A Comparison of Bone Matrix and Tendon with Particular Reference to Glycoprotein Content

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Bone matrix and tendon are compared in terms of their carbohydrate and non-collagenous protein composition. The collagen content of both tissues was similar (90–91%), but bone matrix had at least three times as much sialic acid (0.28%) as tendon (0.08%). Smaller differences were found in the analysis of hexoses and hexosamines. After digestion with bacterial collagenase, about 9% of the total protein from both tissues was non-diffusible on dialysis, and this contained only 0.15% (bone) and 0.7% (tendon) of the original hydroxyproline; recovery of sialic acid was 86–87%. The collagenase-resistant soluble material amounted to about 9% (bone matrix) and 5% (tendon); the insoluble residues were 1 and 4% respectively. There were clear differences in the carbohydrate contents of the digests, but the amino acid compositions were similar. When the soluble digests were chromatographed on DEAE-cellulose, the elution profiles indicated the presence in each tissue of a variety of glycoproteins and a proteoglycan fraction, and showed clearly that an acidic glycoprotein corresponding to bone sialoprotein was not present in tendon.

Glycoproteins make up a considerable proportion of the non-collagenous material present in bone organic matrix, and interest has centred on their ion-binding properties and possible role in mineralization processes (Herring, 1972). The most acidic of these is bone sialoprotein, which has been studied in some detail (Andrews *et al.*, 1967, 1969; Peacocke & Williams, 1967; Williams & Peacocke, 1965, 1967). Earlier studies showed that it did not resemble any of the known plasma proteins (Herring, 1964), but the possibility remained that it might be present in other connective tissues and have no specific function in bone. It was therefore decided to compare the glycoproteins of bone matrix with those of another connective tissue which also has collagen as its main structural component. Tendon was chosen because of its superficial chemical similarity to bone matrix: both contain about 90% by weight of collagen (type I) and about 1% of glycosaminoglycans; both contain serum proteins, particularly albumin, and also glycoproteins that are synthesized in the tissue (Ashton *et al.*, 1974; Owen *et al.*, 1973; Triffitt & Owen, 1973; Anderson & Jackson, 1972; Anderson, 1975). The purpose of this study was to look for differences in the composition of the two tissues, particularly with respect to the non-collagenous components and bound carbohydrates and, more specifically, to see whether or not the well-defined bone sialoprotein could also be found in tendon. Some of

these results have been published in a preliminary form (Herring, 1969).

Materials and Methods

Materials

Clostridium histolyticum collagenase (EC 3.4.24.3) 'collagenase type III, fraction A', bovine serum albumin (crystallized), *N*-acetylneuraminic acid, galactose, glucosamine hydrochloride, glucuronolactone and hydroxyproline were obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K., and DEAE-cellulose (DE-32) was from Whatman Biochemicals, Maidstone, Kent, U.K. All other reagents were analytical grade, where available, from BDH Chemicals, Poole, Dorset, U.K.

Bovine cortical bone, obtained from the long bones of mature animals, was prepared and powdered at low temperature as described previously (Herring *et al.*, 1974). Metatarsal flexor tendons were obtained from similar cattle and were dissected free of synovial sheath and other associated connective tissue. After a brief rinse in ice-cold 0.15M-NaCl, the tendon was frozen and the outer layers (about 0.5 mm thick) were scraped off with a scalpel. The tissue was cut into thin slices perpendicular to the direction of the fibres, and the slices were then chopped into small fragments.

The bone powder and chopped tendon were washed with ethanol and diethyl ether and left to equilibrate with the air at room temperature (21°C) before weighing of samples for moisture determination and subsequent experiments. When not in use the tissues were stored at -20°C.

Preparation of bone organic matrix

Samples (10g) of bone powder were placed in dialysis tubes (25cm×1.5cm) with about 20ml of 2.5M-NaCl. These were suspended in EDTA solution (100g of EDTA and 40.2g of NaOH in 1 litre, adjusted to pH 7.2), which was stirred magnetically. Decalcification was continued at 4°C for 7 days. Ash determinations on washed portions from a test sample showed that less than 1% of the mineral remained after 5 days. The EDTA was then removed by dialysis against water; initially only small volumes of water (about 20ml per dialysis tube) were used to avoid any sharp increase of pressure inside the tubes. Some of the dialysed samples were used to obtain an estimate of the total organic material present in bone and for the analyses comparing this tissue with tendon. After exhaustive dialysis, the contents of the dialysis tube, with washings, were placed in a weighing bottle and dried to constant weight in a desiccator *in vacuo* at 4°C. This material is subsequently referred to as organic matrix or matrix; it represents the total non-diffusible organic material of bone and includes both the soluble and insoluble macromolecules under these conditions.

Collagenase digestion

Dialysis tubes containing decalcified samples of bone powder (approx. 2g of organic matrix) prepared as described above and samples of chopped tendon (approx. 2g), which had been treated in the same way, were further dialysed against a solution (pH 7.4) containing 0.025M-Tris/HCl buffer (pH 7.2) and 0.3M-calcium acetate with 0.001% of phenylmercuric acetate as a bacteriostatic agent. Dialysis was continued with four changes of buffer (200ml per tube) for a total of 48 h. The contents (35–40ml, with washings) of the dialysis tubes were transferred to 100ml flasks. Collagenase (1.5mg, about 750 units as defined in the Sigma catalogue) was added to each flask, which was incubated at 37°C for 96h with gentle shaking. Further portions of collagenase were added after 24h (0.5mg), 48h (0.9mg) and 72h (1.0mg). The digests were cooled to 0°C and centrifuged at 2000g. The clear supernatant solutions were removed and the precipitates washed with 2×5ml of water. The pooled supernatant and washings from each digest were adjusted to a volume of 50ml in a standard flask. Samples of this solution were dialysed as described below to remove the peptides produced by proteo-

lysis of the collagen. The non-diffusible material that remained will be referred to as the soluble collagenase-resistant fraction. The precipitates (insoluble collagenase-resistant fraction) were dried in a desiccator at room temperature.

Analytical methods

Samples of bone matrix and tendon were analysed for moisture content by drying to constant weight at 105°C, and for ash by dry incineration.

The total soluble organic material remaining after digestion and dialysis was determined by dialysing a sample of the soluble collagenase-resistant fraction (5ml) against 0.2M-NaCl (four changes of 1 litre over 2 days), followed by dialysis against water until Cl⁻ ions could not be detected. The contents of the dialysis tube, with washings, were transferred to a weighing bottle and dried to constant weight *in vacuo* at 4°C. Control digests containing collagenase without tissue were treated identically to give blank values.

Samples of soluble digests dialysed in the same way were used for the determination of total protein. The dialysis residue was transferred to a 10ml standard flask and the pH adjusted to about 8.5 with NaOH to ensure that the protein was completely dissolved. A sample (1ml) of this solution was hydrolysed with 1ml of conc. HCl in a sealed, evacuated tube for 48h at 100°C. The hydrolysates were evaporated to dryness *in vacuo* over NaOH pellets, and dissolved in standard volumes of 0.2M-sodium citrate buffer, pH 5.0 (50ml for bone digest; 20ml for tendon digest), for determination of total amino acids as described by Eastoe & Courts (1963). Samples of bovine serum albumin treated in the same way were used as standards.

Hydroxyproline was determined in samples of bone matrix and tendon, and their soluble and insoluble collagenase-resistant fraction. Hydrolysis was in 5.7M-HCl for 24h at 100°C in sealed tubes, by using 5mg of tissue or insoluble collagenase-resistant fraction and 1ml of soluble collagenase-resistant fraction. After evaporation to dryness, the hydrolysates were dissolved in 100ml (tissues), 10ml (insoluble collagenase-resistant fraction) or 4ml (soluble collagenase-resistant fraction) of water. Samples (1ml) were analysed by the method of Hutterer & Singer (1960).

For the determination of sialic acid, 40–50mg of bone matrix and 80–100mg of tendon were hydrolysed in stoppered centrifuge tubes with 2.5ml of 0.05M-H₂SO₄ for 1h at 80°C, the contents of the tubes being swirled gently at intervals. The hydrolysates were centrifuged at 2000g and 0.5ml was taken for each determination by the method of Aminoff (1961), with *N*-acetylneuraminic acid as the standard. Samples (5ml) of soluble collagenase-resistant fraction were dialysed exhaustively against 0.05M-Tris/

HCl buffer (pH 7.2) and made up to a volume of 10 ml; samples (0.5 ml) of these solutions, and 1 mg samples of insoluble collagenase-resistant fraction in 0.5 ml of the Tris buffer, were used for analysis after the addition of 0.1 ml of 0.3 M-H₂SO₄ and hydrolysis at 80°C for 1 h.

The direct Ehrlich reaction was carried out on 10–20 mg of tissue and 1 ml of the soluble collagenase-resistant fraction by the method of Werner & Odin (1952). Care was taken to mix the samples during heating and to centrifuge them before taking spectrophotometric readings.

Determinations of hexoses and hexosamines, after acid hydrolysis at 100°C with 2 M-HCl for 3 h and 4 M-HCl for 16 h respectively, were not satisfactory, because of the small amounts of these sugars present and interference from the other constituents. Much more reproducible values were obtained when the acid ion-exchange-resin hydrolysis procedure of Grant & Jackson (1968) was used. This was scaled down for use with 20 mg of tissue or 1 ml of the soluble collagenase-resistant fraction which had been dialysed against water. Hexose was determined by an anthrone procedure (Dinker *et al.*, 1952), and hexosamines by the method of Gatt & Berman (1966) directly on the 2 M-HCl eluates. Methylpentose was not determined separately and would contribute to the hexose values in the anthrone reaction. However, relatively small amounts of fucose have been found in bone and tendon glycoproteins.

Amino acids were determined with a Locarte amino acid analyser on samples of soluble and insoluble collagenase-resistant fraction after hydrolysis in 5.7 M-HCl at 110°C for 16 h in evacuated tubes. Half-cystine was estimated from the cystine peak in the amino acid elution pattern.

DEAE-cellulose chromatography

The DEAE-cellulose (DE-32) was prepared according to the manufacturer's instructions and was equilibrated with 0.05 M-Tris/HCl buffer (pH 7.2). A column (1 cm × 2.5 cm) was made and washed with 50 ml of the same buffer. A sample (5 ml) of bone or tendon digest, which had been dialysed thoroughly as described under 'Analytical methods', was applied to the column and the effluent was collected in 4 ml fractions. The column was washed with buffer (25–30 ml), followed by elution with a linear gradient of 0–0.7 M-NaCl in the starting buffer. The total gradient volume was 200 ml and a flow rate of about 20 ml/h was used. The E_{280} of each fraction was measured as an indication of protein content, and samples were taken for determination of uronic acid by an orcinol method (Brown, 1946) and of sialic acid by the direct Ehrlich reaction (Werner & Odin, 1952) and by a thio-barbituric acid procedure (Aminoff, 1961). To measure the NaCl concentration in the gradient where the

sialic acid peak corresponding to bone sialoprotein was eluted, Cl⁻ concentrations in fractions in this region were determined by the method of Hamilton (1966).

Electrophoresis

The fractions corresponding to the bone sialoprotein peak shown on the DEAE-cellulose elution pattern of bone digest (Fig. 1, peak 4), and the fractions from the same region when the tendon digest was used (Fig. 1), were pooled and concentrated by dialysis against polyethylene glycol (Carbowax 20M; R. A. Lamb, 6 Sunbeam Road, North Acton, London NW10 6JL, U.K.). The concentrate (1–2 ml) was dialysed against 0.2 M-potassium acetate and then transferred to a 15 ml centrifuge tube with the washings of 0.2 M-potassium acetate to give a volume of not more than 2.5 ml. The dialysis tube was rinsed with ethanol which was added to the concentrate so that the final volume was 15 ml. The mixture was left at 5°C for 24 h. The precipitate which formed was collected by centrifugation (2000g for 30 min), washed with ethanol and ether and left to dry in a desiccator. The samples were dissolved in 100 µl of electrophoresis buffer and portions (about 5 µl) were applied to cellulose acetate strips for electrophoresis. This was carried out in a Shandon Universal electrophoresis apparatus in 0.1 M-sodium barbitone/barbitone buffer (pH 8.6). Four strips (18 cm × 5 cm) were mounted in the apparatus with a bridge width of 12 cm, and a satisfactory separation of the components was obtained after 2–4 h at 50 V and 3.5 mA. The strips were dried at room temperature for 30 min and stained with Alcian Blue [1% (w/v) in 1% (v/v) acetic acid] for 10 min, followed by washing with water, to demonstrate the presence of bone sialoprotein. Ponceau S (Korotser *et al.*, 1961) and Nigrosin (Kohn, 1958) were used to detect proteins.

Results and Discussion

Solubilization procedure

The 'purified' bacterial collagenase which is available commercially usually contains non-specific proteolytic activity (Porter *et al.*, 1971; Benya *et al.*, 1973; Lee-Own & Anderson, 1975). In the studies reported here, no attempt was made to purify the collagenase further, or to use inhibitors against contaminating proteinases (Porter *et al.*, 1971; Benya *et al.*, 1973; Peterkofsky & Diegelmann, 1971). Endogenous proteinases might also be present in the tissues and still be active during collagenase digestion. Therefore the possibility that some degradation of the non-collagenous proteins took place cannot be excluded. However, it was hoped to minimize non-specific proteolysis by using a low enzyme/

Table 1. *Chemical analysis of whole tissues, and the soluble and insoluble collagenase-resistant fractions from bone matrix and tendon*

Details are given in the text. For whole tissues, the results are mean values $\pm$ S.E.M. for the numbers of samples in parentheses. For collagenase-resistant fractions, the results are mean values $\pm$ range for two samples of each tissue. n.d., Not determined.

Composition (g/100g dry wt. of tissue) in								
Bone matrix					Tendon			
	Total	Soluble collagenase-resistant fraction	Insoluble collagenase-resistant fraction		Total	Soluble collagenase-resistant fraction	Insoluble collagenase-resistant fraction	
Dry weight	100	9.55 $\pm$ 0.36	0.99 $\pm$ 0.03	100	5.27 $\pm$ 0.13	4.06 $\pm$ 0.03		
Protein	n.d.	8.25 $\pm$ 0.05	n.d.	n.d.	4.23 $\pm$ 0.03	n.d.		
Hydroxyproline	13.04 $\pm$ 0.11 (5)	0.018 $\pm$ 0.007	0.002 $\pm$ 0	12.90 $\pm$ 0.09 (8)	0.036 $\pm$ 0.001	0.057 $\pm$ 0.004		
Sialic acid*	0.276 $\pm$ 0.006 (5)	0.227 $\pm$ 0.008	0.014 $\pm$ 0.002	0.081 $\pm$ 0.002 (5)	0.039 $\pm$ 0.001	0.032 $\pm$ 0.002		
Sialic acid†	0.55 $\pm$ 0.02 (4)	0.41 $\pm$ 0.01	n.d.	0.26 $\pm$ 0.01 (4)	0.18 $\pm$ 0.01	n.d.		
Hexose	0.73 $\pm$ 0.01 (5)	0.41 $\pm$ 0.01	n.d.	0.60 $\pm$ 0.03 (5)	0.26 $\pm$ 0.03	n.d.		
Hexosamine	0.38 $\pm$ 0.01 (12)	0.34 $\pm$ 0.02	n.d.	0.39 $\pm$ 0.01 (12)	0.22 $\pm$ 0.01	n.d.		

* Thiobarbituric acid method (Aminoff, 1961).

† Direct Ehrlich method (Werner & Odin, 1952).

substrate ratio (1:500, w/w) and a high Ca^{2+} concentration (Mandl *et al.*, 1958). Under the same conditions, but with an enzyme/substrate ratio about 20 times greater, Triffitt & Owen (1973) found that only 1.8% of the radioactivity was released from [^{14}C]-glucosamine-labelled plasma proteins, and 4% from those labelled with [^{14}C]leucine. In the present study, good yields of non-collagenous protein were obtained, relative to the estimated amount calculated from hydroxyproline values (Table 1), and the electrophoretic behaviour of bone sialoprotein obtained from collagenase digests appeared to be identical with that prepared by using extraction procedures. For these reasons the conditions used here were considered satisfactory for the purposes described.

Analyses, yields and recoveries

The analyses for total content of certain components were carried out on a number of samples of each tissue and the mean values $\pm$ S.E.M. calculated (Table 1). Collagenase digests of two samples each of bone matrix and tendon were prepared and analysed. The means of the duplicate values and the range are shown.

As found in previous studies (Oldroyd & Herring, 1967), the bone matrix was solubilized after incubation with collagenase for 24 h, and more than 99% of the hydroxyproline was diffusible after 48 h. The tendon, on the other hand, was apparently resistant, remaining in a fibrous form dispersed in the digestion medium, even after the incubation had been continued for a further 48 h with additional collagenase. However, appearances were misleading, and in terms

of hydroxyproline content (Table 1), only 0.7% of the collagen originally present in the tendon remained, compared with 0.15% of that in bone matrix.

The total hydroxyproline values indicated collagen contents of about 91% for bone matrix and 90% for tendon, and therefore values of 9–10% for the non-collagenous material present. This is in reasonable agreement with the amounts of the total collagenase-resistant fraction obtained for both tissues, being 9–10% for bone and 8–9% for tendon. However, there was a marked difference between the relative amounts of the soluble and insoluble fractions present in each tissue (Table 1).

Another distinctive difference was in the amounts of sialic acid present in the two tissues. As shown in Table 1, bone matrix contained at least three times as much as tendon. Most of this was in the soluble collagenase-resistant fraction of bone, whereas in tendon there was a larger proportion in the insoluble collagenase-resistant fraction. The recovery of total sialic acid in the collagenase-resistant fractions was 87 and 86% respectively for bone matrix and tendon. Much higher values for sialic acid were obtained by the direct Ehrlich procedure. Part of this appeared to be a non-specific reaction which proceeded at room temperature and could be seen as a pink colour on the surface of the tissue fragments before heating. It suggests the presence of pyrrole derivatives associated with the connective-tissue macromolecules; if this is the case, their exact nature and origin could be of some interest. The difference in sialic acid values in the two methods could also be due to the presence of *O*-acetyl groups. Buscher *et al.* (1974) have isolated and identified the 7- and 8-*O*-

acetyl derivatives of *N*-acetyl- and *N*-glycolyl-neuraminic acid from several sources, including bovine submandibular-gland glycoproteins. The colour yield of these in the thiobarbituric acid assay ranged from 5 to 51 % of that given by *N*-acetylneuraminic acid.

Amino acid analyses

In general, the amino acid analyses (Table 2) resembled those for other connective-tissue glycoproteins (Anderson, 1976). It was surprising to find that the soluble collagenase-resistant fractions of the two tissues were so similar, since glycoproteins with higher content of the acidic amino acids have been isolated from bone (Herring, 1972; Ashton *et al.*, 1974). Presumably there is insufficient of these in the total protein to make a noticeable contribution; bone sialoprotein, for example, though it has 403/1000 residues of aspartic acid and glutamic acid, makes up only about 5 % of the total soluble protein (Herring, 1972). Greater differences occur between the insoluble fractions (Table 2), and that from tendon is similar in composition to the structural glycoproteins that have been isolated by similar collagen-solubilizing procedures from other connective tissues (Robert & Comte, 1968; Timpl *et al.*, 1969; Wolff *et al.*, 1971). The fact that this component makes up 4 % of tendon, and appears to retain structural integrity during collagenase digestion (see above), suggests that it

may have an important physiological role, and should be studied in more detail.

DEAE-cellulose chromatography

The elution patterns given by bone matrix and tendon digests on DEAE-cellulose chromatography are shown in Fig. 1. Components 1–3 correspond to the less-acidic glycoproteins, some of which have been isolated and studied in detail from bone (Ashton *et al.*, 1974) and from tendon (Anderson & Jackson, 1972; Anderson, 1975). The sialic acid contents of the whole tissues are reflected in the elution patterns, and it appears that, in general, the glycoproteins in the bone digest have more sialic acid than those in the tendon digest. This is also in accord with the analyses of the isolated glycoproteins. As noted in the tissue analyses, some of the components (Fig. 1) showed higher sialic acid contents in the direct Ehrlich reaction compared with the thiobarbituric acid method. The possibility that this may be due to a non-specific reaction in the direct Ehrlich assay or to the presence of *O*-acetylsialic acids has been discussed above. The main uronic acid-containing peaks (5) correspond to the proteoglycans containing chondroitin 4-sulphate in bone, and chondroitin 6-sulphate and dermatan sulphate in tendon (Meyer *et al.*, 1956; Dörner *et al.*, 1968; Anderson, 1975). Only component 4 was examined in more detail. As shown by the elution pattern, this component present in the bone digest is relatively acidic and rich in sialic acid; previous studies showed that bone sialoprotein is eluted from DEAE-cellulose under the same conditions. On cellulose acetate electrophoresis, a single Alcian Blue-staining band was seen with a mobility identical with that of bovine bone sialoprotein. The elution pattern of the tendon digest, on the other hand, showed no evidence for the presence of component 4 (Fig. 1). When the fractions in the same elution range were concentrated and examined by electrophoresis, a band corresponding to bone sialoprotein could not be detected.

Conclusions

These results indicate that the Ca^{2+} -binding acidic glycoprotein, bone sialoprotein, is not present in tendon and may therefore be specific to bone, though its exact function is open to speculation. Other clear differences, which have been noted here, lie in the larger amount of sialic acid present in the whole tissue and in the other glycoproteins of bone matrix, and in the amount and type of the collagenase-resistant insoluble 'structural glycoprotein' in the two tissues. The study of the chemical composition of bone matrix can probably provide only a limited insight into the function of this tissue. Attempts have been made to implicate collagen and glycosaminoglycans in apatite

Table 2. Amino acid composition of collagenase-resistant fractions from bone matrix and tendon

Experimental details are given in the text.

	Content (residues/1000 residues)			
	Soluble collagenase-resistant fraction		Insoluble collagenase-resistant fraction	
	Bone matrix	Tendon	Bone matrix	Tendon
Asp	113	124	126	107
Thr	49	45	37	49
Ser	77	68	62	71
Glu	133	127	145	111
Pro	67	79	79	54
Gly	110	135	110	139
Ala	69	70	73	78
I				
Cys	21	21	20	14
Val	49	44	41	52
Met	13	12	9	16
Ile	32	29	24	34
Leu	80	72	75	84
Tyr	37	37	37	28
Phe	36	33	35	36
His	28	26	23	25
Lys	42	36	46	41
Arg	43	41	57	60

Fig. 1. Elution profiles of the soluble collagenase-resistant fractions from bone matrix and tendon on DEAE-cellulose

Details of the procedures are given in the text. Linear gradients (0–0.7 M-NaCl) were started at the points marked by arrows. (a) Bone matrix digest. (b) Tendon digest. —, E_{280} , ----, uronic acid (orcinol method), ····, sialic acid (thiobarbituric acid method), - · - ·, sialic acid (direct Ehrlich method). The reasons for the differences between the two methods for sialic acid determination are discussed in the text.

nucleation and formation, though as yet there is no clear evidence that there are any differences in these macromolecules in bone which can be related to a particular function in mineralization. The results described here suggest that the glycoproteins of bone differ considerably from other connective tissues and merit further study. It is hoped that this may advance our understanding of the factors involved in mineral formation in normal and pathological bone, and in abnormal calcification of soft tissues.

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