Characterization of Procollagen-Derived Peptides Unique to the Precursor Molecule†

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ABSTRACT: A disulfide-bonded fragment with a molecular weight of about 100,000 was identified in the medium of cultured chick cranial bone and its derivation from procollagen was established by immunological criteria. The molecular weight of the fragment was reduced to 33,000 after cleavage of disulfide bonds, indicating a triple-stranded structure. The amino acid composition of the fragment lacked hydroxyproline and hydroxylysine and differed markedly from that of collagen in other respects. A similar but somewhat larger fragment was isolated after bacterial collagenase digestion of chick bone procollagen purified by chromatography on DEAE-cellulose. The characterization and comparison of these fragments further define the nature of the additional regions in procollagen and, when combined with information derived from studies of acid-extracted and dermatosparactic procollagens, support a mechanism for the conversion of procollagen to collagen which involves more than one proteolytic step.

Recent attempts to characterize procollagen have been hampered by difficulty in purifying either the intact protein or its constituent pro-α chains (for reviews see Bornstein, 1974; Martin et al., 1975). Studies of dermatosparactic collagen (Lenaers et al., 1971; Furthmayr et al., 1972; Timpl et al., 1973) and of chains derived from acid-extracted procollagen (von der Mark and Bornstein, 1973; von der Mark et al., 1973) demonstrated that the precursor regions had amino acid compositions, conformations, and antigenic properties different from collagen. However, it is now clear that those proteins represent derivatives of procollagen and that the intact precursor is larger and may have other unique properties (Goldberg et al., 1972; Fessler et al., 1973; Monson and Bornstein, 1973; Bankowski and Mitchell, 1973; Tanzer et al., 1974).

Sherr et al. (1973) and Dehm et al. (1974) reported studies of collagenase resistant disulfide-bonded fragments derived from procollagens secreted into the medium of cultured fibroblasts. Antibodies to procollagen were used both to identify and to aid in the purification of those fragments. In this study we characterize and compare a collagenase-resistant peptide obtained from embryonic chick cranial bone procollagen with a procollagen-derived peptide isolated from the culture medium of cranial bone. The use of embryonic cranial bone was prompted by its marked biosynthetic activity in vitro, which permits selective labeling of procollagen without introduction of significant radioactivity into extracellular collagen. In addition, membranous bone contains little or no type III procollagen (Miller, 1973) in contrast to the product of cultured skin fibroblasts, which is a mixture of type I and type III procollagens (Church et al., 1973; Lichtenstein et al., 1975).

The collagenase-produced and medium peptides were found to share a number of properties including recognition by antiprocollagen antibodies and the presence of interchain disulfide bonds, but differed in size and solubility properties; their characterization, together with information obtained from the study of acid-extracted and dermatosparactic procollagens, provides additional evidence for multiple steps in the conversion of procollagen to collagen.

Materials and Methods

Preparation of Procollagen. Cranial bones from 17-day-old chick embryos were removed and prepared for culture as described previously (Bornstein et al., 1972; Monson and Bornstein, 1973). After oxygenation and preincubation for 1 hr, bones were transferred to fresh medium containing 5–10 μCi/ml of [2,3-^{3}H]proline, [G-^{3}H]tryptophan, [G-^{3}H]tyrosine or a mixture of ^{14}C-labeled amino acids obtained from an algal hydrolysate. Routinely, synthesis in the presence of radioactive tryptophan or tyrosine was continued for 2 hr at 37°; incubation with labeled proline was limited to 18 min. In two experiments cranial bones were incubated in glucose-free medium in the presence of [^{3}H]mannose (5 μCi/ml) for 2 hr.

Procollagen was extracted from bone by rapid homogenization in 1 *M* NaCl containing 0.05 *M* Tris-HCl, (pH 7.5), 25 m*M* EDTA,[1] 10 m*M* MalNEt, 1 m*M* iPr_2FP, and 1 m*M* benzamidine-HCl (Monson et al., 1975). The subsequent partial purification of procollagen by successive precipitation with 25% NaCl and 5% Cl_3CCOOH has been described (Monson, 1974; Monson et al., 1975).

DEAE-Cellulose Chromatography of Procollagen. The method used was a modification of the procedure described by Müller et al. (1973). Procollagen, dissolved after precipitation with Cl_3CCOOH in 1 *M* NaCl–0.25 *M* Tris-HCl, (pH 7.5), was dialyzed at 4° against 0.1 *M* Tris-HCl (pH 7.5), containing 2 *M* urea. Any insoluble material which formed at this step was removed by centrifugation and the supernatant was applied to a column (25 × 2.5 cm) of

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[1] Abbreviations used are: EDTA, ethylenediaminetetraacetic acid; MalNEt, *N*-ethylmaleimide; iPr_2FP, diisopropyl fluorophosphate; Cl_3CCOOH, trichloroacetic acid; $PhMeSO_2F$, phenylmethylsulfonyl fluoride.

DEAE-cellulose (Whatman DE-52) equilibrated with the above Tris–urea buffer and eluted at 4° with a linear gradient from 0 to 0.1 *M* NaCl over a total volume of 800 ml. Fractions containing procollagen were concentrated by vacuum dialysis against 0.15 *M* NaCl–0.05 *M* Tris-HCl–5 m*M* $CaCl_2$ (pH 7.5) and stored frozen until further use.

Collagenase Digestion of Procollagen. [^{3}H]Tryptophan-labeled procollagen (1×10^6 cpm), prepared as described above, was diluted to a volume of 4 ml in a siliconized glass vial with 0.15 *M* NaCl–0.05 *M* Tris-HCl–5 m*M* $CaCl_2$ (pH 7.5), and made 10 m*M* in MalNEt. Fifty micrograms of bacterial collagenase (Worthington, CLSPA), purified further by Sephadex G-200 chromatography (Peterkofsky and Diegelmann, 1971), was added and the solution incubated for 2 hr at 37°. The reaction was terminated by addition of 0.1 ml of glacial acetic acid and heating to 65° for 10 min. Aliquots were removed for sodium dodecyl sulfate–polyacrylamide gel electrophoresis and the remainder lyophilized or dialyzed.

Preparation of Procollagen-Derived Peptides from Culture Medium of Bone. After culture the medium was decanted into a chilled flask and protease inhibitors added to yield the following final concentrations: 25 m*M* EDTA, 10 m*M* MalNEt, and 1 m*M* iPr_2FP. Following centrifugation at 39,000*g* for 10 min at 4°, pepstatin was added to a concentration of 5 μg/ml and 100% Cl_3CCOOH added dropwise to a concentration of 10%. The resulting suspension was kept at 0° for 30 min; the precipitate was then recovered by centrifugation at 39,000*g* for 10 min, washed with cold 10% Cl_3CCOOH, and recentrifuged. The pellet was extracted by suspension in 40 ml of 0.25 *M* Tris-HCl (pH 7.5), containing 0.1 m*M* $PhMeSO_2F$. The suspension was stirred overnight at 4° and centrifuged and the clear supernatant concentrated to 5 ml by vacuum dialysis against 0.05 *M* sodium phosphate (pH 7.0) containing 0.1 m*M* $PhMeSO_2F$.

DEAE-Cellulose Chromatography of Medium Proteins. Medium proteins were dialyzed against the starting buffer, 0.025 *M* Tris-HCl (pH 7.5), containing 2 *M* urea, and applied to a column (1.6 × 12 cm) of DEAE-cellulose (Whatman, DE 52) at room temperature. The column was eluted at a flow rate of 60 ml/hr with 50 ml of starting buffer and then with a linear gradient from 0 to 0.5 *M* NaCl over a total volume of 500 ml.

Immunological Studies. Antibodies to procollagen were prepared and purified through the DEAE-cellulose chromatography step, as described recently (Nist et al., 1975). The cross-reactivity of medium peptides and collagenase-produced peptides with antibodies to procollagen was assayed by the double antibody radioimmune procedure described by von der Mark et al. (1973).

Analytical Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. Gel electrophoresis was performed in 7.5% gels as described by Goldberg et al. (1972). Samples were reduced with 5% mercaptoethanol or 50 m*M* dithiothreitol in 1% sodium dodecyl sulfate at 55° for 30 min prior to application to gels. Chains and peptides with intact disulfide bonds were analyzed with the addition of 0.1 *M* iodoacetamide to the sample buffer to prevent haphazard reduction (Parkhouse, 1971). Dansylated collagen chains and CNBr fragments were added to establish internal reference points. Optical density traces at 565 nm were obtained in a Gilford gel scanner. Gels were sliced with a Mickle gel slicer and counted as previously described (von der Mark and Bornstein, 1973). In some cases, the methylene bisacrylamide cross-linker was replaced by an equimolar concentration of *N,N'*-diallyltartardiamide and slices were digested for 20 min at room temperature with 2% periodic acid (Anker, 1970).

Preparative Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. The assembly described by Hagen and Young (1974) was modified by addition of a circulating water cooling jacket around the central gel tube (Savant Instruments, New York). Acrylamide gels (7.5%) were prepared from electrophoresis grade acrylamide (Bio-Rad) in 0.5% sodium dodecyl sulfate, 0.1 *M* sodium phosphate (pH 7.0), containing 0.5 *M* urea and cross-linked with *N,N'*-diallyltartardiamide. Sodium dodecyl sulfate (specially pure, BDH Chemicals Ltd., Poole, England) was purified further by passage through a column of AG 50W-X 8 (H^+ form) at pH 4.0. Gels were poured to a height of 6.5 cm in 10 × 1.5 cm gel tubes. The electrode compartment buffer was 0.1 *M* sodium phosphate (pH 7.0) containing 0.1% sodium dodecyl sulfate and 0.5 *M* urea. The elution buffer contained, in addition, 0.1 m*M* $PhMeSO_2F$.

Following pre-electrophoresis of the gel at a constant current of 65 mA (80 V) for 1–3 hr, the sample containing 3–10 × 10^6 cpm of [^{3}H]amino acid in a volume of 0.4–0.5 ml was layered on the gel and the electrophoresis resumed at a temperature of 23°. Routinely, 0.1 *M* iodoacetamide was added to the sample buffer (1% sodium dodecyl sulfate–0.1 *M* phosphate, pH 7.0) and the sample heated at 55° 30 min prior to application. Peptides were also electrophoresed after reduction with 0.05 *M* dithiothreitol and alkylation with 0.15 *M* iodoacetic acid. The elution cell was purged at 7 ml/hr and fractions of 0.9–1.0 ml were collected.

Scintillation Counting. Samples were counted in a Beckman LS 230 liquid spectrometer. Routinely, aliquots of liquid samples were counted in xylene containing 25% Triton X-114 (Sigma) and 0.3% Omnifluor (New England Nuclear). Gel slices, after digestion, were counted in BBS-3 (Beckman). In the case of immune precipitates, where volumes of 1.0–1.2 ml were required for quantitative transfer, counting was performed in 10 ml of Aquasol (New England Nuclear).

Amino Acid Analysis. Peptides for analysis were hydrolyzed in triply distilled constantly boiling hydrochloric acid. Hydrolysis ampoules were flushed repeatedly with purified nitrogen before being sealed in vacuo. Hydrolysates were analyzed on a Beckman 121 Analyzer modified for single column analysis using a four buffer program and equipped with 12-mm light path cuvettes. When availability of pure material was limited, analyses were conducted with a Durrum analyzer, Model D-500. Provided that rigorous precautions were taken, reliable analyses of samples with a total of as little as 40 nmol of amino acids could be made on the latter instrument.

Results

Partial Purification of Procollagen. Rapid homogenization and extraction of procollagen in the presence of protease inhibitors served to minimize proteolysis by tissue-associated enzymes. Precipitation with Cl_3CCOOH was introduced both as a convenient means of concentrating samples and to denature small amounts of proteases which continued to contaminate the procollagen preparation after salt precipitation (Monson, 1974). The procollagen was readily solubilized following Cl_3CCOOH precipitation and retained the native triple helical conformation as evidenced by

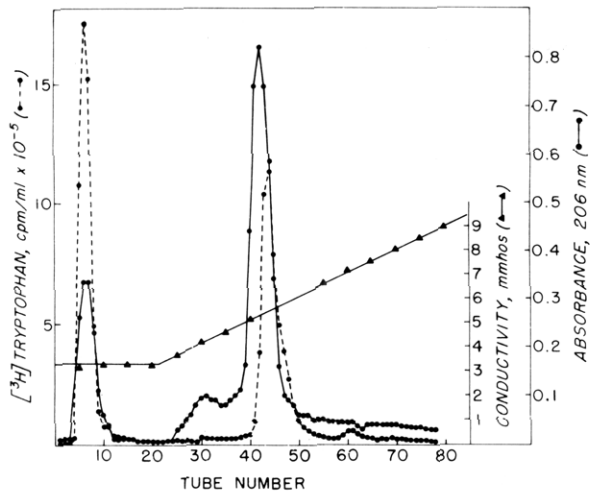

FIGURE 1: DEAE-cellulose chromatogram of a 1 *M* NaCl extract of [^{3}H]tryptophan-labeled procollagen. Conditions of chromatography are provided in the text.

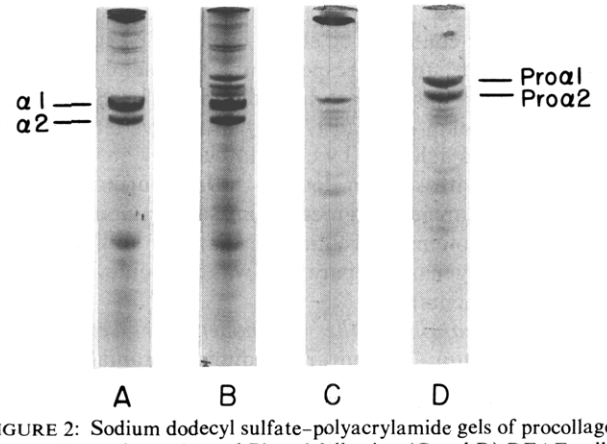

FIGURE 2: Sodium dodecyl sulfate–polyacrylamide gels of procollagen preparations prior to (A and B) and following (C and D) DEAE-cellulose chromatography. The proteins on gels A and C were unreduced and run in the presence of iodoacetamide; the material on gels B and D was reduced with 2-mercaptoethanol; Coomassie Blue stain.

resistance of the collagenous domain to pepsin digestion (unpublished observation).

The partly purified procollagen preparation was chromatographed on DEAE-cellulose under conditions which preserve the collagen triple helix (Figure 1). In this system, collagen eluted unretarded from the column whereas elution of procollagen required a higher ionic strength buffer. Routinely, 65–90% of the counts applied to the DEAE-cellulose column were recovered in effluent fractions.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of procollagen prepared by DEAE-cellulose chromatography indicated substantial purification (Figure 2). Collagen, which represents the major component of the material prior to chromatography, was largely removed by the DEAE-cellulose column. The material migrating in the region of pro-α2 prior to reduction (Figure 2C) and the several lower molecular weight components (Figures 2C and D) represent derivatives of pro-α chains and are susceptible to digestion by bacterial collagenase (Monson, 1974). When gels containing tryptophan-labeled procollagen were sliced and counted, the patterns in Figure 3 were observed. The tryptophan label in this preparation was limited essentially to procollagen.

Collagenase-Resistant Peptides. Bacterial collagenase digests of tryptophan-labeled procollagen were concentrated and placed directly on sodium dodecyl sulfate gels before and after reduction of disulfide bonds (Figure 4). Prior to reduction the majority of the counts migrated as a high molecular weight band with a mobility similar to that of dansylated α1 chains. After reduction, the high molecular weight material was replaced by a broader peak migrating near dansylated α1-CB7 (mol wt 24,500). Qualitatively similar findings have been reported for collagenase-produced peptides from human skin (Sherr et al., 1973) and

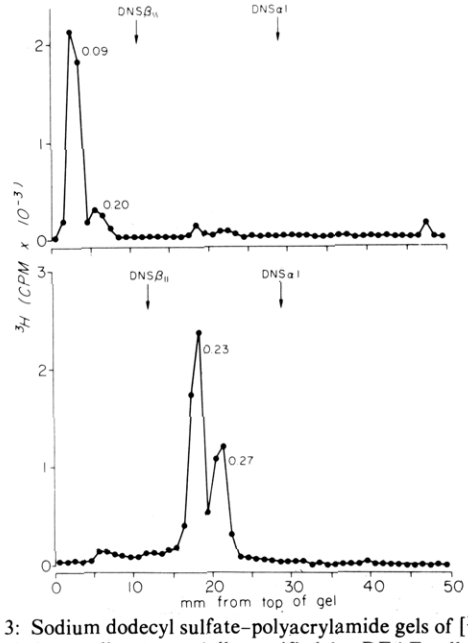

FIGURE 3: Sodium dodecyl sulfate–polyacrylamide gels of [^{3}H]tryptophan-labeled procollagen partially purified by DEAE-cellulose chromatography (Figure 1): (top panel) prior to reduction; (bottom panel) after reduction with 2-mercaptoethanol. R_f values were calculated relative to the position of migration of dansylated α1-CB7. Only half of each 10-cm gel is shown; the lower half contained essentially no counts above background. The arrows indicate the positions of dansylated β and α components used as internal standards.

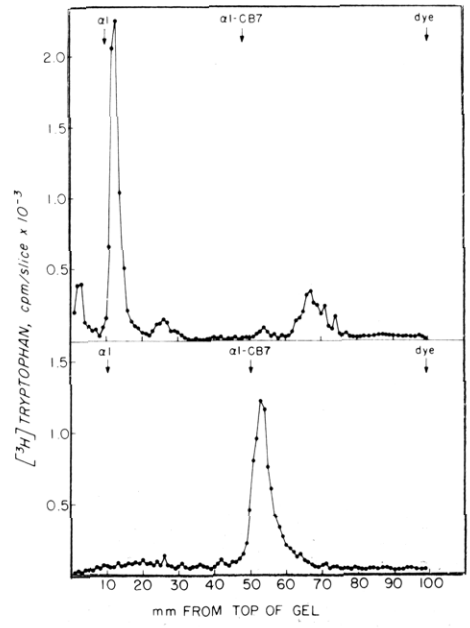

FIGURE 4: Sodium dodecyl sulfate–polyacrylamide gels of peptides obtained after bacterial collagenase digestion of [^{3}H]tryptophan-labeled procollagen: (top panel) prior to reduction; (bottom panel) after reduction with 2-mercaptoethanol. The arrows indicate the positions of dansylated collagen components, used as internal standards, and the tracking dye.

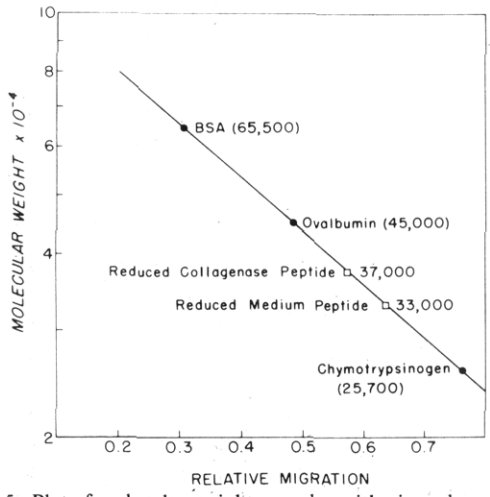

FIGURE 5: Plot of molecular weight, on a logarithmic scale, vs. migration relative to a Bromophenol Blue dye marker in sodium dodecyl sulfate–polyacrylamide gels. The gel was calibrated by plotting the observed migration distance of three globular proteins against their known molecular weights; BSA, bovine serum albumin.

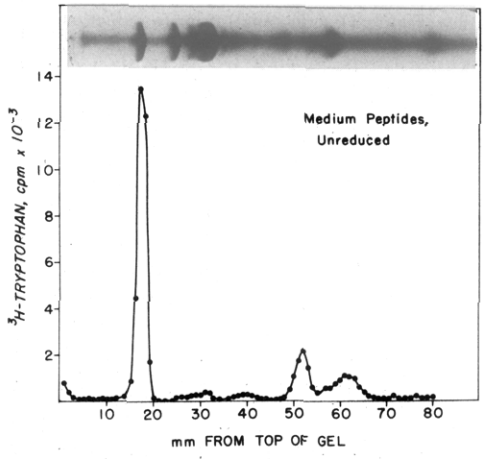

FIGURE 6: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of [^{3}H]tryptophan-labeled cranial bone culture medium. A gel was stained with Coomassie Blue (above) and a parallel gel sliced and counted (below). Similar results were obtained by slicing and counting the stained gel. The preparations were treated with iodoacetamide prior to electrophoresis (see Materials and Methods).

chick tendon (Dehm et al., 1974) fibroblast procollagens. In addition, in this study lower molecular weight peptides migrating between the α1-CB7 and dye markers prior to reduction (Figure 4) were also observed. The nature of these peptides is under investigation.

The molecular weight of the major reduced collagenase-resistant peptide, based on noncollagen standards, was 37,000 (Figure 5). The lack of correspondence between the probable molecular weight of "noncollagenous" sequences in procollagen and that calculated by comigration of collagen chains and fragments in sodium dodecyl sulfate has been noted before (von der Mark and Bornstein, 1973). Although accurate measurement of the molecular weight of the unreduced fragment was difficult, the data are consistent with a 3:1 ratio in size between the unreduced and reduced fragments.

Preparative isolation of collagenase-resistant procollagen-derived peptides was attempted by chromatography on molecular sieve and ion exchange columns without notable success. In the absence of sodium dodecyl sulfate, the peptides adsorbed to supports such as Sepharose and Sephadex

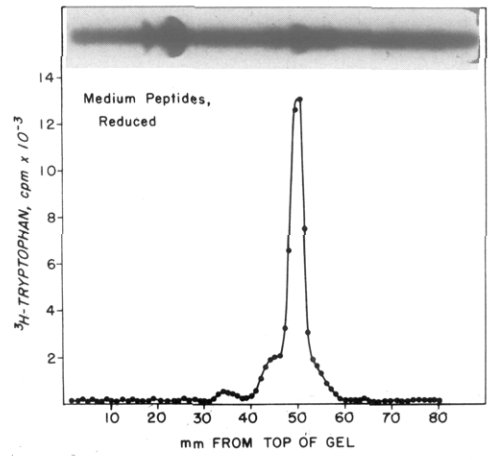

FIGURE 7: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of [^{3}H]tryptophan-labeled cranial bone culture medium. A gel was stained with Coomassie Blue (above) and a parallel gel sliced and counted (below). The preparations were reduced with 2-mercaptoethanol prior to electrophoresis.

and thereafter could not be eluted even by detergents or denaturing agents. In this respect these fragments differed in behavior from the preparations described by Sherr et al. (1973) and Dehm et al. (1974). Quantitative elution of radioactivity was achieved by chromatography on 8% agarose equilibrated with sodium dodecyl sulfate buffers but separation of the labeled fragment from small amounts of contaminating proteins, introduced in part by addition of the bacterial collagenase preparation, was not achieved (unpublished experiments).

Characterization of the Components in Cranial Bone Culture Medium. The culture medium of cranial bone contained a complex mixture of proteins when examined by analytical polyacrylamide gel electrophoresis (Figure 6). However, the majority of these proteins were unlabeled during a 2-hr incubation with [^{3}H]tryptophan. Similar results were obtained when bones were incubated with a ^{14}C-labeled algal hydrolysate except that a small amount of uncleaved procollagen was also identified in the culture medium. Presumably the unlabeled proteins in the medium represent extracellular proteins which had not been completely removed during preliminary washes or proteins which were leached from cells during incubation.

The majority of tryptophan counts was contained in the highest molecular weight band in the medium preparation (Figure 6). After reduction, label and comigrating Coomassie Blue staining material shifted to a position of greater mobility indicative of a marked reduction in molecular weight (Figure 7). Similar results were obtained with a [^{3}H]cystine label (data not shown). Routinely 70–80% of the tryptophan label was found in the high molecular weight component. Two minor components, one migrating at 50–54 mm in the unreduced gel (Figure 6) and the other near the dye marker (not shown), were consistently observed, but were not studied further.

The electrophoretic behavior of the labeled material in the culture medium of bone, before and after reduction, resembled that of the collagenase-resistant procollagen-derived fragment. Molecular weight determinations of the reduced medium peptide, using calibrated sodium dodecyl sulfate–polyacrylamide gel electrophoresis, yielded a value of 33,000, slightly less than the size of the reduced collagenase-resistant fragment (Figure 5).

DEAE-Cellulose Chromatography of Medium Peptides.

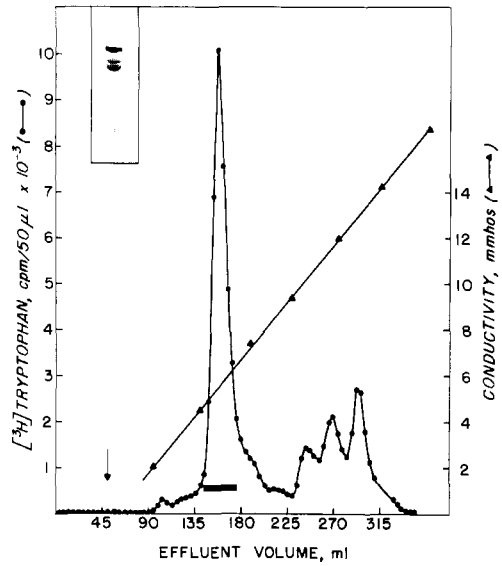

FIGURE 8: DEAE-cellulose chromatogram of [^{3}H]tryptophan-labeled cranial bone culture medium. Conditions of chromatography are described under Materials and Methods. The inset shows an analytical sodium dodecyl sulfate gel (Coomassie Blue stain) of the pooled fractions indicated by the horizontal black bar.

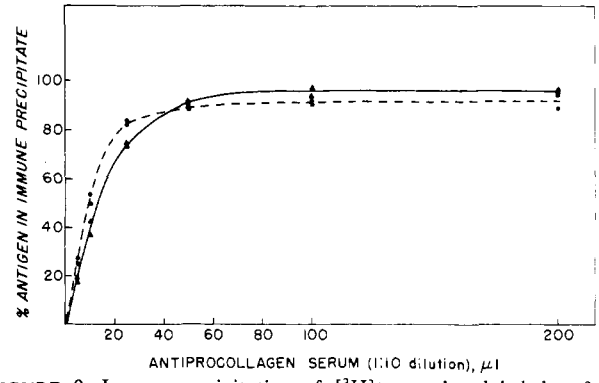

FIGURE 9: Immunoprecipitation of [^{3}H]tryptophan-labeled unfractionated medium proteins (●---●) and a partially purified preparation obtained by DEAE-cellulose chromatography as in Figure 8 (▲-▲) with an antiserum to chick cranial bone procollagen. Duplicate determinations are plotted for each volume of serum used. The immune complex was harvested by precipitation with a sheep anti-rabbit γ-globulin serum (von der Mark et al., 1973).

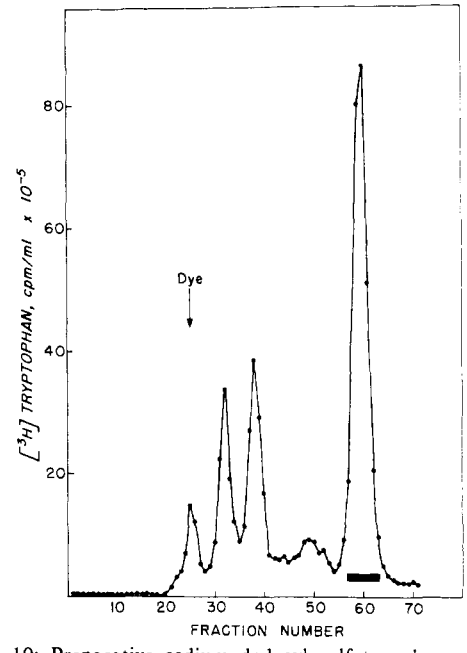

FIGURE 10: Preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis of [^{3}H]tryptophan-labeled cranial bone culture medium. Fractions represented by the horizontal bar were pooled for subsequent analysis.

In order to isolate the medium peptide in amounts suitable for further studies, bone culture medium was chromatographed on DEAE-cellulose in the presence of 2 *M* urea (Figure 8). The yield from the column was 90%. The collagenase-resistant peptide did not elute from DEAE-cellulose under these conditions. The initial peak contained some 50% of the counts recovered from the column and was enriched in the high molecular weight labeled fragment (compare inset to Figure 8 with Figure 6) but contained substantial amounts of more rapidly migrating unlabeled proteins. The labeled proteins eluting at higher ionic strengths were lower in molecular weight as indicated by analytical sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown). When compared with the pattern obtained by direct sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of medium (Figure 6), a larger fraction of the radioactivity appeared to be in low molecular weight fragments, perhaps because of partial degradation or reduction of the material during chromatography.

Immunologic Studies. Antisera to procollagen were used to establish that labeled tryptophan-containing peptides in the medium of cultured bone were, in fact, derived from procollagen. As seen in Figure 9, 90–95% of the tryptophan label present in either an unfractionated medium preparation, or in a partially purified DEAE-cellulose fraction (Figure 8), was precipitable with an antiserum to chick bone procollagen. Only background levels of counts were precipitable by nonimmune sera. Collagenase-resistant procollagen-derived peptides, labeled with tryptophan, were also quantitatively precipitated by antiprocollagen sera (data not shown).

Medium peptides previously treated with sodium dodecyl sulfate were incompletely precipitable by antiprocollagen sera even though attempts were made to quantitatively remove residual sodium dodecyl sulfate by extensive dialysis against ion-exchange resins. This suggests that small amounts of sodium dodecyl sulfate were irreversibly bound and interfered with antigenicity (a possibility supported by the subsequent failure of sodium dodecyl sulfate treated peptides to be eluted from anion-exchange resins) or that antigenic sites in the peptide were irreversibly destroyed by denaturation (Dehm et al., 1974).

Preparative Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis. Because of the success in isolating intact pro-α chains by preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Monson, 1974; Monson et al., 1975) this method was used for purification of the disulfide bonded peptide directly from unfractionated medium. The elution pattern of the gel (Figure 10) again revealed a high molecular weight tryptophan-containing fraction with a variable but somewhat larger proportion of more rapidly eluting, lower molecular weight fractions than seen in comparable analytical gels (see Figure 6).

Characterization of the Procollagen-Derived Medium Peptide. The fractions comprising the major labeled medium peptide (e.g., Figure 10, solid bar) were pooled and examined by analytical sodium dodecyl sulfate-polacrylamide gel electrophoresis and amino acid analysis (Table I). The peptide, in this case obtained from a tyrosine-labeled prepa-

Table I: Amino Acid Composition of a Procollagen-Derived Peptide in the Medium of Cultured Embryonic Chick Cranial Bone.[a]

Hyp	0	Met	7.5
Asp	130	Ile	50.8
Thr	78.3	Leu	72.5
Ser	61.3	Tyr	33.0
Glu	120	Phe	33.0
Pro	51.3	Hyl	0
Gly	89.7	Lys	66.5
Ala	68.5	His	15.0
Val	43.0	Trp	N.D.[c]
Half-Cys[b]	33.5	Arg	46.6

[a] Values are expressed as residues per 1000 residues and represent an average of six determinations performed on three different preparations. The values are not corrected for losses incurred during hydrolysis. [b] Includes cysteic acid and *S*-carboxymethylcysteine. [c] N.D., not determined.

ration, was essentially homogeneous on the basis of distribution of radioactivity (Figure 11, top panel); a scan of the stained gel (Figure 11, top panel, inset) indicated that more than 90% of the protein comigrated with the radioactivity. Analyses (Table I) indicated the absence of hydroxyproline and hydroxylysine and a relatively high content of aspartic and glutamic acids (or their amides). Although the tryptophan content was not determined the peptide was readily labeled by incubation of bones in the presence of radioactive tryptophan. This analysis is consistent with the recently determined composition of the pro-α1 chain of chick cranial bone procollagen (Monson et al., 1975) although direct comparison is not possible since the peptide presumably contains a fragment derived from the pro-α2 chain and the pro-α1 chain contains an additional nonhelical sequence not found in the peptide (Davidson and Bornstein, 1975).

Since purification had been achieved in a single step, an additional experiment was performed to evaluate the homogeneity of the isolated fragment. The peptide obtained by preparative sodium dodecyl sulfate–polyacrylamide gel electrophoresis was reduced and alkylated and reapplied to the preparative gel. Greater than 90% of the counts (in 65% overall yield) eluted in the position expected for the reduced peptide (data not shown). This material was essentially monodisperse both by distribution of radioactivity and protein (Figure 11, bottom panel) and the amino acid composition was not significantly different from that of the unreduced peptide.

When cranial bones were incubated in the presence of [^{3}H]mannose, both procollagen and procollagen-derived medium peptides were radioactive. The patterns of radioactivity in sliced sodium dodecyl sulfate gels of the culture medium did not differ appreciably from those obtained with a labeled amino acid (data not shown). Thus, procollagen contains carbohydrate in addition to the mono- and disaccharides linked to hydroxylysine.

Discussion

The disulfide-bonded procollagen-derived fragment which appears in the medium of cultured cranial bone resembles the collagenase-resistant fibroblast procollagen fragments prepared by others (Sherr et al., 1973; Dehm et al., 1974) in chain composition and other properties. The amino acid composition tabulated in Table I and that reported by Sherr et al. (1973) are similar; small differences may be due in part to species differences between human and chick procollagen or to the presence of additional se-

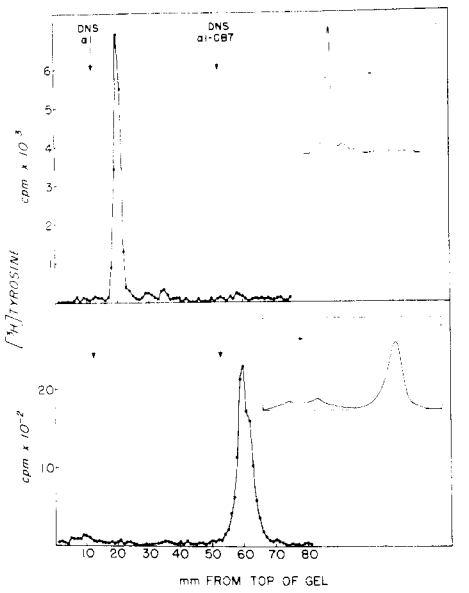

FIGURE 11: Analytical sodium dodecyl sulfate gels of the purified procollagen-derived peptide isolated from the medium of cultured bone: (above) radioactivity patterns of the unreduced peptide; (inset) a densitometric tracing of a parallel gel stained with Coomassie Blue and scanned at 565 nm; (below) radioactivity pattern of the peptide isolated preparatively after reduction and alkylation; (inset) a densitometric tracing of a parallel gel. The arrows indicate the position of migration of dansylated α1 chain and α1-CB7.

quences in the cranial bone fragment. The susceptibility of procollagen to nonspecific limited proteolysis (Monson and Bornstein, 1973; Monson et al., 1975) makes it likely that substrates isolated from different sources by different techniques will differ and therefore the products of reproducible enzymatic digestion may also differ.

The amino acid composition of the medium fragment (Table I) differs significantly, however, from that of the additional sequence at the NH_2-terminal end of the pro-α1 chain of acid-extracted procollagen (von der Mark and Bornstein, 1973). This disparity may be due in part to the presence in the medium peptide of a sequence derived from the pro-α2 chain. However, an additional more attractive explanation suggests itself. Recently, Tanzer et al. (1974) have provided evidence for the existence of extended sequences at the COOH-terminal end of the procollagen molecule. Unpublished experiments from this laboratory[2] have confirmed this finding and suggest that the medium and collagenase-resistant fragments originate from this COOH-terminal nontriple helical domain. In this scheme acid-extracted procollagen, which lacks interchain disulfide bonds (Bornstein, 1974), would represent a truncated precursor containing only NH_2-terminal extensions.

A COOH-terminal assignment for the interchain disulfide-bonded region in procollagen reconciles the conflicting amino acid compositions, deduced from several studies of procollagen, and provides an explanation for other puzzling findings including the observations that (1) antibodies to the collagenase-resistant fragment of chick tendon procollagen do not cross-react with the pro-α1 chain of acid-extracted procollagen; the converse is also true (Nist et al., 1975); (2) preparations of procollagen peptidase (Kohn et al., 1974), capable of converting acid-extracted procollagen to collagen, fail to convert procollagen to a product which

[2] Byers, P. H., Click, E. M., Harper, E., and Bornstein, P., manuscript in preparation.

lacks interchain disulfide bonds (Bornstein et al., 1975); (3) disulfide bond formation occurs relatively late in the assembly of the procollagen molecule (Schofield et al., 1974; Harwood et al., 1973; Fessler, et al., 1973). These findings, coupled with other evidence (Goldberg and Sherr, 1973; Davidson and Bornstein, 1975), suggest that at least two discrete steps, at opposite poles of the procollagen molecule and possibly catalyzed by different enzymes, are involved in conversion of procollagen to collagen.

Pontz et al. (1973) have also described procollagen-derived peptides in the medium of cultured chick cranial bone. These fragments, with molecular weights of 18,000 and 12,000, may represent the NH_2-terminal nonhelical extensions in procollagen and could correspond to the lower molecular weight peptides observed (Figure 6) but not further studied in this work. Pontz et al. (1973) did not find the larger disulfide-bonded fragment described by us, either because the fragment was adsorbed to agarose in the presence of 1 *M* $CaCl_2$ or because of proteolysis during dialysis of medium against acetic acid.

The major fragment obtained by collagenase digestion of chick cranial bone procollagen is somewhat larger than that derived from procollagen secreted by cultured fibroblasts (Sherr et al., 1973; Dehm et al., 1974), although comparisons of molecular weights obtained by different methods and in different laboratories are subject to question. However, in this work the medium fragment was also found to be slightly, but reproducibly, smaller than the collagenase-resistant fragment and there were striking differences in the solubility properties and chromatographic behavior of the two fragments. Routinely, the collagenase-produced fragment from bone adsorbed to column supports, glass surfaces, etc., unless handled in the presence of sodium dodecyl sulfate. In contrast the medium fragment, like the fragments derived from fibroblast procollagens, was soluble at neutral pH in the absence of detergents. Presumably these fragments lack a region (possibly containing a substantial amount of carbohydrate) which confers to the collagenase-produced fragment from bone procollagen the property of adsorption to surfaces. These observations suggest the occurrence of other limited proteolytic steps in the conversion of procollagen to collagen. We cannot distinguish, however, between limited proteolysis of the precursor prior to release of the medium fragment and subsequent proteolytic alteration of the released fragment.

The finding of a large, relatively stable procollagen conversion product in the medium of cultured bone raises the possibility that a fragment functions in feedback regulation of procollagen synthesis and secretion or plays some other physiologic role. An effect of this nature would be consistent with the preliminary observation by Lichtenstein et al. (1973) that human fibroblasts, deficient in procollagen peptidase activity, synthesized excessive amounts of collagen. Studies are under way to examine this possibility but we have thus far (in unpublished experiments) been unable to establish an unambiguous response by cultured chick fibroblasts to addition of the partially purified fragment. The metabolic fate of the fragment is also unknown, although an antigenic activity which cross-reacts with antibodies to procollagen has been observed in the serum of chick embryos (Dehm et al., 1974)

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The Role of the Bound Nucleotide in the Polymerization of Actin†

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ABSTRACT: Three nucleotides, ATP, ADP, and an unsplittable analog of ATP (adenylyl imidodiphosphate (AMPPNP)), were bound to monomeric actin, and their effects on the rate and extent of the actin polymerization were studied. The kinetics of polymerization, assayed by the change in OD_{232}, followed a simple exponential curve. The rates of polymerization were equal for bound ATP and AMPPNP; both of which were three to five times faster than the rate for ADP. The concentration of actin monomers in apparent equilibrium with the polymer, G(∞), was determined. Values of G(∞) in 100 m*M* KCl were found for different nucleotides to be: G·ATP(∞) = 0.7 μM, G·AMPPNP(∞) = 0.8 μM, and G·ADP(∞) = 3.4 μM. The equilibrium constant of the polymerization is given by $K = [G(\infty)]^{-1}$ when no nucleotide is split. The polymerization of actin·ATP is more complex due to the splitting of the nucleotide and our data require that this polymerization involves more than one step. The kinetic parameters for the polymerization of actin·ATP can be explained by a simple scheme in which the nucleotide dephosphorylation occurs in a step following the polymerization step. The conclusions are: (1) the binding of ATP to actin monomer promotes polymerization slightly more than the binding of ADP, (2) actin bound ATP provides less than 4 kJ/mol of free energy to promote polymerization, and (3) the dephosphorylation of the nucleotide is not coupled to polymerization.

The actin polymer forms the backbone of the thin filaments found in a muscle fiber, and it is also found in the cytoplasm of most, if not all, eucaryotic cells. The interaction of myosin with an actin polymer produces mechanical work at the expense of the energy of ATP. The actin filaments are formed by a helical array of monomers, which can be dissociated by lowering the ionic strength. The polymerization–depolymerization cycle of actin is thought to play no role in the contraction of muscle cells, but is hypothesized to play a major role in the assembly of contractile structures in the cytoplasm of nonmuscle cells. This paper investigates the extent to which the polymerization of actin is controlled by a nucleotide which is bound to each actin monomer. The biochemistry of muscle actin has been reviewed by Oosawa and Kasai (1971) and that of cytoplasmic actin has been reviewed by Pollard (1973).

The actin monomer (G-actin) binds one nucleotide and one divalent cation. In G-actin, the bound nucleotide can be either a di- or triphosphate, and rapidly exchanges with unbound nucleotides in the medium. The nucleotide of the actin polymer (F-actin) exchanges with external nucleotides at a rate which is many orders of magnitude smaller than the rate for G-actin. When G-actin with bound ATP is polymerized to F-actin, the ATP is dephosphorylated, and the nucleotide found in the polymer, or in the thin filament of a muscle, is ADP. Although the dephosphorylation of the bound nucleotide accompanies polymerization it is not a requirement for it since it has been shown that G-actin·ADP (Hayashi and Rosenbluth, 1960) or G-actin with no nucleotide (Barany et al., 1966) can also polymerize.

No function has been quantitatively demonstrated for the nucleotide of actin. It has been implicated as playing a role in promoting or regulating polymerization. The evidence is twofold: the dephosphorylation occurs during polymerization, and G-actin·ATP polymerizes faster than G-actin ADP. Both polymerize faster than G-actin with no bound nucleotides (Hayashi and Rosenbluth, 1960). However, these studies have not explored quantitatively the coupling between the bound nucleotide and the polymerization. Other investigators have speculated that the bound nucleotide may play a role in the energy transducing reactions with myosin. These speculations have found little experimental justification. The only known requirement for the bound nucleotide is to maintain G-actin in a native state. The presence of bound nucleotides on proteins associated with other energy transduction systems—mitochondria (Harris et al., 1973), chloroplasts (Yamamoto et al., 1972), and tubulin (Berry and Shelanski, 1972)—prompts the speculation that bound nucleotides do play an important role in energy transduction. The possibility exists that the actin nucleotide may also have an important but as yet un-

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