Heterogeneity of the α Chains of Rat Skin Collagen and Its Relation to the Biosynthesis of Cross-Links*

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ABSTRACT: The $\alpha 1$ and $\alpha 2$ chains from rat skin collagen show heterogeneity when chromatographed on CM-cellulose. Under favorable conditions two forms of $\alpha 1$ can be partially resolved. They differ in that a lysyl residue in one ($\alpha 1^{Lys}$) is present as an aldehyde in the other ($\alpha 1^{Ald}$). Samples of $\alpha 2$ contain equivalent forms ($\alpha 2^{Lys}$ and $\alpha 2^{Ald}$), although they are not well enough resolved to be isolated. $\alpha 1^{Lys}$ has a single N-terminal glycyl residue which is not demonstrable in $\alpha 1^{Ald}$. Neither form of $\alpha 2$ has a free N terminal. These results are consistent with previous studies on peptides from the cross-linking region of collagen at the N-terminal ends of the α chains. Isotope incorporation studies demonstrate that $\alpha 1^{Lys}$ and $\alpha 2^{Lys}$ are labeled more rapidly than $\alpha 1^{Ald}$ and $\alpha 2^{Ald}$, confirming the expected precursor–product relationship. In the extractable collagen fraction the two forms are equally labeled after 1 day in the case of $\alpha 1$ and 3 days in the case of $\alpha 2$. Since the cross-linked β components require 4–7 days to become labeled to the same degree as the α chains, the data are consistent with aldehyde formation being a preliminary step to cross-link formation. In rats receiving a lathyrogen, the rate of appearance of isotope in $\alpha 1^{Ald}$ and $\alpha 2^{Ald}$ is markedly decreased, consistent with inhibition of cross-linking as a result of blocking the lysine-to-aldehyde conversion.

Studies on peptides prepared from the $\alpha 1$ and $\alpha 2$ chains of rat skin collagen by cyanogen bromide (CNBr) cleavage (Bornstein *et al.*, 1966a; Bornstein and Piez, 1966) have shown that a lysyl residue in each chain is converted to the δ-semialdehyde of α-aminoadipic acid in peptide linkage, and suggest that this step is preliminary to the formation of an interchain, intramolecular cross-link. The cross-link has not been completely characterized but may result from an aldol-type condensation of aldehydes on adjacent chains. Since the cross-linking process is relatively slow in rat skin and presumably occurs extracellularly, this evidence must mean that the pools of $\alpha 1$ and $\alpha 2$ chains in recently synthesized collagen are heterogeneous in that each is a mixture of lysine- and aldehyde-containing forms. Although only a single lysyl residue in each α chain of about 1000 amino acids is apparently involved, the loss of a charged group in the conversion to the aldehyde might be expected to result in an observable effect on the chromatographic behavior of the α chains. This is suggested by the ready separation by carboxymethylcellulose chromatography of $\alpha 1$ and $\alpha 2$ (Piez *et al.*, 1961, 1963) which differ in net charge by about 13/1000 amino acids.[1]

On the basis of this reasoning, the previously observed chromatographic heterogeneity of $\alpha 1$ from rat skin collagen (Piez *et al.*, 1963) has been reexamined and shown to be related to the lysine-to-aldehyde conversion. In addition, a previously unexplained observation that the specific activity across chromatographic peaks of radioactive α chains was not always constant (G. R. Martin and K. A. Piez, unpublished results) has been examined in more detail and is also shown to be related to the presence of two forms of the $\alpha 1$ and $\alpha 2$ chains. This evidence permits an estimate of the rate of synthesis of the lysine-derived aldehyde and a comparison with the rate of formation of intramolecular cross-links. The effect of a lathyrogen, β-aminopropionitrile, on the over-all process has also been studied. This agent has been shown to block cross-linking in collagen (Martin *et al.*, 1961, 1963) and peptide studies suggest that the defect results from an inhibition of the lysine-to-aldehyde conversion (Bornstein and Piez, 1966).

Preparation and Chromatography of Collagen. Salt extracted (1 M NaCl) and acid-extracted (0.5 M acetic acid) collagens were prepared from rat skin and chromatographed in the denatured form on CM-cellulose (Whatman microgranular CM 32, Reeve Angel, Clifton, N. J.) as previously described (Piez *et al.*, 1963). Collagen from lathyritic rats to be used for isolation of α chains was obtained by salt extraction of skin from rats that had received β-aminopropionitrile fumarate

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[1] Amino acid data averaged from ten separate analyses of $\alpha 1$ and $\alpha 2$ from rat skin collagen (Piez *et al.*, 1965) show a net charge of +10 for $\alpha 1$ and +23 for $\alpha 2$ per 1000 amino acid residues at about pH 6. In calculating a difference of 13, the number of carboxyl groups protonated at pH 4.8 where chromatography is performed is assumed to be the same for $\alpha 1$ and $\alpha 2$.

(BAPN)[2] in their diet for 3–4 weeks (Bornstein and Piez, 1966). For the preparation of α chains, samples of about 200 mg were chromatographed on columns 1 in. in diameter and 5 in. high at a flow rate of 250 ml/hr. For analytical purposes, slightly better resolution was obtained using a column measuring 18 × 180 mm, a flow rate of 150 ml/hr, and samples of about 75 mg. The effluent was monitored at 230 mμ in a micro flow cell in a Beckman DB spectrophotometer.

Chromatography of CNBr Digestion Products. Samples of α chains isolated by CM-cellulose chromatography were digested with CNBr and the small peptides from the cross-link region were chromatographed on phosphocellulose as described (Bornstein *et al.*, 1966a; Bornstein and Piez, 1966).

Amino Acid Analysis. Samples were hydrolyzed in 6 N HCl at 108° for 24 hr under nitrogen. The hydrolysates were analyzed on an automatic instrument using a high-speed single-column system (Miller and Piez, 1966). Corrections were made for losses of serine, threonine, tyrosine, and methionine and incomplete release of valine using previously determined correction factors (Piez *et al.*, 1960).

N-Terminal Analysis (Sanger). The dinitrophenyl (DNP) derivatives were prepared as described by Fraenkel-Conrat *et al.* (1955). Coupling with fluorodinitrobenzene was done in 4% $NaHCO_3$–50% ethanol at 40° for 6 hr using 10–30 mg (0.1–0.3 μmole) of α chain. Excess reagent was removed by ether extraction. The DNP-protein was isolated by lyophilization in the dark and then hydrolyzed under nitrogen in sealed tubes with 6 N HCl at 108° for 16 hr. The ether-soluble DNP-amino acids were chromatographed on paper using the toluene–pyridine–2-chloroethanol–ammonia solvent in one dimension and the pH 6.0, 1.5 M phosphate buffer in the second dimension. The water-soluble DNP-amino acids were chromatographed on paper using the 1-butanol–acetic acid–H_2O and the *t*-amyl alcohol–phthalate solvents. The DNP-amino acids were eluted and quantitated by their absorbance at appropriate wavelengths. Correction factors were determined by analyzing appropriate model compounds in the presence of the protein being studied. Under these conditions, the recovery of DNP-glycine was about 50%. In the absence of protein, DNP-glycine was nearly all lost during acid hydrolysis.

N-Terminal Analysis (Edman). The phenylthiohydantoin (PTH) derivatives were prepared as described by Fraenkel-Conrat *et al.* (1955) and Sjoquist (1960). Coupling of α chains (10–30 mg) with phenylisothiocyanate was performed in triethylamine acetate buffer, pH 9.0, containing 50% dioxane at 40° for 2 hr. Excess reagent was extracted with benzene and the protein was isolated by lyophilization. Cyclization was performed in 3 N HCl at room temperature. Complete cyclization usually required 4–8 hr. The PTH-amino acids were chromatographed and quantitated as described by Sjoquist (1960). Correction factors were determined from appropriate model compounds. The recovery of PTH-glycine was approximately 50%.

^{14}C-Labeling Experiment. Two groups of 12 rats weighing 135–145 g each were placed on a standard laboratory diet. One group received 100 mg of BAPN/day by intraperitoneal injection beginning 1 day before the administration of isotope. All animals received a single intraperitoneal injection of 40 μc of glycine-*U*-^{14}C. Three rats from each group were killed 6 hr, 24 hr, 3 and 7 days later. Acid-extracted collagen was prepared from the skins without a prior salt extraction. These samples then contained both fractions generally referred to as salt-extracted and acid-extracted collagen representing both recently synthesized and more mature collagen.

Samples weighing 50–100 mg were chromatographed on the 18 × 180 mm column of CM-cellulose as described above. In addition to monitoring the effluent at 230 mμ in a flow cell, 5-ml fractions were collected and their absorbances at 230 mμ in 1-cm cells were measured to provide an accurate concentration measurement for the calculation of specific activities. Aliquots of 1 ml were plated on planchets and counted for 10 min in a low-background gas-flow counter. A circle of lens paper was placed on each planchet before adding the sample and a drop of dilute detergent was added to ensure that the paper would lie flat after drying. Counting of samples to which standards were added demonstrated that self-absorption was constant for all fractions. Activity was expressed as counts per minute per milliliter and specific activity as activity per unit absorbance at 230 mμ.

Double-Labeling Experiment. Three 140-g rats each received 1 mc of glycine-^{3}H 3 days before sacrifice and 100 μc of glycine-^{14}C 1 hr before sacrifice. Collagen was isolated by acid extraction and chromatographed as above. Aliquots of 1 ml from the 5-ml fractions were added to Bray's solution and counted in a scintillation counter using standard techniques to determine the two isotopes.

Results

Chromatographic Heterogeneity of α1. Although the peaks on CM-cellulose chromatograms of denatured collagen from various sources often appear homogeneous (see, for example, Piez *et al.*, 1963; Figures 4–8), the α1 and α2 peaks frequently show evidence of at least two chromatographic components. A second component may appear as a shoulder or, in favorable cases, as a partial separation. This type of behavior is more often seen in salt-extracted collagens but may also appear in samples of acid-extracted collagen. A particularly clear example of a salt-extracted collagen exhibiting heterogeneity is seen in Figure 1. The α1 peak is split and the α2 peak is somewhat broadened. These peaks may be compared to the β_{12} peak which usually appears homogeneous and is frequently sharper. Examination of protein from different parts of the α1 peak by sedimentation velocity,

[2] Abbreviations used: BAPN, β-aminopropionitrile fumarate; PTH, phenylthiohydantoin derivative.

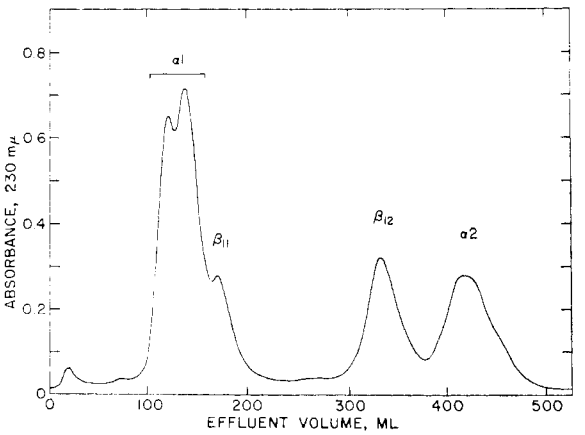

FIGURE 1: CM-cellulose chromatograms of denatured skin collagen from normal rats obtained by extraction with 1 M sodium chloride. The two chromatographic forms of the $\alpha 1$ fraction were taken for amino acid (Table I) and N-terminal (Table II) analysis, and for examination of the peptides from the cross-link region (Figures 2 and 3).

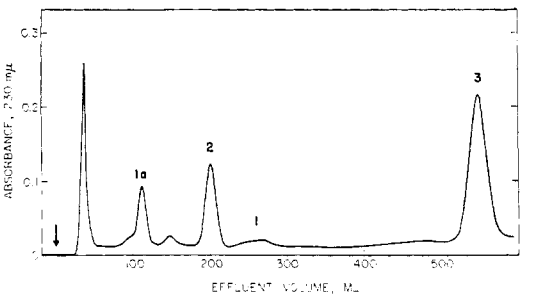

FIGURE 2: Phosphocellulose chromatogram of the low molecular weight peptides in a cyanogen bromide digest of the acidic portion of $\alpha 1$ (see Figure 1) consisting largely of $\alpha 1^{Ald}$. Peptides $\alpha 1$-CB-1a, 1, 2, and 3 are identified. The forepeak consists largely of non-peptide ultraviolet-absorbing contaminants plus a small amount of the minor peptide $\alpha 1$-CB-1b (Bornstein and Piez, 1966). The arrow indicates the point at which the sample was applied and elution was begun with a salt gradient.

gel electrophoresis as described by Nagai *et al.* (1964), and amino acid analysis failed in several attempts to show any significant differences. Amino acid data on the two forms of $\alpha 1$ taken from the sample illustrated in Figure 1 and purified further by rechromatography are shown in Table I. All of the differences are within experimental error.

TABLE I: Amino Acid Composition of Two Forms of the $\alpha 1$ Chain from Rat Skin Collagen.[a]

	$\alpha 1^{Ald}$	$\alpha 1^{Lys}$
3-Hydroxyproline	1.2	1.3
4-Hydroxyproline	95	99
Aspartic acid	48	49
Threonine	21.2	20.4
Serine	38	41
Glutamic acid	76	76
Proline	125	122
Glycine	324	325
Alanine	116	116
Valine	21.8	20.0
Methionine	7.6	8.4
Isoleucine	7.1	6.6
Leucine	19.3	18.6
Tyrosine	2.2	2.2
Phenylalanine	11.8	11.4
Hydroxylysine	4.5	4.1
Lysine	30.3	29.8
Histidine	1.9	1.9
Arginine	49	48
Amide N	(40)	(38)

[a] Residues/1000 total residues. Corrected for incomplete release of valine and loss of threonine, serine, methionine, and tyrosine during hydrolysis using previously determined values (Piez *et al.*, 1960).

The demonstration that CNBr digests of $\alpha 1$ and $\alpha 2$ chains each contain two related peptides, one containing a lysyl residue ($\alpha 1$-CB-1 and $\alpha 2$-CB-1) and the other containing instead a lysyl-derived aldehyde ($\alpha 1$-CB-1a and $\alpha 2$-CB-1a) (Bornstein *et al.*, 1966a; Bornstein and Piez, 1966), suggested that chromatographic heterogeneity of α chains might arise from this difference. To test this possibility, the two forms of the $\alpha 1$ chain were isolated from the sample whose chromatogram appears in Figure 1. On rechromatography they maintained their original positions in the effluent. They were digested with CNBr and the resulting peptides were chromatographed on phosphocellulose. The more acidic (faster moving) form of $\alpha 1$ yielded predominantly $\alpha 1$-CB-1a and almost none of the $\alpha 1$-CB-1 form (Figure 2). The more basic (slower moving) form of $\alpha 1$ yielded $\alpha 1$-CB-1 and $\alpha 1$-CB-1a in a ratio of about 70:30, based on the areas under the peaks (Figure 3). These peptides of 15 amino acids may be compared relative to peptides $\alpha 1$-CB-2 and $\alpha 1$-CB-3 which have molecular weights of about 3500 and 12,000 and do not vary in content. These results would be expected if the heterogeneity of $\alpha 1$ were the result of the lysine to aldehyde conversion. The aldehyde-containing form ($\alpha 1^{Ald}$) would be more acidic and elute first with very little contamination by the lysine-containing form ($\alpha 1^{Lys}$) while $\alpha 1^{Lys}$ would be more basic and elute second with some contamination by $\alpha 1^{Ald}$ owing to the tailing always present in these chromatograms.

N Termini of α Chains. N-Terminal analysis of peptides $\alpha 1$-CB-1 and $\alpha 1$-CB-1a (Kang *et al.*, 1966; A. H. Kang, P. Bornstein, and K. A. Piez, in preparation) has shown that $\alpha 1$-CB-1 has an N-terminal glycine but a free N terminus cannot be demonstrated

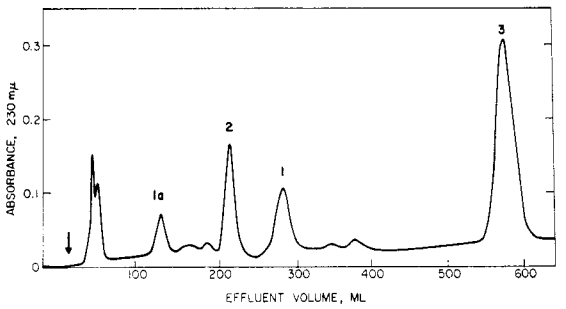

FIGURE 3: Phosphocellulose chromatogram of the low molecular weight peptides in a cyanogen bromide digest of the basic portion of $\alpha 1$ (see Figure 1) consisting largely of $\alpha 1^{Lys}$; see legend to Figure 2.

in α1-CB-1a. The reason for this is not clear, but the apparent loss may be associated with the lysine-to-aldehyde conversion. It is also known (Bornstein *et al.*, 1966a; Bornstein and Piez, 1966) that α1-CB-1a predominates in CNBr digests of $\alpha 1$ from acid-extracted collagen while α1-CB-1 is the major form in digests of $\alpha 1$ from the more recently synthesized salt-extracted collagen. In collagen from lathyritic rats, where cross-linking is inhibited, very little α1-CB-1a is seen. Similar results should be obtained by analysis of whole $\alpha 1$ chains since these peptides come from the N-terminal end (Bornstein *et al.*, 1966a,b). This was determined by analyzing $\alpha 1$ chains isolated by CM-cellulose chromatography of collagen from lathyritic rats and of salt- and acid-extracted collagen from normal rats.

The results (Table II) were in accord with the findings from the peptide studies. The same results were obtained with both the Sanger (DNP) and Edman (PTH) methods and only DNP- or PTH-glycine were found in significant amounts. In $\alpha 1$ from acid-extracted collagen only trace amounts of N-terminal glycine could be demonstrated, while in $\alpha 1$ from salt-extracted collagen an average of two-thirds of an equivalent was present. When the $\alpha 1$ peak from salt-extracted collagen was divided into halves corresponding approximately to $\alpha 1^{Ald}$ and $\alpha 1^{Lys}$, the N-terminal glycine was seen to be predominantly associated with the fraction rich in $\alpha 1^{Lys}$, the more basic form. $\alpha 1$ from collagen from lathyritic rats gave nearly a full equivalent. It is evident, as suggested by the peptide data, that the availability of the N-terminal glycine in $\alpha 1$ is related to the lysine-to-aldehyde conversion and to the chromatographic heterogeneity. The absence of a free N-terminal may also contribute to the less basic character of $\alpha 1^{Ald}$ depending on the chemical form in which the group is bound.

Neither form of the peptide from the cross-linking region of the $\alpha 2$ chain, α2-CB-1, or α2-CB-1a has a demonstrable N-terminal amino group (A. H. Kang, P. Bornstein, and K. A. Piez, in preparation). Similarly, whole $\alpha 2$ chains yielded no significant amounts of DNP- or PTH-amino acids whether the chain was derived from salt- or acid-extracted collagen or from collagen from lathyritic rats (Table II).

Metabolic Relationship of the Two Forms of the α Chains. Since the acidic and basic forms of $\alpha 1$ and $\alpha 2$ show a partial chromatographic separation related to the lysine to aldehyde conversion, it should be possible to demonstrate different rates of isotope incorporation into the two forms and obtain direct evidence for a precursor–product relationship. For this purpose rats were given single injections of glycine-^{14}C and sacrificed at various times after the injection. Acid-extracted collagen without a prior salt extraction was prepared to provide a fraction containing both recently synthesized and cross-linked collagen. A second group of animals received BAPN daily, beginning 1 day before the injection of isotope, and were sacrificed on the same schedule. During at least the first part of the 8-day course of the experiment gross changes of the type observed under chronic lathyritic conditions, including major connective tissue abnormalities, increased extractability of collagen (Levene and Gross, 1959), and decreased content of cross-linked collagen components (Martin *et al.*, 1961, 1963), were not expected. That is, unlike the collagen from chronically lathyritic rats used for the N-terminal studies, the extractable collagen would have been largely made prior to the administration of the lathyrogen and would appear normal except that isotope incorporation, which occurred in the presence of lathyrogen, would reflect any changes. These relatively mild conditions were chosen in an attempt to limit the changes to primary effects of the lathyrogen. The collagens from the normal and lathyritic rats were chromatographed on CM-cellulose and protein concentration and radioactivity were measured in the effluent fractions. The data for some of the time periods appear in Figures 4–8.

After 6 hr the distribution of isotope in the collagen

TABLE II: N-Terminal Analyses of α Chains from Rat Skin Collagen.

Chain	Av Equiv of Glycine/ Chain[a]	Range	Number of Analyses DNP	PTH
$\alpha 1$, acid-extracted collagen	0.06	0–0.11	3	4
$\alpha 1$, salt-extracted collagen				
Whole	0.66	0.50–0.78	1	2
Basic half[b]	1.10	—	1	—
Acidic half[b]	0.40	—	1	—
$\alpha 1$, lathyritic collagen	0.84	0.70–1.10	1	3
$\alpha 2$, all sources	0.03	0–0.12	4	5

[a] No amino acids other than glycine were found. Equivalents were calculated on the basis of a molecular weight of 95,000. [b] Refers to the two chromatographic forms (see Figure 1).

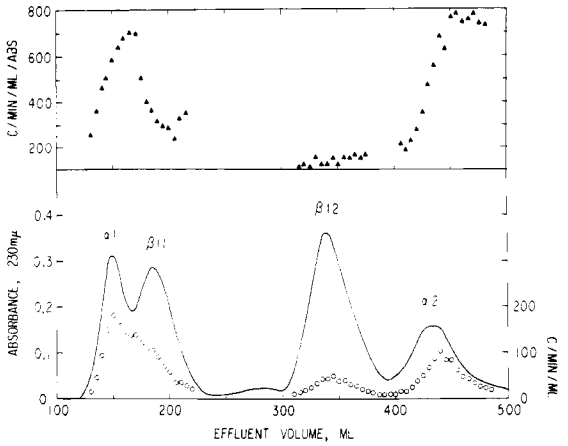

FIGURE 4: CM-cellulose chromatogram of denatured skin collagen from normal rats that received glycine-^{14}C 6 hr before sacrifice. Protein concentration was measured by absorbance at 230 mμ (solid line) and activity as counts per minute per milliliter (O). Calculated specific activities appear above the chromatogram (▲).

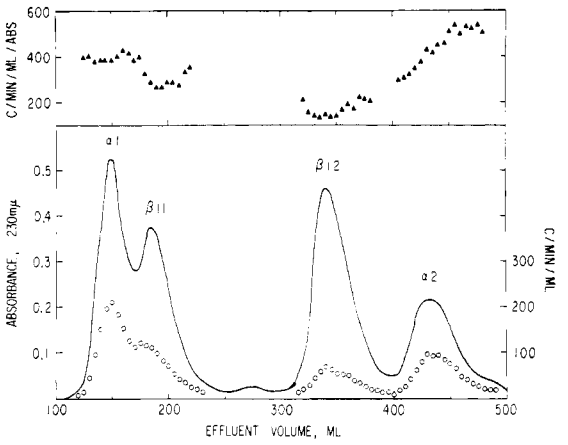

FIGURE 5: CM-cellulose chromatogram of denatured skin collagen from normal rats that received glycine-^{14}C 1 day before sacrifice. See legend to Figure 4.

components from normal animals was clearly not uniform (Figure 4). Two effects were apparent. The β components were labeled only slightly. This was expected from previous studies (Orekhovitch *et al.*, 1959; Martin *et al.*, 1961, 1963) that demonstrated that approximately 4–7 days are required for the α and β components to reach equal specific activities in acid-extractable collagen from rat skin. It could also be seen that the specific activity across both peaks representing $\alpha 1$ and $\alpha 2$ was not constant in spite of the fact that the concentration curves showed no evidence of heterogeneity. The more basic portions of $\alpha 1$ and $\alpha 2$ corresponding to the expected positions of $\alpha 1^{Lys}$ and $\alpha 2^{Lys}$ had three to four times the specific activity of the more acidic portions. In the case of

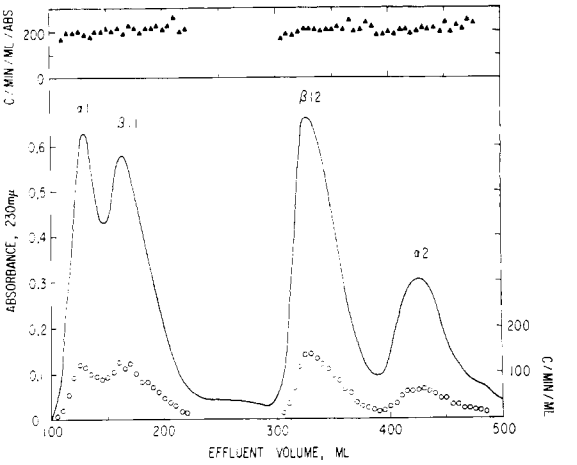

FIGURE 6: CM-cellulose chromatogram of denatured skin collagen from normal rats that received glycine-^{14}C 7 days before sacrifice. See legend to Figure 4.

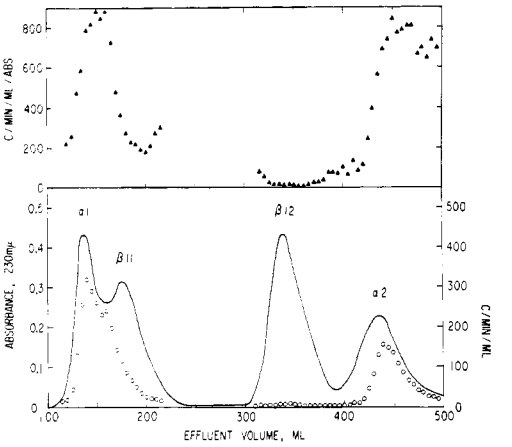

FIGURE 7: CM-cellulose chromatogram of denatured skin collagen from lathyritic rats that received glycine-^{14}C 6 hr before sacrifice. See legend to Figure 4.

$\alpha 1$, the high specific activity region extended into the peak that would have been ascribed entirely to β_{11} on the basis of the concentration curve. Apparently $\alpha 1^{Lys}$ chromatographed in this position but was not present in sufficient quantity to be detected other than by its greater radioactivity at early times.

After 1 day (Figure 5) the β components from collagen of normal animals were labeled at about one-third the level of the α components. The specific activity across the $\alpha 1$ peak was essentially constant, while the slope of the specific activity curve of $\alpha 2$ was much reduced from its value at 6 hr but still showed a significant nonuniform distribution. These findings confirm the expected precursor–product relationship of the two forms of the α chains and reveal that the accumulation of $\alpha 2^{Ald}$ in the extractable pool is not as rapid as the accumulation of $\alpha 1^{Ald}$.

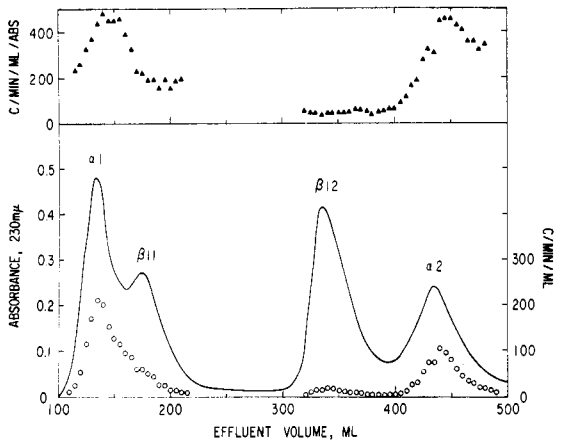

FIGURE 8: CM-cellulose chromatogram of denatured skin collagen from lathyritic rats that received glycine-^{14}C 1 day before sacrifice. See legend to Figure 4.

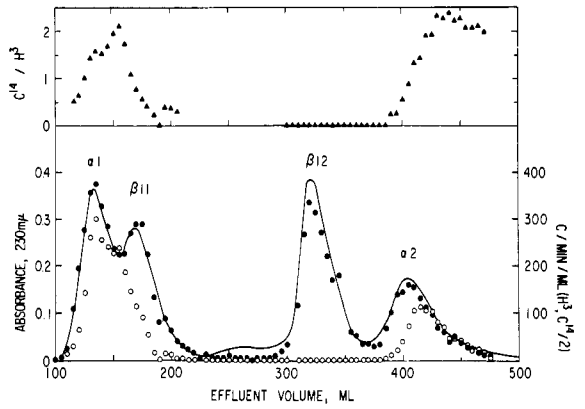

FIGURE 9: CM-cellulose chromatogram of denatured skin collagen from normal rats that received glycine-^{3}H 3 days before sacrifice (●) and glycine-^{14}C 1 hr before sacrifice (○). Protein concentration was measured by absorbance at 230 mμ (solid line). ^{14}C:^{3}H ratios appear above the chromatogram (▲).

After 3 days (not shown) both α chains showed a constant specific activity and the β-components were labeled to only a slightly lesser degree. After 7 days (Figure 6) the specific activity throughout the chromatogram was constant.

The effects seen in the normal animals were accentuated in the animals receiving BAPN. After 6 hr (Figure 7), the incorporation of isotope into the β components was negligible and the slopes of the specific activity curves of α1 and α2 were considerably greater than in the normal case consistent with the expected inhibition of the lysine-to-aldehyde conversion. In the absence of incorporation into the β components, the activity curve was considered to represent only incorporation into α components. Two partially resolved α1 peaks representing $\alpha1^{Ald}$ and $\alpha1^{Lys}$ were seen (Figure 7). Since $\alpha1^{Ald}$ would predominate in this kind of sample (see Discussion), $\alpha1^{Lys}$ must have had a considerably greater specific activity. Only one relatively sharp radioactive α2 peak could be seen apparently representing $\alpha2^{Lys}$, again suggesting a difference in the rates of accumulation of $\alpha1^{Ald}$ and $\alpha2^{Ald}$.

After 1 day (Figure 8) the same effects, somewhat reduced in magnitude, were present. Some $\alpha1^{Ald}$ and $\alpha2^{Ald}$ were produced but at a slower rate than in normal animals. After 3 and 7 days (not shown) very little change from the situation at 1 day was noted. This suggests that the labeled collagen had essentially reached a steady state condition in 24 hr.

To confirm the results from normal animals and to provide information at an earlier time, an additional experiment of a similar nature was performed. A single group of rats was given glycine-^{3}H 3 days before sacrifice and glycine-^{14}C 1 hr before sacrifice. The acid-extracted collagen was chromatographed and differentially counted for the two isotopes. These results appear in Figure 9.

As in the first experiment, the ^{3}H label representing 3-day-old collagen was distributed in a nearly uniform manner throughout the α and β components. However, the ^{14}C label was confined entirely to the α components. The ^{14}C:^{3}H ratio, which is equivalent to specific activity in the first experiment, showed that isotope was incorporated first into $\alpha1^{Lys}$ and $\alpha2^{Lys}$. The discrimination was greater than at 6 hr in collagen from normal rats and was essentially identical with the pattern observed in the collagen from lathyritic rats at 6 hr.

Discussion

The experiments described show clearly that there are two forms of the α1 and α2 chains. In the case of α1 these have been differentiated on the basis of partial chromatographic resolution, N termini, and presence of α1-CB-1 or α1-CB-1a in CNBr digests. In the case of both α chains they have also been differentiated by the rate of incorporation of isotope into the two forms. All of these data are consistent with the conclusion, suggested first from peptide studies (Bornstein *et al.*, 1966a; Bornstein and Piez, 1966), that cross-linking of collagen is preceded by the conversion of a specific lysyl residue in each α chain to an aldehyde.

In the total extractable pool as defined in the present studies, the two forms of α1 were equally labeled within 1 day, while the two forms of α2 required 3 days to reach the same specific activity. These rates are faster than the rate of appearance of isotope in the β components. Since the collagen studied here contained both salt-extractable and acid-extractable collagen but not insoluble collagen, and these fractions have a complex relationship (see the review by Harkness, 1961), the observed rates are net rates which may differ quantitatively from the rates in the whole collagen pool. Additional studies are required to provide more detailed data, but the qualitative aspects of the rela-

tionship of lysine to aldehyde and aldehyde to cross-link seem well established.

The data presented here confirm the indications that in lathyritic animals the lysine-to-aldehyde conversion is inhibited. Under the conditions employed, the inhibition was only partial. In spite of this the formation of β components in the extractable pool was almost completely blocked. This is presumably a reflection of the fact that intramolecular cross-links require the participation of two aldehyde groups on adjacent chains (Bornstein *et al.*, 1966a; Bornstein and Piez, 1966). The absence of either one would prevent the formation of a cross-link.

A number of attempts have been made to find N-terminal amino acids in collagen. Except with collagens that have been partially degraded, it has not been possible to find more than traces of any single N terminal if expressed as equivalents per α chain of about 95,000 mol wt (see, for example, Steven and Tristram, 1962; Hörmann *et al.*, 1965). This was confirmed in the present studies on isolated α chains except that fractions rich in $\alpha 1^{Lys}$ were found to contain a single N-terminal glycine. Since this form of $\alpha 1$ would be a minor component in most collagen samples, the N-terminal would not have been detected in previous studies on whole collagen. The reason for the inability to detect this group following the lysine to aldehyde conversion is not clear but it may become bound in a Schiff's base with the aldehyde which is only five residues away (Bornstein *et al.*, 1966a; Bornstein and Piez, 1966). In the case of the $\alpha 2$ chain there is evidence (A. H. Kang, P. Bornstein, and K. A. Piez, in preparation) that pyrrolidone carboxylic acid is N-terminal in isolated samples, presumably arising from glutamine or glutamic acid.[3]

The finding that $\alpha 1$ from salt-extracted collagen contains more than 0.5 equiv of N-terminal glycine is consistent both with the precursor position of this fraction and the finding from peptide studies (Bornstein *et al.*, 1966a; Bornstein and Piez, 1966) that $\alpha 1$-CB-1 predominates over $\alpha 1$-CB-1a in CNBr digests of $\alpha 1$ from salt-extracted collagen. It follows that $\alpha 1^{Lys}$ must be the predominant form in salt-extracted collagen while acid-extracted collagen contains largely $\alpha 1^{Ald}$. The chromatographic resolution of the two forms is not good enough to obtain quantitative data on this point, but the impression has been gained that this is the case (for example, compare Figure 1 with Figures 4–9). The same pattern presumably exists for $\alpha 2^{Lys}$ and $\alpha 2^{Ald}$. The present studies do not show any other differences between α chains from salt- and acid-extracted collagens.

[3] The possibility that the N terminal is acetylated must be considered since Hörmann *et al.* (1965) have reported the presence of about 18 acetyl groups/collagen molecule. However, initial experiments, utilizing a gas chromatographic procedure, have failed to demonstrate any acetyl groups in rat skin collagen. These experiments are being pursued and will be reported subsequently (A. H. Kang, P. Bornstein, and K. A. Piez, manuscript in preparation).

It was recognized in earlier experiments that $\alpha 1$ was not chromatographically homogeneous. The difficulty in isolating β_{11} in pure form was in part a result of the fact that some of the $\alpha 1$ fraction chromatographed with β_{11} even on rechromatography (Piez *et al.*, 1963). This contaminant can now be identified as $\alpha 1^{Lys}$. Since β_{11} must be the dimer of $\alpha 1^{Ald}$ (Bornstein and Piez, 1966), it will be more acidic than $\alpha 1^{Lys}$. The chromatographic effect of the charge difference is opposite and approximately equal to the retarding effect resulting from the higher molecular weight of β_{11}, explaining why chromatographic separation is difficult.

Electrophoretic heterogeneity of $\alpha 1$ has been reported by Hollmen and Kulonen (1964). This observation may also be the result of the presence of the two forms of $\alpha 1$ described here. The chromatographic heterogeneity of $\alpha 1$ from calf skin and rat skin collagen was examined by Heidrich and Wynston (1965). They reported small but apparently significant differences in amino acid composition of protein from different parts of the $\alpha 1$ peak. The studies reported here do not confirm this finding. The existence of another type of heterogeneity may be indicated but it is also possible that the analytical error in the analyses of Heidrich and Wynston (1965) was larger than expected.

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An Equilibrium Ultracentrifuge Study of the Effect of Ionic Strength on the Self-Association of Bovine Insulin*

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ABSTRACT: Insulin self-associates in aqueous solution. Experiments are described in which the association constants for the association equilibria at pH 2 are determined at ionic strengths 0.05, 0.10, 0.15, and 0.20, by means of equilibrium sedimentation experiments. Calculation of the electrostatic free energy of association for appropriate models using both the Debye–Hückel and Verwey–Overbeek theories has been carried out and the results were compared with the experimental free energies of association. It is suggested that the Verwey–Overbeek theory works better for this system. The results support the thesis that the association equilibria represent a balance between a nonelectrostatic intermolecular attraction and an electrostatic repulsion.

In a recent paper (Jeffrey and Coates, 1966; which we will refer to as paper 1) we have described experiments in which the thermodynamic parameters characterizing the self-association of insulin, at pH 2 and ionic strength 0.1, have been determined by means of the equilibrium ultracentrifuge. This present paper describes experiments in which the methods described in paper 1 are applied to insulin solutions at pH 2 and ionic strengths of 0.05, 0.15, and 0.20. The number of polymeric species, their degree of polymerization, and the equilibrium constant determining their concentrations have been determined for these ionic strengths. In order to test the hypothesis that the ionic strength influences the equilibria largely by altering the electrostatic free energy of the species in solution; calculations of the latter have been attempted using simple models and the Debye–Hückel and Verwey and Overbeek (1948) theories.

Experimental Procedures

Preparation of Insulin Solutions. Bovine crystalline zinc insulin (batch no. A3) was supplied by the Australian Commonwealth Serum Laboratories. The zinc content was stated to be between 0.3 and 0.9%. Zinc is not bound by insulin at pH 2 (Cunningham *et al.*, 1955); thus after dialysis the concentration in the insulin solutions was reduced to about 1/2500 of the original value. The buffer used was an aqueous sodium chloride–glycine–hydrochloric acid solution; its ionic strength was varied by adjusting the concentration of sodium chloride in the mixture appropriately.

Solutions of insulin in the buffer were dialyzed at 2–6° against buffer. The pH values of diffusate and insulin solution were identical after dialysis and were always between 2.00 and 2.04, measured at 25°.

Measurement of Molecular Weights and Determination of Equilibrium Constants. Sedimentation equilibrium experiments and determinations of concentration were carried out in a manner identical with that described in paper 1. From the experimental data, graphs of apparent weight-average molecular weight *vs.* concentration were obtained for a range of solute concentrations. The plots were then analyzed by Steiner's (1952) method as applied in paper 1 with the assumption that the species involved in the equilibria carry no electrostatic charge and are thermodynamically ideal. From this analysis one obtains the set of equilibrium constants which most satisfactorily reproduce the data when expressed in a manner appropriate to the Steiner formulation (see paper 1). To enable a

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