Characterization of Collagen Precursors Found in Rat Skin and Rat Bone†

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ABSTRACT: Two genetic types of collagenous proteins, type I and type III, were isolated by extraction and differential salt precipitation from rat skin. The yield of collagen precursors was increased by injecting animals with colchicine 30 min before sacrifice to inhibit secretion of collagen. DEAE-cellulose chromatography was used to separate collagen from collagen precursors. Although these preparations contained more type I collagen than type III collagen, there were always more type III than type I precursors. The precursor chains of type I fractions were separated on CM-cellulose chromatography after denaturation. Three precursor forms were found for each collagen α chain, a complete chain (proα chain), and a precursor chain with only an amino-terminal (pNα chain) and carboxy-terminal extension (pCα chain). Species differences were demonstrated between rat collagen precursors and other species using rat calvaria (frontal and parietal) bones extracted with either 0.5 N acetic acid or neutral salt buffers containing protease inhibitors. Native rat procollagen elutes earlier than chicken or human procollagen on DEAE-cellulose chromatography and does not separate significantly from the pC collagen form. The collagenase resistant amino terminal peptides of rat pNα1 and pNα2 were the same size (16 000) but could be separated by DEAE-cellulose chromatography.

Collagen is synthesized as a larger, more soluble precursor. In recent years several laboratories have demonstrated collagen precursor forms with a variety of apparent molecular weights, ranging from 115 000 to 145 000 for individual α-chain monomers. In the review by Martin et al. (1975), it was suggested that the largest collagen precursors (with chains of 145 000) be termed procollagen, while precursors intermediate in size between procollagen and collagen be termed p collagen. Recent investigations (Fessler et al., 1975; Byers et al., 1975; Davidson et al., 1975) demonstrate that procollagen has extra peptide extensions at both the carboxy-terminal and the amino-terminal regions. A p collagen form, therefore, could arise from a cleavage at either the amino-terminal extension or the carboxy-terminal extension. In addition the existence of several types of genetically different collagen molecules (Miller, 1971a,b; Kefalides, 1973), each with its own biosynthetic precursor forms (Byers et al., 1974; Grant et al., 1972; Dehm and Prockop, 1973), have been discovered. Therefore, investigations of collagen precursors have been difficult to interpret because of size heterogeneity and mixtures of collagen types as well as small yields of precursor.

In order to isolate procollagen in sufficient quantity and purity to clarify some of the above problems, we have developed techniques to separate and isolate native type I and type III precursors from rat skin (Byers et al., 1974). This paper shows that yields of these precursors can be enhanced by injecting colchicine which slows the secretion. In addition each collagen precursor preparation isolated from rat skins is composed of three different precursor monomers for each α chain, one with only the smaller amino-terminal extension, one with the larger carboxy-terminal extension, and one with both extensions. We will designate these pNα, pCα, and proα chains, respectively. We also report related studies done with type I precursors from rat calvaria.

Experimental Procedures

Preparation of Collagen Precursors from Rat Skins. Colchicine (5 mg/animal) was injected intraperitoneally into 50–75-g Sprague-Dawley rats about 30 min before sacrifice. In certain experiments, an intracardiac injection of 100 μCi of [^{3}H]proline was given to ten animals 20 min before sacrifice to label the procollagen. Skins were removed, ground twice in an electric grinder, homogenized by a polytron, and extracted for 30 min in approximately 2 L of 150 mM Tris-HCl–20 mM EDTA–10 μM $PhCH_2SO_2F$–10 mM PMB (pH 7.4) at 4 °C. The collagenous proteins were purified as described by Byers et al. (1974) with the following modifications. Procollagen was precipitated by slow addition of ammonium sulfate to 20% of saturation at 4 °C (11.2 g/100 mL) rather than 30%. This eliminated some contaminating proteins which otherwise interfered in later steps. The precipitate was then redissolved in 600 mL of the original extraction buffer, rather than 1 M NaCl, allowing more type III collagen to be solubilized. Next, the type III collagen and precursors were precipitated from this solution by adding 10 g of NaCl/100 mL of solution and centrifuged for 20 min at 30 000 rpm. Type I collagen and precursors were then precipitated by bringing the salt concentration of the supernatant up to 20 g/100 mL. Each precipitate was redissolved in approximately 500 mL of 200 mM NaCl–50 mM Tris-HCl (pH 7.6) and dialyzed against the same buffer. Proteoglycans were removed by passage through a DEAE-cellulose column as previously described (Miller, 1971a). The collagen fractions were dialyzed against at least three changes of water overnight. Any precipitation which formed during dialysis could be redissolved by addition of 2–3 drops of acetic acid. The solution was then adjusted to 50 mM Tris-HCl–2 M urea, pH 8 (by addition of solid Tris-HCl and urea), and chromatographed on DEAE-cellulose chroma-

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‡ Abbreviations used: EDTA, ethylenediaminetetraacetic acid; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; PMB, *p*-mercuribenzoate; DEAE-cellulose, diethylaminoethylcellulose; CM-cellulose, carboxymethylcellulose.

tography as previously described (Byers et al., 1974). Fractions from the DEAE-cellulose column chromatography were pooled, desalted by dialysis, and lyophilized. (Recovery of counts at this step varied between 75 and 90%.)

Calvaria (frontal and parietal bones) of newborn rats were removed and washed with Dulbecco-Vogt media without glycine. After two 20-min preincubations with fresh media (100 mL/100 calvaria), radioactive amino acids were added (500 μCi of [^{3}H]glycine for 100 calvaria in 50 mL of modified Dulbecco-Vogt media). After a 20-min incubation the calvaria were extracted and homogenized either in 100 mL of cold 0.5 N acetic acid or in 100 mL of the neutral salt extraction buffer described earlier for the rat skin preparations. After centrifugation at 30 000 rpm for 20 min at 4 °C, the collagenous proteins were precipitated from the supernatant fraction by slow addition of ammonium sulfate to 20% saturation (11.2 g/100 mL) at 4 °C as previously described for rat skin preparations, centrifuged at 30 000 rpm for 20 min, and dialyzed against 100 mM Tris-HCl (pH 8) overnight. The samples were adjusted to 50 mM Tris-HCl-2 M urea, pH 8 (by addition of solid urea and diluting to proper volume), and placed on a DEAE-cellulose column for separation of collagen and precursor forms.

CM-Cellulose Chromatography in 8 M Urea. Prior to separation of precursor chains by CM-cellulose chromatography, preparations were first denatured and reduced by 1 mM 2-mercaptoethanol in 5 mL of 8 M deionized urea-40 mM Tris-HCl (pH 8) for 1 h at room temperature. Samples were then either placed over a Bio-Gel p-2 (200-400 mesh) column (3 × 30 cm) equilibrated in 8 M urea-50 mM sodium acetate (pH 4.8) or adjusted to 50 mM sodium acetate-8 M urea (pH 4.8) by addition of 1 M sodium acetate stock buffer and 10 M deionized urea. The sample was then dialyzed against this buffer for at least 1 h to remove Tris-HCl and excess 2-mercaptoethanol. Chromatography was then performed on a 2 × 7 cm CM-cellulose column at 25 °C using a linear gradient from 0 to 60 mM NaCl in a total volume of 400 mL of 50 mM sodium acetate-8 M urea (pH 4.8) buffer (flow rate 70 mL/h). (Recovery of counts from this column was usually about 75-80%.)

Polyacrylamide Gel Electrophoresis and Cyanogen Bromide Digestions. Precursor preparations from DEAE-cellulose chromatography or precursor chains isolated from CM-cellulose chromatography were always examined for chain composition, apparent molecular weight, and purity by 5% sodium dodecyl sulfate polyacrylamide gel electrophoresis using the method of Furthmayr and Timpl (1971). Some samples were digested by cyanogen bromide in 70% formic acid (Epstein et al., 1971) and the peptides were separated by 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Furthmayr and Timpl, 1971) to give a peptide fingerprint characteristic of the types of collagen present (Byers et al., 1974; Smith et al., 1975).

Bacterial Collagenese Digests of Calvaria Preparations and Separation of Collagenase Resistant Peptides. Fractions from DEAE-cellulose chromatography or precursor chains from CM-cellulose chromatography were digested by pure bacterial collagenase (Advanced Biofactures, form III) in 2.5 mM Tris-HCl-1 mM $CaCl_2$ (pH 7.4) containing 9 mM *N*-ethylmaleimide for 6 h at 35 °C. The reaction was stopped by heating to 90 °C for 15 min. Samples were dialyzed against water overnight.

Collagenase resistant peptides were placed on a 10% agarose column Bio-Gel A-0.5m, 200-400 mesh (2.1 × 120 cm), equilibrated and eluted with 1 M calcium chloride-50 mM Tris-HCl (pH 7.5) as described by Piez (1968). The column was standardized using cyanogen bromide digested type I collagen peptides.

The collagenase resistant peptides were also chromatographed on DEAE-cellulose columns (2 × 7 cm) in 50 mM Tris-HCl-50 mM NaCl-2 M urea (pH 7.4) at 25 °C. A linear gradient was used from 50 to 350 mM NaCl, in a total volume of 400 mL.

Results

The rat skin procollagen preparation as modified in this paper had certain advantages over the previously described methods (Byers et al., 1974). First, injection of colchicine increased the amount of both type I and type III collagen precursors several fold. Approximately 3-9 mg of each type precursor can be isolated from 50 untreated rats, while from 50 colchicine-treated rats between 20 and 40 mg can be recovered. The total collagen extracted is less than 1% of the total collagen in skin and represents newly synthesized un-cross-linked collagen.

Type III collagen and its precursors were partially separated from type I collagen and its precursors by differential salt precipitation. The 10% NaCl precipitate contained predominantly type III collagenous proteins (about 90%) as judged by cyanogen bromide patterns on 7.5% polyacrylamide gels, negligible amounts of α2, and the presence of pepsin-resistant disulfide bonds as shown on 5% polyacrylamide gel electrophoresis (data not shown). The 20% precipitate fraction contained predominantly type I collagen (about 70%) as judged by cyanogen bromide peptide patterns, the negligible amount of pepsin resistant disulfide bonds, and the presence of α2 chains after pepsin treatment (data not shown). An estimate of purity is given since the gel electrophoresis staining procedure used is not sensitive enough nor linear in the upper concentrations to allow accurate α1 to α2 ratios.

DEAE-cellulose chromatography was used to separate the collagens from their precursor forms (Figure 1). Both type I and type III collagen eluted before the gradient under the conditions used, whereas the precursors eluted after the initiation of the gradient. Type I precursors eluted first (P-1, Figure 1), well resolved from type III precursors (P-2, Figure 1). Bacterial collagenase digestion showed that each peak contained collagenous proteins. Amino acid analysis (not shown) agreed with previously published data for collagen precursors (Byers et al., 1974). The type of collagen was first established by cyanogen bromide peptide analysis. Subsequent preparations were continually analyzed before and after pepsin treatment by 5% polyacrylamide gel electrophoresis with and without reduction. Every preparation, whether from colchicine treated or untreated animals, contained more type III than type I precursors, even though the preparations contained more type I than type III collagen (Table I). The specific activity of type I precursors was always at least ten times greater than type III. This could indicate that type III collagen was synthesized or secreted at a slower rate and was processed more slowly than type I collagen precursors at this stage of development.

The collagen precursor fractions have not been termed "procollagen" up to this point because the fractions from DEAE-cellulose contained molecules with several size precursor chains, one with an amino extension of 15 000 molecular weight (pNα chain), one with a carboxy extension of 30 000 (pCα chain), and one with both extensions (proα chain). As shown in Figure 2 these could be separated by a combination of CM-cellulose chromatography and gel electrophoresis. The type I precursor fraction from DEAE-cellulose chromatog-

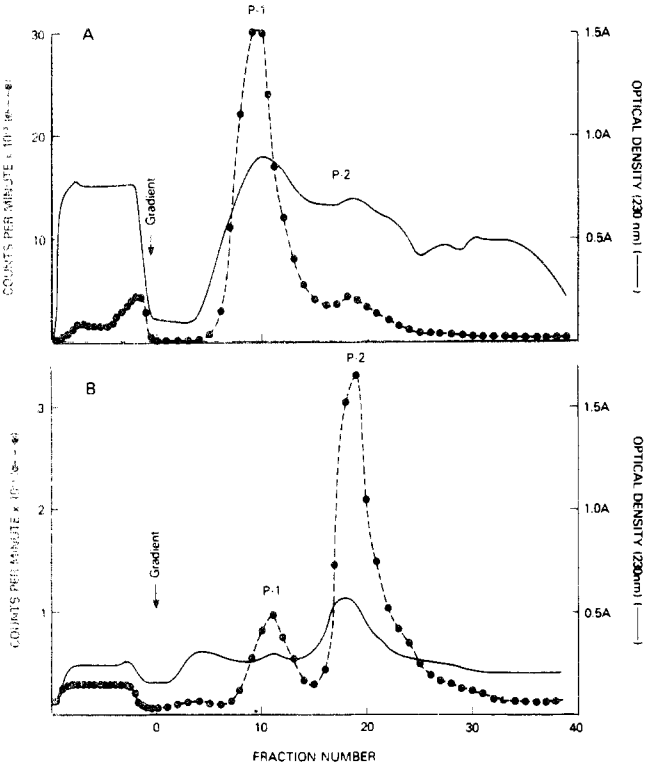

FIGURE 1: DEAE-cellulose chromatography of native collagen precursors from rat skin. Chromatography was performed at 4 °C with 50 mM Tris-HCl-2 M urea (pH 8) and a linear gradient from 0 to 0.2 M NaCl in a total volume of 600 mL. (A) 20% NaCl precipitate on DEAE-cellulose chromatography; (B) 10% NaCl precipitate on DEAE-cellulose chromatography. P-1 is type I collagen precursors and P-2 contains type III collagen precursor (recovery 85%).

raphy contained several bands on 5% polyacrylamide gel electrophoresis (Figure 2, gel numbers 2 and 3). If the sample was reduced with 2-mercaptoethanol, several high molecular weight bands disappeared and at least four distinct bands in addition to $\alpha 1$ and $\alpha 2$ were visible. In order to separate the individual chains, the samples were reduced and chromatographed on CM-cellulose chromatography in 8 M urea. This method separated denatured type I precursors into six major components (Figure 2). The first peak eluting after the start of the gradient (Figure 2, fraction A) had an apparent molecular weight on 5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis of 115 000 using collagen $\alpha 1$ and $\beta 11$ molecules as standards. This fraction had a typical cyanogen bromide pattern for $\alpha 1$(I) chain (Figure 3) and, as will be described later, is also found in acid-extracted calvaria. Therefore, this chain was identified as pN$\alpha 1$. The second peak on CM-cellulose chromatography (Figure 2, fraction B) contained two proteins, one with a molecular weight of 145 000 and the second migrating in the position of the $\alpha 1$ chain. The beginning of this peak contained primarily the higher molecular weight component which, because of its molecular weight and its characteristic $\alpha 1$(I) cyanogen bromide peptides (Figure 3), was identified as pro$\alpha 1$. The third peak (Figure 2, fraction D) contained a component with a molecular weight of 130 000. This component was not present in acid-extracted calvaria but was present in neutral salt extracted calvaria. The cyanogen bromide digest had several interesting features (Figure 3, fraction D). First, the carboxy-terminal peptide CB6 found in digests of collagen was missing. Instead, two additional peptides larger than CB6 were generated on reduction (Figure 3, bands a and b). These peptides were also present to a lesser

TABLE I: Amount of Protein in Each DEAE-Cellulose Fraction from a Typical Colchicine Preparation.

	Collagen		Type I precursors		Type III precursors	
	mg	% of total	mg	% of total	mg	% of total
20% precipitate	69	46	20	13	15	10
10% precipitate	21	14	5	3	21	14
Total	90	60	25	16	36	24

extent in fraction B from CM-cellulose chromatography. The largest peptide (a in Figure 3) has an apparent molecular weight of 46 000 which would correspond to the molecular weight of $\alpha 1$(I)-CB6 (16 000) plus a 30 000 molecular weight peptide. The smaller peptide (b in Figure 3) with an apparent molecular weight of 22 000 may arise from a cleavage in the carboxy-terminal procollagen peptide. This chain was, therefore, the $\alpha 1$ chain with the carboxy-terminal extension identified as pC$\alpha 1$. The peptide from the carboxy-terminal extension peptide has a methionine residue and probably formed interchain disulfide bonds subsequent to CM-cellulose chromatography. In the $\alpha 2$ region of the chromatogram there were two peaks, which contained only $\alpha 2$ cyanogen bromide peptides (Figure 3). In the first of these two fraction (Figure 2, fraction E), there were two proteins as seen on 5% sodium dodecyl sulfate-acrylamide gel electrophoresis, the $\alpha 2$ chain and a precursor chain with an apparent molecular weight of 115 000 when compared with $\alpha 2$ and $\beta 12$. The last fraction contained two molecular weight proteins with either a small amount or no band migrating between $\alpha 1$ and $\alpha 2$ depending on how this fraction was pooled. These slowest migrating bands have apparent molecular weights of 130 000 and 140 000. The $\alpha 2$ chain, therefore, has three precursor forms with different apparent molecular weights, presumably analagous to the $\alpha 1$ chain precursor forms.

Rat Calvaria Preparations. When rat calvaria were acid extracted, the precursor forms isolated had an apparent molecular weight of approximately 115 000 after reduction when electrophoresed on 5% sodium dodecyl sulfate-polyacrylamide gels, with no protein bands observed of higher molecular weight (not shown). On DEAE-cellulose chromatography without prior NaCl fractionation, there was one major peak after the gradient corresponding to type I precursors and a minor peak corresponding to type III precursors. If the type I fraction was chromatographed on a CM-cellulose column for separation of α chains, with or without reduction, the radioactive precursor chains eluted as pNα chains before $\alpha 1$ and with $\alpha 2$. No evidence for pCα, proα chains, or dimers of pN$\alpha 1$ was found. When each of the pNα chains isolated from CM-cellulose were digested with bacterial collagenase, 95% of the proline and glycine counts became dialyzable. The collagenase resistant peptides from both pN$\alpha 1$ and pN$\alpha 2$ acid-extracted precursors had the same molecular weights on 10% agarose column of 16 000 (Figure 4). However, the peptides could be separated by DEAE-cellulose chromatography at room temperature (Figure 5A). The pN$\alpha 2$ collagenase resistant peptide eluted first, indicating that the amino-terminal peptides have different compositions but are the same size. If the native acid-extracted pN collagen precursor was collagenase digested and chromatographed on DEAE-cellulose columns, one heterogeneous peak was found with shoulders corresponding to the collagenase resistant peptides from pN$\alpha 1$ and pN$\alpha 2$ (Figure 5B).

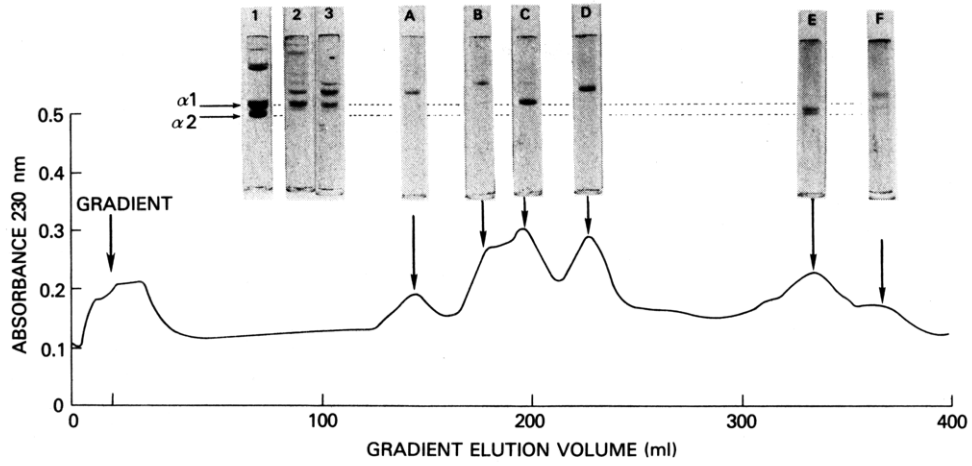

FIGURE 2: CM-cellulose chromatography of denatured type I rat skin precursor chains isolated by pooled fractions from DEAE-cellulose chromatography (Figure 1A) (recovery 80%). Chromatography was performed in 8 M urea 50 mM sodium acetate (pH 4.8) at 25 °C with a linear gradient from 0 to 70 mM NaCl at a flow rate of 70 mL/h. Fractions were examined by 5% polyacrylamide gel electrophoresis (Furthmayr and Timpl, 1971). (Gel 1) Acid-extracted rat skin collagen with the $\alpha 1$ and $\alpha 2$ chains indicated. (Gel 2) The total type I precursors before CM-cellulose chromatography. (Gel 3) The same preparation after reduction with 2-mercaptoethanol. (Gels A through F) Aliquots of pooled fractions taken from CM-cellulose chromatography as indicated by arrows.

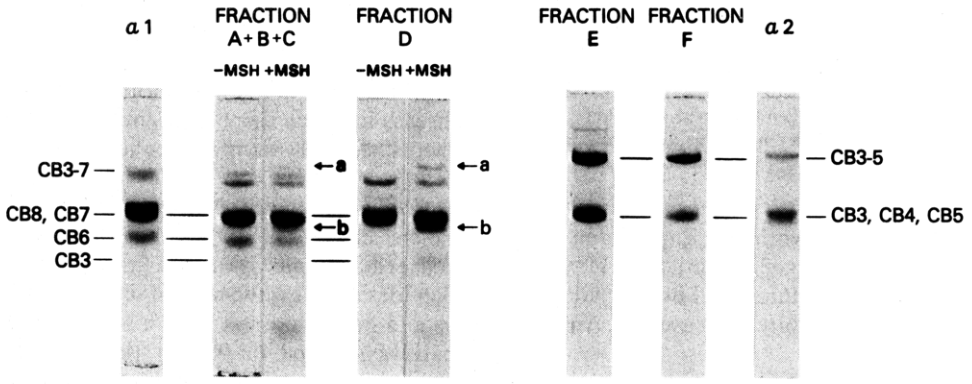

FIGURE 3: Electrophoresis patterns of cyanogen bromide peptides derived from CM-cellulose fractions. The letters A through F are as identified in Figure 3. The letters a and b indicate the peptides which appear after reduction with 2-mercaptoethanol (MSH).

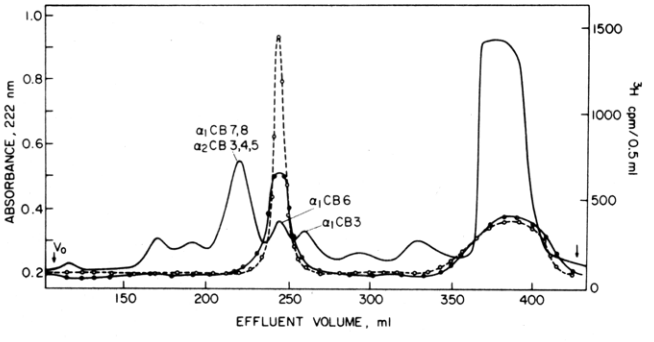

FIGURE 4: Gel filtration on 10% agarose of the bacterial collagenase resistant peptide from acid-extracted pNα1 precursor (O- - -O) and pNα2 precursor (●—●) (not reduced). The solid line is optical density obtained from standard cyanogen bromide digests of acid-extracted collagen used as molecular weight standards (recovery 95%).

After heat denaturation, the major peak decreases and the individual amino-terminal precursor peptides were separated. When an aliquot of these same peptides was placed on 10% agarose column, two peaks resulted with apparent molecular weights of 30 000 and 16 000 (Figure 6). After reduction with 2-mercaptoethanol, the largest peak disappeared with an increase in the 16 000 molecular weight peak.

When rat calvaria were extracted with neutral salt buffers containing protease inhibitors, the precursor forms had exactly the same properties on DEAE-cellulose chromatography, CM-cellulose chromatography, and 5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis as previously described for rat skin type I. Bacterial collagenase digestion of the native neutral extracted collagen precursors contained additional collagenase resistant peptides, not seen in acid extractions. When placed on a 10% agarose column, this additional peak had a molecular weight of 90 000. After reduction this peptide was reduced to a 30 000 molecular weight peptide eluting just before $\alpha 1$(I)-CB7 and $\alpha 1$(I)-CB8. This indicated the existence of a collagenase resistant trimerically linked carboxy-terminal peptide in neutral extracted preparations. These additional peptides could also be labeled with cysteine, tryptophan, and mannose (unpublished observations).

Discussion

In this paper we have described an improved method for isolating collagen precursors from skin by pretreating rats with colchicine. Many investigators (Dehm and Prockop, 1972; Diegelmann and Peterkofsky, 1972; Ehrlich and Bornstein, 1972) have found that colchicine inhibits collagen secretion over short periods of time without significantly stopping collagen synthesis. Since prolonged administration of colchicine

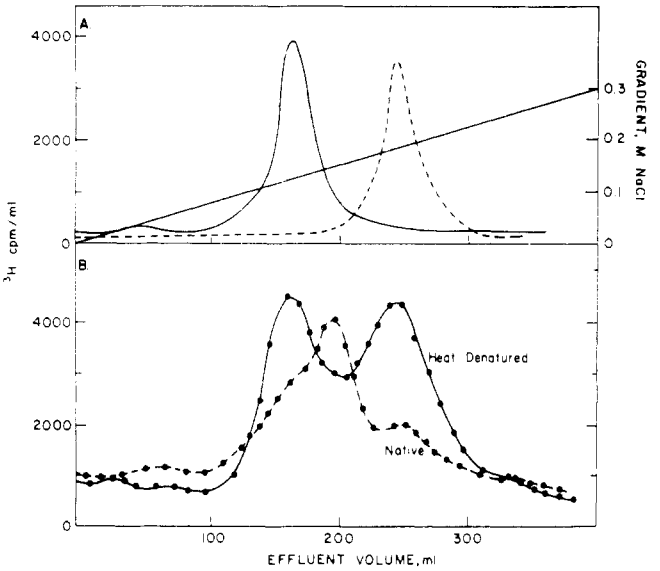

FIGURE 5: DEAE-cellulose chromatography of bacterial collagenase resistant peptides from acid-extracted calvaria. Chromatography was performed in 2 M urea–50 mM NaCl–50 mM Tris-HCl (pH 7.6) at 25 °C with a linear gradient from 50 to 350 mM NaCl at a 200 mL/h flow rate. (A) The individual precursors pNα1 and pNα2 were separated by CM-cellulose chromatography and then digested with bacterial collagenase. Amino-terminal α1 peptide (- - - -); amino-terminal α2 peptide (—) (10 000 cpm of each peptide placed on column) (not reduced). (B) Collagenase resistant peptides of pN collagen from acid-extracted calvaria. Native amino-terminal peptides (●- - -●); heat-denatured amino terminal peptides (●—●) (no change if reduced) (recovery 90%).

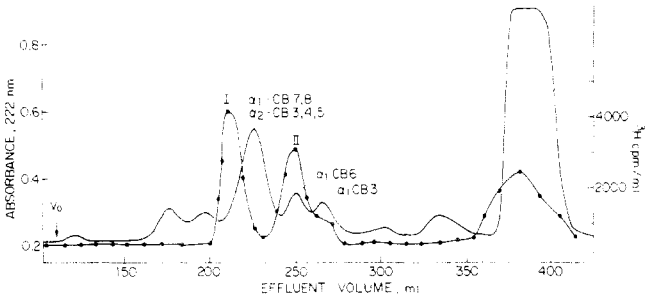

FIGURE 6: Gel filtration on 10% agarose of the collagenase resistant peptides from pN collagen isolated by DEAE-cellulose chromatography at 4 °C from acid-extracted calvaria. Collagenase resistant peptides, not reduced (●—●). Collagen cyanogen bromide peptides used as molecular weight standards (—) (recovery 95%).

can lead to decreased collagen synthesis (Ehrlich and Bornstein, 1972) or to increased collagenase activity (Harris and Krane, 1971), colchicine was administered in a high dose for a short time period. This procedure increased the yields of precursors several fold.

Our results show a difference in synthesis and processing of the two types of collagens found in rat skin. First, at this stage of development, there seems to be more type I being synthesized as judged by the greater amount of type I collagen extracted and by the high proline incorporation into the type I collagen fractions. However, there were always more type III precursors than type I precursors, both in normal rat skin preparations and in colchicine treated rat skins. This suggests that the type III procollagen is converted to collagen at a slower rate than type I prcollagen. Supporting this conclusion, immunofluorescence studies using antibodies to type III p collagen demonstrated that p collagen does remain in reticulum fibers (Nowak et al., 1976) and can be found surrounding human skin fibroblasts in greater quantities than type I p collagen (Gay et al., 1976). The slower processing of type III procollagen leading to accumulation of precursors may effect the synthesis of type III collagen by a feedback mechanism.

Other investigators (Veis et al., 1972; Clark and Veis, 1972; Anesey et al., 1975) have demonstrated the presence of collagen chains larger than α chains in skin preparations. However, their results are ambiguous as to the collagen type and form present. Without separation of the collagen types before CM-cellulose chromatography, interpretation of data is difficult since the α2 and type III precursors all elute in the same region. Recently we have observed (unpublished results) that type II isolated from the Swarm rat chondrosarcoma with the procedure described in this paper also has three precursor chains. The smallest elutes after α1(II) chains where pcα1(I) elutes and the larger precursor elutes, as described (Müller and Jamhawi, 1974) for chicken sternum type II procollagen, in the α2 region. Therefore, it is important to separate the types of collagen in tissues before attempting separation of precursor chains by CM-cellulose chromatography.

Rat skin type I collagen precursors as isolated by this procedure consist of three larger chains for each type of chain synthesized. The precursor chains pNα1 are found in the smallest quantities in all neutral salt preparations containing protease inhibitors, but are the major forms found in acid extractions without protease inhibitors. This precursor form has characteristics similar to dermatosparactic calf collagen, a disease in which there is an enzyme deficiency in a protease that is necessary to complete the conversion of procollagen to collagen (Lenaers et al., 1971; Stark et al., 1971). According to the kinetic studies performed by Davidson et al. (1975) and Fessler et al. (1975), the amino-terminal region of the procollagen molecule is removed first before the carboxy-terminal peptides. If this is the normal sequence of events, pNα chains should not be present. There may be alternate degradative pathways found in skin or the amino-terminal extension cleavage may be slower in skin, so that, before amino-terminal cleavage has been completed, some of the carboxy-terminal extensions are being removed one at a time. This would result in a small amount of chains with amino-terminal extensions only. However, we cannot rule out the possibility that the pN collagen is an artifact produced by degradation during purification procedures even though all possible steps were taken to avoid proteolysis.

The precursor chain, pCα, has an approximate molecular weight of 130 000. The cyanogen bromide pattern of this precursor indicates that there is a carboxy-terminal extension peptide since α1(I)-CB6, the collagen carboxy-terminal cyanogen bromide peptide, is not obtained. This has also been observed in human procollagen chains isolated from fibroblast media (Lichtenstein et al., 1975) but the data were more variable. The additional cyanogen bromide peptides found in digests of pCα1 suggest that there is a methionine in the carboxy-terminal peptide which was partially cleaved. Other investigators (Byers et al., 1975; Fessler et al., 1975) have confirmed the initial observation of Tanzer et al. (1975) that procollagen has a carboxy-terminal extension. These data support the view that the larger extension contains disulfide linkages between all three chains since collagenase resistant peptides from neutral salt extracted collagen precursors have a molecular weight of 90 000 before reduction and 30 000 after reduction with 2-mercaptoethanol.

Certain species differences are apparent when the rat calvaria results are compared with chicken calveria studies already published (Bornstein et al., 1972; Monson and Bonstein, 1973; Fessler et al. 1973; Morris et al., 1975). First rat pro-

collagen elutes earlier than chicken or human procollagen in the position Fessler et al. (1975) and Davidson et al. (1975) have reported for chicken pC collagen. Although chicken or human pC collagen can be separated in our laboratory from the complete molecule by DEAE-cellulose chromatography, we have been unable to separate the native rat precursors by this method, presumably because of species differences. The bacterial collagenase resistant peptides found for rat pNα1 chains, 16 000 molecular weight, are much larger than those isolated from chicken pN collagen (about 3500 for pNα1 peptides; no pNα2 peptide was reported; Bornstein et al., 1972). We were able to demonstrate a pNα2 collagenase resistant peptide, as well as a pNα1 peptide with the same molecular weight on agarose sieve chromatography. The rat amino-terminal extensions may not be as susceptible to bacterial collagenase attack again suggesting species differences in the chemistry of the peptide extensions.

Although the two pN collagen extension peptides could not be separated on the basis of size, they could be separated by DEAE-cellulose chromatography after denaturation. Some peptides were joined by disulfide bonds in these studies. It is not known whether these occur in vivo or are an artifact of the preparation. The pNα2 peptides were not disulfide linked, yet were not dissociated readily, unless heated, which may reflect properties important in chain assembly during the formation of the collagen molecule.

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