THE NATURE OF CROSSLINKING IN COLLAGENS FROM MINERALIZED TISSUES

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SUMMARY

Following reduction with sodium borotritide, bone and dentine collagens are found to contain only a few labelled substances, in contrast with the soft tissue collagens. The reduced aldehydes, ε-hydroxynorleucine (HNL) and δ, ε-dihydroxynorleucine (DHNL) are abundant in the mineralized collagens and their relative proportions differ in fetal and mature tissues. The reduced crosslinks, δ-hydroxylysinonorleucine (HLNL) and δ, δ'-dihydroxylysinonorleucine (DHLNL) together account for a major portion of the radioactivity and their relative abundance varies with age. In contrast with the results of others, we find that DHLNL is the major reducible crosslink of mineralized collagens, comprising up to 60% of the total radioactivity. Moreover, reduction with $NaBD_4$ shows that a portion of the Schiff base precursor of DHLNL becomes reduced *in vivo*. The marked insolubility of bone and dentine collagens may, in part, be attributed to such *in vivo* reduction.

Rapid progress is being made in determining the structure of carbonyl compounds and carbonyl-derived crosslinks in various collagens. Thus, the structure of one aldol compound has been clearly demonstrated (1) and another closely related one has been postulated (2). Three examples of Schiff base crosslinks have been documented (3-7) and it is likely that more complex, polyfunctional amino acids are present, similar to those found in elastin. The relative abundance of these different compounds varies with the tissue and species of animal (8-10) and such comparative studies have led to the detection of previously undescribed substances (7). One of these substances, $\Delta^{6,7}$-dehydrodihydroxylysinonorleucine (Schiff base), is prevalent in bovine Achilles tendon and we now report that it is the major reducible crosslink in acid hydrolysates of bovine bone and dentine collagens. Furthermore, deuterium labelling studies show that *in vivo* reduction of this Schiff base is quantita-

tively important (20-25% reduced *in vivo*); such reduction could be partially responsible for the marked insolubility of bone and dentine collagens.

Finally, we have examined the relative abundance of the reducible substances in fetal and adult mineralized collagens. The results show that substantial differences in abundance occur at different stages of development.

EXPERIMENTAL

Teeth and long bones were obtained from fetal calves and from steers; they were processed as described (11, 12). The demineralized collagens from the isolated tooth dentine and from the bones were treated with a mixture of $NaBD_4$ - $NaBT_4$ which had been calibrated by the method of Paz *et al.* (13). These preparations were hydrolyzed in 3N HCl and analytical and preparative chromatography of the labelled compounds was performed as previously described (7). The relative abundance of specific compounds was established by tracing the chromatographic profile (Fig. 1 and 2) on paper of uniform thickness followed by weighing both the entire cut-out profile and selected peaks from the profile. The peak weights are expressed as percentages of the total weight.

The radioactive components which were isolated by preparative chromatography were converted to volatile derivatives which were then analyzed by mass spectrometry. Sufficient material was obtained to make the acetylated, permethylated derivative (4), the isobutyloxycarbonyl, permethylated derivative (14) and the trifluoroacetyl methyl ester (15). The spectra were recorded using a Hitachi RMU-6E machine and the content of deuterium in the appropriate ions was calculated from the recorder tracings as described (13, 16).

RESULTS AND DISCUSSION

The elution profiles of the radioactive components from dentine and bone collagens are shown in Figures 1 and 2,respectively. Comparison with similar elution profiles obtained from bovine soft tissue collagens (7) shows that

TABLE 1

DISTRIBUTION OF RADIOACTIVITY IN LABELLED COMPOUNDS

COLLAGEN	COMPOUND			
	DHNL[a]	HNL	DHLNL	HLNL
Fetal Dentine	17[b]	5	40	8
Adult Dentine	21	7	33	6
Fetal Bone	8	2	57	11
Adult Bone	10	8	15	15

[a] Abbreviations are: DHNL, Dihydroxynorleucine; HNL, Hydroxynorleucine; DHLNL, Dihydroxylysinonorleucine; HLNL, Hydroxylysinonorleucine.

[b] Expressed as the percentage of the total eluted radioactivity in Figures 1 and 2. See text for method of calculation.

the mineralized collagens contain fewer radioactive components. The major peaks are identified in the legend to Figure 1 and their relative abundance is given in Table 1. The results show: (1) dihydroxynorleucine is more plentiful than hydroxynorleucine in both bone and dentine collagens; interestingly, there is a significant increase of hydroxynorleucine in adult bone collagen; (2) the crosslinks, hydroxylysinonorleucine and dihydroxylysinorleucine together account for about half of the radioactivity and the latter compound is most abundant except in the case of adult bone collagen.

Since it has been previously reported by other workers that the predominant crosslink in mineralized collagens is an aldol (2) rather than dihydroxylysinonorleucine we isolated all of the material eluting in fractions 190-193 (Figure 1) and directly identified it. During the preparative work-up care was taken to quantitatively account for all of the radioactive material. A single substance was obtained and three different derivatives were analyzed by mass spectrometry; all were consistent with only a single compound, dihydroxylysinonorleucine (7). Thus, we disagree with Bailey *et al.* (2) as to

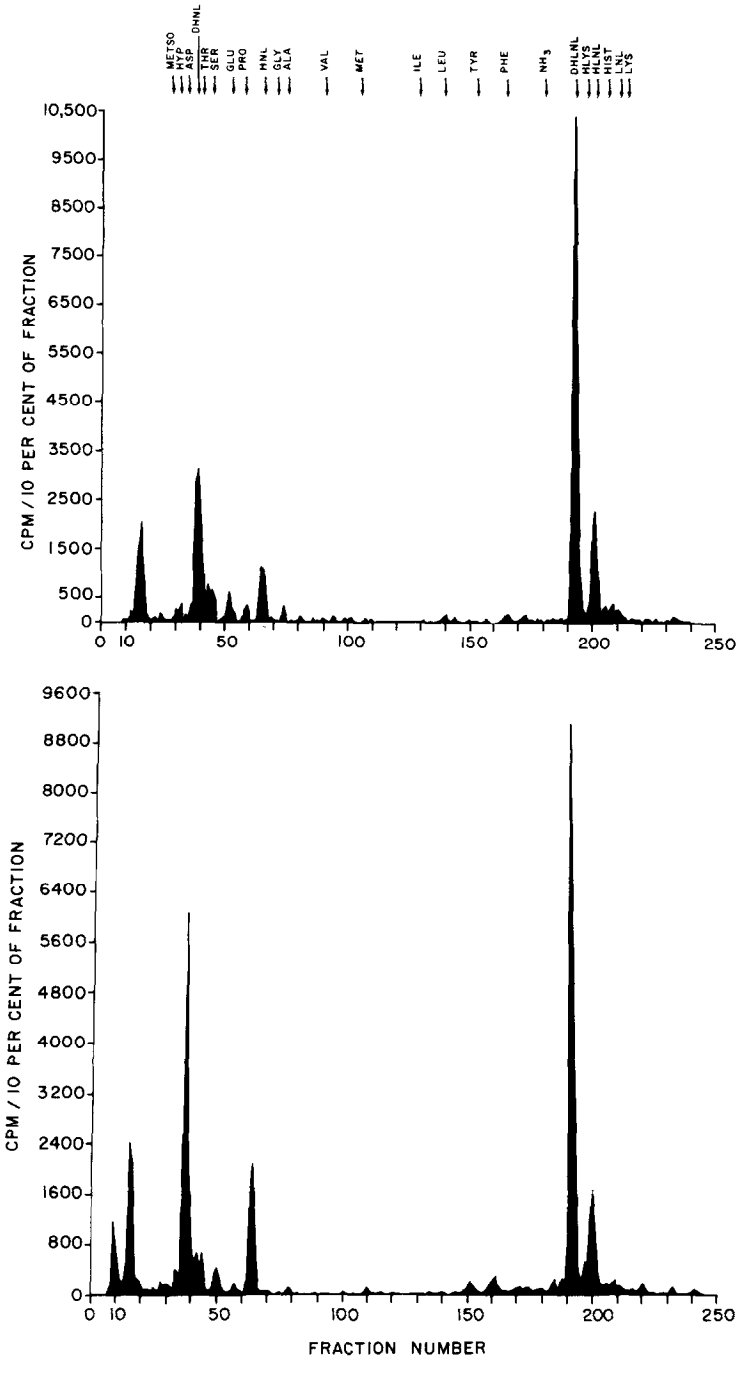

FIGURE 1. Chromatographic fractionation of acid hydrolysates of NaB^3H_4 treated dentine collagens. Upper figure is that of fetal dentine and the lower one is that of adult dentine. The order of elution is: dihydroxynorleucine, fractions 37-40; hydroxynorleucine, fractions 62-65; dihydroxylysinonorleucine, fractions 190-193; hydroxylysinonorleucine, fractions 200-203.

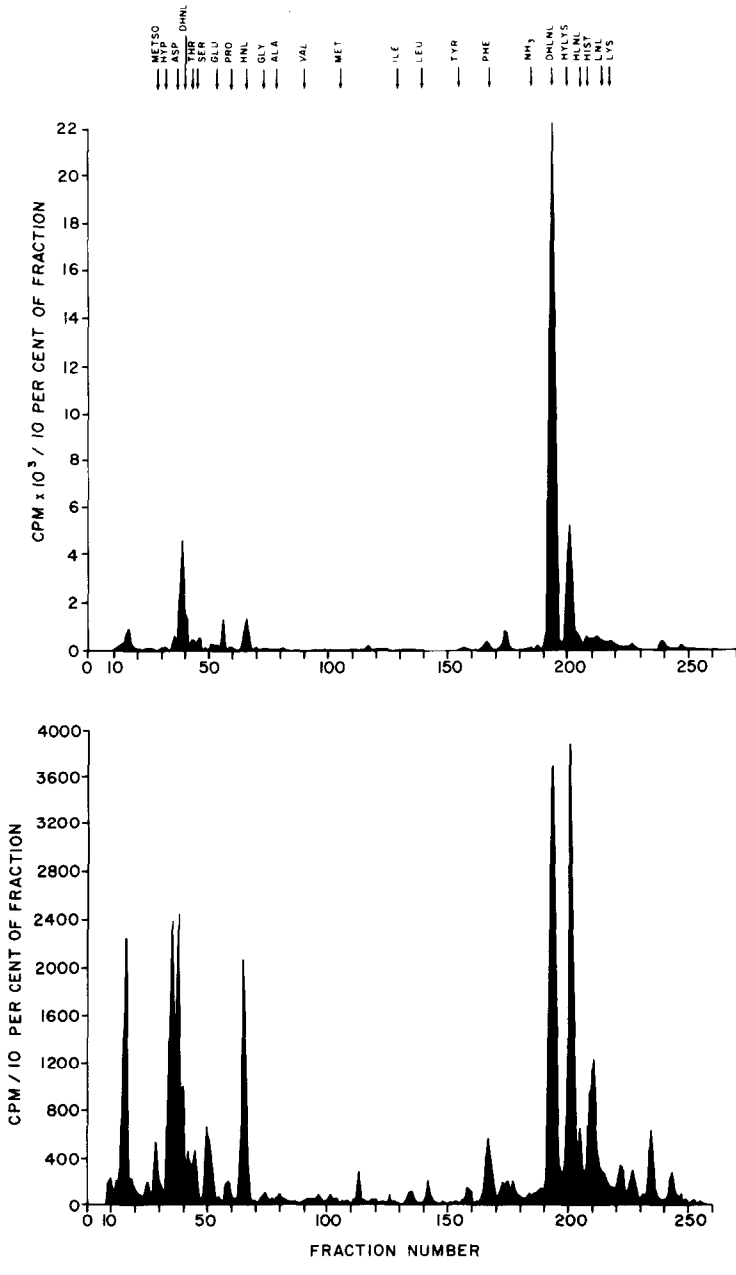

FIGURE 2. Chromatographic fractionation of acid hydrolysates of NaB^3H_4 treated bone collagens. Upper figure is that of fetal bone and the lower one is that of adult bone. Identification of peaks is in the legend to Figure 1.

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the identity of the major reducible crosslink in mineralized tissues. Conceivably, their compound co-elutes as a trace component with dihydroxyly-

sinonorleucine and, unlikely as it seems, was overlooked during our work-up alternatively, their structural identification of the compound was erroneous. We believe this alternative explanation to be the case for the following reasons; 1) the aldol condensation product derived from 2 α-amino adipic semialdehydes does not survive acid hydrolysis (1) whereas the closely related aldol condensation product reported by Bailey et al. was obtained from an acid hydrolysate (2); 2) the reported mass spectrum of the proposed aldol appears to be almost identical with the spectrum of dihydroxylysinonorleucine when the same volatile derivatives are examined (Figure 3). The important features in Figure 3 are the absent parent ion and the predominant neutral losses of one or two molecules of trifluoroacetic acid to yield secondary parents at M/e 702 and 588 respectively. Although the primary parent, M/e 816, is not seen, the highly characteristic radicals at M/e 757, 703, 477, and 478 derive from this parent compound. In similar fashion, radical losses from the secondary parents give rise to strong ions at M/e

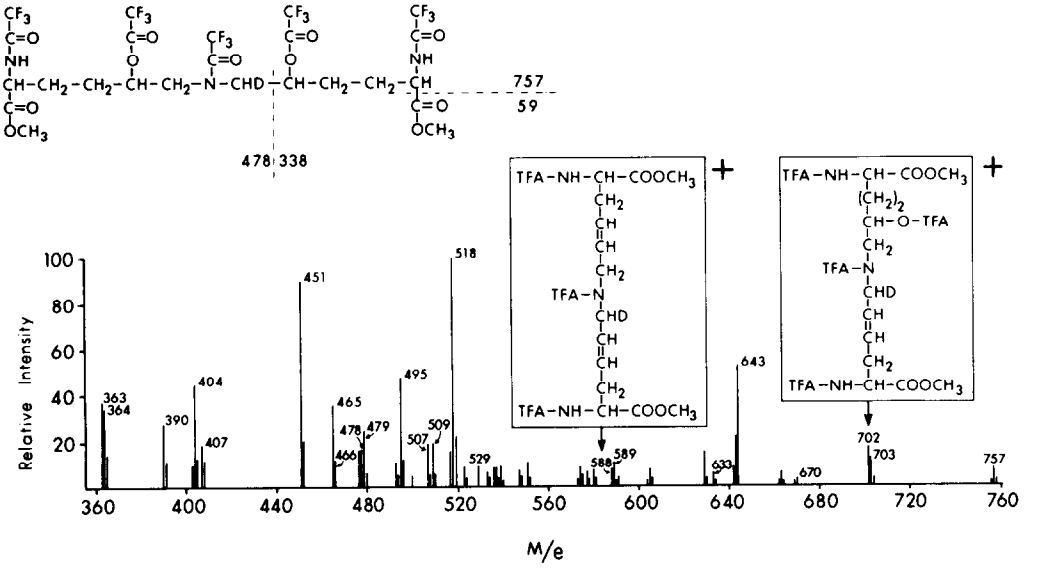

FIGURE 3. Mass spectrum of the trifluoroacetyl methyl ester of δ-hydroxylysino-δ^1-hydroxynorleucine. Note that $NaBD_4$ had been used to reduce this compound prior to derivatization.

643, 518, 465, 404, 390, 364, and 363. Other characteristic ions occur at M/e 670, 633, and 589. Another secondary parent also occurs at M/e 466 which fragments to yield M/e 451 and 407. A less complex spectrum was obtained from the permethylated isobutyloxycarbonyl derivative (Figure 4), and this spectrum complements and supports the proposed structure. Note that, with the exception of the added atomic mass unit due to deuterium, the spectrum in Figure 4 is identical with that of dihydroxylysinonorleucine (7).

The mass spectrum of the dihydroxylysinonorleucine obtained from dentine which had been reduced with $NaBD_4$ provided an estimate of the amount of the crosslink which occurs in the reduced form in vivo. Thus, the intensities of the primary parent group (M/e 692) and the secondary parent group (M/e 660) of the permethylated, isobutyloxycarbonyl derivative (Figure 4) were measured

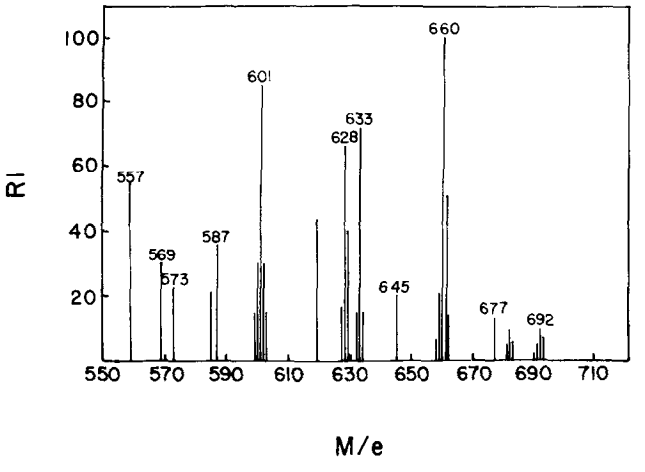

FIGURE 4. Mass spectrum of the same substance as Figure 3 except that the permethylated, isobutyloxycarbonyl derivative was employed.

and corrected for fragmentation and isotope effects (Table II). Only the data for the 660 parent group is shown because the much greater ion intensity in this region allowed more accurate measurements than for the ions of the 692 parent group. However, the results of both groups were in reasonable agreement. The calculations (Table II) showed that the ion intensity at M/e 659 was 24% of that at M/e 660. The probable source of the M/e 660 ion

TABLE II

DEUTERIUM ENRICHMENT IN THE MASS SPECTRAL IONS OF PERMETHYLATED ISOBUTYL-OXYCARBONYL DIHYDROXYLYSINONORLEUCINE

M/e	Relative Intensity, Corrected
662	18*
661	37*
660	100*
659	24†

*Corrected for isotope abundance.

†Reduction of Schiff base compounds with $NaBD_4$ in H_2O introduces one deuterium atom into the reducible molecule (13). Those molecules which contain a hydrogen atom instead of deuterium atom at the reduced locus arise from either reduction in vivo or from the small amount of $NaBH_4$ in the $NaBD_4$ reagent. The latter contribution is determined by a calibration procedure, providing a correction factor of 4% (13) which is applied to obtain the true value of the intensity at M/e 659. Another possible contribution to this ion's abundance, namely fragmentation in the mass spectrometer, was found to be negligible when non- deuterated dihydroxylysinonorleucine was examined.

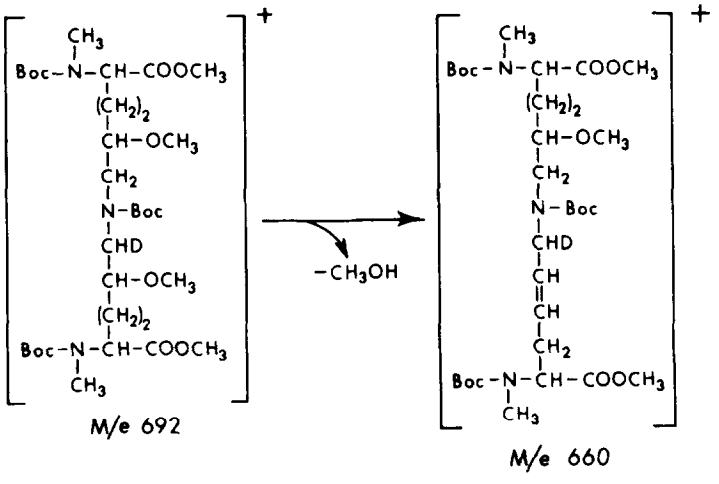

FIGURE 5. Postulated structure of the parent ion and the postulated formation of the secondary parent ion of permethylated isobutyloxycarbonyl deuterodihydroxylysinonorleucine.

is from a neutral fragment loss of methanol (Figure 5). The M/e 659 ion would correspondingly arise from a similar loss from the non-deuterated parent ion (M/e 691). Conceivably, a small portion of the 659 ion could also arise from the neutral loss of CH_3OD from 692 but the mass spectra of other volatile derivatives of deutero-dihydroxylysinonorleucine indicated that the loss of deuterium by this means was minor. Moreover, the parent fragments at M/e 692 and 691 are not subject to this uncertainty, and as noted, support the results in Table II. In addition, preparations of dihydroxylysinonorleucine and hydroxylysinonorleucine from $NaBD_4$ - reduced calf bone collagen have been examined in similar fashion to those of dentine, using the acetylated permethylated derivatives. The results indicate that 25 to 50% of these crosslinks occur in their reduced form in vivo.

Although it has been suspected that, as in the case of elastin, Schiff base reductions may occur naturally in collagen, this seems to be the first instance in which direct evidence has been found to support this concept. Conceivably, the marked insolubility of bone and dentine collagens may partially be attributed to such reduction. Interestingly, dentine collagen is solubilized by periodate oxidation (17) and it is noteworthy that the reduced crosslinks, hydroxylysinonorleucine and dihydroxylysinonorleucine, are rapidly cleaved by this reagent (5, 7).

In summary, we note that the experimental techniques of mild chemical reduction and mass spectrometry provide two powerful, albeit simple, tools for investigating a number of important problems in connective tissue biochemistry. The present work illustrates the analytical strengths and one of the potential weaknesses of the methods if sufficient caution is not exercised. Other limitations of the methods have also been clearly documented (13); recognition of such potential pitfalls strengthens the credibility of the data and aids in its interpretion.

We conclude, from the present study and from our earlier studies, that there is marked biologic variation of the reducible compounds in the collagens

from animals of different ages and from different tissues. Furthermore, it seems likely that a mechanism may exist for *in vivo* reduction of at least one of the Schiff base crosslinks.

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