to $\alpha_2$ ratio would be the reciprocal of the value for Type I collagen.

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and 40% Type III at 7 days, about 35% Type I and 65% Type III from 9 to 12 days, and 90 to 95% Type I and 5 to 10% Type III thereafter.

Synthesis of collagen relative to other proteins during this period

remains constant (11) although the amount of collagen per wet weight (12) or dry weight (13) abruptly increases between 12 and 13 days of development. At this time crossbanded collagen fibers are first seen ultrastructurally (12). Thus the appearance of crossbanded collagen and a dramatic increase in the collagen content of skin occurs at the time when skin collagen synthesis changes from production of both Types I and III to mainly, if not exclusively, Type I collagen. The embryonic period of 7 to 12 days during which skin maximally synthesizes Type III collagen is the key period for skin differentiation as mesenchymal condensations form in the dermis during this period which are essential for development of feathers and scales (14). Collagenase digestion (14,15) or incubation with BAPN or D-penicillamine (14), both which prevent the formation of collagen cross links, likewise prevent the formation of these dermal condensations and subsequent feather and scale development. Thus, Type III collagen synthesis by embryonic chick skin may be correlated with the early development of ectodermal derivatives. Type III collagen would therefore appear to be an embryonic skin collagen necessary for normal dermal development which, in turn, is necessary for normal ectodermal development.

## Acknowledgements

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