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Studies in Calcification*

THE REACTION OF SOME HARD- AND SOFT-TISSUE COLLAGENS WITH 1-FLUORO-2,4-DINITROBENZENE

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It has long been considered that those factors which determine whether mesodermal connective tissue will calcify or not are present in the organic fraction of the tissue, and much attention has been directed to the function of mucopolysaccharides as a local factor in mineralization (Sobel, 1955). However, little work has been done on the properties of the protein fraction of the organic matrix and its relation to calcification. This paper describes some of the chemical and structural properties of collagen in both hard and soft mammalian connective tissues, found by the use of Sanger's (1945) fluorodinitrobenzene method, and shows in which respects the reaction of the hard-tissue collagens with fluorodinitrobenzene differ from those of the soft-tissue collagens. These differences may well be significant in understanding why soft-tissue collagen does not calcify, whereas hard-tissue collagen does so despite the almost identical amino acid composition (Bowes, Elliott & Moss, 1955; Eastoe, 1955), appearance under the electron microscope and X-ray-diffraction pictures of both proteins (Bear, 1952).

Samples of collagen obtained from ox achilles tendon, ox hide, ox bone, ox dentine, rat-tail tendon and human dentine have been studied. The collagen was prepared with a minimum of chemical treatment, as it was thought that the small amounts of reticular and elastic fibres that might be present would lead to less error than the drastic treatments which are necessary for their complete removal. In most of the experiments demineralization of bone and dentine for structural studies with

fluorodinitrobenzene was effected by shaking with ethylenediaminetetra-acetic acid at pH 8.0, but in some experiments demineralization was also carried out with hydrochloric acid or trichloroacetic acid, although it was recognized that treatment with acid may damage the protein. In some experiments the tissue was treated with ethylenediaminetetra-acetic acid and fluorodinitrobenzene simultaneously, thus avoiding any time lag between demineralization and subsequent dinitrophenylation.

Preliminary accounts of some of this work have already been published (Solomons & Irving, 1955, 1956*a, b*).

MATERIALS

Ox-hide collagen. Hide from the thigh region of a freshly killed ox was washed first with water to remove blood, etc., then left to stand overnight in 0.9% NaCl soln. at 4° and then re-washed with water. The grain layer, which contains the greater part of the muscle and elastic tissue as well as hair roots, was split off and the remaining material, excluding adipose tissue, was cut into approx. 0.5 cm. cubes. The cubes were defatted with three changes of light petroleum (b.p. 40–60°).

Ox achilles tendon and rat-tail tendon. These were cut into thin strips, washed in 0.9% NaCl soln. and defatted with light petroleum.

Ox bone. The diaphysis of an ox tibia, soaked in 0.9% NaCl soln. overnight and then washed with water, was cut into sections about 1 in. thick with a hacksaw. These sections were then split into approx. 0.25 cm. cubes by means of root-splitting dental forceps. Care was taken here and in the preparation of the dentine to minimize the generation of heat, as this can lead to denaturation as well as solution of some of the protein fraction.

Human dentine. Approx. 0.25 cm. cubes of human root dentine from healthy incisor and canine teeth were

* Some of the results quoted in this paper were taken from a thesis presented by C. C. S. for the degree of Ph.D. of the University of the Witwatersrand.

obtained with root-splitting forceps. The pulp material was removed and the dentine soaked overnight in 0.9% NaCl soln.

Ox dentine. This was similarly prepared from ox incisor teeth.

METHODS

Demineralization of tissues. This was carried out in ethylenediaminetetra-acetic acid (EDTA) soln. (0.5M) adjusted to pH 8.0, N-HCl or N-trichloroacetic acid. Samples (0.2 g.) of tissue were placed in 15 ml. of the decalcifying agent maintained at 4°. At regular intervals pieces of tissue were removed, allowed to dry in air, and reweighed in order to calculate the percentage demineralization. The moisture content was determined on a separate sample by drying at 105° for 16 hr. This method was shown to be valid by estimations of calcium and phosphorus on the supernatant fluid. In order to ensure true comparisons, the soft tissues were similarly treated with demineralizing agents before dinitrophenylation.

Dinitrophenylation of tissues. The technique was as follows (Sanger, 1945): samples (0.2 g.) of tissue were treated with a mixture of 0.1 g. of NaHCO₃ and 0.5 g. of fluorodinitrobenzene (FDNB) in 15 ml. of 66% (v/v) ethanol for 36 hr. at room temperature. Prolonging the period of dinitrophenylation to 3 weeks had no effect on the amounts of dinitrophenyl (DNP) amino acids recovered. The DNP proteins were then washed with water and shaken with changes of acetone until no more yellow-coloured substances could be extracted. The DNP amino acids were liberated by hydrolysing the DNP protein in a sealed tube at 105° with 20 ml. of constant-boiling HCl which had been redistilled over SnCl₄. A hydrolysis time of 16 hr. was used since it was found that this time resulted in complete hydrolysis of the protein with minimal destruction of ϵ -DNP amino acids.

By repeated extraction of the acid hydrolysate with 5 ml. portions of peroxide-free ether, the ether-soluble α -DNP amino acids were separated from the water-soluble ϵ -DNP amino acids which remained in the acid aqueous phase. The α -DNP amino acids were separated on paper chromatograms by developing with *tert.*-amyl alcohol saturated with 0.2M-potassium hydrogen phthalate buffer, pH 6.0 (Blackburn & Lowther, 1951). The spots thus obtained were eluted with 1% NaHCO₃ soln. and the optical density of the solutions was measured at 360 m μ with the Beckman spectrophotometer model B. It was found that the absorption maximum of ϵ -DNP lysine and hydroxylysine in N-HCl occurs at 360 m μ . The α -DNP amino acids (with the exception of α -DNP arginine and the DNP prolines) absorb maximally at 355 m μ in 1% NaHCO₃ soln. In this work both α - and ϵ -DNP amino acids were colorimetrically determined at 360 m μ and their concentrations obtained from a standard calibration curve. Interference from *O*-DNP tyrosine was negligible at this wavelength. The total concentrations of ϵ -DNP lysine and hydroxylysine in the acidic watery layer were similarly determined by recording the total optical density at 360 m μ . A portion of this solution was placed on a paper chromatogram and developed with butanol-acetic acid-water (4:1:5). The spots corresponding to ϵ -DNP lysine and ϵ -DNP hydroxylysine were discrete and their *R_F* values were 0.59 and 0.48 respectively. The spots were eluted separately with N-HCl and their optical densities measured at 360 m μ . α -DNP

arginine, which would remain in the acid aqueous layer, was not detected.

Recovery of dinitrophenyl amino acids. Correction factors for the destruction of DNP amino acids during hydrolysis and subsequent chromatographic procedures were obtained by treating known quantities of pure DNP amino acids for various lengths of time with 6N-HCl at 105° in the presence of protein, and measuring the amounts remaining after chromatographic separation. After hydrolysis for 2, 8, 16 and 24 hr. the recoveries of both ϵ -DNP lysine and ϵ -DNP hydroxylysine were 98, 90, 80 and 75% respectively. The recoveries of α -DNP aspartic and glutamic acids after hydrolysis for 16 hr. were both 64%. Free lysine and hydroxylysine in hydrolysates of protein or DNP protein were determined by making use of the fact that copper ions will protect the α - but not the ϵ -amino groups of dibasic amino acids from reacting with FDNB (Porter, 1950). A measured volume of the hydrolysate was neutralized with excess of solid NaHCO₃. The solution was brought to the boil and CuCO₃ powder was added until no more dissolved. The solution was boiled for 2 min., filtered and the filtrate treated with FDNB (0.1 g.) for 24 hr. The mixture was then acidified and extracted with ether. The optical density of the aqueous layer, containing ϵ -DNP lysine and ϵ -DNP hydroxylysine, was measured at 360 m μ and the concentrations were obtained from a calibration curve. Care was taken to keep acidic solutions of the α -DNP amino acids in the dark, as they decompose in ultraviolet light. The ϵ -DNP amino acids were found to be relatively stable to light.

Combined demineralization and dinitrophenylation of hard tissues. Demineralization of hard tissues by means of EDTA and simultaneous dinitrophenylation of the protein fraction with FDNB were both carried out in one operation.

Samples (0.2 g.) of hard tissue were treated with 15 ml. of 0.5M-EDTA (pH 8.0) containing 0.1 g. of NaHCO₃, to which was added 0.5 g. of FDNB dissolved in 5 ml. of ethanol. This solution was renewed daily for 5 days, after which demineralization was complete. The subsequent procedures are described above.

RESULTS

Demineralizing agents, under the conditions used, did not cause the liberation of free amino acids from any of the tissues studied, as judged by treating the supernatant solution with ninhydrin or FDNB.

Human dentine. The first experiments were carried out on human dentine, dinitrophenylation following the various stages of decalcification. In fully calcified dentine, no free N-terminal groups were detected. However, during demineralizing small amounts of aspartic and glutamic acids were detected and these increased in a linear manner to a maximum of 10 μ moles/g. of protein, when related to the percentage demineralization (Table 1). Hydrolytic decomposition products of DNP proline were not detected (Scanes & Tozer, 1956) and no DNP glycine was found after hydrolysis for 4 hr.

Fully calcified dentine possessed small amounts of ϵ -amino groups, about 25–35 μ moles/g. of protein,

i.e. about 9% of the total concentration. As demineralization progressed, the number of ϵ -amino groups available to FDNB increased in a linear manner to 278 μ moles/g. of protein, when plotted against the percentage decalcification (Table 1 and Fig. 1). Thus in the fully demineralized state, over 96% of the ϵ -amino groups reacted with FDNB, accepting the figure for total lysine and hydroxylysine obtained by Hess & Lee, 1954. Similar results were obtained with all three of the demineralizing agents employed.

Ox bone. Fully calcified ox bone had extremely small amounts of terminal aspartic acid, about 1–2 μ moles/g. of protein, and these remained constant during demineralization. Fully calcified ox bone had 35 μ moles of ϵ -amino groups/g. of protein. When decalcification was followed by dinitrophenylation, the number of ϵ -amino groups available to FDNB at complete decalcification varied very considerably from sample to sample, ranging from 55 to 100% of the total number present. It seemed possible that the procedure of simultaneous decalcification and dinitrophenylation would yield more consistent results, as the reaction of the ϵ -amino groups would follow immediately on decalcification. When this was done, the availability of ϵ -amino groups to FDNB increased in a linear manner to 330 μ moles/g. of protein, when plotted against the percentage demineralization (Fig. 1). Thus in the fully demineralized state, over 93% of the ϵ -amino groups reacted with FDNB, by accepting the value for the total lysine and hydroxylysine content of bone obtained by Eastoe (1955).

Table 1. α - and ϵ -amino groups of human dentine collagen

Liberated groups were estimated by reaction with FDNB during decalcification with EDTA at room temperature (pH 8.0).

Percentage demineralization	Groups (μ moles/g. of protein)
α -Amino groups	
0	0.0
20.0	2.1
38.8	4.0
55.0	5.8
75.0	7.7
100.0	10.2
ϵ -Amino groups*	
0	28
18.8	60
32.6	100
45.0	135
57.0	165
75.0	210
90.1	245
100.0	278

* Total content = 289 μ moles/g. (Hess & Lee, 1954).

In view of these findings, simultaneous decalcification and dinitrophenylation were carried out later on human dentine, but the results with this procedure were the same as those obtained when the processes were successive.

Ox dentine. The results obtained were the same if decalcification and dinitrophenylation were simultaneous or successive. No free *N*-terminal amino acids were detected in the calcified tissue, but after demineralization 2–4 μ moles of terminal aspartic acid/g. of protein were found. Fully calcified ox dentine had about 25–30 μ moles of ϵ -amino groups/g. of protein, and as with human dentine these rose linearly during demineralization to a maximum of 340 μ moles/g. of protein when decalcification was complete (Fig. 1).

Soft tissues. No *N*-terminal amino acids were detected in ox-hide collagen, ox achilles tendon or rat-tail tendon in the native state, and no *N*-terminal amino groups were liberated when these tissues were treated with demineralizing solutions under the same conditions as were used to decalcify the hard tissues.

Between 64 and 70% of the total number of lysyl and hydroxylysyl ϵ -amino groups present in these soft tissues were recovered as their DNP

Fig. 1. Availability of ϵ -lysyl and hydroxylysyl amino groups of hard-tissue collagens to FDNB during demineralization. $\circ$, $\bullet$, Denote successive demineralization and dinitrophenylation of ox dentine and human dentine respectively; +, $\square$, $\triangle$, denote simultaneous demineralization and dinitrophenylation of ox dentine, ox bone and human dentine respectively.

Table 2. *Free ϵ -amino groups of soft-tissue collagens*

Tissue	Total content (μ moles/g. of protein)	Percentage of total content reacting with FDNB		
		Mean	S.E.	No. of determinations
Ox hide	341*	65.0	± 1.2	20
Ox achilles tendon	341†	67.0	± 1.5	10
Rat-tail tendon	340†	68.0	± 1.3	12

* Bowes, Elliott & Moss (1955).

† Results obtained by the present writers with the copper method (see text).

derivatives after treatment with FDNB (Table 2). The percentage of ϵ -amino groups available to FDNB was not changed either by increasing the pH to 10 during coupling with FDNB or by performing the reaction in aqueous medium. As has been reported by other investigators, modification of ox-hide collagen by hydrothermal shrinkage, or treatment with urea or acetic acid or alkali, did not change the availability of ϵ -amino groups.

The remaining lysyl and hydroxylysyl residues reacted quantitatively with FDNB only after hydrolysis of the DNP protein. In this way it was possible to account for over 95 % of the lysine and hydroxylysine originally present in each protein. Treatment with demineralizing agents did not affect these results.

DISCUSSION

The most significant findings from these investigations are that the collagens of soft and demineralized hard tissues can be readily distinguished from each other by the reactivity of their ϵ -lysyl and hydroxylysyl amino groups with FDNB. That a linear relationship exists between the release of these groups and the degree of demineralization (with several different decalcifying agents) strongly suggests that there is a fundamental connexion between free ϵ -amino groups of collagen and calcification. The lower reactivity of the soft-tissue collagens is probably not due to steric factors, as swelling the protein with urea solutions had no effect on the yield of DNP amino acids. Bowes & Moss (1953) treated ox-hide collagen with FDNB and observed the low yield of DNP lysine. They postulated that this might be due to ϵ -DNP lysine being less stable to acid when combined in the collagen than when present as the free amino acid. On hydrolysis of the DNP protein they recovered only small amounts of free lysine; this result is at variance with those of the present authors, who were able to account for almost all the lysine and hydroxylysine originally present in the protein. It appears that ϵ -lysyl groups may take part in peptide cross-links, since Mechanic (1957) has recently isolated an ϵ -lysyl tripeptide from a partial acid hydrolysate of ox achilles tendon. This

possibly explains the failure of some of the ϵ -amino groups to react with FDNB.

The absence of *N*-terminal amino groups in all the soft-tissue collagens studied is in agreement with the results obtained by others on ox-hide collagen (Bowes & Moss, 1953). This finding indicates that the molecule either is extremely large or is cyclic or that the terminal amino groups may be masked in some way. The amounts of *N*-terminal amino groups present in ox bone and ox dentine are so small that it is difficult to attach any significance to them. Demineralized human dentine contains amounts which, though not large, are appreciable. Only in human dentine was there any correlation between the concentration of free terminal amino groups and the degree of demineralization. Too much significance should not, however, be attached to the absolute values for the concentrations of *N*-terminal residues in human dentine until more is known about the nature of the polypeptide chains that form dentine collagen. It is possible that human dentine may have a chain length smaller than that of bovine dentine or bone.

It is not unlikely that dentine and bone collagens differ in some details of their intimate structure since consistent values for the full availability of the ϵ -amino groups to FDNB could only be obtained with bone if demineralization and dinitrophenylation were carried out simultaneously.

In previous studies of calcification, attention has been directed more to the role that mucopolysaccharides play in this process (Sobel, 1955). Such work has been done almost entirely with rachitic hypertrophic cartilage in which the content of chondroitin sulphate is high. Bélanger (1954) has found that ^{35}S is incorporated into bone and dentine during calcification, and has interpreted his results to indicate that chondroitin sulphate is also concerned with calcification in these tissues, though their content of mucopolysaccharide is very low. Whereas it is possible that such substances as chondroitin sulphate are concerned with calcification, the present work indicates that the nature of collagen is also an important factor. Since calcification is a process of crystallization rather than precipitation (Neuman & Neuman, 1953), hard-tissue collagens, in which all the ϵ -lysyl and

hydroxylslyl amino groups are free to react with FDNB, may act as a suitable 'seeding template' for the growth of hydroxyapatite crystals. The fact that apatite crystals are deposited on collagen fibres predominantly at the cross-banded regions (Robinson & Watson, 1955), where it is thought that the content of basic and acidic amino acids is high, supports this concept.

SUMMARY

1. A variety of hard and soft connective tissues of bovine and human origin were treated with fluorodinitrobenzene before, during and after treatment with demineralizing agents.

2. It was found that the collagens of soft and demineralized hard tissues could readily be distinguished from each other on the basis of the reactivity of their ϵ -lysyl and hydroxylslyl amino groups with fluorodinitrobenzene. In intact soft-tissue collagen only about two-thirds of these ϵ -amino groups reacted with fluorodinitrobenzene, but the concentration of reactive ϵ -amino groups of hard-tissue collagens increased from very low levels almost to the theoretical value at complete decalcification.

3. The significance of these findings is discussed in relation to calcification *in vivo*.

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Studies in Carotenogenesis

24*. THE CHANGES IN CAROTENOID AND CHLOROPHYLL PIGMENTS IN THE LEAVES OF DECIDUOUS TREES DURING AUTUMN NECROSIS

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It has long been stated that the change in colour of the leaves of deciduous trees in early autumn is due to the preferential destruction of the green chlorophylls, which unmasks the much more slowly disappearing yellow carotenoids. Early work indicated that the carotenoids also undergo qualitative as well as quantitative changes. From 70 to 80 % of the total carotenoids in all green leaves are unesterified xanthophylls (Goodwin, 1952), which are hypophasic when partitioned between light petroleum and 90 % (v/v) aqueous methanol (Goodwin, 1955). Tswett (1911) found that the carotenoids from autumn leaves were almost entirely epiphasic, but that the major fraction could be separated from β -carotene; he named these pigments 'autumn xanthophylls'; Palmer (1922), considering their partition properties,

suggested the name 'autumn carotenes'. Kuhn & Brockmann (1932) found that these autumn pigments were not hydrocarbons but esterified xanthophylls, which behaved like carotenes in the partition test. Karrer & Walker (1934), however, considered that they were unidentified oxidation and degradation products of lutein. On the other hand, Strain (1938) stated that the major pigment in yellow-shed leaves was zeaxanthin (3:3'-dihydroxy- β -carotene). Kuhn & Brockmann (1932) agreed with Willstätter & Stoll (1918) that the total amount of carotenoids did not alter in autumn leaves; however, Karrer & Walker (1934) and Nagel (1939) found that both the carotenes and xanthophylls disappeared, but that the carotenes were destroyed more quickly than xanthophylls. The carotenoids are reported to undergo these same autumnal changes in leaves of tropical evergreens (Egle, 1944). The *aurea* varieties of *Acer*

* Part 23: Goodwin, Land & Sissins (1956).