Isolation of Peptides Containing the Cross-link, Hydroxylysinonorleucine, from Reconstituted Collagen Fibrils*

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ABSTRACT: Reconstituted calfskin collagen fibrils were treated with sodium borotritide and then sequentially digested with bacterial collagenase and trypsin. Subsequent purification of the labeled peptides involved gel filtration, papain digestion, and ion-exchange chromatography. Three purified peptides containing stoichiometric amounts of the cross-link, hydroxylysinonorleucine, were isolated and their compositions are reported.

A portion of the structure of these peptides may originate from the N-terminal region of collagen.

The formation and isolation of the intermolecular cross-links, lysinonorleucine and hydroxylysinonorleucine, from both native and reconstituted collagen fibrils have been described (Tanzer *et al.*, 1970; Tanzer and Mechanic, 1968, 1970; Bailey and Peach, 1968; Kang *et al.*, 1970). These results indicate that *in vivo* α-aminoadipic semialdehyde residues in one polypeptide chain condense with lysine or hydroxylysine residues in a neighboring chain to yield a Schiff base which can be reduced and labeled simultaneously using sodium borotritide. We wished to study the local peptide regions in which the reduced cross-links occur in order to determine if any unusual features exist which might favor Schiff base formation. We now report the isolation and composition of three hydroxylysinonorleucine containing peptides obtained after proteolytic digestion of reconstituted collagen fibrils.

Materials and Methods

Collagen Digestion and Isolation of Radioactive Peptides. Reconstituted calfskin collagen fibrils (5 g) were treated with calibrated sodium borotritide as described (Tanzer, 1968; Blumenfeld and Gallop, 1966). The lyophylized product was suspended in 250 ml of 0.05 M Tris-Cl–0.01 M $CaCl_2$, pH 7.4, and digested with chromatographically purified bacterial collagenase (Worthington) at 40° for 24 hr. The pH was then adjusted to 8.0 and the mixture digested with 30 mg of trypsin (TPCK treated, Worthington) for another 24 hr. The digestions were carried out under toluene to retard bacterial growth. This procedure is similar to that used for insoluble guinea pig skin collagen (Cunningham and Ford, 1968). Following the tryptic digestion the mixture was adjusted to pH 4 and centrifuged at 19,000*g* for 15 min. The small precipitate was washed twice with water and the fluid added to the original supernatant. The precipitate was then lyophylized and weighed. The supernatant was concentrated by rotary evaporation at 40° and portions equivalent to 1.5 g of original substrate were applied to a 2.5 × 100 cm column of Sephadex G-25, superfine, and eluted at room temperature using 0.1 M acetic acid. The radioactive fractions from this column were pooled and lyophylized; the resultant powder was weighed and then subjected to digestion by papain (Worthington). The conditions of papin digestion were: enzyme–substrate, 1:50 (w/w); 0.2 M pyridine acetate, pH 5.5, containing 0.1 M dithiothreitol; substrate concentration 10 mg/ml; incubation at 37° for 16 hr. Following digestion the pH was lowered to 3 and the mixture was lyophylized. The dried material was dissolved and passed through a 2 × 150 cm column of Sephadex G-25, superfine, using 0.13 M ammonium acetate, pH 5.9, as developer. The emerging radioactive fractions were pooled into four separate regions and then lyophylized. These peptide mixtures were then further resolved by cation- and anion-exchange chromatography using volatile buffers, as outlined by Schroeder (1967). Amino acid composition was used to evaluate peptide purity; the analyses were carried out using either high-sensitivity Spinco 121 or Spinco 116 amino acid analyzers, and approximately 10 nmoles of peptide was routinely analyzed.

High-voltage electrophoresis on Eastman Kodak thin-layer plates (6064) was carried out at pH 6.4 using a pyridine–acetate buffer of the composition, pyridine–acetate–water, 25:1:225. The moistened plate, containing the spotted samples, was subjected to 50 V/cm in a Savant flat plate machine, using wicks of Whatman No. 3MM paper. Approximately 75 mA of current was flowing during the run and the temperature was maintained at about 15° by a thermostated circulating bath. Following electrophoresis, the plate was dried in an air stream and sprayed with ninhydrin solution. Each of the colored spots was cut out and eluted in 0.2 ml of 0.13 M NH_4 acetate directly into scintillation vials. Omnifluor solution (New England Nuclear) containing Beckman Biosolv-3 was used as the scintillation fluid.

Calculation of Hydroxylysinonorleucine Content. The molar content of hydroxylysinonorleucine in the peptides was measured both by its reaction with ninhydrin and by its radioactivity content. We determined the ninhydrin color yield of hydroxylysinonorleucine in two ways: (1) [^{14}C]hydroxylysinonorleucine was synthesized by initially converting [^{14}C]-hydroxylysine (Schwarz) into its α-acetyl methyl ester, then

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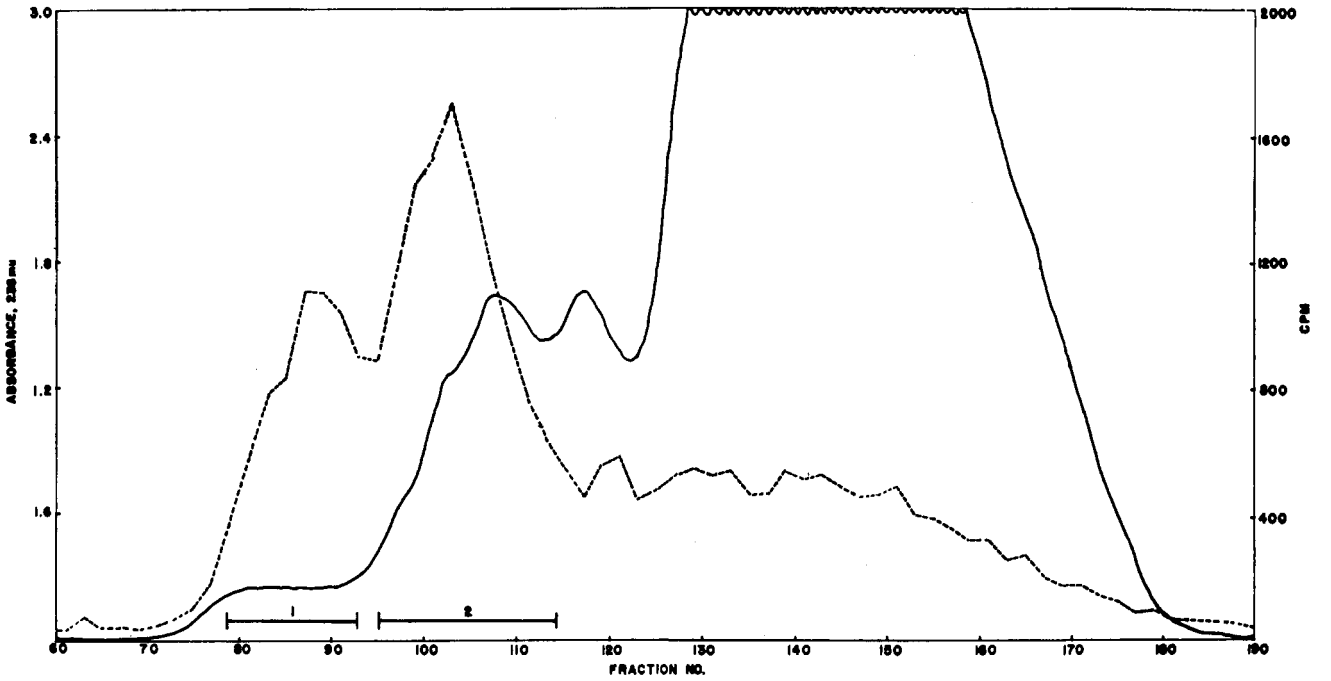

FIGURE 1: Gel filtration of collagenase–trypsin digested collagen. Conditions were: Sephadex G-25, superfine, 2.5 × 100 cm column, room temperature, 0.1 M acetic acid, flow rate 20 ml/hr, 2-ml fractions. The material denoted by the bars labeled 1 and 2 was pooled together and lyophylized: (----) radioactivity; (——) absorbance, 238 mμ.

condensing this derivative with bromobutylhydantoin, followed by base hydrolysis, and isolation of the product by ion-exchange chromatography (Tanzer *et al.*, 1970). The specific activity of the purified, homogeneous product was used to determine the amount applied to the amino acid analyzer. (2) Hydroxylysinonorleucine was quantitatively oxidized with periodate (Tanzer and Mechanic, 1970) and the resultant lysine measured on the amino acid analyzer. The color values obtained by these two methods were in close agreement and similar to that for lysinonorleucine (Franzblau *et al.*, 1965).

As an independent approach, the radioactivity content in each peptide was measured and converted into molar content of tritium (Blumenfeld and Gallop, 1966). This value was always found to agree with the molar content of hydroxylysinonorleucine as determined by amino acid analysis (see Results).

Results

Collagen Digestion and Peptide Isolation. Digestion of the reduced insoluble reconstituted collagen fibrils by bacterial collagenase proceeded rapidly and a turbid solution resulted within 10–12 hr. Following the digestion with trypsin, the amount of insoluble material remaining was about 2–3% of the initial mass, including the added enzymes. Gel filtration of the concentrated digest yielded a clear separation between the radioactive peptides and the bulk of the nonradioactive peptides (Figure 1). Approximately 70–80% of the applied radioactivity was recovered from the column and it represented approximately 10% of the total mass originally placed on the column. Thus, the gel filtration step yielded about a tenfold purification of the radioactive peptides. The amino acid composition of fractions taken across the radioactive elution profile showed, in general, that the content of basic amino acids was 5–10 mole %, the acidic amino acids constituted 15–20 mole %, and the neutral amino acids made up the remainder. Hydroxylysinonorleucine was present in some fractions and both hydroxyproline and hydroxylysine were prominent in all fractions.

Partial purification of the mixture of radioactive peptides could be obtained by ion-exchange chromatography on both SE-Sephadex and QAE-Sephadex; these resins gave good yields in contrast to the sulfonated polystyrene anion and cation exchangers which were also tried. Despite our success with the Sephadex ion exchangers, adequate purification of the peptides was not obtained and we turned to further enzymatic digestion. Treatment with subtilisin, chymotrypsin, nagarse, and pronase were all relatively ineffective but papain had a substantial action on the peptides (Figure 2). It can be seen that, in addition to decreasing the size of the radioactive peptides, papain digested a considerable proportion of the nonradioactive peptides. The radioactive profile was divided into four regions as noted in the figure legend and the peptide mixtures in each region were subjected to high-voltage electrophoresis on thin-layer plates. The peptides were stained with ninhydrin and eluted, and their radioactivity content was measured. The results showed that both radioactive and nonradioactive peptides were in the mixtures (Figure 3). Amino acid analysis of several of the partially resolved labeled peptides showed that they were still heterogeneous and that hydroxylysinonorleucine was not present in stoichiometric amounts. The radioactive peptides in mixtures PA-1 and PA-2 were found to be primarily enriched in an unidentified presumptive cross-link, "post-histidine" (Franzblau *et al.*, 1970), which eluted near lysine on the amino acid analyzer; only small

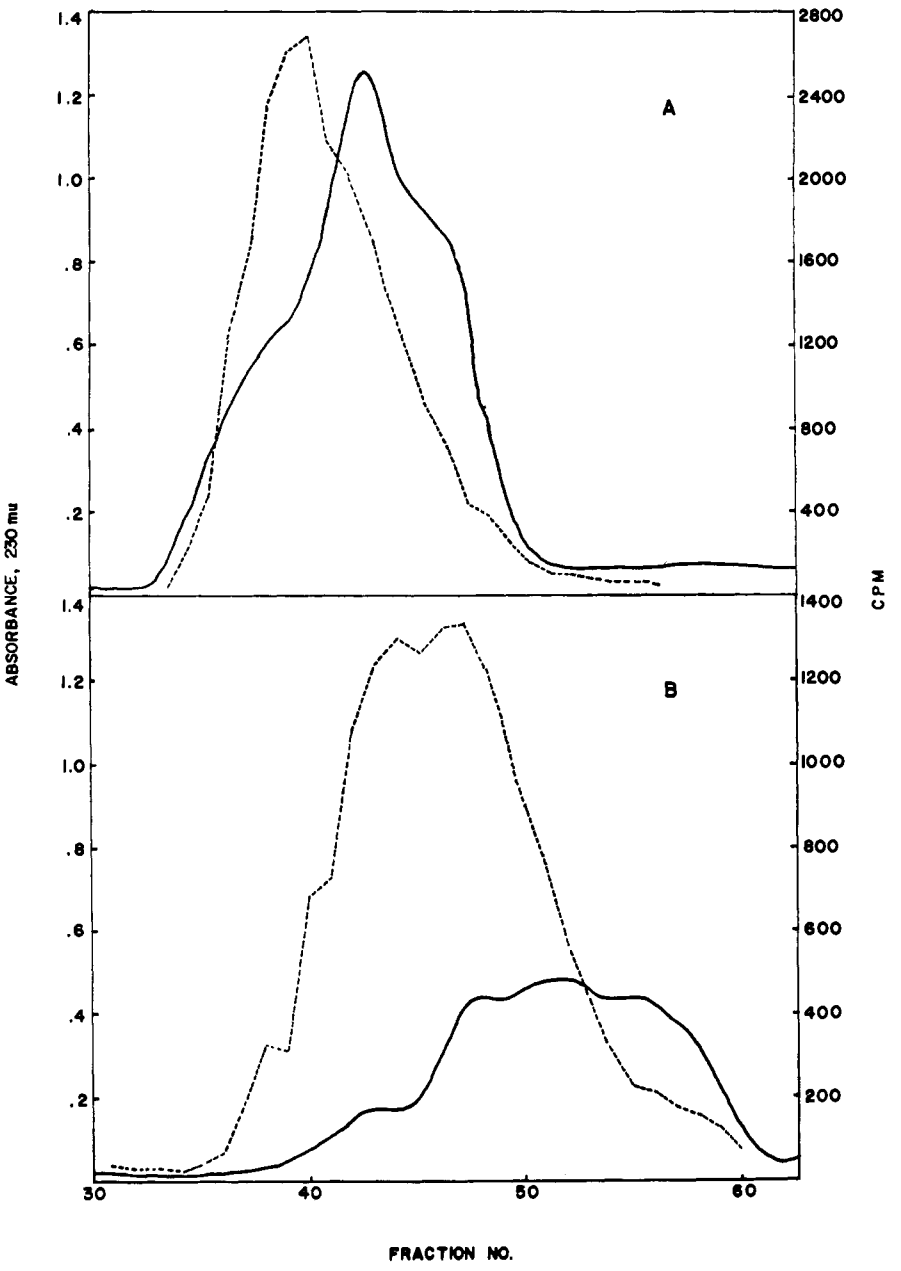

FIGURE 2: Gel filtration of peptides which had been isolated as shown in Figure 1 and then digested with papain (see text). Conditions were: Sephadex G-25, superfine, 2 × 150 cm column, room temperature, 0.13 M NH_4 acetate, pH 5.9, flow rate 12 ml/hr, 2-ml fractions: (----) radioactivity; (——) absorbance, 230 mμ; A, control; B, papain treated. The following fractions from B were pooled and lyophylized: 36–40 (PA-1); 41–44 (PA-2); 45–49 (PA-3); 50–55 (PA-4).

amounts of hydroxylysinonorleucine were present in these peptides. They were not purified further. The radioactive peptides in mixtures PA-3 and PA-4 were found to be enriched in hydroxylysinonorleucine and, to a much lesser extent, lysinonorleucine. These peptides were purified as described below.

Peptide Ion-Exchange Chromatography and Peptide Composition. In order to obtain sufficient material for analysis, the radioactive peptides in mixtures PA-3 and PA-4 were isolated and purified by chromatography on a cation-exchange resin (Figures 4 and 6) followed by chromatography of the individual peaks on an anion-exchange resin (Figures 5 and 7). The average increase in molar content of hydroxylysinonorleucine in the peptides following these two steps was about 30- to 40-fold. The amino acid compositions of three peptides isolated in this fashion are listed in Table I. Two other radioactive peptides were also isolated, but were of insufficient quantity to accurately determine their composition. The content of hydroxylysinonorleucine was determined both by reaction with ninhydrin in the amino acid analyzer and by the content of radioactivity in the peptide. The two methods were always in close agreement and we found all of the tritium to be in hydroxylysinonorleucine when the amino acids were separated on the Spinco analyzer. In a typical example, 0.013 μM of

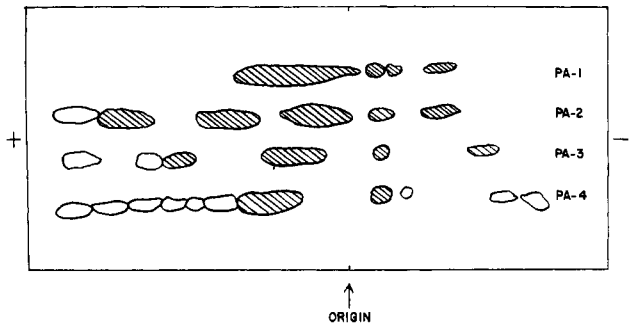

FIGURE 3: High-voltage electrophoresis of peptides upon a thin-layer plate (Eastman 6064). Conditions were: pyridine acetate, pH 6.4, 50 V/cm, 20-min duration. The shaded spots represent radioactive peptides. The symbols PA-1 through PA-4 refer to the fractions described in the legend to Figure 2.

tritium was applied to the analyzer column and 0.0125 μM of hydroxylysinonorleucine was eluted from the column.

The approximate number of residues corresponding to each amino acid is listed in Table I. The largest peptide, PA-3, was subjected to gel filtration on a calibrated Bio-Gel P-2 column (Figure 8). This peptide eluted as a slightly asymmetrical peak and had an approximate molecular weight of 800 daltons, which is consistent with its calculated molecular weight of 835 daltons.

For all three peptides, prolonging the time of hydrolysis to 72 hr did not alter the content of leucine and isoleucine at all but did diminish the amount of hydroxy amino acids (Table II). Rechromatography of the peptides prior to hydrolysis and analysis did not alter their composition. Of interest is the fact that the sum of leucine and isoleucine is approximately one residue and that the leucine is about one-third of this total. It may be that the isolated peptides are actually a mixture of two homologous peptides which are identical except for the presence of leucine or isoleucine. The ratio of leucine and isoleucine could be accounted for on the basis that two of the

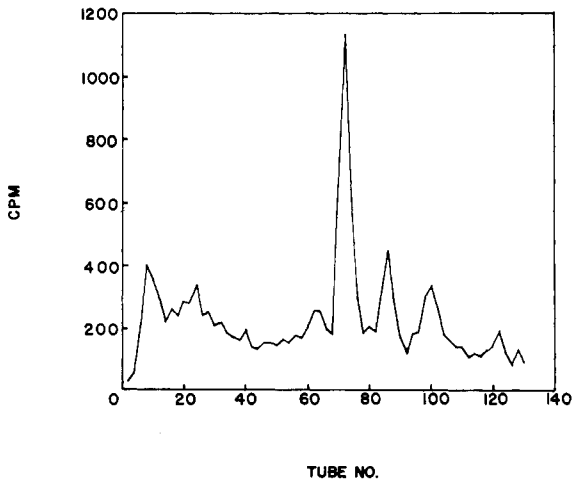

FIGURE 4: Cation-exchange chromatography of the peptides from region PA-3 in Figure 2. Conditions were: 0.9 × 23 cm column of Bio-Rad aminex A-5, 50°, 60 ml/hr, linear gradient of pyridine acetate from 0.2 M, pH 3.5 to 0.8 M, pH 5.2. The radioactive peaks were individually pooled and lyophylized.

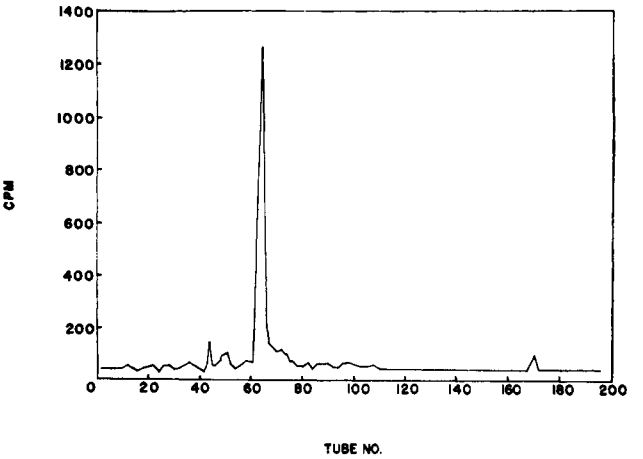

FIGURE 5: Anion-exchange chromatography of the material eluting in fractions 70–74 in Figure 4. Conditions were: 0.6 × 30 cm column of Dowex 1-X2, 50°, 50 ml/hr, buffer systems as described by Schroeder (1967). Isolated peptide is referred to as PA-3 in Table I.

collagen chains are of the same structure and one is dissimilar (Piez, 1968). Our fractionation procedure would not be expected to separate a mixture of such homologous peptides as their size, charge, and charge to mass ratio would be identical.

Discussion

The manner in which collagen forms a continuously cross-linked polymeric network has been of widespread interest. Although a variety of different cross-links have been proposed, it is now clear that at least one class of covalent intermolecular bonds are of the Schiff base variety, involving reactive groups which are intrinsic to the collagen molecule (Tanzer, 1968; Bailey, 1968). For example, the structure of hydroxylysino-

TABLE I: Amino Acid Composition of Hydroxylysinonorleucine-Containing Peptides.[a]

	Residues Per Peptide		
Amino Acid	PA-3	PA-4A	PA-4B
Asp		1.12 (1)	
Thr	0.86 (1)		
Ser	0.85 (1)		
Glu	1.16 (1)	1.26 (1)	1.06 (1)
Gly	2.27 (2)	1.83 (2)	1.20 (1)
Ile	0.67 (1)	0.58 (1)	0.56 (1)
Leu	0.28	0.37	0.30
OHLNL[b]	0.90 (1)	0.84 (1)	0.88 (1)

[a] Hydrolysis in 6 N HCl for 22 hr, not corrected for losses. All values of 0.2 or less are omitted. Estimated number of residues in parentheses. Results are averages of two or more analyses. Nomenclature of the peptides is in the legends to Figure 5 and 7. [b] Abbreviation used is: OHLNL, hydroxylysinonorleucine.

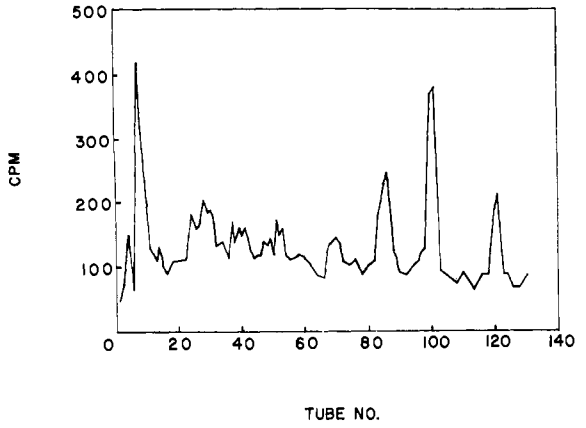

FIGURE 6: Cation-exchange chromatography of the peptides from region PA-4 in Figure 2. Conditions were identical with those described in the legend to Figure 4. The radioactive peaks were individually pooled and lyophylized.

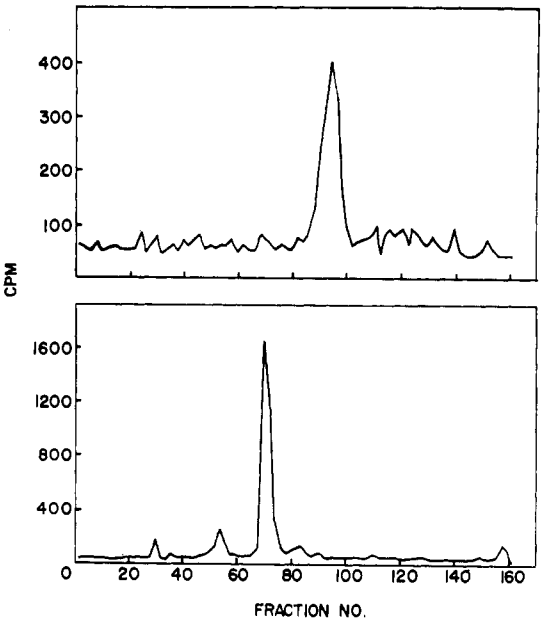

FIGURE 7: Anion-exchange chromatography of the material eluting in fractions 98–102 and 118–122 in Figure 6. Conditions were identical with those described in the legend to Figure 5: upper figure, fractions 98–102 from Figure 6; lower figure, fractions 118–122 from Figure 6. Isolated peptides are referred to as PA-4A and PA-4B, respectively.

norleucine has been independently established in two laboratories (Tanzer *et al.*, 1970; Bailey and Peach, 1968) and the distribution of tritium in this molecule has been partially localized by examination of the products of periodate cleavage (Tanzer and Mechanic, 1970). The resultant tritium distribution is consistent with borotritide reduction of the Schiff base between an ϵ-amino group of hydroxylysine and the carbonyl group of α-aminoadipic semialdehyde. Additional support for the presence of Schiff bases in collagen has been afforded by the isolation and identification of radioactive lysinonorleucine from the protein (Tanzer and Mechanic, 1970). Lysinonorleucine has been shown to arise from a Schiff base precursor in elastin and apparently, in this protein, undergoes reduction *in vivo* (Lent and Franzblau, 1967). Of importance is the fact that both lysinonorleucine and hydroxylysinonorleucine are present in both reconstituted collagen fibrils and in natural, insoluble fibrils. This finding supports the generally accepted concept that *in vitro* polymerization and time-dependent insolubility closely simulate the *in vivo* process. Furthermore, indirect studies of borotritide-reduced collagenous tissues indicate that the relative proportions of some radioactive components vary with animal age or collagen maturation (Tanzer and Mechanic, 1968; Bailey and Peach, 1968). Similarly, reconstituted collagen gels incubated for various durations also undergo a progressive change in the nature of some of their reducible components (Franzblau *et al.*, 1970). Perhaps, in these different cases, transient intermediates are being

TABLE II: Effect of Prolonged Hydrolysis upon Amino Acid Composition of Hydroxylysinonorleucine-Containing Peptides.[a]

Amino Acid	Residues Per Peptide		
	PA-3	PA-4A	PA-4B
Asp		1.09 (1)	
Thr	0.87 (1)		
Ser	0.67 (1)		
Glu	1.25 (1)	1.32 (1)	1.03 (1)
Gly	2.33 (2)	1.76 (2)	1.27 (1)
Ile	0.68 (1)	0.60 (1)	0.62 (1)
Leu	0.29	0.33	0.28
OHLNL[b]	1.00 (1)	0.89 (1)	0.78 (1)

[a] Hydrolysis in 6 N HCl for 72 hr, not corrected for losses. All values of 0.2 or less are omitted. Estimated number of residues in parentheses. Results are averages of two or more analyses. [b] Abbreviation used is: OHLNL, hydroxylysinonorleucine.

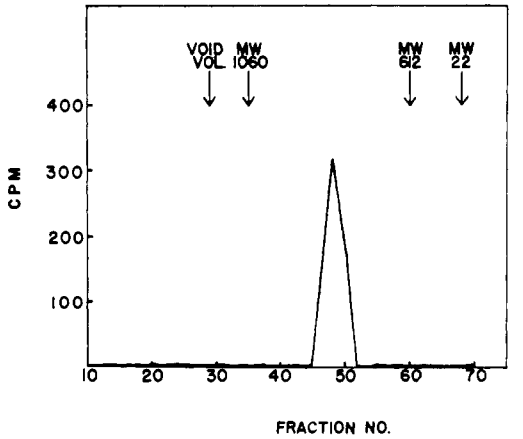

FIGURE 8: Gel filtration of peptide PA-3. Conditions were: Bio-Gel P-2, 200–400 mesh, 0.9 × 63 cm column, room temperature, 0.13 M NH_4 acetate, pH 5.9, flow rate 4 ml/hr, 0.5-ml fractions. The markers were: cytochrome *c* (void volume); Bradykinin (mol wt 1060); oxidized glutathione (mol wt 612); and tritium oxide (mol wt 22).

detected, or conceivably, *in vivo* and *in vitro* reductions are occurring.

The molar uptake of tritium by collagen following borotritide reduction varies with the experimental conditions, the source of collagen, and, perhaps, its degree of maturation (Tanzer, 1968; Paz *et al.*, 1969). The relative proportions of the individual radioactive components in the labeled protein have not been definitively established and seem to vary with the tissue source and animal age (Mechanic, 1970). However, it is apparent from the chromatographic patterns presented by several investigators that hydroxylysinonorleucine is a major component of acid-hydrolyzed, reduced collagen fibrils (Tanzer and Mechanic, 1970; Bailey *et al.*, 1970; Franzblau *et al.*, 1970). Quantitative studies have shown that almost all of the tritium in these acid hydrolysates is eluted from the amino acid analyzer columns and that hydroxylysinonorleucine is stable to acid hydrolysis (M. L. Tanzer, unpublished). In alkaline hydrolysates, hydroxylysinonorleucine is partially destroyed (M. L. Tanzer, unpublished) while acid-labile radioactive compounds appear in the chromatographic patterns of such hydrolysates (Kang *et al.*, 1970). A definitive balance sheet encompassing these technical problems has not been established, making it impossible to currently account for all of the tritium in the collagen. As a consequence one cannot ascertain what proportion of the total tritium in collagen is present in known structures such as lysinonorleucine, hydroxylysinonorleucine, and the reduced aldol condensation product of α-aminoadipic semialdehyde. Furthermore, isolation of peptides containing these moieties is subject to the same reservation, as is the determination of peptide yields at each fractionation step. As a specific case in point, it has not been shown what proportion of the total α-aminoadipic semialdehyde in collagen is represented by the characterized peptides which contain this moiety (Bornstein and Piez, 1966). A similar statement applies to other characterized peptides isolated from collagen which contain some specific component such as aldehyde or carbohydrate (Rojkind *et al.*, 1966; Muir and Lee, 1970; Katzman and Jeanloz, 1970) and also applies to glycopeptides isolated from other proteins (Jackson and Hirs, 1970; Scocca and Lee, 1969). Nevertheless, similar to these other examples we have chosen to focus our attention upon peptides containing a specific moiety, in this case hydroxylysinonorleucine, and to isolate and characterize such peptides.

Since the highly purified reconstituted collagen fibrils contain the same Schiff base compounds as the naturally insoluble protein, we chose to use the former material for the isolation of peptides because we could start with a well-defined, soluble protein, minimizing any contaminants. The next requirement was to digest the reconstituted, borohydride-treated, insoluble fibril material by specific means, yielding a completely solubilized preparation. Initial studies using cyanogen bromide were unsuccessful in that a considerable proportion of the protein remained insoluble even though all of the methionine had been completely converted into homoserine (M. L. Tanzer, unpublished). Consequently, we used the sequential collagenase–trypsin method of Cunningham and his associates (1968) because naturally insoluble collagen is thoroughly digested by this technique. Proceeding on the assumption that, on the average, cross-linked peptides would be larger than noncross-linked peptides, gel filtration was employed to partially fractionate these two classes of peptides, and this method yielded about a tenfold increase in specific activity of the radioactive peptides. At this point, although ion-exchange chromatography was partially successful in purifying the labeled peptides the contamination by nonradioactive peptides was still substantial and another means of eliminating them was sought. Digestion with papain was found to accomplish this quite well and also decreased the size of the labeled peptides. However, since papain cleaves in a nonspecific manner, it was no longer possible to anticipate any specific structural features of the resultant peptides, *e.g.*, the N- or C-terminal residues. Nevertheless, sequential cation- and anion-exchange chromatography was successful in yielding several purified peptides containing stoichiometric amounts of hydroxylysinonorleucine. The paradox concerning the partial leucine and isoleucine contents of these peptides can be adequately explained, we feel, by the rationale that a homologous mixture is present as a consequence of the original α-chain composition. We have been unsuccessful in further fractionating these peptides and anticipate that determination of their covalent structure should clear up the paradox.

Whether the three peptides derive from the same region of the original collagenous network cannot be ascertained by the available data. Since papain cleaves peptide bonds in a nonspecific fashion, the isolated peptides could well be from the same locus, and the smallest peptide may have arisen by further digestion of either or both of the larger ones. Similarly, although the two larger peptides qualitatively differ in 3 residues, they may be overlap portions of the same polypeptide region, although this seems unlikely. Again, determination of their covalent structures should help settle this point.

The available evidence indicates that, in calfskin tropocollagen, the predominant aldehyde is α-aminoadipic semialdehyde and that this compound markedly diminishes when the protein is reconstituted into fibrils which are then reduced with sodium borohydride (Tanzer and Mechanic, 1968; Tanzer, 1970). In many collagens α-aminoadipic semialdehyde has been localized to only one site, near the N-terminus of the α chains (Piez, 1968), although it may occur at other loci. In native-type fibrils, condensation of this aldehyde with hydroxylysine to yield the corresponding Schiff base might be expected to occur at only a limited number of sites as a consequence of the specificity imparted by the quarter-stagger packing (Tanzer, 1968). Assuming homology of calfskin collagen to other collagens, the compositions of the isolated peptides could be consistent with their partial origin from an α-aminoadipic semialdehyde moiety near the N-terminus, or, alternatively, from other loci. In order to resolve this point, precise comparison with the available sequence information of collagen will require knowing the complete covalent structure of the isolated hydroxylysinonorleucine-containing peptides.

Acknowledgments

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Isolation of Two Distinct Collagens from Chick Cartilage*

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ABSTRACT: Two different molecular species of collagen have been isolated from neutral extracts of lathyritic chick xiphoid cartilage, supporting the suggestions and extending the observations of Miller and Matukas (1969). In the first extract the ratio of $\alpha 1$ to $\alpha 2$ is 4, whereas in later extracts it is as much as 30, indicating that an excess of $\alpha 1$ chains are present in cartilage and that the collagen in the later extracts is comprised only of $\alpha 1$ chains. Amino acid and carbohydrate analyses of this $\alpha 1$ chain ($\alpha 1$ type II) indicate it is different from the $\alpha 1$ chain ($\alpha 1$ type I) of skin and bone. In order to separate the two collagen molecules without dissociation into their component α chains, dilute solutions were treated with formaldehyde to introduce intramolecular cross-linkages. The cross-linked molecules were denatured and separated by chromatography on carboxymethylcellulose. The chain composition of the two molecules as determined by amino acid composition and chromatographic behavior is $[\alpha 1(I)]_2\alpha 2$ and $[\alpha 1(II)]_3$. Over 90% of the extractable collagen from the lathyritic xiphoid cartilage is $[\alpha 1(II)]_3$. Electron micrographs of segment long spacings of cartilage collagen revealed a different and reproducible band pattern from that observed for chick skin collagen.

Changing staining and solubility characteristics of collagen during embryonic growth and wound repair, as well as differences in properties of this structural protein among the different tissues, had led us to search for the existence of different types of collagen, the changing distribution of which might be analogous to that of other proteins such as hemoglobin. However, it remained for Miller and Matukas (1969) to make the first definitive observation of two distinct $\alpha 1$ chains in cartilage differing in amino acid composition and suggesting the existence of two molecular species. In this paper we wish to add to their observations and to use cartilage as a model for further studies on other tissues.

Soluble collagen from a variety of vertebrate tissues yields three α chains of identical size (mol wt ~100,000) and their covalently cross-linked β components (mol wt ~200,000) when the collagen is denatured and chromatographed on carboxymethylcellulose. In most collagens so far examined two of the chains ($\alpha 1$) are identical with each other, but differ significantly from the other ($\alpha 2$) in amino acid composition and chromatographic behavior. The most notable exception to date of this pattern has been codfish skin collagen

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