IN VITRO FORMATION OF INTERMOLECULAR CROSSLINKS IN CHICK SKIN COLLAGEN.

II. KINETICS*

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Summary

The intramolecular aldol condensation product present in acid soluble collagen disappears during in vitro fibril formation at 37°C. Data presented in this communication suggest that during fibril formation this intramolecular crosslink is incorporated into another substance designated previously as the "post-histidine peak". Formation of the "post-histidine peak" begins after the fibril, as measured by opacity, is almost completed. Evidence is also presented that rat tail tendon contains this same substance in vivo.

Recent studies have shed considerable light on the chemistry of several cross-links in collagen. Because of the nature of the tropocollagen molecule, and the fibril it forms in vivo, one has distributed these compounds among intramolecular and intermolecular cross-links. Thus, Bailey and Peach (1) and Tanzer et al. (2) have noted the presence of hydroxylysinonorleucine in preparations of insoluble collagen reduced with NaB^3H_4 and designated it as an intermolecular crosslink. More recently, lysinonorleucine has been detected as well (3,4) and it probably serves a similar function. In addition, Bailey et al. (5) have isolated an aldol condensation product from reduced bone collagen which presumably resulted from the interaction of one residue of α-aminoadipic acid-δ-semialdehyde and one residue of α-amino-δ-hydroxyadipic acid-δ-semialdehyde, the latter arising from the deamination of the ε-amino group of hydroxylysine. Because of its abundance

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in insoluble reduced bone collagen, it too, was assigned to the role of an intermolecular crosslink. Studying the crosslink between α-chains (β_{12}) from reduced acid-soluble chick skin collagen, Kang et al. (6) identified the reduced aldol condensation product of two residues of α-aminoadipic acid-δ-semialdehyde, a compound found in relatively large quantities in reduced elastin (7). A purified preparation of β_{12} indicated the presence of approximately one mole of intramolecular aldol condensate per β_{12} chain. Rojkind et al. (8) found similar results using rat skin collagen. These data confirmed the structure originally suggested by Bornstein et al. (9) and Kang et al. (10) for such an intramolecular crosslink.

We addressed ourselves to the general question of how and when these crosslinks are formed, and more specifically, whether the intramolecular crosslink in β_{12} remains intact during fibril formation. Earlier, it was reported (4) that the integrity of the aldol condensation product present in β_{12} is lost during in vitro fibril formation at 37°C. Analysis of a reduced fibril made from soluble chick skin in vitro did not reveal the presence of the aldol originally present in soluble collagen. This communication deals with the fate of this aldol condensate and the role it plays in intermolecular crosslink formation in collagen.

Methods and Results

Fibril Formation In Vitro - Purified acid soluble chick skin collagen was dissolved in 0.05M acetic acid (0.1-0.2%) and dialyzed overnight versus 0.16M NaCl, buffered with 0.05M Tris, pH 7.5. The dialysate was spun at 30,000 x g for 30 min. and 5 ml aliquots of the supernatant were placed in a 37°C. water bath to induce fibril formation as previously described by Gross and Kirk (11). Opacity was measured in a Klett densitometer employing a 570 mμ filter. At various times of incubation, the contents of an individual tube were reduced with NaB^3H_4 as described previously (4). The reduced reaction mixture was then dialyzed versus 0.05M acetic acid and lyophilized. An aliquot of each lyophilized preparation was hydrolyzed independently in 6N HCl and in 2N NaOH. The hydroly-

sates were analyzed both for amino acids and radioactivity employing an amino acid analyzer equipped with a stream divider as described previously (6).

Two major radioactive fractions are present in the soluble chick skin collagen reduced at zero time; one is the intramolecular reduced aldol condensate previously described (6) and the other, which has not yet been characterized, elutes just before hydroxylysine on the amino acid analyzer. The latter, designated "pre-hyly peak", is obtained from the 6N HCl hydrolysate and the former from the 2N NaOH hydrolysate. The radioactive elution profile from an acid hydrolysate of the reduced soluble collagen is shown in Figure 1a. Reduction

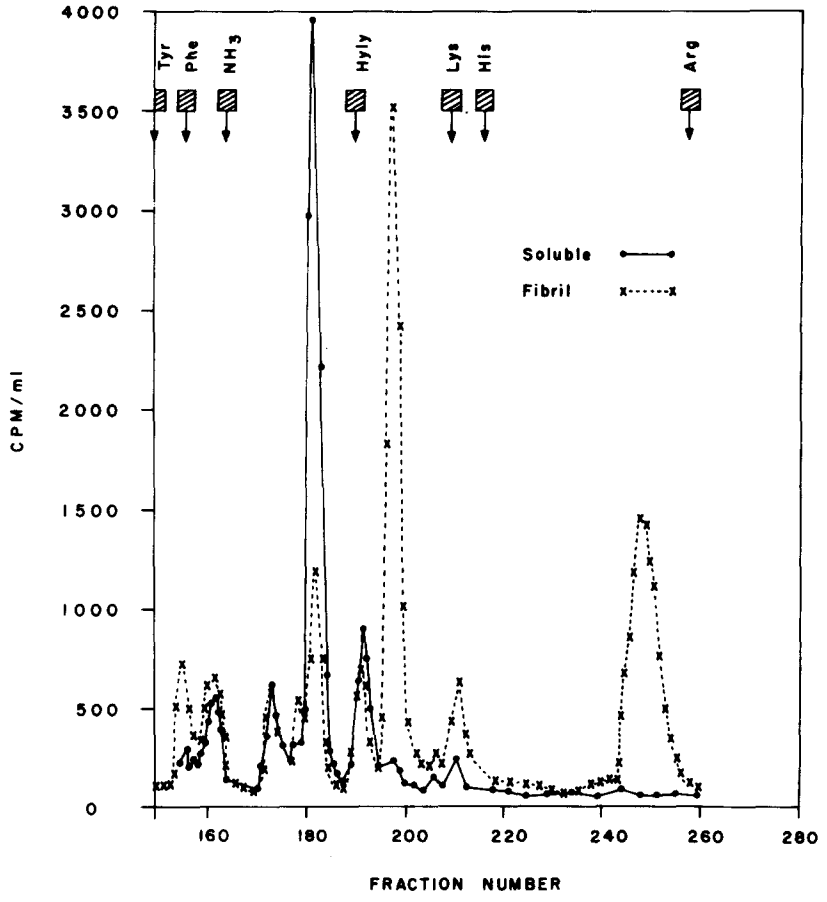

Figure 1a and b. Radioactive elution pattern of chick skin collagen reduced with NaB^3H_4 before (o—o) and 24 hours after (x—x) fibril formation was initiated. Both samples were hydrolyzed in 6N HCl. The buffer system utilized for the amino acid analyzer is a modification of Hamilton (12). The elution position of several amino acids are indicated.

of the chick skin collagen at various times during fibril formation indicate that both of these radioactive fractions decrease in content, while two other major radioactive peaks appear in the fibril being formed. One peak has been identified as hydroxylysinonorleucine (1,2) and the other, still unidentified, appears after histidine and therefore designated as the "post-histidine peak". The radioactivity of the reduced compounds are calculated from 6N HCl hydrolysates, the elution profile of which is illustrated in Figure 1b. The radioactivity obtained in hydroxylysinonorleucine, the reduced intramolecular aldol condensate, the "pre-hyly peak", and the "post-histidine peak during fibril formation are given in Table I. In Figure 2 the radioactivity data from the reduced intramolecular aldol condensate, the "post-histidine peak" and the Klett optical density values are plotted versus time. The radioactivity of the intramolecular

TABLE I

THE DISTRIBUTION OF RADIOACTIVITY OBTAINED FROM CHICK SKIN COLLAGEN REDUCED WITH NaB^3H_4 DURING FIBRIL FORMATION

Time min	Klett O.D.	Intramol Aldol*	"Post-Hist"	"Pre-Hyly"	OH-Lysinonor
			cpm/μM Lys**		
0	25	23,250	0	25,907	< 500
15	40	-	0	28,125	< 500
20	60	23,375	0	25,810	2,750
23	240	18,562	1,344	13,747	5,537
25	350	17,375	2,231	12,241	11,326
32	400	12,350	6,425	10,262	12,132
45	400	7,837	10,987	7,250	-
24 hr.	400	1,887	23,125	6,500	23,500

* The intramolecular aldol is determined separately after alkaline hydrolysis (6). All other radioactive fractions are determined from an acid hydrolysate.

** All cpm values are adjusted to the total concentration of lysine in the sample placed on the amino acid analyzer. There are 27 lysyl residues per 1000 amino acid residues in chick skin collagen (10).

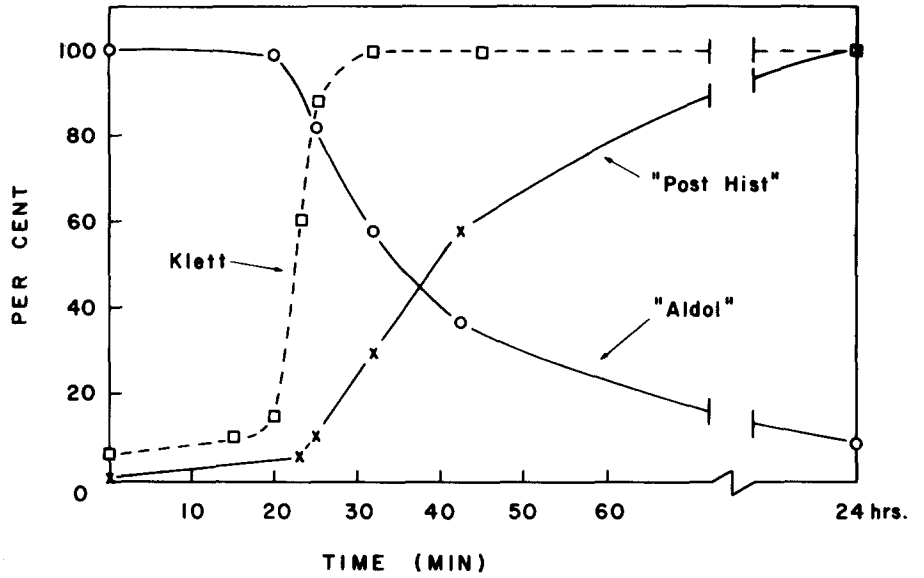

Figure 2. The percent of radioactivity in the intramolecular aldol condensate and "post-histidine" peak versus time during fibril formation. "Aldol" refers to the reduced intramolecular aldol condensate (6). See text for details.

aldol condensate from soluble chick skin collagen reduced before incubation at 37°C. was assigned a value of 100%. An arbitrary value of 100% was also assigned to the radioactivity of the "post-histidine peak" obtained from the 24 hour sample.

Rat Tail Tendon - Rat tail was stripped of its skin and the tendon was reduced directly with NaB^3H_4 after washing with cold saline but without prior solubilization of the collagen. The 2N NaOH hydrolysate contained no reduced intramolecular aldol condensate. Analysis of a 6N HCl hydrolysate revealed a significant number of counts in those fractions containing hydroxylysinonorleucine while the fractions with the highest counts were eluted from the amino acid analyzer in the "post-histidine peak". The radioactivity elution profile of the acid hydrolysate is given in Figure 3.

Discussion

The aldol condensation product originally assigned the role of an intramolecular crosslink (6) disappears during fibril formation. As seen in Figure 2,

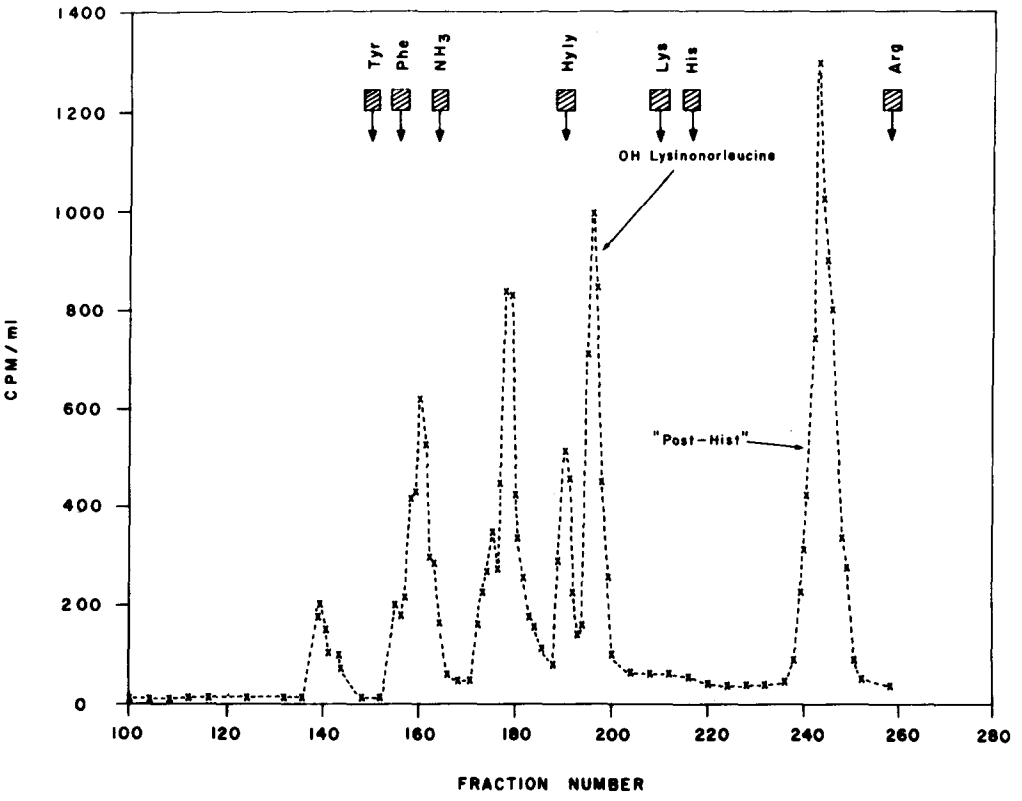

Figure 3. Radioactive elution profile of rat tail tendon reduced with NaB^3H_4. Tendon collagen was not solubilized prior to reduction.

the Klett reading, which corresponds to visible fibril formation, reaches almost to a maximum before the aldol condensate begins to disappear. The "post-histidine peak" begins to appear at the time the aldol condensate begins to disappear, both curves crossing at their approximate 50% values. These data suggest that the aldol condensate originally present in the acid soluble collagen is in some manner converted to the "post-histidine peak" during the later stages of fibril formation. On the other hand, if one allows reduced soluble collagen to form a fibril at 37°C. and then reduces the fibril as well, the aldol condensate is still present in toto, while there is no evidence for the presence of the "post-histidine peak" suggesting again a possible precursor-product relationship (4). Material similar to the "post-histidine peak" was detected by Bailey et al. (5) in hydrolysates of reduced rat tail tendon. These authors suggested that this

substance is quite complex and probably crosslinks several molecules. This is consistent with the idea that the aldol condensation product present in β_{12} is converted to a larger compound by possible addition of lysyl or lysine aldehyde residues or both. The formation of hydroxylysinonorleucine during fibril formation occurs at an earlier time than the "post-histidine peak". Formation of hydroxylysinonorleucine appears to follow closely the increase in Klett values, while during the same time period the "pre-hyly peak" disappears. (Although the data are quite suggestive, it is not absolutely clear whether these two peaks, the "pre-hyly peak" and the hydroxylysinonorleucine, are in fact precursor and product respectively.)

It is also of interest that the reduced rat tail tendon revealed the presence of both hydroxylysinonorleucine and the "post-histidine peak", the latter containing twice as much radioactivity. Since no prior treatment to the tissue was made before reduction, with NaB^3H_4, it suggests that this structure exists in an *in vivo* system as well as the *in vitro* system just described. Although no intramolecular aldol condensate could be detected in the reduced tendon before extraction, one finds the reduced aldol condensation product described by Kang *et al.* (6) when soluble collagen fractions, obtained by extraction of the tendon with dilute acetic acid are reduced with NaB^3H_4. One interpretation of this observation is that the β subunits are formed as a result of the extraction procedure. Under the mild acid extraction procedure used, the cross-link corresponding to the non-reduced "post-histidine peak" is disrupted forming the aldol condensate among other products. The aldol condensate then represents the intramolecular linkage between two α chains. This aldol condensate could arise in part from the disruption of the Schiff base of hydroxylysinonorleucine as well. The question that arises is whether formation of the β chains, or more directly intramolecular crosslinks, is a necessary prerequisite to the formation of insoluble fibrils. Investigations are currently in progress to clarify this point.

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