Characterization of the α Chains of Chick Skin Collagen and the Nature of the NH_2-Terminal Cross-Link Region*

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ABSTRACT: Carboxymethylcellulose chromatography of neutral salt-extracted collagen from normal and lathyritic chicks indicates that chick skin collagen contains two identical α chains (α1) and one α chain (α2) which is different from the other two. Acid-extracted collagen, however, contains another species of α chain (α1′) which is chromatographically separable from the α1 chain but has a similar amino acid composition and gel electrophoretic mobility. Studies of the peptides produced by cyanogen bromide digestion of α1 and α1′ show that both yield the same peptides except that α1-CB0 and α1-CB1 (and α1-CB1^{Ald}) are lacking in digests of α1′. Since the missing peptides represent the NH_2-terminal portion of the α1 chain, it appears that α1′ is a form of α1 from which a small part of the NH_2-terminal sequence is absent. Whether the absence of the short sequence in the older (acid-extracted) collagen but not in more newly synthesized (salt-extracted) collagen reflects the presence *in vivo* of a physiologic proteolytic mechanism or is an artifact arising from limited proteolysis of the protein during extraction and purification is not known. These findings may explain, in part, observations of heterogeneity in the chain structure of other collagens. From cyanogen bromide digests of isolated α chains, a septadecapeptide (α1-CB1) was isolated from α1 and a pentadecapeptide (α2-CB1) from α2 which predominate in the α chains of recently synthesized (salt-extracted) collagen and lathyritic collagen in which the formation of interchain cross-links had been blocked while in the digests of the α chains of more highly cross-linked (acid-extracted) collagen these peptides are largely replaced by aldehyde-containing peptides (α1-CB1^{Ald} and α2-CB1^{Ald}) of identical amino acid composition except for the absence of a lysyl residue and the presence of a residue of allysine (α-aminoadipic δ-semialdehyde). These peptides are absent from CNBr digests of β_{12} but a new peptide is present which is a dimer of α1-CB1^{Ald} and α2-CB1^{Ald} and contains a functional group with the properties of an α,β-unsaturated aldehyde presumably arising by an aldol condensation of two residues of allysine. These findings suggest that intramolecular cross-link formation by aldol condensation previously shown to occur in rat skin collagen is common to many vertebrate collagens.

The collagen molecule consists of three α chains of molecular weight about 95,000, some of which become covalently cross-linked to form the β components. Because of its great size, the amino acid sequence of collagen has been difficult to establish. Recently, however, specific scission with CNBr of the isolated α chains of collagen into a limited and predictable number of peptide fragments of molecular weight below 30,000 has provided an essential initial step in ultimately establishing the primary structure of the entire molecule (Piez *et al.*, 1969) and in elucidating some unusual structural features. The work reported here on chick skin collagen parallels that under way on rat skin collagen (Piez *et al.*, 1969; Fietzek and Piez, 1969), rat tail tendon collagen (Bornstein, 1969), and chick bone collagen (Miller *et al.*, 1969; Lane and Miller, 1969) and was undertaken to obtain a more general picture of collagen primary structure. We have previously reported the isolation and characterization of the peptides obtained by CNBr digestion of the whole α1 chain of chick skin collagen (Kang *et al.*, 1969a). A similar study on the CNBr peptides of the α2 chain of chick skin collagen is being reported separately (Kang *et al.*, 1969b).

In this paper we describe the NH_2-terminal region of chick skin collagen chains to shed further light on the question whether chick skin and certain other collagens contain two or three different α chains (Francois and Glimcher, 1967; Heidrich and Wynston, 1965; Miller *et al.*, 1967, 1969; Kang *et al.*, 1969a) and also to characterize the mode of intramolecular cross-linking in this particular collagen for comparison with rat skin collagen (Bornstein *et al.*, 1966a; Bornstein and Piez, 1966; Kang *et al.*, 1967).

Experimental Section

Source of Collagen. Chicks 1-day old, White Leghorn, were obtained from a local hatchery and fed a commercial diet for 3 weeks. The chicks were then decapitated and the feathers were carefully removed. The skin was dissected from the body and the subcutaneous fatty tissue was scraped away with a scalpel. In some instances 8-week-old chicks were used with no difference in the results obtained. Lathyrism was induced by feeding 1-day-old chicks a commercial diet containing 0.1% β-aminopropionitrile for 3 weeks.

Preparation of Collagen. All operations were performed at 4°. The skin was ground by a mechanical meat grinder with chips of Dry Ice, washed with a large volume of cold water, and extracted overnight with five volumes of 0.05 M Tris buffer

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(pH 7.0) containing 1 M NaCl, with occasional stirring. A few drops of toluene were used to retard bacterial growth. The extract was filtered through several layers of cheesecloth and a layer of Whatman No. 1 filter paper with the aid of a Celite cake in a Büchner funnel under vacuum, and further clarified by centrifugation at 13,000*g*. The residue was washed with water and extracted overnight with five volumes of 0.5 M acetic acid (acid extract I). The acid extract I was separated by filtration and the residue was reextracted with another five volumes of 0.5 M acetic acid (acid extract II). A total of three successive acetic acid extractions was performed. The acid extracts were similarly clarified as described above. The neutral salt extracts and the acid extracts were then purified by the methods previously described (Kang *et al.*, 1966, 1969a).

CM-cellulose Chromatography. The method utilized was essentially as previously described (Piez *et al.*, 1963; Bornstein and Piez, 1966). Collagen samples were dissolved in a buffer containing 0.06 M sodium acetate and 0.06 M acetic acid (pH 4.8), and denatured for 15 min at 45°. Denatured samples were applied to 2.5 × 10 cm columns of CM-cellulose (CM-32, Whatman) equilibrated with the same buffer. The column was jacketed at 42°, since the midpoint of the melting curve of the native collagen was at 41° (0.15 M potassium acetate, pH 4.8; see Miller *et al.*, 1967). Fractionation was carried out by superimposing a linear gradient of NaCl from 0 to 0.1 M over a total volume of 800 ml. The effluent was monitored continuously at 230 mμ by the use of a flow cell (10-mm path length) in a Gilford or Beckman DB spectrophotometer. Fractions of 10 ml were collected. Combined fractions representing each peak were desalted on columns of Sephadex G-25 (Pharmacia) equilibrated with pyridine acetate (pH 4.8). The $\alpha 1$ and $\alpha 1'$ chains obtained from acid-extracted collagen were further purified by rechromatography on CM-cellulose columns.

Acrylamide Gel Electrophoresis. Disc electrophoresis of the fractions obtained by CM-cellulose chromatography was performed as described by Nagai *et al.* (1964).

CNBr Cleavage. CNBr digestion products of α and β components were prepared as reported earlier (Bornstein and Piez, 1966; Kang *et al.*, 1967, 1969a).

Chromatography of CNBr Peptides. The lyophilized CNBr peptides were chromatographed on 2.5 × 20 cm columns of phosphocellulose as described previously (Bornstein and Piez, 1966; Kang *et al.*, 1969a). The chromatography was performed at 42° in 0.001 M sodium formate (pH 3.6) by superimposing a linear gradient of NaCl from 0 to 0.6 M over a total volume of 1600 ml. The peptides not resolved on the initial fractionation on phosphocellulose were eluted with 1 M NaCl and desalted on columns of Bio-Gel P-2 equilibrated with 0.15 M acetic acid. These peptides were further fractionated on columns of CM-cellulose at pH 3.6 as previously described (Kang *et al.*, 1969a).

Azine Formation with N-Methylbenzothiazolone Hydrazone. The procedure of Paz *et al.* (1965) was employed. The reaction was monitored in a Cary Model 11 spectrophotometer with the cell compartment jacketed at 40°.

Reduction with Sodium Borohydride. A 0.1 M solution of $NaBH_4$ in cold 0.1 M Tris buffer, pH 8.5, was prepared immediately before use. To 0.03–0.1 μmole of peptides dissolved in 1 ml of water was added 1 ml of the $NaBH_4$ solution. The reaction was allowed to proceed at room temperature for 2 hr, and was terminated by addition of 2 N acetic acid to pH 4. The peptides were separated from salts by chromatography on Bio-Gel P-2 columns equilibrated with 0.15 M acetic acid.

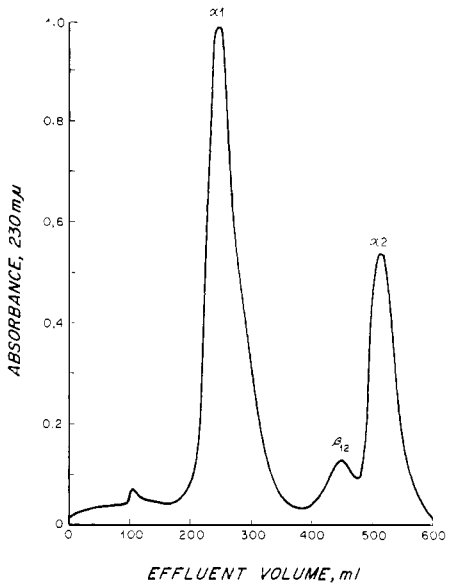

FIGURE 1: CM-cellulose elution pattern of denatured chick skin collagen prepared from lathyritic chicks.

Amino Acid Analysis. Samples were hydrolyzed under nitrogen in constant-boiling HCl at 108° for 24 hr. Analyses were performed on a single-column automatic amino acid analyzer modified for high-speed analysis (Miller and Piez, 1966). Correction factors for loss of labile amino acids (threonine, serine, methionine, and tyrosine) and incomplete release of valine were used as determined previously (Piez *et al.*, 1960).

Results

CM-cellulose Chromatography of Denatured Chick Skin Collagen. A CM-cellulose chromatogram of the collagen extracted with 1 M NaCl from lathyritic chicks is presented in Figure 1. The peaks were identified as α chains or β components by their migration on acrylamide gel electrophoresis. The small forepeak consists of nonprotein ultraviolet-absorbing material. The chromatogram resembles closely those obtained from CM-cellulose chromatography of collagens from other vertebrates, such as rat skin and tail tendon (Piez *et al.*, 1963), dogfish skin (Lewis and Piez, 1964), chick bone (Miller *et al.*, 1967), and human skin (Bornstein and Piez, 1964) collagens.

The β_{11} component observed in other collagens was not present in sufficient amounts to be readily apparent in any of the collagen samples, although acrylamide gel electrophoresis indicated the presence of a small amount of material with the mobility of the β component at the trailing edge of the $\alpha 1'$ peak (see Figure 3). Amino acid analysis established β_{12} as the dimer of $\alpha 1$ and $\alpha 2$ (see Table I). A chromatogram of the collagen obtained by 1 M NaCl extraction from normal chicks is presented in Figure 2. In addition to $\alpha 1$, $\alpha 2$, and β_{12}, another component was observed frequently on the trailing edge of $\alpha 1$. This component was much more striking in chromatograms of acid-extracted collagens and the relative proportion of the component as compared to the $\alpha 1$ chain increased

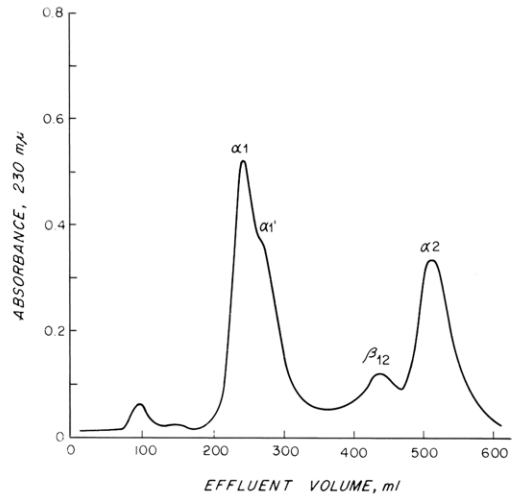

FIGURE 2: CM-cellulose elution pattern of denatured, neutral salt-extracted chick skin collagen.

TABLE I: Amino Acid Composition[a] of Chick Skin Collagen and Components Derived from Denatured Collagen.

	α1	α1′	α2	β12	Collagen
3-Hydroxyproline	0.9	1.0	1.0	0.6	1.0
4-Hydroxyproline	107	108	97	100	106
Aspartic acid	42	42	50	47	45
Threonine	19	19	20	20	19
Serine	27	28	31	30	29
Glutamic acid	77	77	67	69	73
Proline	120	120	117	119	118
Glycine	328	328	328	333	332
Alanine	129	129	104	116	116
Valine	14	14	28	21	18
Methionine	8.4	8.4	4.7	6.9	7.4
Isoleucine	6.4	6.3	16	10	10
Leucine	20	20	32	25	24
Tyrosine	1.7	0.6	1.8	1.6	1.6
Phenylalanine	12	12	13	12	12
Hydroxylysine	5.2	5.4	10	8.4	6.9
Lysine	30	30	21	25	27
Histidine	1.9	1.9	7.6	5.2	4.2
Arginine	50	50	50	51	51

[a] Residues/1000 total residues. An α chain of mol wt 95,000 contains about 1040 residues.

with successive acid extractions (Figure 3). On acrylamide gel electrophoresis it migrated as an α component, and amino acid analysis showed it to be very similar to α1 (Table I). Thus this component was designated α1′.

Amino Acid Composition of Chick Skin Collagen and Its Chains. The amino acid composition of α1, α2, and β_{12} components is presented in Table I. These data confirm the identification of β_{12} as the dimer of α1 and α2. The α2 chain differs from the α1 chain in that it contains larger amounts of valine, isoleucine, leucine, and histidine, and smaller amounts of glutamic acid and alanine. These differences are in general similar to the differences observed between the corresponding chains in other vertebrate collagens (Piez *et al.*, 1963). The amino acid composition of chick skin collagen and its constituent chains is, within experimental error, identical with that of chick bone collagen and its chains (Miller *et al.*, 1967). The amino acid composition of α1 and α1′ do not differ significantly except for the lower tyrosine content of α1′. There appear to be two residues of tyrosine in α1, but none in α1′. The partial residue in the latter is consistent with cross-contamination of α1 and α1′.

Phosphocellulose Chromatography of CNBr Peptides of α1, α2, and β_{12}. When CNBr digests of the α chains from lathyritic, neutral salt, and acid-extracted collagens were compared, the only detectable differences were noted in the peptides from the cross-link region which are resolved by phospho-

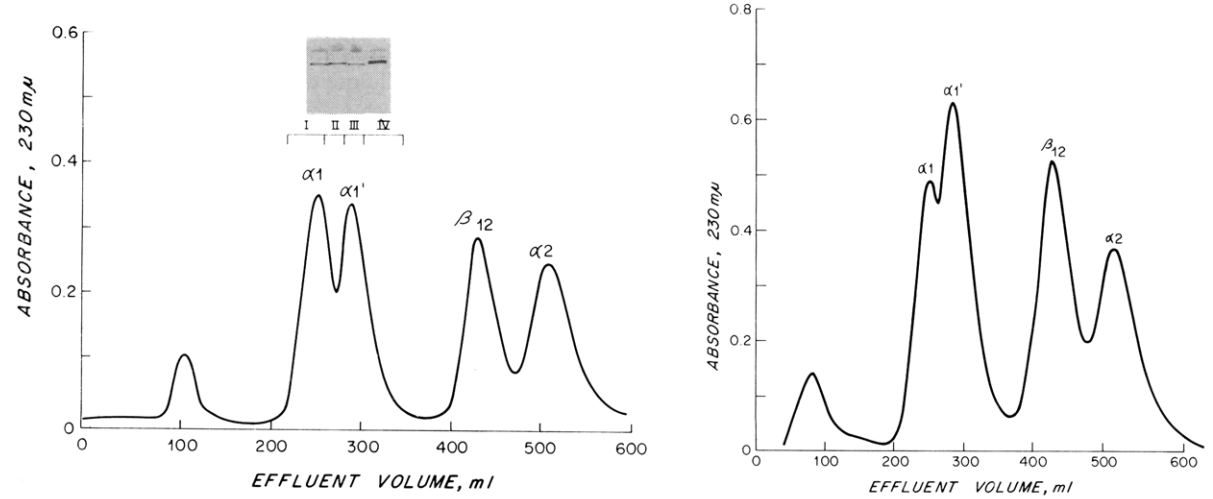

FIGURE 3: CM-cellulose elution pattern of denatured, acid-extracted chick skin collagen. The left-hand figure is acid extract I and the right-hand figure acid extract III. The gel electrophoresis patterns show the composition of the pooled fractions as indicated in the figure for acid extract I. The electrophoretic patterns of the similarly pooled fractions of acid extract III were identical.

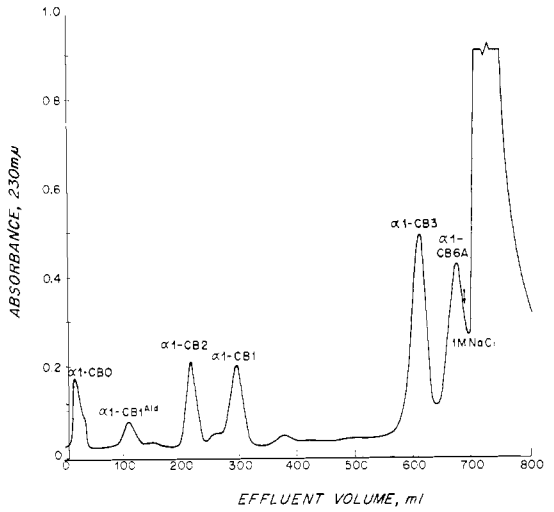

FIGURE 4: Phosphocellulose elution pattern of peptides obtained by CNBr cleavage of the $\alpha 1$ chain of salt-extracted collagen from lathyritic chicks.

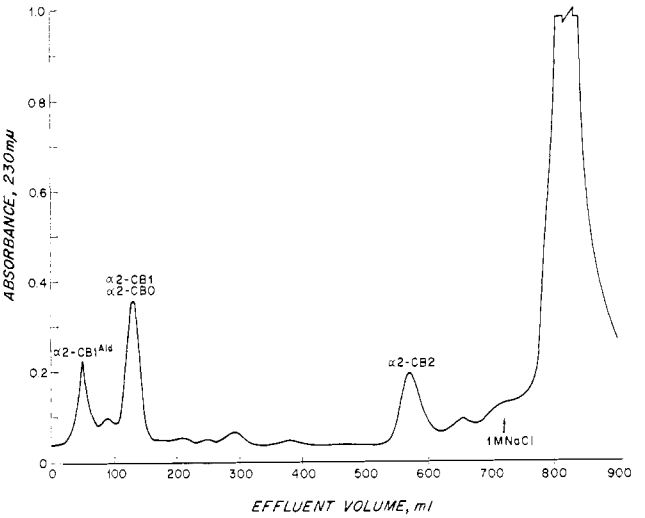

FIGURE 6: Phosphocellulose elution pattern of peptides obtained by CNBr cleavage of the $\alpha 2$ chain of salt-extracted collagen from lathyritic chicks.

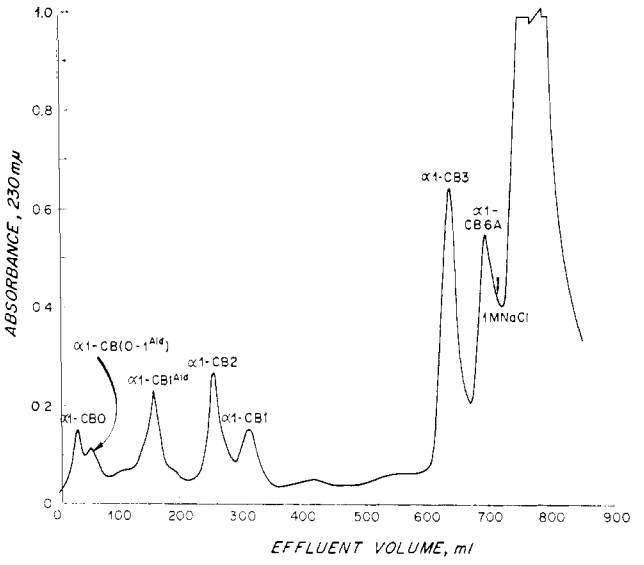

FIGURE 5: Phosphocellulose elution pattern of peptides obtained by CNBr cleavage of the $\alpha 1$ chain of acid-extracted chick skin collagen.

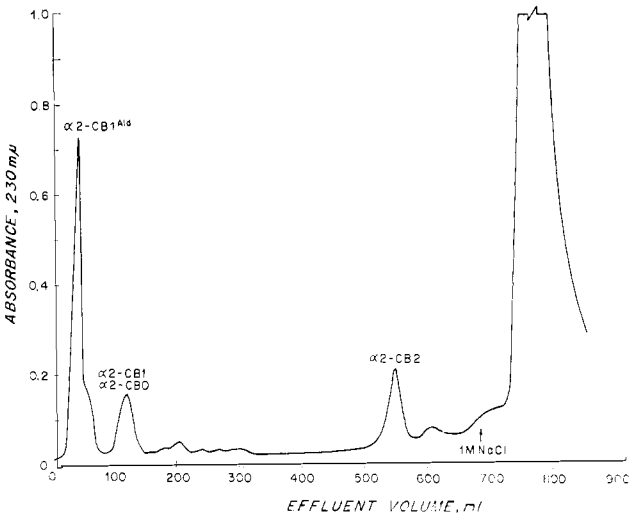

FIGURE 7: Phosphocellulose elution pattern of peptides obtained by CNBr cleavage of the $\alpha 2$ chain of acid-extracted collagen from normal chicks.

cellulose chromatography. In Figures 4 and 5 are presented typical phosphocellulose elution patterns of CNBr peptides of the $\alpha 1$ chain derived from lathyritic and acid-extracted collagens, respectively. A comparison of the elution patterns shows that the relative amounts of $\alpha 1$-CB1 and $\alpha 1$-CB1^{Ald} vary depending on the source. Thus, in digests of lathyritic collagen $\alpha 1$, $\alpha 1$-CB1 predominates whereas in digests of acid-extracted collagen $\alpha 1$, $\alpha 1$-CB1^{Ald} is the predominant form. In digests of neutral salt-extracted collagen $\alpha 1$, the peptides are present in approximately equal amounts (Kang *et al.*, 1969a).

The corresponding peptides from $\alpha 2$, $\alpha 2$-CB1, and $\alpha 2$-CB1^{Ald} also show the same pattern of dependence upon source. Figures 6 and 7 represent the phosphocellulose chromatograms of the CNBr peptides of $\alpha 2$ prepared from lathyritic and acid-extracted collagens, respectively. Neutral salt-extracted collagen $\alpha 2$ is intermediate between these two sources (Kang *et al.*, 1969b).

Figure 8 presents a phosphocellulose elution pattern of the CNBr peptides derived from β_{12}. The peptides from the cross-link region, $\alpha 1$-CB1, $\alpha 1$-CB1^{Ald}, $\alpha 2$-CB1, and $\alpha 2$-CB1^{Ald}, are absent but a new peptide, β_{12}-CB1, is present. The presence of $\alpha 1$-CB2, $\alpha 1$-CB3, and $\alpha 2$-CB2 is as expected since β_{12} is a dimer of $\alpha 1$ and $\alpha 2$.

The reciprocal source-dependent variations of the peptides $\alpha 1$-CB1, $\alpha 1$-CB1^{Ald}, $\alpha 2$-CB1, and $\alpha 2$-CB1^{Ald} in different collagens with varying degree of intramolecular cross-linking as well as their absence in CNBr digests of the cross-linked β_{12} component provide further evidence that these peptides are involved in the formation of the intramolecular cross-link.

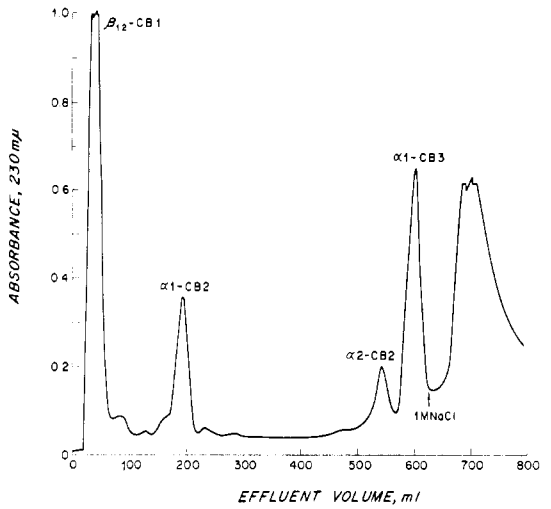

FIGURE 8: Phosphocellulose elution pattern of peptides obtained by CNBr cleavage of the β_{12} component of acid-extracted chick skin collagen.

Similar findings have been reported previously for rat skin collagen (Bornstein *et al.*, 1966a; Bornstein and Piez, 1966).

Comparison of CNBr Peptides from α1 and α1′. To investigate further the differences between α1 and α1′, CNBr digests of rechromatographed α1 and α1′ (both from acid-extracted collagen) were chromatographed on phosphocellulose and CM-cellulose successively as described previously (Kang *et al.*, 1969a). Figure 9 illustrates a typical elution pattern of the CNBr digest of α1′. The chromatogram is similar to that of the CNBr digest of α1 (Figure 5) except that the amounts of the NH_2-terminal peptides, α1-CB0, α1-CB1^{Ald}, and α1-CB1 are markedly diminished (compare Figure 9 with Figure 5). Chromatography on Bio-Gel P-2 and amino acid analysis indicated that the first peak in Figure 9 contained no significant

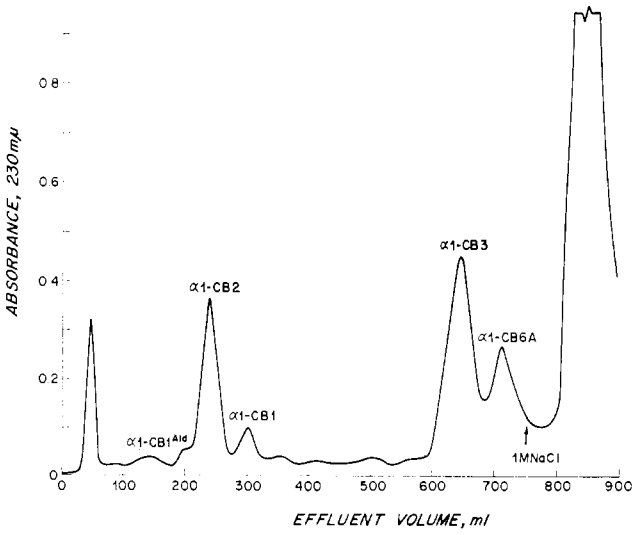

FIGURE 9: Phosphocellulose elution pattern of peptides obtained by CNBr cleavage of α1′ from acid-extracted chick skin collagen.

amount of peptide material. The peptides remaining on the phosphocellulose column were then eluted with 1 M NaCl and, after desalting on Bio-Gel P-2, were fractionated on columns of CM-cellulose as previously described (Kang *et al.*, 1969a). Characterization of these peptides by amino acid composition and molecular weight did not reveal any differences between the CNBr peptides derived from α1 and those from α1′.

These data indicate that α1′ differs from α1 only in that it lacks that part of the sequence of the α1 chain represented by α1-CB0 and α1-CB1 (or α1-CB1^{Ald}). The small amounts of α1-CB1 and α1-CB1^{Ald} observed in the CNBr digest of α1′, about 20% of normal, can be explained by the presence of small amounts of α1 owing to incomplete separation of α1 and α1′ (Figure 3.) These results explain the different tyrosine contents of α1 and α1′ since α1-CB1 contains the two tyrosyl residues in α1 (Kang *et al.*, 1969a).

Characterization of α1-CB1, α1-CB1^{Ald}, α2-CB1, and α2-CB1^{Ald}. The amino acid compositions of α1-CB1 and α1-CB1^{Ald} have been presented (Kang *et al.*, 1969a). They are identical except for the absence of a lysyl residue from the latter. Similarly, as shown in Table II, the amino acid composition of α2-CB1^{Ald} is identical with that of α2-CB1 except for the absence of lysine. These peptides are identical in composition to the corresponding peptides of chick bone collagen except for the partial hydroxylation of the lysyl residue in the latter case (Miller *et al.*, 1969).

TABLE II: Amino Acid Composition[a] of CNBr Peptides from the Cross-Link Region of Chick Skin Collagen.

	α1-CB1	α1-CB1^{Ald}	α2-CB1	α2-CB1^{Ald}	β_{12}-CB1
Aspartic acid	1	1	2 (2.1)	2 (2.2)	3 (2.9)
Serine	2	2	1 (1.0)	1 (0.9)	3 (2.7)
Glutamic acid	1	1	1 (1.0)	1 (1.3)	2 (2.0)
Proline	2	2	3 (3.1)	3 (2.8)	5 (5.0)
Glycine	3	3	2 (2.0)	2 (2.1)	5 (4.8)
Alanine	2	2	2 (2.0)	2 (1.8)	4 (3.8)
Valine	2	2	0	0	2 (2.1)
Tyrosine	2	2	1 (0.9)	1 (0.9)	3 (2.5)
Phenylalanine	0	0	1 (1.0)	1 (0.9)	1 (0.9)
Lysine	1	0	1 (1.0)	0	0
Homoserine[b]	1	1	1 (1.1)	1 (0.9)	2 (2.1)

[a] Residues per peptide. Integer values are presented with the experimental values in parentheses. Detailed data on α1-CB1 and α1-CB1^{Ald} have been given previously (Kang *et al.*, 1969a). [b] Includes homoserine lactone.

Evidence was previously presented (Bornstein *et al.*, 1966a: Bornstein and Piez, 1966) that in rat skin collagen, the lysyl residues of α1-CB1 and α2-CB1 are converted in peptide linkage into allysine[1] prior to the formation of the intramolec-

[1] The trivial name allysine, introduced by Pinnell and Martin (1968) for the lysine-derived δ-semialdehyde of α-aminoadipic acid, is used in this paper.

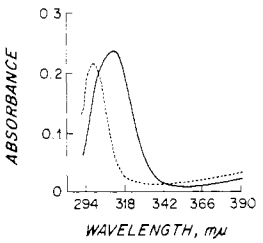

FIGURE 10: Ultraviolet absorption spectra of the azine derivative of α1-CB1^{Ald} at pH 4 (solid line) and pH 1 (dashed line). The cuvet contained 0.033 μmole of the peptides in 1 ml of H_2O, 1 ml of 0.1 M glycine-HCl buffer (pH 4.0), and 0.2 ml of 0.1% *N*-methylbenzothiazolone hydrazone; 0.04 ml of 6 N HCl was added to obtain spectra at pH 1.

ular, interchain cross-link. To investigate this possibility, α1-CB1^{Ald} and α2-CB1^{Ald} were treated with *N*-methylbenzothiazolone hydrazone and assayed by the spectrophotometric method of Paz *et al.* (1965). Figure 10 depicts the ultraviolet absorption spectra of the azine derivative of α1-CB1^{Ald}. α1-CB1 did not react. The absorption maximum of α1-CB1^{Ald} is at 309 mμ, shifting to 298 mμ at pH 1.0. These data are identical with comparable data obtained for α1-CB1^{Ald} of rat skin collagen and is consistent with the presence of one saturated aldehyde in α1-CB1^{Ald}. Entirely analogous results were obtained for α2-CB1^{Ald} and α2-CB1.

In order to investigate further the structure of aldehydes present in α1-CB1^{Ald} and α2-CB1^{Ald}, these peptides were reduced with $NaBH_4$. The resulting products were desalted on Bio-Gel P-2 columns and hydrolyzed in constant-boiling HCl and the hydrolysates were analyzed on an amino acid analyzer. In duplicate analyses, 0.25 and 0.30 equiv for α1-CB1^{Ald} and 0.30 and 0.36 equiv for α2-CB1^{Ald} of ϵ-hydroxynorleucine were obtained. Since it is known that approximately 70% of the ϵ-hydroxynorleucine is converted to chlorononorleucine during acid hydrolysis (Franzblau and Lent, 1969), these values are consistent with the presence of 1 equiv of allysine in α1-CB1^{Ald} and α2-CB1^{Ald}.

These data suggest that, in chick skin collagen, the lysyl residues of α1-CB1 and α2-CB1 undergo conversion into allysine in the process of cross-link formation. An identical conclusion has been reached previously for rat skin collagen (Bornstein *et al.*, 1966a; Bornstein and Piez, 1966).

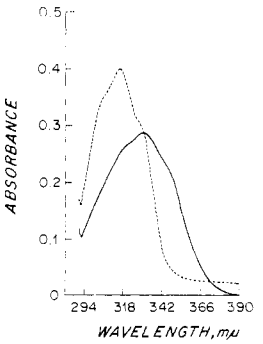

FIGURE 11: Ultraviolet absorption spectra of the azine derivative of β_{12}-CB1 at pH 4 (solid line) and pH 1 (dashed line). See legend to Figure 10 for detail.

Characterization of β_{12}-CB1. The amino acid composition of this peptide, presented in Table II, requires that it contain two residues of homoserine. It must, therefore, be a double chain peptide. Since its composition is the sum of α1-CB1^{Ald} and α2-CB1^{Ald} and these latter peptides together with their precursors, α1-CB1 and α2-CB1, are not present in digests of β_{12}, β_{12}-CB1 must be the cross-linked dimer of α1-CB1^{Ald} and α2-CB1^{Ald}. The spectrophotometric assay of its azine derivative is presented in Figure 11. The absorption maximum at pH 4.0 was 330 mμ shifting to 316 mμ at pH 1.0. These data are consistent with the presence in β_{12}-CB1 of an α,β-unsaturated aldehyde (Paz *et al.* 1965). Similar data have been obtained for rat skin collagen by Bornstein *et al.* (1966a) and Bornstein and Piez (1966). They suggested that the aldehyde might arise by an aldol condensation of the two residues of allysine in α1-CB1^{Ald} and α2-CB1^{Ald} producing a cross-link.

Discussion

We have characterized native collagen extracted from the skin of 3-week-old chicks with 1 M NaCl and 0.5 M acetic acid. Chromatographic fractionation of the chains of acetic acid extracted collagen on CM-cellulose suggested the presence of a third α chain which is chromatographically separable from the other two (Figure 3). Studies of peptides obtained by CNBr digestion of this component (α1′), however, indicate that it is fundamentally identical with α1 except for the absence of the portion of the chain represented by α1-CB0 and α1-CB1 (or α1-CB1^{Ald}). Since these peptides are located at the NH_2 terminus of the α1 chain (Bornstein *et al.*, 1966b; Miller *et al.*, 1969; Kang *et al.*, 1969a), α1′ represents α1 in which the NH_2-terminal peptides, representing a sequence of 19 amino acids, have been removed. At least two explanations seem possible. (1) These sequences may be cleaved by an *in vivo* physiologic enzyme system. Since α1′ is seen mostly in older (acid-extracted) collagen but not in younger (salt-extracted) collagen, it is possible that the removal of these sequences may be related in some way to the physiologic maturation of collagen. The NH_2-terminal region of the molecule is involved in the formation of intramolecular cross-link and may participate in intermolecular cross-link formation. Alteration in this region of the molecule may also influence the aggregative properties of the collagen as well as the extent of cross-link formation. Conceivably, the removal of the NH_2-terminal region of the molecule might be an early step in physiologic degradation. (2) The sequences may be cleaved *in vitro* during extraction and purification by nonspecific proteolytic enzymes. However, they would have to act at low pH below 5°. There are insufficient data to choose between these two possibilities.

The point at which cleavage occurs is not known. It may be at or near the methionyl residue that is cleaved by CNBr to form α1-CB1 since remnants of a cleavage occurring closer to the NH_2 terminus were not found. Chymotrypsin *in vitro* cleaves native collagen in a similar manner (Bornstein *et al.*, 1966b).

Although codfish skin collagen has been shown to contain three distinct α chains (α1, α2, and α3), all other vertebrate collagens so far examined seem to contain only two kinds of α chains. These include dogfish skin collagen (Lewis and Piez,

1964), rat skin and tail tendon collagens (Piez *et al.*, 1963; Butler *et al.*, 1967), carp swim bladder collagen (Piez *et al.*, 1963), human skin collagen (Bornstein and Piez, 1964), and chick bone collagen (Miller *et al.*, 1967, 1969). The criteria applied in the past to distinguish α chains have been chromatographic separation and amino acid composition. By applying these criteria, conclusions have been reached that chick bone collagen (Francois and Glimcher, 1967) and calf skin collagen (Heidrich and Wynston, 1965) contain three distinct α chains. However, it has been shown that chromatographic heterogeneity of the α chains can arise for reasons other than differences in primary structure of the main body of the polypeptide chains (Piez *et al.*, 1966; Bornstein, 1967; Butler, 1968). The difference in amino acid composition between "α1" and "α3" as presented by Francois and Glimcher (1967) is relatively small and could very well be within the limits of error of the method. The present study on chick skin collagen clearly demonstrates yet another way in which heterogeneity of the α chains may arise. A better criterion of a different α chain is the demonstration that the primary structure differs in such a way that the α chains must be a separate gene product.

Previous studies (Bornstein *et al.*, 1966a; Bornstein and Piez, 1966; Kang *et al.*, 1967; Piez *et al.*, 1966) on rat skin collagen indicated that intramolecular cross-link formation involves a specific region of the molecule near the NH_2 termini of the α chains, that specific lysyl residues in this region, one per chain, are converted to allysine and that allysine residues in adjacent chains undergo a condensation to form a covalent cross-link. Although the chemistry of the cross-link was not confirmed then, indirect evidence suggested that an aldol condensation occurs.

In the present study, the reasoning applied was similar to that used in the study of rat skin collagen, and is as follows. If the cross-link is unique in its location on the molecule and is single in number, then there should be a peptide in the CNBr digest of α1 and another in the digest of α2 which are not present in β_{12}. Furthermore, there should be a peptide in the CNBr digest of β_{12} which is not present in digests of α1 and α2. Indeed, from α1 and α2 derived from newly synthesized (neutral salt-extracted) collagen and collagen in which cross-link formation had been blocked (lathyritic collagen), peptides α1-CB1 and α2-CB1 were isolated. From α1 and α2 derived from more highly cross-linked (acid-extracted) collagen, peptides α1-CB1^{Ald} and α2-CB1^{Ald} were isolated. The latter peptides contain all of the amino acids present in α1-CB1 and α2-CB1, respectively, except for the lysyl residues and in addition contain a residue each of allysine. These peptides were absent in CNBr digest, of β_{12} but another peptide β_{12}-CB1 was isolated from β_{12}. Amino acid composition of this peptide showed that it is a dimer of α1-CB1^{Ald} and α2-CB1^{Ald}. The ultraviolet spectrum of its azine derivative suggested that it contained an α,β-unsaturated aldehyde. Data obtained in this study in regard to the intramolecular cross-link of chick skin collagen are in most respects identical with those obtained for the intramolecular cross-link of rat skin collagen, and suggest that the mechanism and the structure of intramolecular cross-linking described may generally apply to other vertebrate collagens.

An enzyme system capable of converting lysyl residues in peptide linkage in elastin to allysine has been recently prepared from chick bone by Pinnell and Martin (1968). They presented evidence that lysyl residues in peptide linkage are substrates for the enzyme and that free lysine and small lysine-containing peptides are not acted on by the enzyme system. These data support the hypothesis that the formation of aldehydes and the cross-link occur after the synthesis of the collagen molecule is complete.

In regard to the chemistry of the cross-link, although they did not then have definitive data, Bornstein *et al.* (1966a) and Bornstein and Piez (1966) made the reasonable suggestion that it may be formed by an aldol condensation of two aldehydes on adjacent chains. An aldol condensation product between two residues of allysine has been isolated in reduced form from elastin after reduction with $NaBH_4$ and alkaline hydrolysis (Franzblau and Lent, 1969). Using similar techniques, Paz *et al.* (1969) recently isolated the same aldol condensation product in small amounts from calf skin collagen. The aldol product is unstable under the condition of acid hydrolysis and this accounts for our inability to observe such a product during amino acid analysis of the hydrolysates of β_{12}-CB1. Recently, however, direct evidence has been obtained that the intramolecular cross-link in chick skin collagen is indeed an aldol condensation product between the two residues of allysine and that it is located in β_{12}-CB1 (Kang *et al.*, 1969c).

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Characterization of Rat Heart Myosin. I. Isolation and Physical Properties*

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ABSTRACT: Cardiac myosin from rats was isolated and characterized by physical methods. The myosin appeared homogeneous by sedimentation velocity and sedimentation equilibrium centrifugation. The calculated value for $s^0_{20,w}$ was 6.35 S, and $D^0_{20,w}$ obtained by the porous disk method was 1.56×10^{-7} cm²/sec.

The apparent molecular weight from sedimentation equilibrium runs was of the order of 436,000. Conformational studies gave a value of 53% for the helical content of myosin isolated from rat cardiac tissue.

Extensive experimental data are now available concerning both the enzymatic and structural properties of rabbit skeletal myosin. Brahms and Kay (1963) reported certain variations in enzymatic properties between dog cardiac and rabbit skeletal myosins. On the other hand, Mueller *et al.* (1964) did not report any drastic differences between dog cardiac, dog skeletal, and rabbit skeletal myosins. The observed variations were ascribed to differences due to species and not due to inherent variations in the size and shape of the myosin molecule from different tissues.

The observation (McCarl *et al.*, 1965) that cortisol acetate had a positive inotropic effect on rat heart cells in culture led us to isolate and purify myosin from mature rat cardiac tissue. In this present paper, a detailed analysis of the hydrodynamic properties of rat cardiac myosin will be presented.

Experimental Procedures

Materials

ATP, the disodium salt, was obtained from Calbiochem, Los Angeles, Calif.

The animals used throughout these experiments were mature albino rats (obtained from the small animal laboratory in Frear Laboratories at The Pennsylvania State University). In each isolation the hearts from 30 animals were pooled and extracted. The weight of rat cardiac tissue in each experiment was 37 ± 3 g.

Methods

Isolation of Rat Cardiac Myosin. The rats were killed by a blow on the head and the hearts were immediately excised and chilled. Blood was washed away with cold, deionized water and the hearts were minced. The minced tissue was homogenized in a Virtis homogenizer for 30 sec at half-maximal speed, at 0°. The homogenization was performed in three volumes of extracting solvent which consisted of 0.4 M KCl–0.075 M KH_2PO_4–0.075 M K_2HPO_4–0.002 M ATP (pH 6.7). Following homogenization the extraction of myosin was continued for 10 min with constant stirring. Our method of isolating myosin was that described by Mueller (1964) except for a final purification by chromatography on a DEAE-cellulose column equilibrated with 0.2 M KCl–0.01 M Tris (pH 7.6) (Brahms, 1959). Elution from the DEAE-cellulose column was performed by applying an ascending KCl gradient (0.2–1.0 M KCl). The protein concentration in each 10-ml effluent fraction was then determined. The flow rate was regulated to 0.7 ml/min.

Protein Determination. Protein was determined by a modification of Lowry's method as described by Oyama and Eagle

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