Characterization of the Aldehydes Present on the Cyanogen Bromide Peptides from Mature Rat Skin Collagen*

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ABSTRACT: Mature rat skin collagen extractable by 0.2 M cysteamine at pH 7.0 at 4° has a higher aldehyde content than recently synthesized neutral salt-soluble collagen (NSS). Mild treatment of tropocollagen with cyanogen bromide under conditions which removes only the N-terminal peptides depletes NSS collagen from all its aldehydes, whereas, the cysteamine fraction retains about 70% of its aldehydes. Complete cleavage with cyanogen bromide allowed us to locate these aldehydes along the whole length of the collagen molecule. In addition to the peptides from the N-terminal region α_1-CB_1 and α_2-CB_1 and the dimeric peptides β_{11}-CB_1 and β_{12}-CB_1, peptides from the helical region α_1-CB_7, α_1-CB_8, α_2-CB_8, α_2-CB_4, and α_2-CB_5 showed significant amounts of reactive aldehydes. Reduction of these compounds prior to hydrolysis has led us to identify these aldehydes as α-aminoadipic δ-semialdehyde, a product of the oxidative deamination of peptide-bound lysine. These experiments suggest the possibility of multiple intermolecular cross-linking sites along the length of tropocollagen, possibly involving Schiff base intermediates of the types already isolated from collagens reduced with sodium borohydride. The fact that these intermediates are quite labile and do not seem to progress at a significant rate toward more stable structures may explain why rat skin collagen undergoes such a slow maturation process.

The structural role of collagen is associated with the presence of intra and intermolecular cross-links that stabilize the macromolecular assembly of the triple-stranded tropocollagen subunits.

Studies involving a collagenase digest of ichthyocol (Rojkind *et al.*, 1964) led to the characterization of a N-terminal peptide containing an aldol-like aldehyde in the form of a 2,4-dinitrophenylhydrazone derivative. Subsequent studies on peptides prepared from the α_1 and α_2 chains of rat skin collagen cleaved with CNBr have shown that lysyl residues located near the N-terminal region are converted into the δ-semialdehyde of α-aminoadipic acid and that this step is followed by an aldol condensation which yields an α-β-unsaturated aldehyde which serves as the intramolecular cross-link (Bornstein *et al.*, 1966b; Bornstein and Piez, 1966). Isolation of the dimeric peptide containing such a cross-link as well as of the reduced aldol compound from elastin (Lent *et al.*, 1969) and from collagen (Paz *et al.*, 1969) has afforded definite proof of such mechanism.

Although the nature and the biosynthetic pathway of the intramolecular cross-link seems quite clear, the intermolecular cross-linking mechanism, which is of primary significance as it relates to the overall stability of the collagen fiber, is not well understood. Weak acids and nucleophilic reagents such as the α-amino-β-thiols are able to depolymerize a maturing form of insoluble collagen from rat skin, by cleaving the intermolecular cross-links but leaving intact the more stable intramolecular cross-link (Nimni *et al.*, 1967). This collagen has a larger number of readily detectable aldehydes than does NSS[1] collagen and rapidly polymerizes *in vitro* to re-form stable fibers (Deshmukh and Nimni, 1968). The presence in collagen of "indirectly reactive aldehydes," exposed by nucleophilic agents has been described by Gallop *et al.* (1970).

Tanzer (1968) has shown that sodium borohydride reduction causes this form of collagen to become insoluble in these reagents, and Bailey (1968) has shown that the bond stabilized by reduction is of the aldimine type. Bailey *et al.* (1968, 1970) were able to isolate the reduced product of the Schiff base formed by the condensation of α-aminoadipic semialdehyde with hydroxylysine from a neighboring molecule in native collagen fibers. A similar compound was isolated by Tanzer *et al.* (1970) after reduction of reconstituted calf skin tropocollagen. These Schiff base intermediates present in polymeric collagens are quite labile, and explain the facility with which they can be dispersed prior to the formation of covalent cross-links, which are acid and heat stable.

The isolation from elastin of polyfunctional cross-linking amino acids, desmosine and isodesmosine, by Partridge *et al.* (1963) and of lysinonorleucine by Franzblau *et al.* (1965) have encouraged the search for such types of stable cross-links but as yet none has been positively identified as being present in native collagen.

We have observed that the amounts of aldehyde groups associated with rat skin collagen increases during maturation and aging (Deshmukh and Nimni, 1969) and that aminothiols at neutral pH and at the cold temperatures are able to render significant amounts of this collagen soluble in neutral-salt solutions (Nimni, 1966).

The present paper describes the location and the nature of the aldehydes on the individual chains of NSS as well as in the more mature fraction of collagen extractable from rat skin by cysteamine.

Materials and Methods

Preparation of NSS and Cysteamine-Soluble Collagen. Dorsal skins of rats (80–100 g for NSS and 100–150 g for cysteamine-soluble collagen) were cleaned, cut into small pieces, washed with 0.15 M NaCl, and then extracted with 0.45 M NaCl (pH 7.0), 0.5 M sodium citrate buffer (pH 3.6), and

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[1] Abbreviations used are: NSS, neutral salt soluble; MBTH, *N*-methylbenzothiazolone hydrazone hydrochloride.

0.2 M cysteamine in 0.45 M NaCl (pH 7.0) (Nimni *et al.*, 1967). The 0.45 M NaCl extract and 0.2 M cysteamine extract were purified (Deshmukh and Nimni, 1968) and centrifuged at 105,000*g* for 2 hr. All operations were carried out at 4°.

Lathyritic collagen was obtained from rats fed a standard diet containing 0.4% β-aminopropionitrile (fumarate) for 2 weeks.

Limited Cleavage of Native Collagen. This procedure which involved the use of cyanogen bromide (Eastman Organic) was carried out as described by Bornstein *et al.* (1966a). Lyophilized collagen was dissolved in 0.1 N HCl to a concentration of 3–4 mg/ml. Nitrogen was bubbled through the solution and solid CNBr (100-fold molar excess relative to methionine) was added. The flask was incubated at 15° for 24 hr. The solution was diluted 10-fold with cold water and lyophilized to remove excess CNBr. The residue was suspended in a small volume of cold water and dialyzed.

The effectiveness of limited cleavage was confirmed by electrophoresis on acrylamide gel.

Complete Cleavage with CNBr. Complete cleavage was accomplished by the procedure of Bornstein and Piez (1965). Collagen solutions were made in 0.1 N HCl as described above and denatured at 60° for 10 min. The flask was flushed with nitrogen and solid CNBr (100-fold molar excess over methionine) was added. The contents were incubated at 30° for 4–6 hr. At the end of the incubation period, the peptide mixture was diluted 10-fold with cold water and lyophilized. To ensure complete removal of CNBr, the residue was dissolved in water and relyophilized.

Chromatography on CM-cellulose and Phosphocellulose. The peptide mixture obtained after cleavage with cyanogen bromide was dissolved in 0.02 M sodium citrate buffer (pH 3.6) and applied to a CM-cellulose column (2.5 × 18 cm) maintained at 40°. Elution was accomplished with a 0.02 M sodium citrate buffer (pH 3.6) and a linear gradient between 0.02 and 0.14 M NaCl (1 l. of each solution) (E. Miller, personal communication). The flow rate was maintained at 100–120 ml/hr and the effluent was monitored at 236 mμ through a flow cell attached to a Beckman DB-G spectrophotometer with a Sargent recorder. Fractions of 10 ml were collected. An improved separation of α_2-CB_3, α_2-CB_4, and α_2-CB_5 was achieved by rechromatography under the same conditions. At the conclusion of the run the column was washed successively with 0.5 M NaCl, 0.01 M NaOH, 0.2 M sodium citrate buffer (pH 3.6), and finally, with the starting buffer.

The smaller fragments which originate from the N-terminal region and which appear in the initial peaks with CM-cellulose chromatography were pooled, desalted using Bio-Gel P-2, as described below, dissolved in 0.001 M sodium acetate buffer (pH 3.8), and chromatographed on a phosphocellulose column (2.5 × 18 cm) at 40°. A linear gradient between 400 ml each of 0.001 M sodium acetate buffer (pH 3.8) and 0.001 M sodium acetate–0.3 M NaCl (pH 3.8) was used for elution of peptides. The flow rate was maintained at 100–120 ml/hr and 6.0-ml fractions were collected.

At the end of the gradient, 100 ml of 0.001 M sodium acetate–0.3 M NaCl (pH 3.8) was passed through the column for complete recovery of peptide α_1-CB_3. At the end of each run the phosphocellulose was redispersed, regenerated, and a new column packed.

Molecular Sieve Chromatography. The peptide fractions were lyophilized and desalted by passing through a column of Bio-Gel P-2 (Bio-Rad Laboratories; 100–200 mesh; column size 1 × 100 cm), equilibrated with 0.15 M acetic acid at room temperature. The flow rate was maintained at 20–25 ml/hr and 3.0-ml fractions were collected.

Further separation of the fractions containing a mixture of α_1-CB_4, α_1-CB_5, and α_1-CB_6 was accomplished by chromatography on Sephadex G-50 (medium grade, Pharmacia). Two columns (1 × 100 cm) attached in series were used and the elution was carried out with 0.15 M acetic acid at the flow rate of 20 ml/hr. Fractions of 3.0 ml were collected and the absorbance was read at 230 mμ.

Acrylamide Gel Electrophoresis. The purity of the individual peptides was checked by disc electrophoresis using the method of Nagai *et al.* (1964) with modifications in the acrylamide concentration (12.5%) and *N,N'*-methylenebisacrylamide (0.53%) in running gels.

Amino Acid Analysis. The peptides were hydrolyzed with 6.0 N HCl in sealed tubes at 110° for 24 hr. The amino acid analysis was carried out with a Jeol amino acid analyzer (JLC-5AH).

Aldehyde Estimation. The aldehyde content of the collagen samples and the peptide fractions was estimated by the spectrophotometric method of Paz *et al.* (1965), using MBTH.

Reaction with Dinitrophenylhydrazine. Native collagen and the peptides were treated with dinitrophenylhydrazine as reported by Rojkind *et al.* (1964). The samples were treated with [U-^{14}C]2,4-dinitrophenylhydrazine (sp act. 1 mCi/mmole; 50-fold molar excess over peptide or α chain) in 2 N HCl at room temperature for 5–10 min. The excess 2,4-dinitrophenylhydrazine was removed by passing the diluted mixture through a Sephadex G-25 column (1.7 × 30 cm) using 1 N acetic acid for elution. Aliquots of the 2,4-dinitrophenylhydrazine derivatives were counted in a Beckman liquid scintillation counter. The specific activity of each product was obtained after hydrolysis and reaction with ninhydrin (Rosen, 1957).

Reduction with [^{3}H]$NaBH_4$ and Analysis of Reduced Products. The aldehyde-containing peptides and the native collagen samples were reduced with 500-fold molar excess of [^{3}H]$NaBH_4$ (9.4 mCi/mmole) at 4° for 2 hr maintaining the pH between 7.0 and 7.5. The reaction was stopped by lowering the pH to 3 with 0.5 N HCl. The reduced samples were hydrolyzed with 2.0 N KOH in sealed tubes at 110° for 24 hr. After hydrolysis, the contents were chilled and perchloric acid was added drop by drop to adjust the pH to 4.0. After standing in the cold for 2 hr, the samples were centrifuged at 10,000 rpm for 30 min to remove the potassium perchlorate. The supernatants were chilled again overnight and centrifuged. The final supernatants were analyzed on the amino acid analyzer, the fractions collected using a split stream divider, and the tritium activity of individual fractions measured.

Results

Limited Cleavage with CNBr. The aldehyde content of lathyritic collagen, NSS, and cysteamine-soluble collagen before and after the removal of the N-terminal peptide is reported in Table I. Lathyritic collagen and NSS collagen showed very low aldehyde content after limited cleavage, whereas additional aldehyde residues seemed to be present in the helical part of cysteamine-soluble collagen fraction. Figure 1 describes the changes in the absorption spectra of NSS and cysteamine-soluble collagen with MBTH, before and after limited cleavage. NSS collagen shows the presence of very small amounts of aldehyde after removal of N-terminal peptide, while in the case of cysteamine collagen the shoulder at 350 mμ, an indication of the presence of α,β-unsaturated aldehydes disappears completely. There is a decrease in

TABLE I: Aldehyde Content of Various Types of Collagen before and after Treatment with CNBr under Conditions which Cause Selective Removal of the N-terminal Peptide (Limited Cleavage).

Type of Collagen	μmoles of Aldehyde/100 mg of Collagen[a] Intact	Minus N-Terminal Peptide
NSS		
Normal	0.88	0.28
Lathyritic	0.58	0.20
$NaBH_4$ treated	0.15	0.15
Cysteamine soluble		
Normal	2.81	2.00
$NaBH_4$ treated	0.20	0.17

[a] Measured by spectrophotometric method of Paz *et al.* (1965).

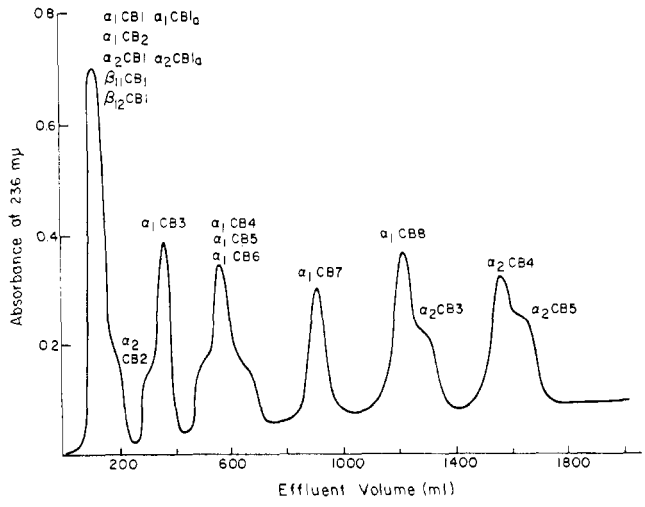

FIGURE 2: Elution pattern of CNBr peptides from NSS collagen or cysteamine-soluble collagen on CM-cellulose at 40°, using a linear gradient between 0.02 M sodium citrate + 0.02 M NaCl and 0.02 M sodium citrate + 0.14 M NaCl (pH 3.6).

absorbance at 308 mμ, but the presence of significant amounts of residual aldehydes is quite apparent. After reduction with sodium borohydride, both NSS and cysteamine-soluble collagen showed only trace amounts of reactive aldehydes and this was not changed by the CNBr-cleavage procedure. The finding that additional aldehyde groups exist in the helical part of cysteamine-collagen was confirmed by treatment of NSS and cysteamine soluble collagen with [U-^{14}C]2,4-dinitrophenylhydrazine. After removal of the excess of dinitrophenylhydrazine by dialysis (or by passing through Sephadex G-25 column), complete cleavage was carried out with cyanogen bromide under the conditions described in the Experimental Section. In the case of NSS collagen, 90–95% of the yellow color and radioactivity appeared in the initial peak containing the N-terminal peptides, while with cysteamine-soluble collagen, only 30–40% of the yellow color and radioactivity was present in this region of the molecule. It should be stated that cysteamine "*per se*" did not affect the parameters measured, since incubation of NSS collagen with 0.2 M cysteamine at 4° did not affect its aldehyde content nor alter the ratio of α to β subunits detectable after denaturation.

Chromatography of Peptides Obtained by Complete Cleavage with CNBr. Chromatography of the peptides obtained from NSS and cysteamine-soluble collagen on CM-cellulose is illustrated in Figure 2. The general pattern of appearance of various peaks was quite similar for both types of collagen. The first peak was comprised of the N-terminal peptides of individual chains along with α_1-CB_2. The following peak contained α_2-CB_2 and α_1-CB_3. As these peaks appeared very close to each other, the individual peptides were separated with phosphocellulose column. The third large peak consisted of a mixture of α_1-CB_4, α_1-CB_5, and α_1-CB_6 which were separated on Sephadex G-50. α_1-CB_6 appeared in the void volume region while α_1-CB_4 and α_1-CB_5 eluted as well-defined peaks (Figure 3). The fractions containing α_2-CB_3, α_2-CB_4, and α_2-CB_5, when screened by acrylamide gel electrophoresis, showed one major band with traces of either one or two small bands. These fractions were purified further by rechromatography on CM-cellulose, using the same conditions.

Figure 4a,b represents the analysis of the initial peaks, obtained with CM-cellulose, rechromatographed on phosphocellulose. The first peak contained α_2-CB_{1a} and some unidentified material, possibly similar to that described as α_1-CB_{1b} by Bornstein and Piez (1966). This mixture, while de-

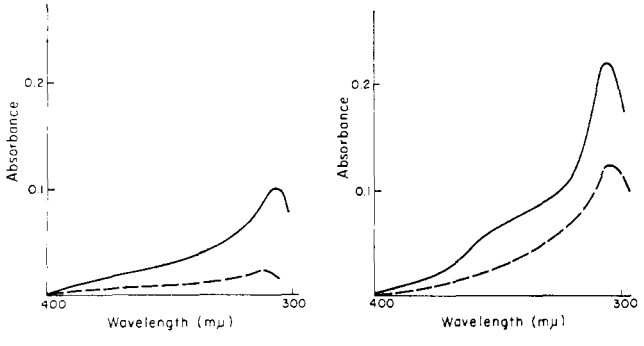

FIGURE 1: Absorption spectrum of NSS and cysteamine-soluble collagen after reaction with MBTH. (——) Untreated collagen. (----) After removal of N-terminal peptide. (A) NSS collagen (1.8 mg/2 ml of reaction mixture); (B) cysteamine-soluble collagen (1.4 mg/2 ml of reaction mixture).

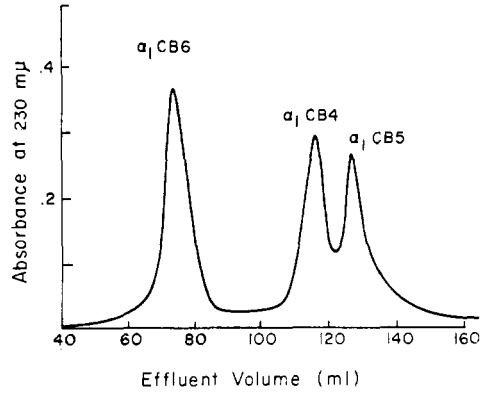

FIGURE 3: Separation of peptides α_1CB_4 and α_1CB_5 and α_1CB_6 on Sephadex G-50. Elution was carried out with 0.15 M acetic acid.

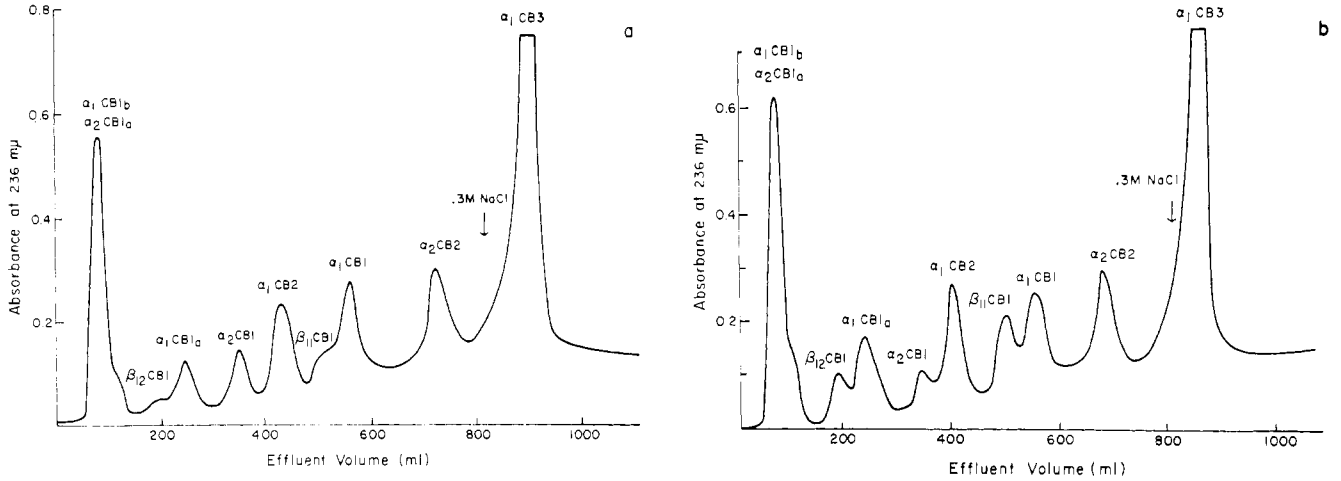

FIGURE 4: Fractionation on phosphocellulose of the mixture of early eluting peptides isolated from the CM-cellulose column. Column temperature was maintained at 40° and the linear gradient established between 0.001 M sodium acetate and 0.001 M sodium acetate + 0.3 M NaCl (pH 3.8). (a) NSS collagen, (b) Cysteamine-soluble collagen.

salting on Bio-Gel P-2, appeared as two close peaks. One contained α_2-CB_{1a} and the other showed a high absorbance at 230 mμ and after hydrolysis a low magnitude of reaction with ninhydrin. In some runs β_{12}-CB_1 and α_1-CB_{1a} and also β_{11}-CB_1 and α_1-CB_1 could not be well separated on phosphocellulose. These small peptides were obtained in a more pure form with Bio-Gel P-2. The β_{12}-CB_1 and β_{11}-CB_1 peaks were more prominent in cysteamine soluble collagen than salt-soluble collagen.

The amino acid analysis of the individual peptides isolated is given in Table II. No change was observed in amino acid composition of the corresponding peptides of NSS and cysteamine-soluble collagen. The analysis of β_{11}-CB_1 and β_{12}-CB_1 is not reported. As one could expect, they showed the same composition as that of N-terminal peptides of α_1 and α_2 chains.

The amino acid composition agrees within the experimental limits with that reported by Bornstein and Piez (1966), Butler *et al.* (1967), and Fietzek and Piez (1969) for CNBr peptides of α_1 and α_2 chains of rat skin collagen. In our analysis, α_1-CB_2 was found to contain 7 residues/1000 of lysine, 10 of aspartic acid, and 5 of threonine. It has been reported that α_1-CB_2 does not contain lysine or aspartic acid. It is possible that in our α_1-CB_2 peptide there was a trace contaminant. Peptide α_1-CB_5 showed less histidine than the previously reported value, whereas α_1-CB_6 contained a trace of histidine.

Table III describes the aldehyde content of peptides of NSS and cysteamine-soluble collagen estimated by the MBTH spectrophotometric procedure (Paz *et al.*, 1965) and also by measuring the binding of [U^{14}C]2,4-dinitrophenylhydrazine to each peptide (Rojkind *et al.*, 1964). α_1-CB_{1a}, α_2-CB_{1a}, β_{11}-CB_1, and β_{12}-CB_1 contain 0.8–1.09 moles of aldehyde/mole of peptide and α_1-CB_7, α_1-CB_8, α_2-CB_3, α_2-CB_4, and α_2-CB_5 contained 0.7–0.9 mole of aldehyde/mole of peptide. These values were slightly lower when determined by binding of 2,4-dinitrophenylhydrazine. It should be noted that the summation of the total aldehyde residues on the individual peptides of tropocollagen (2 α_1 chains + α_2 chain) agrees quite well with the number of aldehyde residues estimated on whole collagen, before and after CNBr treatment. Cleavage of methionyl bonds by CNBr treatment did not "*per se*" affect the net content of detectable aldehyde residues since the amounts present in native collagen, as measured spectrophotometrically with MBTH or by DNP-hydrazone formation, were similar before and after cleavage and removal of excess CNBr by repeated lyophilization. Figure 5 shows the absorption spectra of CNBr peptides after reaction with MBTH at pH 4.0. Peptides α_1-CB_{1a} and α_2-CB_{1a} show a peak at 308 mμ, while β_{11}-CB_1 and β_{12}-CB_1 show a peak at 320 mμ, with a shoulder at 350 mμ. All the other peptides from the helical region of the molecule have a spectrum identical with that of α_1-CB_{1a}. Therefore, to calculate moles of aldehydes, acetaldehyde was used as a standard for all the peptides except for the cross-linked N-terminal peptides of β_{11} and β_{12} chains, where crotonaldehyde was used.

Reduction with Sodium Borohydride. The reduction of native NSS and cysteamine-soluble collagen was carried out with [^{3}H]$NaBH_4$. The reduced collagen was dialyzed extensively against distilled water at 4°, hydrolyzed with 2 N KOH, and chromatographed on the amino acid analyzer. Table I indicated that aldehyde groups in both types of collagen could be reduced with $NaBH_4$, under the conditions employed, to almost undetectable limits. The values of radioactivity incorporated into both types of collagen are almost of a magnitude which is of proportion to their aldehyde content measured by the various techniques discussed (Table IV). On the amino

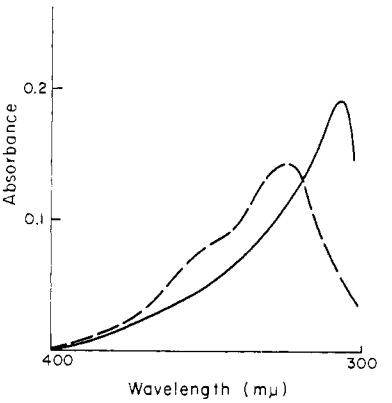

FIGURE 5: Absorption spectra of CNBr peptides after reaction with MBTH. (-----) $\beta_{11}CB_1$ and $\beta_{12}CB_1$. (——) All other peptides containing aldehydes.

TABLE II: Amino Acid Composition of CNBr Peptides of Cysteamine Collagen.

	Residues per 1000												
	α_1 Chain								α_2 Chain				
Amino Acid	CB_1[a]	CB_2	CB_3	CB_3	CB_5	CB_6	CB_7	CB_8	CB_1[a]	CB_2	CB_3	CB_5	CB_5
Hydroxylysine	−(0)[b]	−(0)	2 (0)	− (0)	20 (27)	5 (10)	4 (3)	4 (4)	− (0)	− (0)	3 (8)	6 (10)	6 (8)
Lysine	70 (66)	7 (0)	30 (34)	45 (44)	56 (54)	17 (15)	27 (30)	29 (35)	70 (71)	− (0)	25 (29)	22 (20)	17 (19)
Histidine	− (0)	− (0)	− (0)	− (0)	15 (27)	− (5)	4 (0)	2 (0)	− (0)	− (0)	− (3)	Trace (6)	10 (18)
Arginine	− (0)	27 (27)	45 (40)	85 (88)	30 (27)	50 (55)	37 (42)	56 (56)	− (0)	85 (100)	48 (50)	48 (51)	48 (51)
Hypro	− (0)	135 (139)	91 (94)	130 (130)	80 (81)	85 (85)	95 (100)	99 (102)	Trace (0)	100 (100)	90 (84)	86 (90)	89 (84)
Aspartic acid	85 (66)	10 (0)	39 (37)	60 (65)	85 (81)	60 (50)	42 (40)	35 (38)	75 (71)	89 (100)	41 (45)	38 (36)	39 (40)
Threonine	− (0)	5 (0)	18 (13)	23 (22)	30 (27)	17 (20)	23 (25)	19 (19)	5 (0)	25 (33)	23 (16)	22 (24)	13 (24)
Serine	130 (136)	60 (55)	22 (20)	15 (0)	60 (54)	65 (60)	32 (33)	49 (38)	130 (143)	40 (33)	41 (45)	34 (39)	48 (42)
Glutamic acid	55 (66)	110 (111)	105 (107)	60 (66)	90 (81)	70 (65)	65 (59)	66 (72)	78 (71)	40 (33)	80 (77)	78 (75)	85 (60)
Proline	130 (136)	170 (194)	96 (94)	123 (130)	50 (54)	140 (155)	134 (142)	123 (118)	135 (143)	100 (100)	100 (106)	95 (93)	105 (100)
Glycine	205 (198)	335 (333)	340 (342)	330 (326)	330 (324)	335 (330)	355 (337)	332 (330)	205 (214)	335 (330)	338 (339)	345 (321)	337 (318)
Alanine	70 (66)	64 (55)	140 (134)	70 (66)	80 (81)	100 (95)	122 (106)	126 (136)	67 (71)	80 (67)	120 (116)	129 (111)	120 (100)
Valine	106 (136)	− (0)	22 (26)	− (0)	− (0)	15 (10)	23 (28)	13 (19)	75 (71)	30 (33)	30 (26)	36 (39)	− (0)
Isoleucine	− (0)	7 (0)	2 (0)	5 (0)	− (0)	10 (15)	6 (10)	6 (4)	− (0)	− (0)	20 (19)	12 (12)	38 (37)
Leucine	− (0)	24 (27)	22 (20)	40 (44)	22 (27)	19 (20)	16 (13)	17 (16)	− (0)	40 (33)	26 (23)	39 (39)	20 (21)
Tyrosine	56 (66)	− (0)	− (0)	− (0)	− (0)	2 (5)	− (0)	1 (0)	75 (71)	− (0)	− (0)	− (0)	36 (40)
Phenylalanine	14 (0)	23 (27)	20 (20)	− (0)	30 (27)	10 (5)	8 (10)	8 (12)	− (0)	7 (0)	11 (13)	8 (12)	5 (6)
Homoserine[c]	72 (66)	25 (27)	5 (6)	20 (22)	25 (27)	− (0)	4 (3)	5 (4)	70 (71)	30 (33)	4 (3)	3 (3)	11 (9)

[a] α_1-CB_{1a} and α_2-CB_{1a} have amino acid composition similar to α_1-CB_1 and α_2-CB_1, except for the absence of lysine residues. β_{11}-CB_1 has same composition of α_1-CB_{1a}, while β_{12}-CB_1 behaves like an equimolar mixture of α_1-CB_{1a} and α_2-CB_{1a}. [b] Values in parentheses are calculated from the data reported by Bornstein and Piez (1966), Butler *et al.* (1967), and Fietzek and Piez (1969) for CNBr peptides of α_1 and α_2 chains of rat skin collagen. [c] Values for homoserine include homoserine lactone.

TABLE IV: Total Reactive Aldehydes, Tritium Uptake, and Relative Concentration of α-Aminoadipic Semialdehyde and Aldol Condensation Product Present in NSS and Cysteamine-Soluble Collagen.

	Aldehyde Residues/Tropocollagen			% of Radioactivity Eluted as	
Type of Collagen	MBTH	DNP-hydrazine	Tritium Uptake (cpm/100 mg of Collagen)	ε-Hydroxy-norleucine	Reduced Aldol Condensation Product
NSS	2.4	2.2	8.1×10^9	94	6
Cysteamine	8.7	9.0	24.3×10^9	85	15

TABLE III: Aldehyde Content of the CNBr Peptides Originating from NSS and Cysteamine- (CYS) Soluble Collagen Fractions.

	α_1 Chain					α_2 Chain			
	Aldehyde Residues[a,b]		DNP-hydrazine Bound[c]			Aldehyde Residues		DNP-hydrazine Bound	
Peptide	NSS	CYS	NSS	CYS	Peptide	NSS	CYS	NSS	CYS
CB_{1a}	0.85	0.80	0.86	1.08	CB_{1a}	0.86	0.94	0.83	1.09
CB_1				0.01	CB_1		0.02		
CB_2		0.05		0.07	CB_2				
CB_3		0.03		0.02	CB_3		0.76		0.57
CB_4		0.01			CB_4		0.79		0.61
CB_5					CB_5		0.91		0.84
CB_6					$\beta_{12}CB_1$[d]		0.98		0.79
CB_7		0.75		0.54					
CB_8		0.71		0.63					
$\beta_{11}CB_1$[d]		0.94		0.82					

[a] Measured spectrophotometrically and expressed as residues of acetaldehyde equivalent per mole of peptide. [b] These values were corrected in order to account for the background obtained with peptide from $NaBH_4$-reduced collagen. [c] Moles of [^{14}C]-dinitrophenylhydrazine bound per mole of peptide. [d] The amounts of these ppetides available from NSS collagen were small and insufficient for aldehyde estimation.

acid analyzer, the major radioactive peaks appeared near the regions where glycine and norleucine appear (between leucine and tyrosine). It has been reported previously that the reduced product of α-aminoadipic semialdehyde elutes in the same area where glycine appears and reduced aldol condensation product elutes as a post leucine peak. The reduction of native NSS and cysteamine-soluble collagen with $NaBH_4$ at pH 7.0–7.5 did not result in any loss of collagen during exhaustive dialysis. On the other hand, reduction at pH 9.0–10.0 incorporated more tritium into collagen, but caused 15–20% of the hydroxyproline to become dialyzable and the presence of small molecular weight peptides could be observed on acrylamide gel electrophoresis. No such effect was seen when reduction was conducted at pH 7.0–7.5.

The individual peptides obtained from NSS and cysteamine collagen were also reduced with [^{3}H]$NaBH_4$ at pH 7.0–7.5, hydrolyzed, and chromatographed on the amino acid analyzer. The excess [^{3}H]$NaBH_4$ eluted as tritiated H_2O. In all the peptides, except in the case of α_1-CB_6 and α_2-CB_5, an additional radioactive peak was found under homoserine most likely due to an exchange reaction occurring during hydrolysis of peptide-bound homoserine lactone in the presence of tritiated water.

All the peptides which contained aldehydes, except the dimeric peptides, showed radioactivity in the glycine region, reflecting the presence of peptide-bound α-aminoadipic semialdehyde. In the case of the N-terminal cross-linked peptides β_{11}-CB_1 and β_{12}-CB_1 the major peak appeared under norleucine, an indication of the presence of reduced aldol condensation product (Table V).

Although native collagen seems to be relatively resistant to peptide-bond cleavage by action of $NaBH_4$, denatured collagen (and presumably derived peptides) is quite sensitive to this reductive process and may yield α-amino alcohols of the precursor peptide-bound amino acids (Paz *et al.*, 1970). In view of this difficulty, uptake of tritium from [^{3}H]$NaBH_4$ by the CNBr peptide was used exclusively as a tool to identify α-aminoadipic semialdehyde and the aldol condensation product. No radioactive peaks eluted among the basic amino acids after hydrolysis with 6 N HCl, and no further attempts were made to specifically detect compounds that would behave like α-amino alcohols, since all procedures used to quantitate aldehydes did not involve $NaBH_4$ reduction of peptides.

TABLE V: Incorporation of ^{3}H into α-Aminoadipic Semialdehyde (Preglycine Peak) and the Aldol Condensation Product (Postleucine Peak) after Reduction and Base Hydrolysis of CNBr Peptides.

	cpm/Mole of Peptide × 10^9	
Peptide	Reduced α-Aminoadipic Semialdehyde	Reduced Aldol
Cysteamine-Soluble Collagen		
α_1CB_{1a}	5.32	
CB_1	0.07	
CB_2	0.50	
CB_3	0.20	
CB_4	0.20	
CB_5	0.12	
CB_6	0.32	
CB_7	3.60	
CB_8	3.36	
α_2CB_{1a}	5.60	
CB_1	0.05	
CB_2	0.37	
CB_3	4.00	
CB_4	4.50	
CB_5	3.70	
$\beta_{12}CB_1$	0.05	5.4
$\beta_{11}CB_1$	0.03	4.9
Neutral-Salt-Soluble Collagen		
α_1CB_{1a}	5.8	
α_2CB_{1a}	5.6	

Discussion

The isolation of a cross-linking peptide from the N-terminal region of tropocollagen has greatly substantiated our present understanding of the nature of the intramolecular cross-linking process (Bornstein *et al.*, 1966b; Rojkind *et al.*, 1966). The evidence presented by Bornstein and Piez (1966) made it clear that the aldehyde in such a peptide is of an α,β-unsaturated nature and derived from the condensation of two peptide-bound α-aminoadipic semialdehyde residues from different chains. A detailed analysis of the various experiments performed to support this assumption can be found in the review of Piez (1968). Since no other dimeric peptides with such characteristics and containing an α,β-unsaturated aldehyde has been found, it can be concluded that the intramolecular cross-linking process seems to be restricted to the N-terminal region of the molecule.

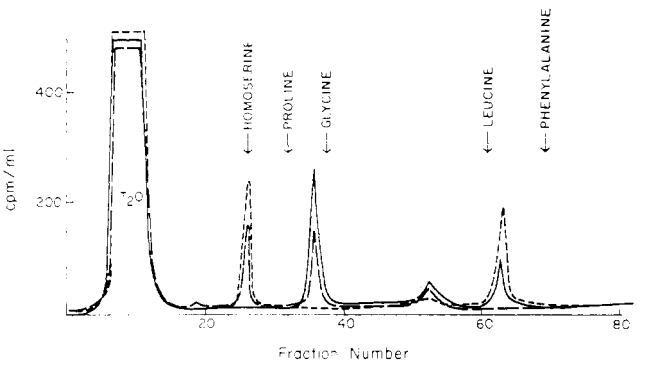

FIGURE 6: Distribution of radioactivity on fractions collected from the amino acid analyzer after reduction and alkaline hydrolysis of cysteamine-soluble collagen and the derived CNBr peptides. (——) Native collagen, (-----) β_{11}-CB_1 and β_{12}-CB_1, and (— —). All other peptides containing aldehydes (no radioactive peak under homoserine for α_1-CB_6 and α_2-CB_5.)

The collagen extracted from rat skin by cysteamine at neutral pH, after the more soluble fractions of collagen have been removed, represents a more mature form of collagen (Nimni *et al.*, 1967). In addition to containing a larger proportion of aldehydes than NSS collagen when reacted with MBTH it reflects the presence of a significant amount of α,β-unsaturated aldehydes (Deshmukh and Nimni, 1969). This is in agreement with the fact that it has a large proportion of β components (50–60%). Removal of the N-terminal peptide by limited CNBr cleavage leaves about 70% of the total aldehydes attached to the main body of the tropocollagen molecule with an absorption spectrum similar to that of saturated aldehydes. In contrast, limited cleavage of NSS collagen removes all the aldehydes on the molecule. The presence of these aldehydes on the helical part of cysteamine-soluble collagen was confirmed by DNP-hydrazone formation, tritium uptake after reduction with [^{3}H]$NaBH_4$, and elution of radioactivity from the amino acid analyzer as ϵ-hydroxynorleucine, the reduced product of peptide bound α-aminoadipic semialdehyde. Cysteamine-soluble collagen yielded larger amounts of α_1-CB_{1a} and α_2-CB_{1a} peptides than NSS collagen. Also, cysteamine collagen contains significantly more N-terminal cross-linked peptides β_{11}-CB_1 and β_{12}-CB_1, an observation consistent with the fact that this collagen shows a much larger proportion of β chains, reflection of the more advanced maturation of this collagen, as well as the presence of labile intermolecular cross-links of the Schiff base type, which are readily cleaved by cysteamine or weak organic acids.

The distribution of aldehydes along the helical region of collagen seems to be random, α_1-CB_7, α_1-CB_8, α_2-CB_3, α_2-CB_4, and α_2-CB_5 having the higher aldehyde content (after the N-terminal peptides which are the first ones to become oxidatively deaminated). Reduction of these peptides with $NaBT_4$ under carefully controlled conditions that prevented peptide bond hydrolysis allowed most of the radioactivity to be recovered as reduced α-aminoadipic semialdehyde, indicating that peptide-bound lysine must be the source of these aldehydes, such as was shown to be the case with α_1-CB_1 and α_2-CB_1 (Bornstein and Piez, 1966).

The reduced product of α-aminoadipic semialdehyde, ϵ-hydroxynorleucine, was was identified by Lent *et al.* (1969) from a base hydrolysate of reduced elastin. They observed that ϵ-hydroxynorleucine elutes near glycine, while the aldol condensation product of two peptide-bound α-aminoadipic semialdehydes appears between leucine and tyrosine. These investigators showed that acid hydrolysis partially converts reduced α-aminoadipic semialdehyde into ϵ-chloronorleucine (which elutes near leucine) and destroys the aldol condensation product to a significant extent. We have been able to confirm these observations and have restricted ourselves to the use of alkaline hydrolysis.

The amounts of radioactivity eluted as ϵ-hydroxynorleucine, after hydrolysis of the peptides reduced with [^{3}H]$NaBH_4$ shows close correlation with the aldehyde content estimated spectrophotometrically and by [^{14}C]DNP-hydrazine binding. Peptides β_{11}-CB_1 and β_{12}-CB_1 showed a peak at the location where the reduced aldol condensation product elutes and no radioactivity at the glycine position.

Cysteamine-soluble collagen contains about 9 aldehyde residues/tropocollagen unit, whereas, NSS and lathyritic collagen contain 2.5 and 1.5 aldehyde residues per tropocollagen, located exclusively in the α_1-CB_1 and α_2-CB_1 peptides in the latter two types of collagen.

The role of these aldehydes isolated along the main body of the mature collagen subunit is not yet clear. Hydroxylysinonorleucine, as mentioned earlier, has been isolated from reduced native or reconstituted collagen. The reduced form of $\Delta^{6,7}$-dehydrolysinonorleucine has never been detected in native collagen suggesting that there is either no physiological mechanism for reduction or other means of stabilization occurring, such as addition to the double bond, complex ring formation, etc.

Rat skin collagen may be a unique species in this respect, in the sense that new aldehydes are formed during the process of maturation, some of which may be involved in Schiff base interactions with opposing hydroxylysine residues without progressing any further to form stable covalent cross-links.

Based on studies involving the formation of segment long-spacing fibrils after pepsin treatment and on the reduction of reconstituted calf Achilles' tendon collagen, Kuhn *et al.* (1970) postulated the appearance of functional groups responsible for side to side bonding during maturation, since they seemed to increase as one goes from NSS collagen to acid-soluble collagen. Although these observations did not seem to apply to rat skin collagen, where end to end bonding appeared to be of primary significance, side-to-side alignment *in vitro* may not locate the proper functional groups in register for cross-linking to occur. There is no doubt that our observations on the more random distribution of aldehydes will facilitate the formation of cross-links based solely on the increased probability of interaction.

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Isolation of a Nickel α_2-Macroglobulin from Rabbit Serum*

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ABSTRACT: Measurements of nickel were performed by atomic absorption spectrometry upon serum protein fractions which were separated successively by ultrafiltration and by column chromatography on Sephadex G-200 and on DEAE-cellulose. Rabbit serum was used as the starting material, since measurements showed that nickel concentrations in serums from rabbits (mean = 9.3 μg/l.; std dev $\pm$ 2.2; range 6.5–14.0; N = 24) were higher than in serums from man or ten other mammalian species. Nickel in rabbit serum was found to exist in three forms: (a) ultrafiltrable nickel (16% of total Ni), (b) albumin-bound nickel (40% of total Ni), and (c) in a nickel–metalloportein which was named "nickeloplasmin" (44% of total Ni). Disc gel and immunoelectrophoresis showed that purified nickeloplasmin migrates as a single protein band in the α_2-globulin region. Sedimentation equilibrium studies indicated that nickeloplasmin is a macroglobulin, with estimated molecular weight of 7.0×10^5. Following dialysis of rabbit serum against buffer containing Ni(II), the albumin-bound nickel became increased, but nickel content of the nickeloplasmin fraction was not affected.

Himmelhoch *et al.* (1966) separated human serum proteins by gradient chromatography on DEAE-cellulose columns, and measured the trace metal content of the eluted protein fractions by means of emission spectrography. Their data suggested the presence in serum of a metalloprotein which is rich in nickel, and which does not contain any detectable calcium, magnesium, strontium, barium, iron, zinc, manganese, aluminum, chromium, lead, cadmium, molybdenum, or tin. The present study confirms the existence of a serum nickel metalloprotein (which we have named "*nickeloplasmin*"), and furnishes additional information regarding its chemical properties. Rabbit serum was selected as the starting material for isolation of serum nickeloplasmin, since pre-

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