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Biochemistry

Biosynthesis of A,B procollagen

(processing/type V collagen/chicken embryo/muscle)

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ABSTRACT **Interest in cell-associated collagens led others to isolate A and B collagen chains, also known as type V collagen, from many tissues, but only after pepsin cleavage. Soluble precursors of these chains are synthesized *in vitro* by crop, a chicken embryo muscle tissue, and are converted by at least two processing steps from procollagens via intermediates to final forms which are larger than the pepsin-derived A and B chains. Heterotrimeric, disulfide-bridged procollagen molecules corresponding to B_2A exist. Components were separated by ion exchange chromatography, velocity sedimentation, and electrophoresis, and the relationships between them were established by sequential radioactive labeling and comparison of peptides generated by protease cleavage.**

Collagens form a family of related gene products composed of two general groups: the interstitial and the basement membrane collagens. Two gene products, known as A and B chains or type V collagen, show similarities in amino acid composition to collagens associated with basement membranes. Knowledge of A and B chains is confined to polypeptides solubilized from various tissues by pepsin digestion, but proper comparisons with other collagens require the full gene products. Here we report the isolation of soluble precursors of A and B chains* and their processing in chicken embryo crop. The composition of individual triple helical collagen V molecules is disputed.

From reconstitution experiments with separate pepsin-solubilized A and B chains, Bentz *et al.* (1) concluded that the trimer A_3 would be unstable at body temperatures, that B_3 was possible, and that B_2A was a likely, stable configuration consistent with the proportions in which the chains were recovered by Burgeson *et al.* (2) and others (e.g., ref. 3). Miller and associates (4, 5) reported different ratios of these chains and also pepsin-resistant B chains, indicating B_3 trimers. We find proαA chains disulfide-linked to proαB chains, which establishes the heterotrimer [(proαB)$_2$proαA]. However, an excess of B chains suggests that homotrimer B_3 also exists. Because type V collagens have been located immunologically in muscle tissue (6, 7), have been found in chicken embryo tissues (7–9), and have been shown to be made by cultured smooth muscle (10) and chicken cells (7, 11), we chose a rapidly growing muscle tissue of the chicken embryo, the crop. Because this thin-walled diverticulum of the esophagus does not produce digestive enzymes, accidental proteolysis was minimized; furthermore, other studies support our conclusion of stepwise processing.†

MATERIALS AND METHODS

Typically, crops from 50 19-day chicken embryos were incubated in 15 ml of Dulbecco's modified Eagle's medium (DME medium) by described procedures (12) with the modifications given below. The medium lacked lactalbumin hydrolysate and the amino acids used for labeling; it contained 2-aminopropionitrile (64 μg/ml) and ascorbate (100 μg/ml). Pulse labels with [^{3}H]proline and [^{3}H]leucine (each at 50 μCi/ml; Schwarz/Mann; 1 Ci = 3.7×10^{10} becquerels) were chased in complete DME medium containing proline (100 μg/ml), 10% heat-inactivated (56°C, 30 min) fetal calf serum (GIBCO), and, sometimes, cycloheximide (250 μg/ml). During 2.5 hr of labeling with these amino acids and [^{35}S]methionine (33 μCi/ml; New England Nuclear) the medium was changed once. During long-term (5 hr) labeling with [^{3}H]proline in complete DME medium with 10% heat-inactivated fetal calf serum, the medium was changed every 1.5–2 hr.

Preparation and Analysis of A,B Chains. All procedures were done at 4°C. Crops were homogenized in 1 M NaCl/50 mM Tris/10 mM EDTA, pH 7.5 (buffer H), and centrifuged (40,000 rpm, 15 min). The pellet was rehomogenized in 0.5 M acetic acid and the suspension was digested with pepsin (100 μg/ml; Worthington) for 18 hr at 4°C. The supernatant was diluted with 1 vol of 4 M urea/0.2% Triton X-100/50 mM Tris, pH 7.5, and, after dialysis against 0.5 M acetic acid/0.2 M NaCl/0.1% Triton X-100 (Buffer P), it was digested with pepsin as above. Further purification of A and B chains was as described (2, 7) except that NaCl was added to 5% by dialysis and type I and III carrier collagens (0.1 mg/ml) were added to help precipitate material from the buffer H extract. CNBr cleavage and peptide separation were as described (2).

Extraction of Type V Precursors, DEAE-Cellulose Chromatography, and Velocity Sedimentation. Tissue was homogenized in buffer I [buffer H plus 1250 μg *N*-ethylmaleimide (MalNEt) and 150 μg of phenylmethylsulfonyl fluoride (PheMeSO$_2$F) per ml] and centrifuged (40,000, rpm, 15 min). The supernatant was diluted with 0.5 vol of 6 M urea/50 mM Tris/0.3% Triton X-100, pH 7.5, and dialyzed against column buffer (4 M urea/50 mM Tris/75 mM NaCl/0.1% Triton X-100, pH 7.8) for 3 hr. During the first hour of dialysis the buffer contained 250 μg of MalNEt and 10 μg of PhMeSO$_2$F per ml and 4 mM EDTA. After clarification (15,000 rpm, 20 min) the sample was applied to a DEAE-cellulose column (Whatman DE-52) and eluted with column buffer and either a linear salt gradient (75–275 mM NaCl) followed by 1 M NaCl or with a step gradient of 150 mM NaCl followed by 1 M NaCl. Aliquots were analyzed for type V collagen by pepsin digestion in buffer P as above.

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Abbreviations: proα, largest isolated precursor chain; pα, partly processed procollagen chain; fα, processed, final form, distinct from pepsin-cleaved chains; DME medium, Dulbecco's modified Eagle's medium; MalNEt, *N*-ethylmaleimide; PhMeSO$_2$F, phenylmethylsulfonyl fluoride.

* We name the largest recovered chains proαB and proαA; the trimeric molecule is named procollagen V. Processing gives intermediates pαB and pαA and final forms fαB and fαA, which are distinctly larger than the pepsin fragments known heretofore and established as B and A chains [suggested alternate names are proα, pα, α and α(pepsin); 1,2(V)].

† Experiments in this laboratory with chicken embryo blood vessels extracted and analyzed by different techniques showed identical processing of type V procollagen (unpublished data).

Collagens were precipitated at 7% NaCl, after addition of carrier collagen, and analyzed electrophoretically. Pooled fractions containing type V collagen were dialyzed against 7% NaCl/50 mM Tris/0.1% Triton X-100, pH 7.5; then carrier collagen was added and acetic acid was added to 0.5 M. The precipitate was redissolved in 4 M urea/0.5 M Tris/0.1% Triton X-100, pH 8.5, and dialyzed against 1 M NaCl/50 mM Tris/0.1% Triton X-100, pH 7.5. For velocity sedimentation, samples were dialyzed against 2 M urea/0.5 M NaCl, 50 mM Tris/0.1% Triton X-100, pH 7.5; 200 μl was loaded onto a 3.5-ml 5–20% (wt/vol) linear sucrose gradient in the same buffer, above a pad of 60% sucrose, and centrifuged in a Beckman SW 60 rotor (56,000 rpm, 19 hr, 4°C) (12).

Protease Digestions and Electrophoresis. Worthington CLSPA collagenase was purified (13, 14) and incubated with samples in 1 M NaCl/50 mM Tris/5 mM $CaCl_2$/0.1% Triton X-100, pH 7.5, for 3 hr at 35°C. Controls were incubated identically but without enzyme. Digestion with *Staphylococcus aureus* V8 protease (Miles) was as described (15). Gel bands located by fluorography were reswollen in the buffer used for enzyme digestion. $NaDodSO_4$/polyacrylamide gel electrophoresis with uniform or gradient gels (16) was followed by fluorography (17) with preflashed film (18) or Coomassie blue staining. Bands were reduced and reelectrophoresed as described (19).

RESULTS

Identification of A,B Chains from Crop. Radioactive crop collagens soluble in buffer H were digested with pepsin and purified. When NaCl was added to 5%, the supernatant contained chains with the electrophoretic mobilities of A and B chains and the precipitate contained types I and III collagens (not shown). Fig. 1 compares the CNBr cleavage products of these materials with those of A,B chains prepared both from human fetal membranes (ref. 2; kind gift of R. Burgeson) and from nonradioactive crop by pepsin solubilization. We conclude that these pepsin-digested chicken crop collagens are type V because of similarities with human fetal membrane type V collagen in solubility characteristics, electrophoretic mobility, and CNBr peptide pattern. Most of the type V collagens synthesized during 5 hr were extracted by buffer H and constituted about 6% of the radioactive soluble collagens (predominantly types I and III).

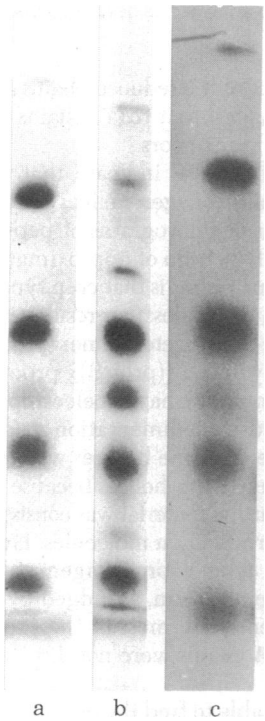

FIG. 1. Comparison of CNBr peptides of crop and human A,B chains. CNBr peptides of A,B chains were isolated from pepsin digests of salt-extracted chicken crop (lane a) and human fetal membranes (lane b). Radioactive crop collagens soluble in buffer H were digested with pepsin and fractionated into A,B chains which gave the CNBr peptides shown in lane c. Crops were labeled with [^{3}H]-proline and [^{3}H]leucine for 2.5 hr. All samples were electrophoresed on one 10% polyacrylamide slab gel which was subsequently cut and processed separately by Coomassie blue staining for lanes a and b and by fluorography for lane c, which caused small differential distortion.

DEAE-Cellulose Chromatography. The buffer I extract of labeled crops was chromatographed on DEAE-cellulose without prior pepsin digestion. Aliquots of individual fractions were treated with pepsin, and proteins that could be digested to yield A and B chains were found only in fractions eluting at approximate conductivity 6.4 mmho/cm (low-salt fraction) and in the 1 M NaCl step (high-salt fraction) (Fig. 2). To purify the non-pepsin-digested type V collagen of the low-salt region further, fractions 52–54 of Fig. 2 were pooled, concentrated by salt precipitation at acid pH after addition of carrier collagen, and sedimented through 5–20% sucrose gradients under nondenaturing conditions. The type V collagen occurred in one peak sedimenting about 1.2 times faster than native procollagen I. This material was free of other collagens as shown by electrophoretic analysis after pepsin digestion (Fig. 3, lane e). Two prominent bands, both digestible with collagenase, were seen when this sedimented type V collagen was electrophoresed after reduction (Fig. 3, lanes a and b). Sensitivity to this enzyme suggests that the bands are collagen chains, and they are identified as proαA and proαB on the basis of peptide analysis and pulse–chase experiments (given below). Electrophoresis without reduction showed four collagenase-sensitive bands (Fig. 3, lanes c and d). The bands labeled L1 and L2 were shown to be disulfide-linked oligomers of proαA and proαB chains by reduction and reelectrophoresis of the individual bands (Fig. 3, lanes f–i). Based on nonreduced electrophoretic mobility and densitometric analysis of the reduction products, band L2 is interpreted as a [proαA, proαB] dimer and band L1 as a [proαA(proαB)$_2$] trimer.

Type V collagens in the high-salt fraction were resolved electrophoretically without further purification. There were three major bands before reduction (M1, M2, and M3) and four bands after reduction (Fig. 3, lanes j and k). All were collagenase sensitive (not shown). The reduced bands were, in order of increasing mobility, pαB, fαB, pαA, and fαA on the basis of peptide analyses and biosynthesis experiments. Before pepsin digestion, type V collagen bands comigrating with A and B chains were not seen in any DEAE-cellulose fractions. The high-salt fraction could be released at 12 mmho/cm with a longer salt gradient.

Peptide Analysis with V8 Protease. To find the relationship of the electrophoretic components of Fig. 3 to marker A and B chains, the bands were cut out of the reduced gels and partly digested with *Staphylococcus aureus* V8 protease, and the resulting peptides were resolved by electrophoresis. The evidence for assignment of proαB and proαA to the reduced components of the low-salt fraction is shown in Fig. 4 *Left*. The patterns in Fig. 4 *Right* show that bands pαB and fαB are related to B and bands pαA and fαA are related to A.

Pulse–Chase Experiments. Pulse–chase experiments showed that labeled type V collagens appeared first in the low-salt fraction and subsequently in the high-salt fraction. Crops were labeled for 45 min and then chased in the presence of nonradioactive amino acids and cycloheximide. After extraction and chromatography on DEAE-cellulose, the low- and high-salt fractions were digested with pepsin and then electrophoresed (Fig. 5). The radioactive type V collagen, which is seen predominantly in the low-salt fraction at the end of the pulse (Fig. 5, lanes a and b) appeared predominantly in the high-salt fraction after the 135-min chase (Fig. 5, lanes g and h). The low-salt fraction of the pulse contained a large amount of ma-

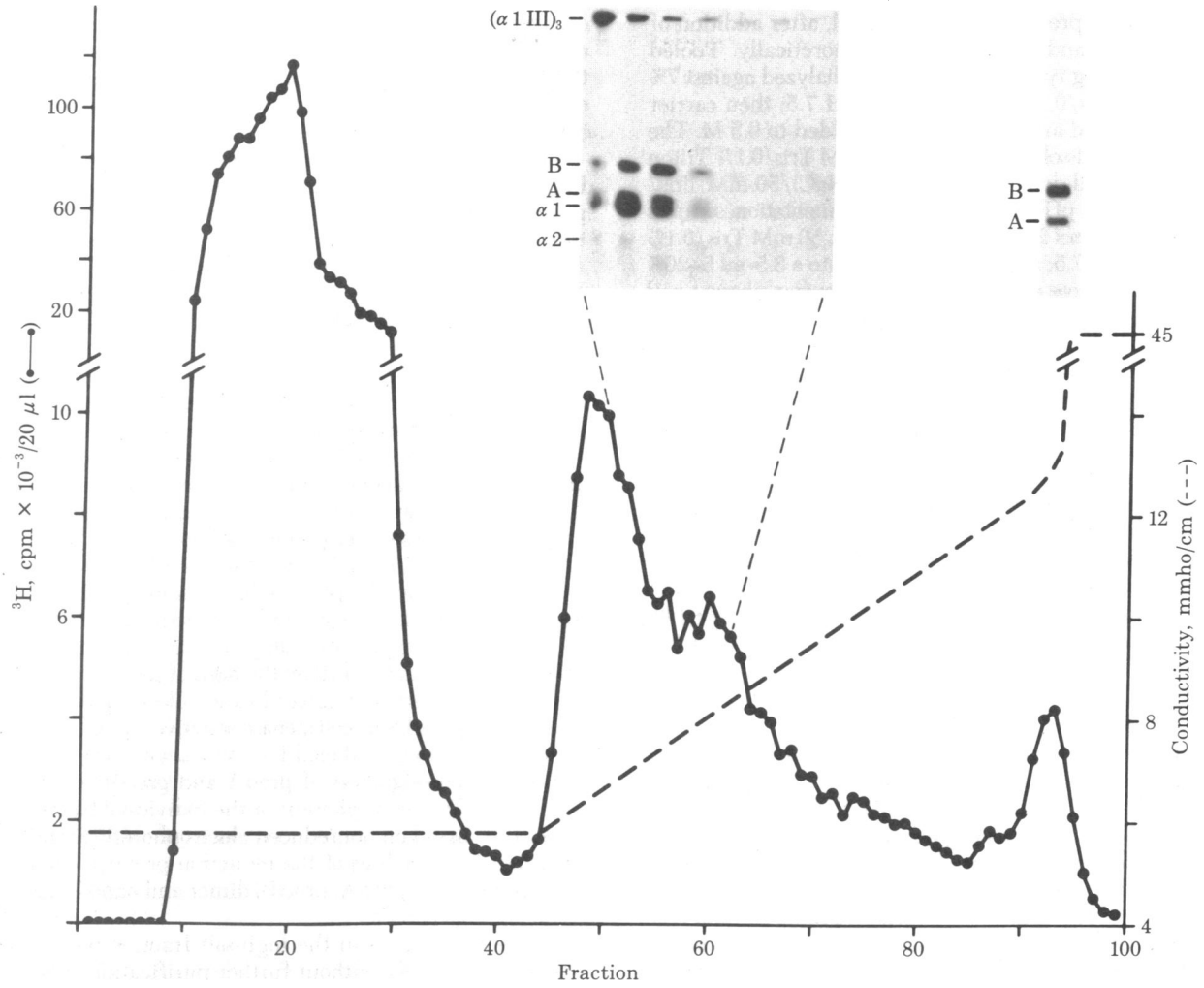

FIG. 2. DEAE-cellulose chromatography. Crops were labeled for 5 hr with [^{3}H]proline. The extract was chromatographed on a DEAE-cellulose column (16 × 140 mm) with a 100-ml linear 75–275 mM NaCl gradient; 1.5-ml fractions were collected. The column was then washed with 1 M NaCl in column buffer. Subsequent washing with 0.25 M HCl, 1 M NaCl, and 2 M urea did not remove additional radioactivity. Aliquots (200-μl) of alternate fractions from fraction 50 to fraction 62 were digested with pepsin and salt precipitated. Equal portions of these samples were analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis in 5% acrylamide gels. A smaller aliquot of fraction 93 was also analyzed as described above. (*Insets*) Corresponding fluorograms.

terial migrating in the position of α1(I), which may be derived from [proα1(I)]$_3$. This band partially overlapped with A (Fig. 5, lanes a and c).

Additional processing was seen in the high-salt fraction when the pooled high-salt peak from each time point was electrophoresed without prior pepsin digestion (not shown). To demonstrate this more clearly, crops were labeled for 2.5 hr and then chased for 5.75 hr with nonradioactive amino acids. The high-salt fractions were isolated by DEAE-cellulose chromatography and electrophoresed. pαB and pαA were labeled first, followed by fαB and fαA (Fig. 6). Because label was still present in pαB and pαA even after this long chase period, this processing step is quite slow. Similar slow conversions of pα(V) and pNα(III) chains have been found in blood vessels.† Conversion was not due to tissue deterioration: identical type V materials, mostly procollagen, were obtained after a 1-hr labeling of either fresh crops or crops first incubated for 5 hr.

DISCUSSION

Pepsin, CNBr, and V8 protease cleavages show that crop contains one set of procollagen type A chains and another of type B. The time-dependent studies show successive conversions within each set. Although all conversions need not be proteolytic, both electrophoretic mobilities of the reduced chains and velocity sedimentation strongly suggest that fα(V) chains are derived by cleavage from proα(V) precursors.

The actions of pepsin and collagenase indicate that the molecules contained the triple-chain collagen helices of type V collagen. Densitometric analysis of fluorograms of pepsin-digested high-salt fraction gave a B/A ratio of approximately 3:1. An interim model which fits our results is that crop type V collagen is a mixture of B_2A and B_3 molecules. Our results (Fig. 3) clearly demonstrate the disulfide-linked heterodimer (proαB, proαA) and a heterotrimer likely to be [(proαB)$_2$ proαA]. Corresponding homooligomers would not have coelectrophoresed. These results are supported by sedimentation studies under denaturing conditions. The disulfide bridges were not between components of two adjacent triple helices because the sedimentation coefficient of the starting material was consistent with individual, native, helical, procollagen molecules. However, although all the recovered type V procollagen of the low-salt fraction was in a triple helical form, as judged by resistance to pepsin and by sedimentation, most of the proαB chains and a portion of the proαA chains were not disulfide-linked (e.g., Fig. 3, lane d).

Furthermore, we have been unable to find the expected di-

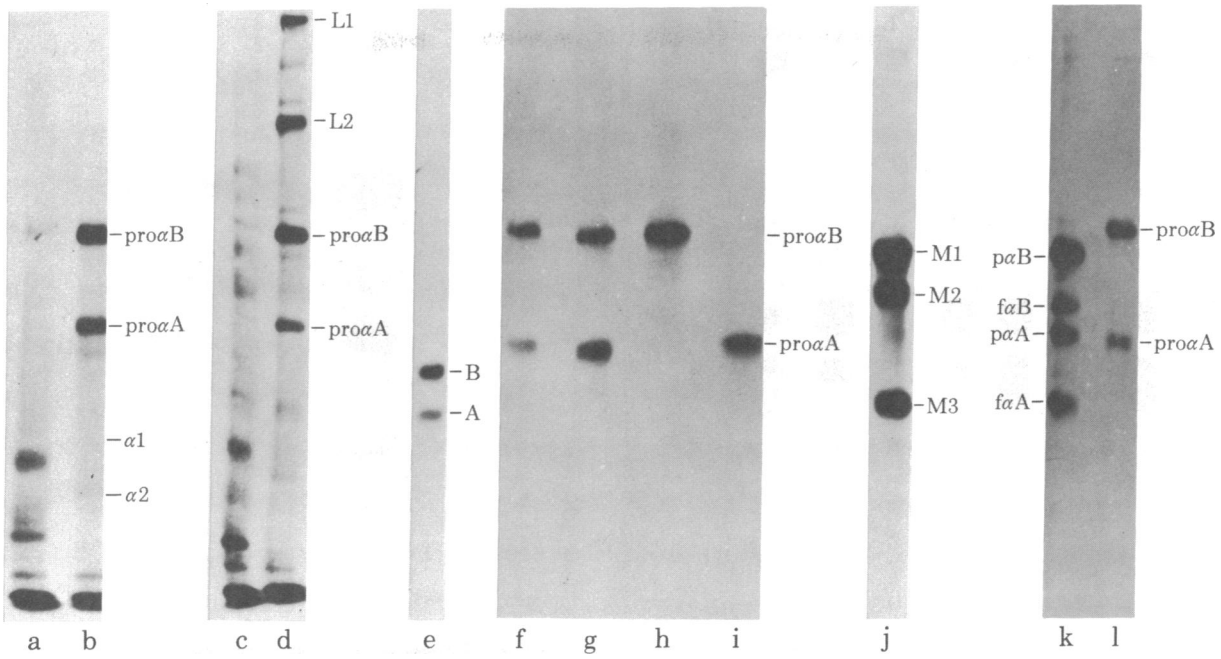

FIG. 3. Crops were continuously labeled for 5 hr with [^{3}H]proline (lanes a–e and j–l) or for 2.5 hr with [^{3}H]proline, [^{35}S]methionine, and [^{3}H]leucine (lanes f–i). Components were separated by DEAE-cellulose chromatography and the low-salt fraction was further purified by sedimentation under nondenaturing conditions. Electrophoretograms on 4% polyacrylamide were made after incubation with or without enzymes. Low salt fraction: lane a, collagenase-treated and reduced; b, control, reduced; c, collagenase-treated, not reduced; d, control, not reduced; e, pepsin-treated. The components of low-salt fraction, which gave an electrophoretogram like that shown in lane d, were cut from the gel and reelectrophoresed on one slab gel after reduction: f, L1; g, L2; h, proαB; i, proαA. High-salt fraction: j, not reduced; k, reduced; l, low-salt fraction, as in b, co-electrophoresed with k for comparison. Type I α chain markers (as indicated) were coelectrophoresed with the single slab gel used for lanes a–e. ProαB migrated almost identically with the dimer of α1(I). Electrophoretic mobilities relative to α1(I), taken as unity, were: L1, 0.05; L2, 0.28; proαB, 0.56; proαA, 0.75; B, 0.87; A, 0.97.

sulfide-linked (proαB)$_3$. Either interchain disulfide bridges do not readily form between type V procollagen chains and form not at all between two proαB chains, or there are experimental reasons. Our methods strongly select for native folded molecules, both during concentration by salt precipitation and by the test of pepsin digestion in searching for type V chains. Earlier forms could exist that are disulfide-linked but not yet fully folded, and this would be consistent with our pulse–chase studies. However, proteolytic modifications that would give the low-salt fraction would have to be small because the reduction products of the disulfide-linked procollagen V chains comigrate electrophoretically with the corresponding monomer

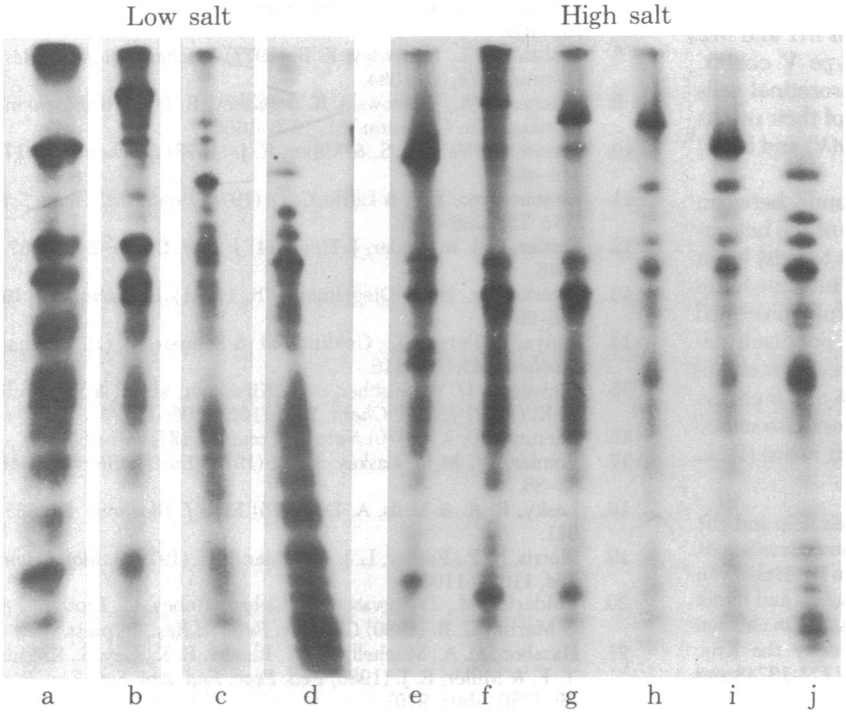

FIG. 4. V8 protease digestion of type V polypeptides. Reduced, [^{3}H]proline-labeled, low-salt and high-salt type V polypeptides were prepared as in Fig. 3. The A and B chains of pepsin-digested, [^{3}H]proline-labeled crop extracts were purified and separated electrophoretically. Bands identified by fluorography were cut from the polyacrylamide gel, reswollen in buffer, and digested (for 30 min at 22°C in the stacking gel) with V8 protease at 25 μg/ml (lanes, a, d, and e–j) or at 10 μg/ml (lanes b and c). Peptides were resolved on two 7.5/15% gradient polyacrylamide gels as shown. The substrates were as follows: lane a, B; b, proαB; c, proαA; d, A; e, B; f, pαB; g, fαB; h, pαA; i, fαA; and j, A. To allow for differences in substrate concentration, enzyme concentrations were varied. Use of higher or lower enzyme concentrations changed the relative intensities but not the mobility of peptides generated from a given substrate (not shown). Parent peptides incubated without enzyme gave weak traces of faster migrating material.

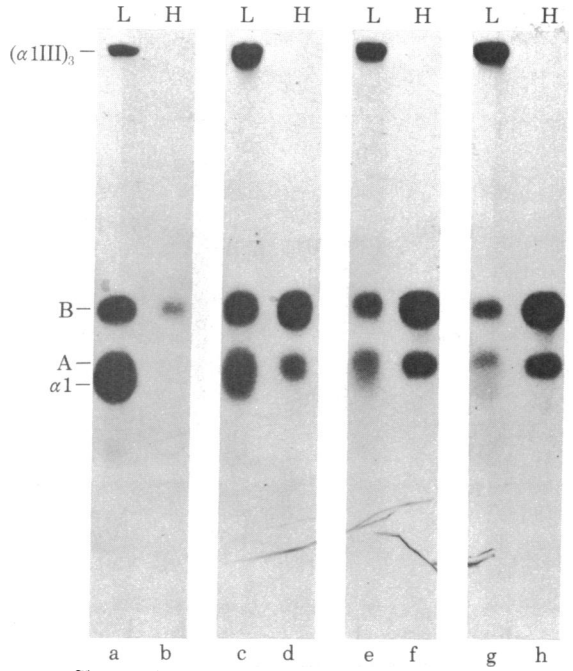

FIG. 5. Short pulse–chase experiment. Crops were labeled for 45 min and chased for an additional 45, 90, or 135 min in the presence of cycloheximide. After DEAE-cellulose chromatography with step elution (150 mM NaCl followed by 1 M NaCl), 80% of the total volume in each pooled peak was pepsin digested and salt precipitated. Then, 24% of the redissolved precipitates was applied to a $NaDodSO_4$/5% polyacrylamide gel as follows. With 45-min pulse: lane a, low-salt; b, high-salt. With 45-min chase: c, low-salt; d, high-salt. With 90-min chase: e, low-salt; f, high-salt. With 135-min chase, g, low-salt; h, high-salt.

chains (Fig. 3). Precautions were taken against adventitious disulfide exchange or proteolytic cleavage. Observations of procollagens V in other chick tissues, by other methods, give similar results.†

The electrophoretic mobilities of pαB and fαA chains of the high-salt fractions are changed little by reduction and therefore they are single chains. Any disulfide-linked dimers or trimers should migrate more closely than pαB but have not been found. Thus, the disulfide bridges of materials in bands M1 and M2 of Fig. 3, lane j, do not link two complete type V chains. Linsenmayer and Little (11) reported that neuroretinal cells elaborated some novel collagens. The similarity of their results to some of ours suggests that these chains were pα(V) and fα(V) collagens.

In summary, disulfide bridges have been found between proαB and proαA chains, proving the existence of the heterotrimer B_2A. The homotrimer $(proαB)_3$ is likely to exist but a disulfide-linked form of it has not been found. Intermediates pαB and pαA arise during processing. The most fully processed chains that we have observed, fαB and fαA, are not disulfide-linked and are smaller than their procollagen precursor chains but significantly larger than the pepsin-derived A and B chains. Recent reports show some similar chains (20), cell-associated B_3 (21), and differential losses influencing A/B ratios (R. L. Trelstad, personal communication).

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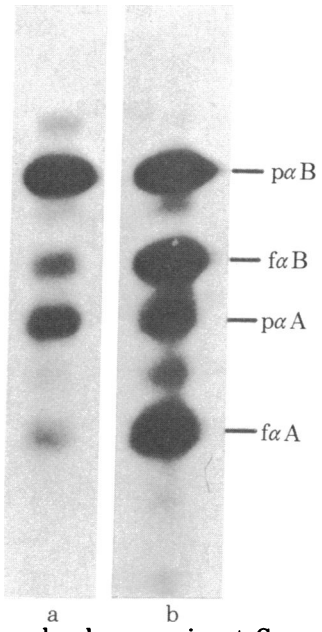

FIG. 6. Longer pulse-chase experiment. Crops were labeled for 2.5 hr with [^{3}H]proline and [^{3}H]leucine. Half were chased in the presence of nonradioactive amino acids and 10% heat-inactivated fetal calf serum for an additional 5.75 hr. DEAE-Cellulose chromatography with a linear 75–375 mM NaCl gradient was used to separate low- and high-salt forms of type V collagen. High-salt fractions were concentrated by salt precipitation and electrophoresed after reduction on a 4% acrylamide gel. Lanes: a, pulse; b, chase.

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