ylation system. These findings suggest that the COOH-terminal tryptophan residue is involved in binding to cytochrome $P450_{cam}$ (Sligar et al., 1974). Second, the chemical modification of the tyrosine residue rearranges the structure of the binding site towards the reductase, resulting in an increase in the dissociation constant towards the reductase. Our results failed to weight these two possibilities. Studies on the location of the tyrosine residue in the adrenodoxin molecule together with that of tryptophan residue in the reductase will provide a clue for understanding the nature of the complex between adrenodoxin and its reductase.

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NH_2-Terminal Extensions on Skin Collagen from Sheep with a Genetic Defect in Conversion of Procollagen into Collagen†

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ABSTRACT: A modified form of procollagen was extracted with 10 M urea from the skin of lambs with dermatosparaxis, a disease which is produced by a genetic defect in the conversion of procollagen to collagen. The extracts contained little if any $\alpha 1$ and $\alpha 2$ chains of normal type I collagen, and instead they contained the larger polypeptides $p\alpha 1$ and $p\alpha 2$ together with higher polymers. $p\alpha 1$ was purified by ion-exchange chromatography and gel filtration. The polypeptide was shown to be related to $\alpha 1$ by its chromatographic behavior, its amino acid composition, and the peptides obtained after cleavage with cyanogen bromide. The molecular weight of $p\alpha 1$ by gel filtration was 112 300 ± 6300. After digestion of $p\alpha 1$ with bacterial collagenase, a fragment of about 100 amino acid residues was obtained which was similar in amino acid composition and antigenic activity to a comparable fragment previously obtained from the NH_2-terminal region of $p\alpha 1$ chains from dermatosparaxic cattle. However, after cleavage of $p\alpha 1$ with cyanogen bromide, a larger NH_2-terminal fragment of about 160 amino acid residues was obtained. The larger cyanogen bromide fragment contained 8 residues of hydroxyproline, 12 residues of proline, and 19 residues of glycine not found in the NH_2-terminal fragment isolated after digestion with bacterial collagenase. The results indicated that, in addition to containing amino acid sequences similar to those found in globular proteins, the peptide extensions on the NH_2-terminal end of the $p\alpha 1$ chain of procollagen also contain amino acid sequences similar to those found in the triple-helical region of the collagen molecule. The molecular weight of $p\alpha 2$ by gel filtration was 102 400 ± 6800. No additional peptide fragment was recovered after digestion of $p\alpha 2$ with bacterial collagenase.

Collagen is first synthesized as a precursor molecule which has been called procollagen and which is larger than collagen because of peptide extensions on the three polypeptide chains of the molecule (for recent reviews, see Schofield and Prockop, 1973; Bornstein, 1974; Martin et al., 1975; Gallop and Paz, 1975; Veis and Brownell, 1975). The three pro α chains of procollagen have been shown to be linked by interchain disulfide bonds, and initially it was thought that all the additional peptides and the interchain disulfide bonds of procollagen were located at the NH_2-terminal end of the protein (Dehm et al., 1972). More recent results (Tanzer et al., 1974; Anesey et al., 1975; Fessler et al., 1975; Byers et al., 1975; Olsen et al., 1976), however, have shown that, although each pro α chain contains

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a peptide extension of about 15 000 daltons at the NH_2-terminal end, a larger fragment of about 35 000 daltons, which was previously isolated by several laboratories (Sherr et al., 1973; Dehm et al., 1974; Murphy et al., 1975), originates from the COOH-terminal ends of the molecule. Also, it now appears that the interchain disulfide bonds are found at the COOH-terminal ends of the pro α chains (Fessler et al., 1975; Olsen et al., 1976).

Studies on the nature of procollagen were stimulated by the discovery of genetic defects in the conversion of procollagen to collagen. The first such defect discovered was among cattle in a Belgian herd, and it was named dermatosparaxis because the skin of affected calves was readily torn (Lenaers et al., 1971). Part of the collagen consisted of polypeptides larger than α chains, and extracts of the skin lacked enzymatic activity for converting the larger polypeptides to α chains (Kohn et al., 1974). Similar defects in the conversion of procollagen to collagen were subsequently discovered in a flock of Norwegian sheep (Helle and Ness, 1972; Fjolstad and Helle, 1975) and in man (Lichtenstein et al., 1973). The defect in sheep resembled bovine dermatosparaxis, but clinically the defect in sheep appeared to be more severe (Helle and Ness, 1972; Prockop et al., 1973; Fjolstad and Helle, 1975). Also, all the collagenous polypeptides in extracts of skin from the sheep were larger than α chains (Prockop et al., 1973). In the defect reported in man the skin was not affected as much as joint ligaments and part of the collagen in affected tissues consisted of α chains (Lichtenstein et al., 1973).

We here report on the purification of collagenous polypeptides from the skin of dermatosparactic sheep and the characterization of the additional NH_2-terminal extension on the $\alpha 1$ chain. The data suggest that the peptide extension contains collagen-like amino acid sequences in addition to the globular structure previously shown to be present in similar polypeptides from dermatosparactic calves (Furthmayr et al., 1972).

Materials and Methods

Skin from Normal and Affected Sheep. Skin from the affected sheep was obtained from a flock maintained at the School of Veterinary Medicine, University of Oslo, Oslo, Norway. Affected lambs were killed from a few hours to 2 days after birth, and the skin was stored frozen at −20 °C. Control samples of skin from normal sheep were obtained from lambs killed on the first day of birth and from an 11-week-old lamb.

Procedures for Extracting Collagen from the Skin. The frozen skin was thawed and most of the wool, fat, and subdermal connective tissue was removed with a scissors. About 100 g (wet weight) was homogenized in a few milliliters of 10 M urea that had been freshly deionized by stirring with an ion-exchange resin (Amberlite MB-3; Serva, Heidelberg). The homogenate was then diluted up to 1 l. of 10 M urea and stirred in the solution for 2 h at room temperature. The suspension was centrifuged at 18 000*g* for 30 min and the residue was extracted again with 1 l. of 10 M urea for 24 h. The two urea extracts were separately dialyzed at 4 °C against the starting buffer used for chromatography on carboxymethylcellulose. The insoluble residue was suspended in distilled water, dialyzed for 2–3 days at 4 °C against distilled water, and lyophilized.

Procedures for Purification of $p\alpha$ and α Chains. Two different methods were used to purify collagen polypeptides from the skin of normal and diseased sheep. In one procedure, the skin was chromatographed on carboxymethylcellulose in the presence of 8 M urea, and in the other procedure the collagen was chromatographed on carboxymethylcellulose without urea (Piez et al., 1963).

For chromatography in the presence of 8 M urea, the urea extract of skin was dialyzed against 8 M urea in 0.01 M sodium acetate buffer, pH 4.8, for 3 days at 4 °C. A precipitate which formed during dialysis was removed by centrifuging the sample at 24 000*g* for 30 min. The precipitate was found by amino acid analysis to consist primarily of noncollagenous proteins. In several experiments the precipitate was digested with collagenase and examined for its content of $p\alpha 1$-CB0,1[1] (see below). From 100 to 200 ml of the supernatant was chromatographed on a 2.5 × 12–18 cm column of carboxymethylcellulose (CM52, Whatman Company, Kent, England). The column was equilibrated with 8 M urea in 0.01 M sodium acetate buffer, pH 4.8, and then eluted with a concave gradient prepared with 800 ml of starting buffer and 860 ml of buffer containing 0.08 M NaCl. Appropriate fractions of the chromatograph were pooled as described below and concentrated to 6–20 ml by ultrafiltration at 4 °C with a PM-30 membrane (Diaflow). From 6 to 8 ml of the concentrated samples were then chromatographed on a 2.5 × 87 cm column of 6% agarose (Bio-Gel A-5m; 200–400 mesh, Bio-Rad) which was equilibrated and eluted with 1 M $CaCl_2$ in 0.05 M Tris-HCl buffer, pH 7.5 (Piez, 1968). Appropriate fractions from the agarose column were desalted on a column of polyacrylamide (Bio-Gel P-2) that was equilibrated and eluted with 0.1 M acetic acid. Samples were then lyophilized. In some experiments, the $p\alpha 2$ chains were further purified by chromatography on carboxymethylcellulose without urea (Piez et al., 1963).

To carry out ion-exchange chromatography in the absence of urea, the urea extract of skin was dialyzed against 0.06 M sodium acetate buffer, pH 4.8. The precipitate that appeared during the dialysis was removed by centrifuging at 15 000*g* for 30 min and discarded. The supernate was denatured by heating the sample at 40 °C for 30 min, and it was chromatographed on carboxymethylcellulose at 40 °C with the procedure originally described by Piez et al. (1963). Appropriate fractions from the column were chromatographed on 6% agarose in 1 M $CaCl_2$, as described above. Normal sheep skin was extracted with 10 M urea and collagen $\alpha 1$ and $\alpha 2$ chains were purified from the extract by chromatography on carboxymethylcellulose and gel filtration (Piez et al., 1963; Piez, 1968).

Cleavage of $p\alpha$ Chains with Cyanogen Bromide and Purification of the Released Fragments. Cleavage of $p\alpha$ chains with cyanogen bromide was carried out with the method of Epstein et al. (1971). About 100 mg of $p\alpha 1$ chains purified as described above was dissolved in 10 ml of 70% formic acid, and 100 mg of cyanogen bromide was added. The sample was then incubated at 30 °C for 4 h. Most of the cyanogen bromide was removed on a water pump and the sample was lyophilized. Peptides were isolated by chromatography of the sample on a 2.5 × 18 cm column of phosphocellulose (P11; Whatman) that was equilibrated at room temperature with 6 M urea in 1 mM sodium acetate buffer, pH 3.6. The column was eluted with a linear gradient prepared with 400 ml of starting buffer and 400 ml of starting buffer containing 0.3 M NaCl. The peak containing the NH_2-terminal peptide from the $p\alpha 1$ chain was dialyzed at 4 °C against 2 M urea in 50 mM Tris-HCl buffer, pH 8.6, and further purified by chromatography on a 2.5 × 15 cm column of DEAE-cellulose (DE-52; Whatman) that was

[1] The nomenclature for cyanogen bromide peptides is according to Traub and Piez (1971). $p\alpha 1$ and $p\alpha 2$ denote the procollagen polypeptides from lambs or calves with dermatosparaxis.

[2] Abbreviation used: DEAE, diethylaminoethyl.

equilibrated with the same buffer. The DEAE-cellulose column was eluted with a linear gradient prepared with 300 ml of starting buffer and 300 ml of starting buffer containing 0.3 M NaCl. The NH_2-terminal peptide was desalted on a Bio-Gel P-2 column equilibrated and eluted with 0.1 M formic acid and lyophilized. For comparison, the NH_2-terminal cyanogen bromide peptide from the α1 chain of normal sheep skin collagen (α1-CB-0,1)[1] was isolated with the procedures described previously (Rauterberg et al., 1972).

Digestion of the Isolated pα Chains with Bacterial Collagenase and Isolation of the Collagenase-Resistant Peptides. Purified bacterial collagenase was obtained from a commercial source (Worthington Biochemical Corp.) and was further purified by gel filtration on Sephadex G-200 (Peterkofsky and Diegelmann, 1971). The enzymatic digestion was carried out at 37 °C in 5 mM $CaCl_2$ and 2.5 mM *N*-ethylmaleimide dissolved in 50 mM Tris-HCl buffer, pH 7.4. The concentration of protein substrate was held constant at 10 mg/ml but the enzyme concentration and time of digestion were varied. For digestion of pα chains, an enzyme–substrate ratio of 1:100 was used and the digestion was carried out for 4 h. For digestion of insoluble residues, the enzyme–substrate ratio was 1:2000–5000 and the digestion was carried out for 24 h. In each case, the digestion was terminated by adding a few drops of glacial acetic acid, and the samples were then centrifuged at 18 000*g* for 30 min. The supernatant from the pα1 digest was then chromatographed without further treatment (see below). The supernatant fractions from the digested insoluble residue were dialyzed against 0.01 M acetic acid at 4 °C for 1 or 2 days and lyophilized.

To purify the peptides obtained after the collagenase digestion, about 20 mg of each sample was chromatographed either on a 1.5 × 87 cm column of Bio-Gel P-30 at 4 °C or on a similar column of Bio-Gel P-10 at 37 °C. In each case the column was equilibrated and eluted with 0.03 or 0.05 M sodium acetate buffer, pH 4.5. To purify the NH_2-terminal peptide (pα1-CB0,1-Col 1), the peak obtained from the Bio-Gel column was chromatographed on a 1 × 8–10 cm column of phosphocellulose (P11; Whatman) that was equilibrated with 6 M urea in 1 mM sodium acetate buffer, pH 3.6. The column was eluted with a linear gradient prepared with 100 ml of starting buffer and 100 ml of buffer containing 0.2 M NaCl. The similar collagenase-resistant peptide was prepared from the skin of calves with dermatosparaxis, as described previously (Furthmayr et al., 1972).

Immunological Procedures. Previously published procedures were used to obtain purified antibodies against the procollagen in the skin of calves with dermatosparaxis (Timpl et al., 1973). Specific antibodies both to the procollagen and to the NH_2-terminal peptide (Nowack, H., and Timpl, R., unpublished observations) from the procollagen were used in passive hemagglutination assays with red cells coated with the calf procollagen (Beil et al., 1972; Timpl et al., 1973). The titrations were performed with the microtitration system from Cooke Engineering Co., Alexandria, Va. For assays involving hemagglutination inhibition, constant amounts of antibodies (8 agglutinating units) were preincubated at 37 °C for 1 h with serial dilutions of the inhibitor before adding the coated red cells. The samples were then incubated at 4 °C for 24 h and the minimum amount of inhibitor that completely prevented agglutination was recorded.

Other Assays and Procedures. For reduction and alkylation of protein samples, 20 mM dithiothreitol and 80 mM iodoacetate in 6 M guanidine-HCl were employed (Furthmayr et al., 1972). Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was carried out as described previously after the samples were dissolved in either 2 or 8 M urea (Furthmayr and Timpl, 1971). For estimating molecular weights, 10% polyacrylamide gels were used and the runs were standardized by using either reduced and alkylated globular proteins (Furthmayr et al., 1972) or cyanogen bromide peptides from α1 chains of calf skin collagen (Furthmayr and Timpl, 1971). To estimate molecular weights by gel filtration, the agarose column was standardized with α chains, β components, and γ components from calf skin collagen (Piez, 1968).

TABLE I: Distribution of Hydroxyproline in Urea-Extracted Skin from 1 Day Old Normal and Dermatosparactic Sheep.

Fraction	% of Total Hydroxyproline[a]	
	Normal skin	Dermatosparactic skin
Urea extract, supernatant[b]	37	57
Urea extract, precipitate[b]	12	5
Insoluble residue	51	38

[a] Hydroxyproline was assayed after acid hydrolysis with an amino acid analyzer. [b] Two times extracted with 10 M urea and subsequently dialyzed against 0.01 M sodium acetate, pH 4.8, 8 M urea, and separated by centrifugation.

For amino acid analysis, samples were hydrolyzed under an atmosphere of nitrogen in 6 M HCl and 0.06% mercaptoethanol at 110 °C for 24 h, and analyses were performed with a Durrum D-500 amino acid analyzer. The observed values for threonine and serine were corrected by factors of 1.08 and 1.21, respectively (Rauterberg and Kühn, 1971). To assay tryptophan content, samples were hydrolyzed with methanesulfonic acid (Liu and Chang, 1972). To assay half-cystine residues, samples were hydrolyzed in 6 N HCl without mercaptoethanol. Half-cystine residues were also assayed as carboxymethylcysteine after reduction and alkylation of the peptides. Homoserine content was assayed by treating acid hydrolyzed samples with 1% aqueous piperidine at 37 °C for 1 h so that the homoserine lactone ring was opened before the samples were placed on the amino acid analyzer.

Experimental Results

Extraction of Collagen from Sheep Skin. The skin of normal and affected sheep was extracted with 10 M urea and the completeness of extraction was followed by assaying peptide-bound hydroxyproline in the extracts and residue (Table I). A slightly larger fraction of the total skin collagen was extracted with the urea from the skin of diseased sheep than from the skin of normal sheep. About one-third of the collagenous polypeptides, however, remained insoluble after extensive extraction with 10 M urea (Table I) or 6 M guanidine-HCl (not shown).

As reported previously (Prockop et al., 1973), an abnormal banding pattern was seen when extracts from the skin of diseased sheep were examined by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (see below). With extracts of all four skins examined here one band was seen that had about the same mobility as the α1 chain of normal collagen and a second band was seen that had a slightly lower mobility than α1. (As indicated below, these components are probably pα2 and pα1, respectively.) In addition, several bands of considerably lower mobility were seen, and these apparently corre-

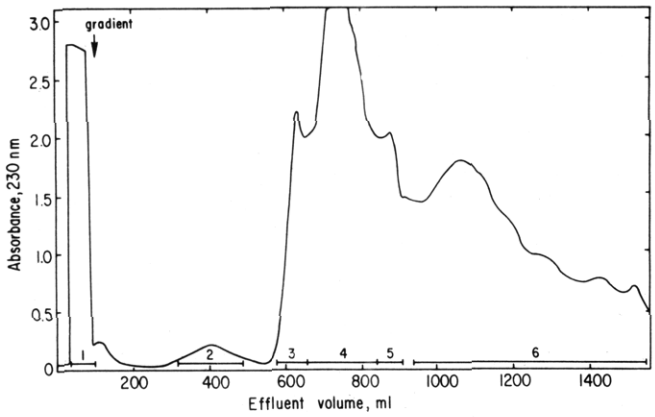

FIGURE 1: Chromatography on carboxymethylcellulose of a 10 M urea extract from the skin of a lamb with dermatosparaxis. The chromatography was carried out in the presence of 8 M urea, as described in Methods. Horizontal lines and numbers indicate how fractions were pooled for further examination.

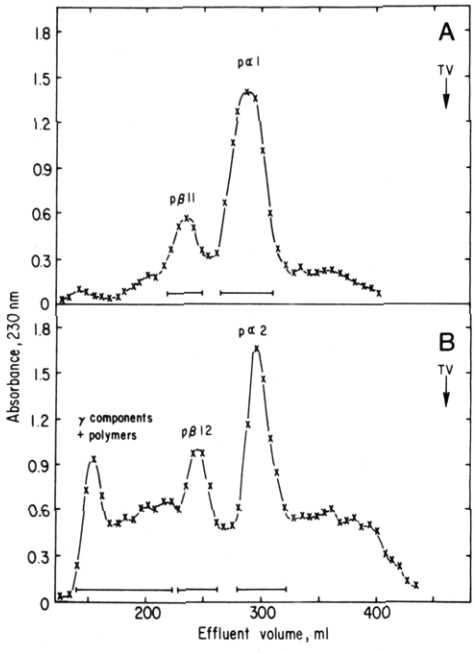

FIGURE 2: Purification of pα1 and pα2 chains by gel filtration on 6% agarose. Chromatographic conditions were as described in Methods. TV indicates total volume of the column. (A) Gel filtration of pool 3 from the chromatogram shown in Figure 1. (B) Gel filtration of pool 5 from Fig. 1.

sponded to dimers, trimers, and higher aggregates of the collagen polypeptide chains. Only about 5% of these higher polymers were converted to protein of high mobility when the samples were reduced and alkylated (not shown). With extracts from skin of three of the diseased lambs, there was no evidence of any α2 chains. With extracts of the fourth lamb, a small amount of α2 was seen by polyacrylamide gel electrophoresis, and subsequent chromatography of the extract (see below) showed that it differed from extracts from the other three lambs in that it contained small amounts of both α1 and α2 chains. We have no explanation as to why this lamb differed from the other three.

Purification of Pα Chains from the Skin Collagen. Urea extracts of the skin from diseased sheep were chromatographed on carboxymethylcellulose in the presence of 8 M urea (Figure 1). An irregular elution profile of protein was obtained and essentially all of the peptide-bound hydroxyproline in the

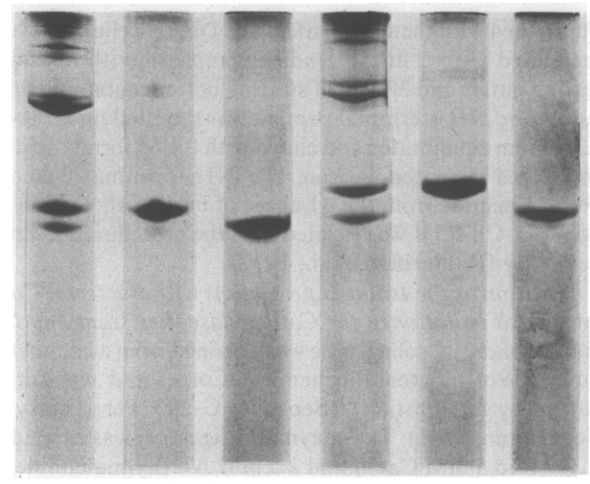

FIGURE 3: Polyacrylamide gel electrophoresis of purified pα1 and pα2. Electrophoresis was carried out on 5% gels in sodium dodecyl sulfate, as described in Methods. Left to right: acid-soluble collagen from normal calf skin; α1 from normal sheep skin; α2 from normal sheep skin; 10 M urea extract from skin of dermatosparactic sheep; purified pα1 from sheep; purified pα2 from sheep.

chromatogram was recovered in the fractions, which were designated as pools 3, 4, and 5. Gel filtration of pool 3 indicated that it largely consisted of a single component (Figure 2A), which was subsequently identified as pα1 (see below). The peak of pα1 obtained by gel filtration (Figure 2A) was shown by polyacrylamide gel electrophoresis not to contain any significant amount of α1 chains (Figure 3). Since the elution position of pool 3 (Figure 1) corresponded to the elution position of α1 chains from normal skin, the results suggested that the skin used for the chromatogram shown did not contain α1 chains.

Gel filtration of pool 5 from the chromatogram revealed the presence of one major and a number of minor components (Figure 2B). The major component was subsequently shown to consist of pα2 (see below). Several of the minor components appeared to be dimers and higher polymers. Gel electrophoresis in sodium dodecyl sulfate also revealed the presence of a small amount of α2 chains, but it was not apparent whether the α2 chains were present in initial skin extract or whether they arose by degradation of pα2 chains during the purification procedures. The nature of the components in pool 5 which eluted after pα2 was not examined.

The other protein fractions in the carboxymethylcellulose chromatogram shown in Figure 1 appeared to be heterogeneous and were not investigated further. For example, pool 4 was found by gel electrophoresis to consist mainly of pβ components and higher polymeric components, and only a small amount of pα chains (not shown).

Chromatography in the presence of 8 M urea gave high recoveries of protein in that 85–95% of the protein applied to the column was recovered in the fractions indicated (Figure 1). When the same type of chromatography was carried out in the absence of urea, the recoveries fell to 50–60%, but a better separation of protein fractions was obtained (Figure 4). The better resolution of the protein apparently was achieved because a large amount of the polymeric material present in the 10 M urea extract of skin was lost as a precipitate when the sample was dialyzed against the starting buffer. The elution positions of the collagen components also differed, probably for the same reason. As indicated in Figure 5, pool 2 from the chromatograph in Figure 4 consisted primarily of pα1 chains and pool 6 consisted primarily of pα2 chains. Most of the α1

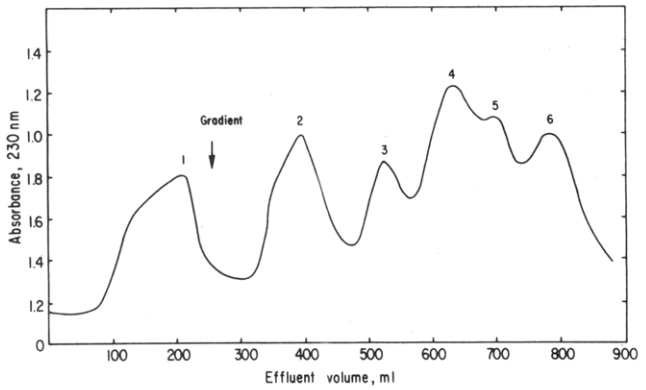

FIGURE 4: Chromatography on carboxymethylcellulose of 10 M urea extract from skin of a lamb with dermatosparaxis. Chromatography was carried out in the absence of urea, as described in Methods. The peak fractions were taken for examination by polyacrylamide gel electrophoresis (see Figure 5).

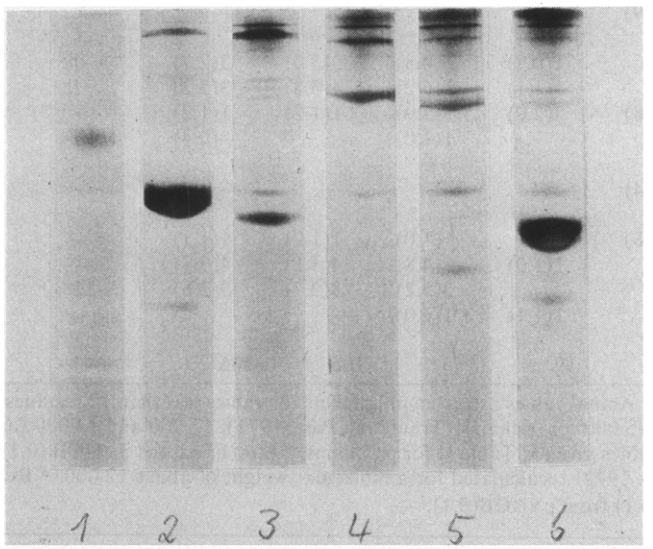

FIGURE 5: Polyacrylamide gel electrophoresis on 5% gels in sodium dodecyl sulfate of the peak fractions from Figure 4. Similar results were obtained when lesser amounts of protein were applied to the gels (not shown). Left to right: peak fractions 1–6.

chains in the chromatograph appeared in pool 3 together with higher molecular weight material. The procedure may therefore be useful to separate pα1 from α1 in samples that contained both these polypeptides. Similar results were obtained with a more complex elution procedure by Lenaers et al. (1971).

Identification of the pα1 and pα2 Chains. The identity of the components referred to above as pα1 and pα2 was suggested by the observation that they eluted in the same position as α1 and α2 chains when ion-exchange chromatography was carried out in urea (Figure 1), but they were larger than α1 and α2 by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (Figure 3). Further proof of their identity was obtained by amino acid analysis (see below) and by cleaving the components with cyanogen bromide. The banding pattern obtained after cleavage of pα1 was similar to the banding pattern obtained by cleavage of α1 chains from normal sheep skin, and the banding pattern obtained after cleavage of pα2 was similar to the pattern obtained by cleavage of α2 (Figure 6). The results indicated, therefore, that the components tentatively identified as pα1 and pα2 had amino acid sequences similar to α1 and α2, respectively.

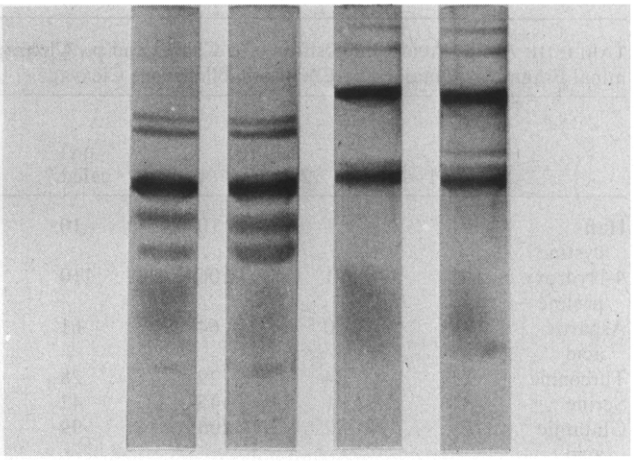

FIGURE 6: Polyacrylamide gel electrophoresis on 7.5% gels in sodium dodecyl sulfate of cyanogen bromide peptides from pα1 and pα2. Left to right: cyanogen bromide peptides from α1 of normal sheep skin; pα1 from dermatosparactic sheep; pα2 from dermatosparactic sheep; α2 from normal sheep skin.

TABLE II: Apparent Molecular Weights of pα Chains and pβ Components.[a]

Chain	Mol Wt[b] (±SD)
pα1	112 300 ± 6 300
pα2	102 400 ± 6 800
pβ11	219 700 ± 14 000
pβ12	209 000 ± 13 400

[a] Determined by molecular sieve chromatography on agarose A-5. [b] Average from 5–7 different runs.

Molecular Weights and Amino Acid Composition of the Isolated Polypeptides. The molecular weights of the purified fractions were estimated by gel filtration in the presence of 1 M $CaCl_2$ (Piez, 1968). As indicated (Table II), the molecular weight of the pα1 chain was about 112 000 and the molecular weight of the pα2 was about 102 000. The pβ11 and pβ12 components were tentatively identified by their behavior in polyacrylamide gel electrophoresis in sodium dodecyl sulfate.

Amino acid analyses of the isolated components are presented in Table III. For comparison, α1 and α2 chains were isolated from skin of normal sheep and analyzed, and they were found to be similar in amino acid composition to α chains of type I collagen from other mammalian species (Traub and Piez, 1971; Fietzek and Kühn, 1975). The amino acid composition of pα1 clearly differed from that of pα2, and it resembled the amino acid composition of the α1 chain more closely than the α2 chain. Similarly, the pα2 chain resembled the α2 chain more closely than the α1 chain.

Isolation of the NH_2-Terminal Peptide from pα1 after Cleavage with Cyanogen Bromide. The NH_2-terminal peptide from the pα1 chain was isolated by cleaving the chain with cyanogen bromide and then identifying the NH_2-terminal peptide by its reactivity with antisera specifically directed against the NH_2-terminal extension of pα1 from calves with dermatosparaxis.

The peptides obtained after cleavage with cyanogen bromide were separated by chromatography on phosphocellulose (Figure 7), and the eluted peaks were examined by polyacrylamide gel electrophoresis in sodium dodecyl sulfate and by

TABLE III: Amino Acid Composition of α Chains and pα Chains from Normal and Dermatosparactic Sheep Skin and of Their NH_2-Terminal Fragments Obtained by CNBr or Collagenase Cleavage.[a, 1]

	α1	α2	pα1	pα1 calcd.[b]	pα2	α1-CB (0.1)	pα1-CB (0.1)	pα1-CB (0.1) Col 1	pα1-CB (0.1) col 1 calf[c]	pα1-CB (0.1) diff.[d]
Half-cystine	—	—	10	10	2(1.6)	—	10	10	10	—
4-Hydroxy-proline	102	91	106	110	96	—	8(7.6)	—	—	8
Aspartic acid	44	50	64	61	56	1(1.0)	18	17	16	—
Threonine	21	24	29	28	26	1(1.0)	8(8.3)	7(6.9)	6(6.0)	—
Serine	41	41	45	42	42	3(2.8)	5(5.3)	2(2.3)	2(1.8)	—
Glutamic acid	78	72	106	99	83	2(2.1)	23	21	22	—
Proline	135	116	155	155	129	2(2.0)	22	10	13	10
Glycine	344	346	352	368	360	3(3.1)	28	9(8.9)	10	16
Alanine	121	112	131	125	118	—	4(4.2)	2(2.2)	2(1.7)	2
Valine	15	29	27	26	34	1(0.8)	12	11	10	—
Methio-nine	7(6.9)	4(3.7)	6(5.9)	7	4(3.9)	—	—	—	—	—
Isoleucine	9(9.0)	14	13	13	16	1(0.9)	5(4.8)	3(3.0)	3(2.7)	1
Leucine	23	34	28	28	37	1(1.1)	6(6.2)	4(3.8)	3(3.2)	1
Tyrosine	4(3.8)	4(4.4)	6(5.5)	5	5(4.8)	2(2.0)	3(2.9)	1(1.2)	1(1.2)	—
Phenyl-alanine	12	14	12	13	15	—	1(1.0)	—	—(0.4)	1
Hydroxy-lysine	5(5.2)	8(8.4)	5(4.9)	5	9(9.4)	—	—	—	—	—
Histidine	3(2.7)	9(8.7)	4(3.6)	4	8(7.8)	—	1(1.0)	1(1.2)	1(1.1)	—
Lysine	34	24	39	38	25	1(1.0)	5(4.8)	4(4.1)	4(3.8)	—
Arginine	53	53	58	58	59	—	5(5.2)	3(2.7)	3(2.6)	2
Homose-rine	—	—	—	—	—	1(1.1)	1(1.0)	—	—	—
Total	1051	1045	1196	1195	1124	19	165	105	106	41

[a] Given as residues/peptide rounded off to the nearest whole number. Actual values are given in brackets for values less than 10 residues. A dash denotes less than 0.2 residue. Molecular weights assumed are 95 000 for α chains (Traub and Piez, 1971); 17 000 and 12 000 for pα1-CB (0.1) and pα1-CB (0.1)Col 1, respectively (see text), and the values given in Table II for pα chains. [b] Sum of α1 and pα1-CB (0.1) minus the composition of α1-CB (0.1). [c] Data from (Furthmayr et al., 1972) recalculated for a molecular weight of about 12 000. [d] Remaining composition after subtracting pα1-CB(0.1) Col 1 and α1-CB(0.1) from pα1-CB(0.1).

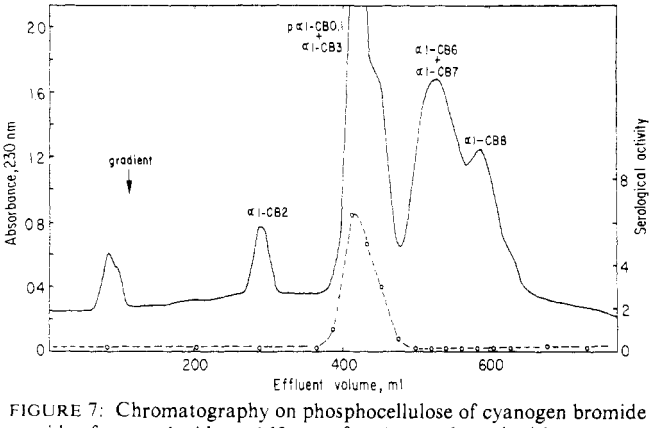

FIGURE 7: Chromatography on phosphocellulose of cyanogen bromide peptides from pα1. About 160 mg of pα1 was cleaved with cyanogen bromide and chromatographed as described in Methods. The arrow indicates the start of the gradient. The absorbance (—) at 230 nm was monitored using a Beckman spectrophotometer DB-T. Serologic activity (O - - - - - O) of the amino-terminal extension was measured in a hemagglutination-inhibition assay after diluting fractions 1:20 with phosphate-buffered saline, pH 7.2. Results are expressed as minimal dilutions ($-\log_2$ units) which prevented agglutination by antibody (8 agglutinating units).

amino acid analysis. Of special importance was the observation that the peptide normally obtained from the NH_2-terminal end of the α1 chain (α1-CB0,1) was not recovered from the column. The peptide α1-CB0,1 elutes just before α1-CB2 (Bornstein and Piez, 1966; Rauterberg and Kühn, 1971), and α1-CB0,1 from normal sheep skin was found here to elute in this position. However, no evidence of α1-CB0,1 was found when skin of diseased sheep was used and the fractions eluting ahead of α1-CB2 were assayed by amino acid analysis. Also of importance was the observation that after cleavage of pα1 with cyanogen bromide, the COOH-terminal peptide normally obtained from α1 chains (α1-CB6) was recovered by chromatography of the digest on phosphocellulose (Figure 7) followed by gel filtration on Bio-Gel P-150 (Rauterberg and Kühn, 1971). Estimates of recoveries (see below) indicated that the peptide was recovered in the expected amount and amino acid analyses indicated that it had the expected amino acid composition (cf. Rauterberg and Kühn, 1971), including the fact that it did not contain homoserine (not shown). It appeared, therefore, that the COOH-terminal end of pα1 had the same structure as the COOH-terminal end of α1, but the NH_2-terminal end differed.

To identify the peptide from the NH_2-terminal end, the peaks obtained from the phosphocellulose column were assayed with antisera specific for the NH_2-terminal end of pα1 from dermatosparactic calves (Timpl et al., 1973). As indicated in Figure 7, antigenic activity was only found in one peak and this peak corresponded to elution position of the α1-CB3 peptide from α1. The antigenically active peak from the phosphocel-

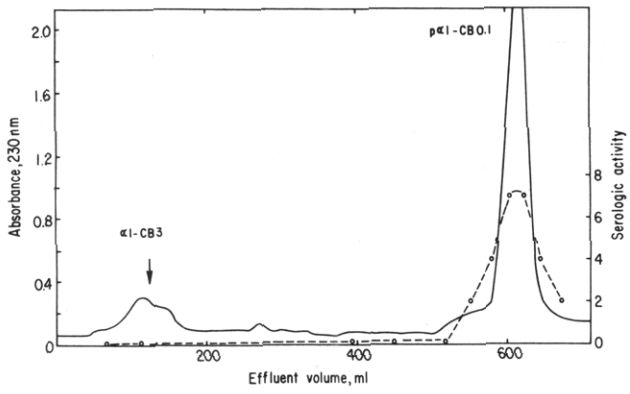

FIGURE 8: Further purification of pα1-CB0,1 on DEAE-cellulose. The serologically active peak obtained from the phosphocellulose column (Figure 7) was chromatographed on DEAE-cellulose as described in Methods. Absorbance at 230 nm (—) and serologic activity (O - - - -O) were measured as indicated in Figure 7.

lulose column was further purified by chromatography on DEAE-cellulose (Figure 8). As indicated, the second chromatographic step separated the antigenically active peptide from α1-CB3.

The purified, antigenically active peptide (pα1-CB0,1) was then examined by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. Most of the peptide was found to migrate as a single band if it was dissolved in 8 M urea, or if it was dissolved in 2 M urea and heated to 100 °C for 5 min before being applied to the polyacrylamide gel (Figure 9). Two faint additional bands were seen, suggesting trace quantities of contaminants may have been present. A distinct slower moving band was seen when the peptide was dissolved in 2 M urea and applied to the gel without any heat treatment. This observation suggested that the peptide had some tendency to associate with itself. Reduction and alkylation did not change the mobility of the peptide. Standardization of the polyacrylamide gels with cyanogen bromide peptides from collagen suggested that the molecular weight of the peptide was 17 400. A higher value of 27 800 was obtained when the gels were standardized with reduced globular proteins.

Amino acid analysis of the isolated peptide pα1-CB0,1 indicated that it contained amino acids not found in α1-CB0,1 (Table III). On the basis of the amino acid composition and the assumption that the peptide contained 1 residue of homoserine, its molecular weight was about 16 950. The peptide contained less than 0.2 residue of tryptophan and about 10 residues of cysteine. Also, the peptide contained about 8 residues of hydroxyproline, 22 residues of proline, and 28 residues of glycine (see below).

The recovery of pα1-CB0,1 was estimated on the basis of amino acid analysis of initial pαl used for cyanogen bromide cleavage and amino acid analysis of the isolated peptide. Because of variable losses during the isolation procedures, the amount of α1-CB2 recovered from the phosphocellulose column (Figure 7) was used as an internal standard. The results indicated that for each mole of α1-CB2 recovered from the phosphocellulose column, 1.04 mol of pα1-CB0,1 was recovered as a homogeneous peak from the DEAE-cellulose column (Figure 8). For purposes of comparison, the peptide α1-CB0,1 was isolated after cyanogen bromide cleavage of α1 from normal sheep skin. Its amino acid composition (Table III) was identical with the amino acid composition of α1-CB0,1 from calf skin collagen (Rauterberg et al., 1972).

Cleavage of the pα1 Chains with Bacterial Collagenase and Isolation of the Collagenase-Resistant NH_2-Terminal Peptide. The isolated pα1 chain was also cleaved with bacterial collagenase and the collagenase-resistant peptide, pα1-CB0,1-Col 1, was isolated first by gel filtration (Figure 10) and then by ion-exchange chromatography on a phosphocellulose column (not shown). The peptide eluting in the first peak from the gel filtration column was not present in a similar collagenase digest of α1 from normal sheep skin. Reduction and alkylation did not alter the position at which pα1-CB0,1-Col 1 eluted from the gel filtration column (not shown). The elution position from the phosphocellulose column was the same as the elution position of the comparable peptide obtained after collagenase digestion of pα1 from dermatosparactic calves (Furthmayr et al., 1972).

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate

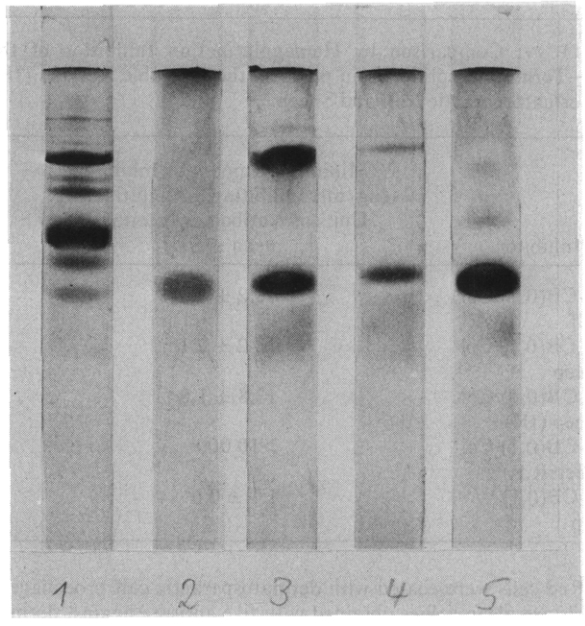

FIGURE 9: Polyacrylamide gel electrophoresis of NH_2-terminal fragments from pα1. Electrophoresis was carried out on 7.5% gels as described in Methods. Left to right: cyanogen bromide digest of collagen from normal calf skin in 2 M urea; pα1-CB0,1-Col 1 in 2 M urea; pα1-CB0,1 in 2 M urea; pα1-CB0,1 reduced and alkylated, in 2 M urea; pα1-CB0,1 in 8 M urea.

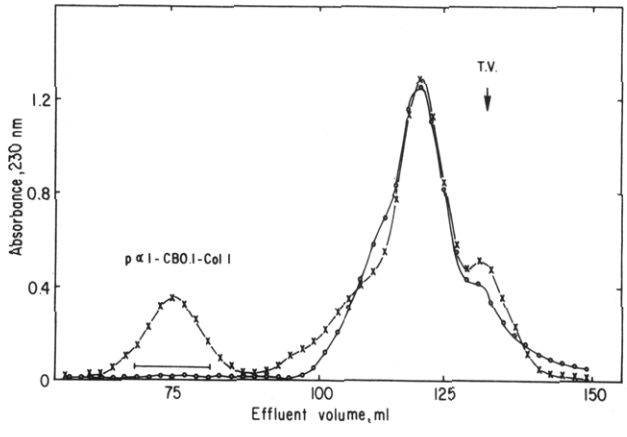

FIGURE 10: Gel filtration of collagenase digests of pα1 and pα2. Samples were digested with bacterial collagenase and chromatographed on a 1.5 × 87 cm column of Bio-Gel P-30 equilibrated and eluted at 4 °C with 0.03 M sodium acetate, pH 4.5. TV indicates the total volume of the column. The peak labeled pα1-CB0,1-Col 1 was taken for further purification by chromatography on phosphocellulose (see text). Symbols: digest of pα1 (x—x); digest of α1 (O—O).

TABLE IV: Comparison by Hemagglutination Inhibition of the NH_2-Terminal Peptides from pα1 and the Insoluble Residue (IR) of Dermatosparactic Calf and Sheep.[a]

Inhibitor	Minimum Amount of Inhibitor (ng/ml) Inhibiting 8 Agglutinating Units of Antibodies (geometric mean ± SD)
pα1-CB(0,1) Col 1 calf	18.2 ± 1.8
pα1-CB(0,1) Col 1 sheep	18.2 ± 2.1
pα1-CB(0,1) Col 1 sheep (IR)	13.8 ± 1.8
pα1-CB(0,1) Col 1 sheep R,A[b]	>10 000
pα1-CB(0,1) sheep	1.1 ± 0.6

[a] Red cells were coated with dermatosparactic calf procollagen. Values are mean values obtained with five antisera against dermatosparactic calf procollagen or the peptide pα1-CB(0.1) Col 1 from calf procollagen. [b] Reduced and alkylated.

suggested that the isolated peptide was homogeneous (Figure 9). The peptide clearly arose from the NH_2-terminal end of pα1, since it reacted with specific antisera to the NH_2-terminal peptides (Table IV). However, amino acid analysis indicated that the peptide was smaller than the peptide obtained after cyanogen bromide cleavage (Table III). The peptide lacked hydroxyproline and methionine. Also, if it was compared to the cyanogen bromide peptide by adjusting its content to the same number of cysteine residues, it apparently lacked 12 residues of proline and 19 residues of glycine found in pα1-CB0,1. As indicated, the number of residues of other amino acids was close to the observed values for the cyanogen bromide peptide, but it contained about 105 amino acid residues compared to about 165 residues for the cyanogen bromide peptide.

The peptide pα1-CB0,1-Col 1 had about the same electrophoretic mobility as the same collagenase-resistant peptide from pα1 from dermatosparactic calves, and reducing and alkylating the peptide did not change its electrophoretic mobility. Comparison of gels from several experiments (not shown) indicated that the mobility in sodium dodecyl sulfate of the collagen-resistant peptide was slightly greater than the mobility of the NH_2-terminal peptide obtained after cyanogen bromide cleavage of pα1. The difference in mobility, however, was not as great as was expected from the difference in amino acid content (Table IV); we have no explanation for this apparently anomalous behavior.

The recovery of the collagenase-resistant peptide pα1-CB0,1-Col 1 was estimated by using amino acid analysis to compare the amount of the peptide recovered from the collagenase digest with the total amount of small peptides in the digest (Figure 10). The results indicated that pα1-CB0,1-Col 1 accounted for 8.6% of the total amino acids in the digest. Since the molecular weight of the peptide was about 10% of the molecular weight of pα1, the results indicated that about 1 mol of pα1-CB0,1-Col 1 was recovered per mol of pα1.

Collagenase digestion was also carried out on the insoluble residue that remained after extraction of the skin with 10 M urea (see Table I). After digestion of the residue, the supernatant solution was examined by gel filtration and a peptide was recovered that had the same elution position as pα1-CB0,1-Col 1 (see Figure 10). The peptide was also similar to pα1-CB0,1-Col 1 in amino acid composition, electrophoretic mobility (not shown), and antigenic activity (Table IV). The yield of the peptide was 1–3 mg/g of collagen in the insoluble residues and considerably less than the value of 70 mg/g that one would expect if all the collagen in the residue consisted of pα1 and pα2 chains.

Attempts to Obtain NH_2-Terminal Peptides from pα2. As shown in Figure 2B, pα2 chains were purified from polymers by gel filtration of pool 5 from the chromatogram shown in Figure 1. It was difficult, however, to obtain peptides from pα2 which were not obtained from α2. pα2 was cleaved with cyanogen bromide, but no peptides which could be attributed to the peptide extensions were found when the cleavage products were chromatographed on phosphocellulose. The search for such peptides was handicapped by the fact that the antisera to dermatosparactic calf procollagen did not cross-react with the pα2 chain (Timpl et al., 1973) and, therefore, could not be used to assay the column fractions. Similarly, no distinctive peptides were found after pα2 was digested with bacterial collagenase. The estimated molecular weight of pα2 (Table II) suggested that it might contain an additional peptide of about 7000 daltons. A peptide of this size should elute between pα1-CB0,1-Col 1 and the broad peak of smaller peptides shown in the chromatogram in Figure 10. However, no peptide was found in this region when collagenase digests of pα2 or α2 were examined.

Immunological Studies on the NH_2-Terminal Peptides from pα1. The antisera produced with procollagen from the skin of calves with dermatosparaxis was used to study further the NH_2-terminal peptides (Table IV). The collagenase-resistant peptide from the sheep pα1 was found to have about the same antigenic activity as the comparable peptide from calf pα1. As noted previously (Timpl et al., 1973), the antigenicity of the NH_2-terminal peptides was largely destroyed by reducing and alkylating the peptides. Of special interest was the observation that the antigenicity of the NH_2-terminal peptide obtained after cyanogen bromide cleavage of pα1 was greater than the antigenicity of the peptide obtained after collagenase digestion.

Discussion

The pα1 and pα2 chains isolated from skin of the diseased sheep were comparable to similar pα1 and pα2 chains previously isolated from dermatosparactic calves in terms of their size and amino acid composition (Lenaers et al., 1971; Furthmayr et al., 1972). In addition, pα1 reacted with the specific antisera for pα1 from dermatosparactic calves, and after collagenase digestion of pα1 with bacterial collagenase, an NH_2-terminal fragment (pα1-CB0,1-Col 1) was obtained that was similar in amino acid composition and immunologic activity to a comparable collagenase fragment previously obtained from pα1 of dermatosparactic calves. The size of the NH_2-terminal peptide (pα1-CB0,1-Col 1) was originally estimated to be about 19 500 by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (Furthmayr et al., 1972). The present estimate of about 12 000 daltons for a similar peptide from sheep skin is probably more accurate because it is largely based on amino acid analysis indicating it contains about 100 amino acid residues. It is not entirely apparent, however, why the apparent molecular weight is larger when estimated by electrophoresis in sodium dodecyl sulfate and by comparison with standards of globular proteins.

Of primary interest here was the demonstration that the NH_2-terminal peptide obtained after cyanogen bromide

cleavage of pα1 (pα1-CB0,1) contained about 60 amino acids not found in peptide extension isolated after collagenase digestion of pα1 (pα1-CB0,1-Col 1). This observation was consistent with the previous observation by von der Mark et al. (1973) that a fragment obtained after cyanogen bromide digestion of pro α1 from chick bone procollagen was larger than comparable fragment obtained after digestion of pro α1 with bacterial collagenase. The results presented here clearly demonstrated that pα1-CB0,1 originated from the NH_2-terminal end of pα1, since it reacted with antisera specific for the NH_2-terminal peptide of pα1 from dermatosparactic calves. Also, the cyanogen bromide digest of pα1 contained the same COOH-terminal peptide (α1-CB6) obtained from α1 chains, but did not contain the NH_2-terminal peptide α1-CB0,1 normally obtained from α1 chains. The peptide pα1-CB0,1 contained a number of amino acids not found in α1-CB0,1 (Table III), the cyanogen bromide peptide from the NH_2-terminal end of α1, and therefore it is very likely that the extension of pα1 did not contain methionine. The distinguishing feature of pα1-CB0,1 was that it contained about 8 residues of hydroxyproline, 10 residues of proline, and 16 residues of glycine which were not found in pα1-CB0,1-Col 1 and α1-CB0,1 (see Table III). The results demonstrated, therefore, that the NH_2-terminal end of pα1 contains amino acid sequences similar to those found in the triple-helical region of the collagen molecule itself.

It was not conclusively demonstrated whether these collagen-like or "helical sequences" were present on the NH_2-terminal or the COOH-terminal side of the fragment resistant to collagenase (pα1-CB0,1-Col 1), but two observations suggest that the collagenase-resistant fragment is at the NH_2-terminal end and that the helical sequences are found almost entirely between the collagenase-resistant fragment and the NH_2-terminal end of the α1 chain. One observation which supports this conclusion comes from amino acid sequencing that Fietzek and Hörlein (unpublished data; reviewed by Fietzek and Kühn, 1975) carried out on a 30 amino acid peptide that was released by digestion of calf pα1 with bacterial collagenase. The peptide had the partial sequence Gly-Pro-Hyp-Gly-Leu-Gly-Gly-Asp-Phe-Ala-Ala-Gln-Leu-Ser-Tyr-Gly. The sequence beginning with glutamine corresponds to the "nonhelical" NH_2-terminal end of the α1 chain (Fietzek and Kühn, 1975). The sequence to the left of the Gln indicates that in pα1 the nonhelical sequence extends for five to eight amino acids but then continues with a sequence which is typical of the helical region of collagen and which would be expected to provide a cleavage site for bacterial collagenase. A second observation which supports the conclusion that the helical sequences are to the right of the collagenase fragment comes from trypsin digestion of pα1-CB0,1. Preliminary results (Becker, U., and Timpl, R., unpublished data) of experiments suggest that trypsin releases a large fragment that corresponds to pα1-CB0,1-Col 1 and a second, smaller fragment that contains the helical sequences joined to the 30 amino acid peptide sequenced by Fietzek and Hörlein (see above).

The presence of helical sequences in NH_2-terminal end of pα1 poses an interesting question as to the function of such sequences. Several different functions have already been suggested for the additional peptides in procollagen: the extensions may keep the molecule soluble and prevent premature fiber formation, they may promote association of the three polypeptide chains, they may facilitate passage of the chains into the cisternae of the rough endoplasmic reticulum, and they may help orient the molecules for proper fiber formation. (For discussion of these and related suggestions, see Schofield and Prockop, 1973; Veis et al., 1973; Bornstein, 1974; Prockop et al., 1976). However, the presence of collagen-like sequences in the peptide extensions suggests still another function. Extensive work on the self-assembly and folding of biological molecules indicates that such processes demonstrate cooperativity and proceed in two discrete steps of nucleation followed by propagation of the structure of the nucleus (for review, see Engel and Schwarz, 1970). As discussed elsewhere (Engel, J., Uitto, J., and Prockop, in preparation), it seems unlikely that the chain association and interchain disulfide bonds provided by the peptide extensions can in themselves explain the observations that during biosynthesis procollagen becomes triple helical within less than 10 min, and that the folding proceeds at this rapid rate at 37 °C (Uitto and Prockop, 1973; Prockop et al., 1976). However, the phenomena could be explained if the peptide extensions provided a nucleus of triple-helical structure from which the helical structure would propagate along the chains. The presence of extra collagen-like sequences in pα1 and the high proline and hydroxyproline content of these sequences raises the possibility that such sequences do in fact provide a site at which a stable nucleus of triple helix first forms and initiates folding of procollagen chains during biosynthesis of the protein. It will be important, however, to provide more direct evidence for such a function.

Unfortunately, it was not possible to isolate an NH_2-terminal fragment from the pα2 chain. Because no specific antisera are available, it will probably be necessary to carry out extensive work on cyanogen bromide digests of both pα2 and α2 in order to identify a fragment comparable to pα1-CB0,1. The failure to obtain the expected fragment of about 7000 daltons after collagenase digestion is in itself of interest, since it suggests that the NH_2-terminal extension on pα2 contains collagenase sensitive sequences. It is possible, however, that a collagenase-resistant fragment is present and is lost during the purification procedures.

Until recently, there was confusion about the genetic defects in the conversion of procollagen to collagen because it was generally assumed that the only peptide extensions on the procollagen molecule were at the NH_2-terminal ends of the three chains. The assumption was made largely because segment-long-spacing aggregates prepared from procollagen were seen by electron microscopy to have extensions only at the NH_2-terminal ends (Stark et al., 1971; Dehm et al., 1972). It is now apparent, however, that the procollagen isolated from embryonic tendon cells and other sources was contaminated by acid proteases that removed the COOH-terminal extensions when the protein was dialyzed against acetic acid in order to prepare segment-long-spacing segments (Hoffmann, H.-P., Olsen, B. R., and Prockop, D. J., submitted for publication). Also, procollagen molecules that remained intact either did not form segment-long-spacing aggregates, or formed thin aggregates in which the COOH-terminal extensions were irregular in appearance and similar to noncovalent attachments occasionally seen in segment-long-spacing aggregates prepared from impure collagen solutions. The nature of procollagen and the manner in which procollagen is converted to collagen have been clarified by the recent demonstrations that the NH_2-terminal end of pro α chains contains additional peptide sequences of about 15 000 daltons and the COOH-terminal end contains peptide sequences of about 35 000 daltons (Tanzer, et al., 1974; Anesey et al., 1975; Fessler et al., 1975; Byers et al., 1975; Olsen et al., 1976). On the basis of this information, it seems reasonable to suggest that the defects in the conversion of procollagen to collagen which have been described in cattle, sheep, and man are limited to defects in removing the NH_2-

terminal peptides. These diseases may or may not involve deficiencies of the same peptidase. It seems apparent, however, that in all three diseases the enzymatic activities for removing the COOH-terminal ends are present.

The defect in the conversion of procollagen is more severe in the sheep examined here than the similar defects reported in cattle (Lenaers et al., 1971) and in man (Lichtenstein et al., 1973), but the results do not establish that the defect in conversion is a complete one. Only trace amounts of $\alpha 1$ and $\alpha 2$ chains were present in urea (Table I), salt, or acetic acid (Prockop et al., 1973) extracts of the skin, and essentially all the collagenous peptides in the extracts were larger than α chains. The smallest collagenous components in the urea extracts were polypeptides identified as $p\alpha 1$ and $p\alpha 2$ chains, but the extracts also contained larger polypeptides that were not dissociated by reduction and that may well, therefore, consist of α chains joined by the types of covalent cross-links found in normal collagen. Also, about one-third of the collagen polypeptides remained insoluble in 10 M urea. The insoluble fraction of the skin may well, therefore, contain fibers assembled from normal collagen that is extensively cross-linked. This conclusion is consistent with microscopic studies indicating that collagen fibers are present in skin from the sheep, and although the fibers are thin and irregular in cross-section, they contain an essentially normal cross-striated pattern (Fjolstad and Helle, 1975). Because of the difficulty of dealing with cross-linked collagen, it was not possible, however, to obtain more direct evidence that some conversion of procollagen to collagen occurred in the skin.

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