The Isolation of Glycoproteins from Bovine Achilles Tendon and their Interaction with Collagen

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Two glycoprotein fractions, A and B, were isolated from bovine achilles tendon. Glycoprotein A was prepared from a 0.2M-sodium chloride extract and glycoprotein B was isolated from a 3M-magnesium chloride extract. They were free from serum proteins. Glycoprotein A was essentially free of collagen, but glycoprotein B contained about 8% collagen. Both glycoproteins gave several bands on isoelectric focusing. This technique was also used to demonstrate that both glycoprotein fractions interacted strongly with acid-soluble calf skin tropocollagen. It is concluded that these fractions are true components of tendon, and that they may have some function in the macromolecular organization of the tissue.

Since the finding by Bowes et al. (1957) that the difference in composition between crude collagen and purified collagen could be accounted for by the presence of non-collagenous proteins, interest in connective-tissue glycoproteins has been growing. It has been suggested that glycoproteins have some function in stabilizing the tissue, and that they may be involved in maintaining the structural stability of collagen fibrils that are above a certain diameter (Jackson & Bentley, 1968). In addition, glycoproteins may be the components of connective tissue that are primarily responsible for its antigenicity, for example, in tissue transplants (Radhakrishnamurthy et al., 1964; Robert et al., 1968).

Several workers have prepared glycoproteins from soft connective tissues (Robert & Comte, 1968; Radhakrishnamurthy et al., 1964; Barnes & Partridge, 1968). Some of the isolation methods make use of high temperatures and extremes of pH, and hence may cause chemical changes in the glycoproteins. Glycoprotein fractions have, however, been isolated by milder procedures during proteoglycan preparation (Dunstone & Franek, 1967; Hascall & Sajdera, 1969). The use of similar mild methods is desirable for the isolation of connective-tissue glycoproteins.

Materials and Methods
Reagents

All reagents were of analytical grade, except carbazole, chloramine-Î, ethanolamine, Folin–Ciocalteu reagent, glucuronolactone, guanidinium chloride, hexosamine hydrochlorides, methylcellulose, neutral sugars and periodic acid. Pyridine and acetylaceton were redistilled.

Hexuronic acid. Hexuronic acid was determined by a modification (Bitter & Muir, 1962) of the method of Dische (1947), with glucuronolactone as standard.

Hexosamine. Weighed samples (approx. 2mg for glycoprotein material) in 1ml of 4M-HCl were hydrolysed in vacuo in sealed tubes at 110°C for 4h. Collagen samples (approx. 50mg) were hydrolysed in 5ml of 4M-HCl. Acid was removed from the hydrolysates by using a rotary evaporator. Total hexosamine was determined by the distillation procedure of Cessi & Piliego (1960), with glucosamine hydrochloride as a standard. Hexosamine values were not corrected for any losses during hydrolysis. Hexosamine ratios of hydrolysates were measured on the 2h Technicon autoanalyser system, by using a starting buffer at pH2.80 (Tsiganos & Muir, 1969).

Protein. Total protein was determined by the method of Lowry et al. (1951), with bovine serum albumin as a standard.

Sialic acid. Sialic acid was determined by the method of Aminoff (1961), with N-acetylneuraminic acid as standard.

Amino acids. Hydrolysates were done in vacuo in sealed tubes with 6M-HCl at 110°C for 16h. Acid was removed from the hydrolysates by using a rotary evaporator. Amino acid analysis was performed by using the 2h Technicon autoanalyser system, with norleucine as internal standard. The half-cystine content was calculated from the area of the cystine peak.

Hydroxyproline. Hydroxyproline present in the hydrolysate for amino acid analysis was determined by the method of Woessner (1961).

Sulphate. Weighed samples (approx. 3mg) were
dissolved in 0.45 ml of water. Then 2M-HCl (0.45 ml) was added and the solution was heated in a sealed tube at 110°C for 5 h. After the solution was cooled, sulphate was measured by the method of Dodgson & Price (1962), with K2SO4 as standard. Two 0.2 ml samples were used for assay with BaCl2-gelatin, and two similar samples were used for the gelatin blank.

**Phosphate.** Phosphate was determined by the method of Chen et al. (1956), with ATP as standard.

**Hexose.** The procedure was that described by Oates & Schrager (1967), as modified by Lote & Weiss (1971). Weighed samples (approx. 2 mg) in 1 ml of 0.5M-HCl were hydrolysed in sealed tubes in vacuo at 110°C for 16 h. A portion (0.05 ml) of sorbitol solution (0.4 mg/ml) was added and the hydrolysate was passed, first down a column (1 cm x 8 cm) of Amberlite IR-4B (OH- form), and then down a column (1 cm x 4 cm) of Dowex 50 (H+ form; 4 % cross-linking; 200-400 mesh). The sample was washed on to the column with water and 20 ml of eluate was collected. The aqueous solution of sugars so obtained was evaporated to dryness on a rotary evaporator and stored in a vacuum desiccator overnight. The sample was silylated in anhydrous pyridine (0.1 ml), hexamethyldisilazane (0.09 ml) and trifluoroacetic acid (0.01 ml) by shaking for 20 min. Analysis for various sugars by g.l.c. was done with a Pye Series 104 Chromatograph, as described by Grant et al. (1969).

**Ouchterlony double gel diffusion.** The presence of precipitating antibodies was investigated by double gel diffusion (Ouchterlony, 1948) in 1 % agar (I onagar no. 2) in barbitone-saline buffer, p H 7.2 (Oxoid complement-fixation diluent tablets), containing sodium azide (0.1 %, w/v). Solutions of glycoproteins at concentrations of 1, 5 and 10 mg/ml were placed in circumperipheral wells, as was bovine plasma. The central well contained precipitating antibodies to bovine serum proteins (Burroughs Wellcome and Co., London N.W.1, U.K.). The plate was kept in a humid atmosphere at room temperature for 5 days.

**Isoelectric focusing.** The 110 ml LKB column was used, containing Ampholine pH 3-10 buffers in a sucrose gradient, in which approx. 2 mg of material had been dissolved. The cathode solution (low density) was an aq. 2 % (v/v) solution of ethanolamine, and the anode solution (high density) was made up of H2PO4 (2 ml) and sucrose (120 g) dissolved in water (140 ml). The sucrose gradient was prepared by filling the column from the bottom by means of a Technicon Autograd apparatus. The first chamber contained LKB carrier ampholytes (pH 3-10, 0.62 ml) in 56.9 ml of water. The second chamber contained LKB carrier ampholytes (pH 3-10, 1.88 ml) and sucrose (28 g) dissolved in 37.6 ml of water. Both solutions contained the same quantity of material to be examined. When the interaction of collagen with glycoprotein was under investigation, the solution of acid-soluble calf skin tropocollagen (2.5 mg/ml in 0.2M-acetic acid), prepared by the method of Steven & Jackson (1967), was added to the solution in the second chamber, and the solution in the first chamber contained the glycoprotein. After focusing, the bands were seen as opaque zones.

**Preparation of glycoproteins**

Twelve bovine Achilles tendons (approx. 170 g dry wt.), that had been obtained within 1 h after the animals had been killed, were dissected free of adhering tissue. They were cut into 3-5 mm cubes and then pulverized by hammering them in a steel die cooled in liquid N2.

**Extraction with 0.2M-NaCl.** The pulverized tendon was suspended in 2 litres of 0.2M-NaCl which contained 0.1 % (w/v) Na2SO4 as a bacteriostatic agent. The suspension was homogenized briefly with a Turrax homogenizer and then was left, with occasional stirring, for 2 days at 4°C. The suspension was centrifuged and the resulting supernatant was dialysed and freeze-dried. Repetition of this extraction procedure nine times gave a crude combined extract of 5.7 g. This material was stirred in 1 litre of 0.05M-sodium acetate, pH 5.8, overnight at 4°C. The supernatant from centrifugation was dialysed and freeze-dried (2.7 g).

**Fractionation on SE-Sephadex.** The soluble extract was dissolved in 500 ml of water and passed down a column (2.5 cm x 6 cm) of SE-Sephadex C-50 [Sulphoethyl-Sephadex; Na+ form; Pharmacia (Great Britain) Ltd.]. The column was washed with two 50 ml portions of water and then with 100 ml of 1M-NaCl. The combined aqueous eluates were freeze-dried (2.15 g). The NaCl eluate was dialysed and freeze-dried (114 mg). The material not retained on SE-Sephadex was processed as shown in Scheme 1.

**Extraction with 3M-MgCl2:** The residue from exhaustive extraction with 0.2M-NaCl was suspended in 6 litres of 3M-MgCl2 by brief homogenization with a Turrax homogenizer. After being left for 2 days at 4°C, the dispersion was centrifuged at 1500 g at 4°C for 1 h, and the supernatant was dialysed. During the dialysis, a collagenous precipitate formed and was subsequently removed by centrifugation; the supernatant was freeze-dried (460 mg). A crude glycoprotein fraction was obtained by extraction of the collagenous precipitate with 0.2M-NaCl-0.1 % (w/v) Na2SO4. After five extractions, the protein content of the extracts had fallen to a constant low value, so the extracts were combined, dialysed and freeze-dried (690 mg). The entire extraction procedure, beginning with further extraction of the tendon residue with 3M-MgCl2, was repeated five times.

**Readsorption of supernatant material on precipitated collagen.** The precipitated collagen (after five
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ex Extractions with 0.2M-NaCl–0.1% NaN₃, obtained from dialysis of the first 3M-MgCl₂ extraction, was dispersed in 3M-MgCl₂ (600 ml). The freeze-dried material from the supernatant (460 mg), dissolved in 100 ml of 3M-MgCl₂, was added to the collagen dispersion. The mixture was kept at 4°C for 5 days and then was centrifuged. Dialysis and freeze-drying of the supernatant gave 230 mg of material. Exhaustive extraction of the precipitated collagen with 0.2M-NaCl–0.1% (w/v) NaN₃ gave 230 mg of material after dialysis and freeze-drying.  

Fractionation of 3M-MgCl₂ extract on SE-Sephadex. The combined material from extraction of precipitated collagen from the first 3M-MgCl₂ extract (920 mg; 690 mg plus 230 mg from the readsorption experiment) was stirred with 250 ml of water. The suspension was centrifuged, and the supernatant was passed down a column (2.5 cm x 15 cm) of SE-Sephadex C-50 (Na⁺ form). The column was washed through with 2 x 50 ml of water and then with 250 ml of 1M-MgCl₂ in which the water-insoluble material had been dissolved. The combined aqueous eluates were freeze-dried (513 mg) and processed as shown in Scheme 2. The MgCl₂ eluate was dialysed and centrifuged, giving 212 mg of insoluble material; the supernatant was freeze-dried and gave 42 mg of material.  

Density-gradient ultracentrifugation. The two solutions described by Hascall & Sajdera (1969) were used: the first (density 1.69 g/ml, 0.4M-guanidinium chloride) is here designated solution UC₁, and the second (density 1.5 g/ml, 4.0M-guanidinium chloride) solution UC₂. Some centrifugations in solution UC₂ were done at a lower loading density. In these cases, the density is given in the Scheme. Density gradients

<table>
<thead>
<tr>
<th>Partially purified material, 2.15 g</th>
</tr>
</thead>
<tbody>
<tr>
<td>UC₁</td>
</tr>
<tr>
<td>T₅₀%, 533 mg B₅₀%, 782 mg</td>
</tr>
<tr>
<td>UC₁</td>
</tr>
<tr>
<td>T₅₀%, 290 mg B₅₀%, 150 mg</td>
</tr>
<tr>
<td>UC₂</td>
</tr>
<tr>
<td>T₁₀%, 110 mg M₃₀%, 108 mg B₆₀%, 65 mg</td>
</tr>
<tr>
<td>UC₂</td>
</tr>
<tr>
<td>T₅₀%, 51 mg B₅₀%, 35 mg</td>
</tr>
<tr>
<td>UC₂</td>
</tr>
<tr>
<td>T₃₃%, 103 mg B₆₇%, 34 mg</td>
</tr>
</tbody>
</table>

Glycoprotein A

Scheme 1. Fractionation by caesium chloride-density-gradient ultracentrifugation of material extracted from bovine tendon with 0.2m-NaCl–0.1% NaN₃

Fractions are designated top (T), middle (M) and bottom (B), followed by their percentage of the total solution volume, and their dry weight in mg. Solutions UC₁ and UC₂ are the first and second ultracentrifugation solutions respectively of Hascall & Sajdera (1969).

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were formed by centrifugation at 100000g for 40h at 20°C. Material was usually centrifuged at a concentration of less than 5mg/ml. Samples centrifuged at higher concentrations often produced a gel or residue. Either a Christ Omega 2 preparative ultracentrifuge with Christ angle rotor 9730 was used, or an MSE Superspeed 65 with MSE angle rotors 59592 or 59594. Fractions corresponding to the proportion of tube volume indicated in Schemes 1 and 2 were isolated by careful withdrawal with a Pasteur pipette having a bent tip. After dialysis, the fractions were assayed for protein and hexuronic acid, and the material was obtained in solid form by freeze-drying. When a gel was formed at the top of a gradient, it was removed as carefully as possible with a spatula, then transferred to water and stirred overnight at 4°C. After centrifugation, the aqueous supernatant was dialysed, freeze-dried and then submitted to the next fractionation step.

Results

Isolation of crude glycoproteins

The first series of extracts was made with 0.2M-NaCl-0.1% NaN₃. Exhaustive extraction removed most of the proteoglycan and some of the glycoprotein from the tissue. When the combined freeze-dried extracts were stirred with 0.05M-sodium acetate, pH5.8, only half of the material dissolved. The insoluble residue contained 33% collagen, 1.2% hexuronic acid, and was not fractionated further. The soluble material was chromatographed on SE-Sephadex and then fractionated as shown in Scheme 1.

After exhaustive extraction with 0.2M-NaCl-0.1% NaN₃, it was found that extraction with other dilute extractants, e.g. 4% (w/v) EDTA, pH7.5, did not remove any appreciable amount of protein from the residual tendon. Extraction with 3M-MgCl₂ removed more protein, and dialysis of the super-

Scheme 2. Fractionation by caesium chloride-density-gradient ultracentrifugation of material extracted from bovine tendon by 3M-MgCl₂

Fractions are designated top (T) and bottom (B), followed by their percentage of the total solution volume, and their dry weight in mg. Solutions UC1 and UC2 are the first and second ultracentrifugation solutions respectively of Hascall & Sajdera (1969). A value quoted after solutions UC2 gives the loading density, if this differed from 1.5g/ml.
natant after centrifugation produced a collagenous precipitate. Amino acid analysis of the precipitate (299 residues of glycine/1000 amino acid residues) suggested that it was mostly collagen, but also contained some non-collagenous protein. This was confirmed when much of the non-collagenous protein was removed by repeated extraction with 0.2M- NaCl–0.1% NaN₃. The extracted precipitated collagen was used to adsorb more protein from the supernatant remaining after precipitation of collagen during dialysis of the 3M-MgCl₂ extract. The subsequent fractionation procedures are described in the Materials and Methods section and in Scheme 2.

Subsequent extractions (five) with 3M-MgCl₂ again gave collagenous precipitates after dialysis, which yielded protein on extraction with 0.2M-NaCl–0.1% NaN₃. However, these protein fractions were so heavily contaminated with collagen (40–50%) that they were not fractionated further.

**Density-gradient ultracentrifugation**

Crude glycoprotein preparations were rendered almost free from proteoglycans by repeated density-gradient ultracentrifugation. Assay of fractions confirmed that in all cases most of the protein was found at the top, and most of the hexuronic acid was found at the bottom, of the gradient. Graphs of protein and hexuronic acid content plotted against fraction number for initial ultracentrifugations of crude material were very similar to those published by Hascall & Sajdera (1969), Franek & Dunstone (1967) and Toole & Lowther (1968).

**Residual collagen**

A sample of residual collagen dispersed in 3M-MgCl₂ was taken after the last 3M-MgCl₂ extraction, then was exhaustively dialysed before being freeze-dried. Hence the approximate dry weight of the original tendons was calculated (170g). The hexosamine content was 0.10%, equivalent to not less than 2 hexosamine residues/3000 amino acid residues. Portions of the residual collagens from extraction of the precipitated collagens with 0.2M-NaCl–0.1% NaN₃ were similarly obtained in a dry state. Hexosamine analyses were: 1st extract, 1.1%; 3rd., 2.5%; 4th., 1.5%; 5th., 0.4%; 6th., 0.3%.

**Analysis of glycoproteins**

Amino acid analyses for glycoproteins A and B are shown in Table 1. Their contents of other components are shown in Table 2.

**Isoelectric focusing and interaction of glycoproteins with collagen**

Isoelectric-focusing patterns obtained from glycoproteins A and B are shown in Fig. 1, together with the results of interaction with collagen. The glycoproteins associated with calf skin tropocollagen to form an aggregate of isoelectric point intermediate between those of the glycoprotein and collagen.

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**Table 1. Amino acid composition of glycoproteins A and B**

For experimental details see the text.

<table>
<thead>
<tr>
<th></th>
<th>Glycoprotein A</th>
<th>Glycoprotein B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyp</td>
<td>&lt;1</td>
<td>24</td>
</tr>
<tr>
<td>Asp</td>
<td>107</td>
<td>113</td>
</tr>
<tr>
<td>Thr</td>
<td>65</td>
<td>35</td>
</tr>
<tr>
<td>Ser</td>
<td>63</td>
<td>70</td>
</tr>
<tr>
<td>Glu</td>
<td>122</td>
<td>96</td>
</tr>
<tr>
<td>Pro</td>
<td>67</td>
<td>108</td>
</tr>
<tr>
<td>Gly</td>
<td>57</td>
<td>106</td>
</tr>
<tr>
<td>Ala</td>
<td>75</td>
<td>61</td>
</tr>
<tr>
<td>Cys</td>
<td>41</td>
<td>14</td>
</tr>
<tr>
<td>Val</td>
<td>72</td>
<td>46</td>
</tr>
<tr>
<td>Met</td>
<td>9</td>
<td>11</td>
</tr>
<tr>
<td>Ile</td>
<td>33</td>
<td>37</td>
</tr>
<tr>
<td>Leu</td>
<td>83</td>
<td>101</td>
</tr>
<tr>
<td>Tyr</td>
<td>32</td>
<td>32</td>
</tr>
<tr>
<td>Phe</td>
<td>38</td>
<td>30</td>
</tr>
<tr>
<td>Hyl</td>
<td>0</td>
<td>Trace</td>
</tr>
<tr>
<td>Lys</td>
<td>71</td>
<td>42</td>
</tr>
<tr>
<td>His</td>
<td>21</td>
<td>21</td>
</tr>
<tr>
<td>Arg</td>
<td>44</td>
<td>53</td>
</tr>
</tbody>
</table>

**Table 2. Percentage chemical composition of glycoproteins A and B**

For experimental details see the text.

<table>
<thead>
<tr>
<th></th>
<th>Glycoprotein A</th>
<th>Glycoprotein B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>85</td>
<td>61</td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td>0.2</td>
<td>1.2</td>
</tr>
<tr>
<td>Hexuronic acid</td>
<td>0.6</td>
<td>0.7</td>
</tr>
<tr>
<td>Sulphate</td>
<td>2.1</td>
<td>2.4</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>2.8</td>
<td>3.2</td>
</tr>
<tr>
<td>Galactosamine</td>
<td>1.7</td>
<td>0.8</td>
</tr>
<tr>
<td>Galactose</td>
<td>1.9</td>
<td>2.2</td>
</tr>
<tr>
<td>Mannose</td>
<td>0.6</td>
<td>1.2</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.4</td>
<td>0.2</td>
</tr>
<tr>
<td>Fucose</td>
<td>0.3</td>
<td>0.5</td>
</tr>
<tr>
<td>Sialic acid</td>
<td>0.2</td>
<td>1.2</td>
</tr>
<tr>
<td>Phosphate</td>
<td>0.1</td>
<td>0.1</td>
</tr>
</tbody>
</table>
Numbers
Experimental protein (c) glycoprotein A+tropocollagen; (d) glycoprotein A; (e), glycoprotein B+tropocollagen. Experimental details are given in the text. In (c) and (e) equal weights (1.3mg) of each component were used. Numbers refer to the pH of each band.

Tests for serum proteins by Ouchterlony double diffusion

No trace of any precipitin lines was observed between the wells containing various concentrations of glycoproteins A and B and that containing the rabbit antiserum. A precipitin line was observed between rabbit antiserum and material eluted (1M-NaCl) from SE-Sephadex during chromatography of the 0.2M-NaCl–0.1% NaN₃ extract of tendon.

Discussion

The extraction procedures used to isolate glycoproteins from the tendons were mild, avoiding high temperatures and extremes of pH. After all the extractions, the residual collagen contained not less than 2 hexosamine residues/3000 amino acid residues. Polymeric collagens purified by various procedures still contain a little hexosamine, but this value is within the range of hexosamine content found by Grant & Jackson (1968) for pure polymeric collagens prepared by different methods. However, hexosamine analysis of the precipitated collagen fractions from 3M-MgCl₂ extracts (total dry weight was 6% of the original tendon dry weight) showed that these still contained appreciable amounts of hexosamine (0.3–2.5%). Therefore there was still proteoglycan and/or glycoprotein associated with these collagen fractions.

Glycoproteins A and B had broadly similar compositions. Their amino acid compositions were similar to those of glycoproteins isolated by other workers (Hascall & Sajdera, 1969; Dunstone & Franek, 1967; Robert & Comte, 1968), aspartic acid, glutamic acid and leucine being predominant. However, bearing in mind that glycoprotein B contained some collagen, there was a significant difference between glycoproteins A and B in their content of threonine, proline, glycine, half-cystine, valine and lysine. The hexose and hexosamine compositions are similar in some respects to those of serum glycoproteins, e.g. fetuin and α₁-acid glycoprotein (Graham, 1966), since the two major hexose components were galactose and mannose, galactose being predominant, and fucose also being present. However, glycoproteins A and B differed from the serum glycoproteins, in containing glucose. There was more glucosamine than galactosamine.

The galactose, glucosamine and sulphate content of the glycoproteins could be accounted for by the presence of proteokeratan sulphate. It is reported that in skeletal tissues keratan sulphate is always associated on the same protein core with chondroitin sulphate (Rodén, 1970; Hofmann & Mashburn, 1970), and thus keratan sulphate-containing material should be isolated from the bottom of density gradients together with hexuronic acid-containing components. Indeed, the free glycosaminoglycan, keratan sulphate, is reported to have a buoyant density of 1.8–2.0g/ml (Dunstone, 1969). However, this sort of argument is almost certainly too simple. Tsiganos et al. (1970) report that there is not only an increase of protein, but also of keratan sulphate, towards the top of a caesium chloride gradient of starting density 1.5g/ml. In our case, the small content of hexuronic acid in the glycoproteins may be due to some chondroitin sulphate associated with keratan sulphate. But since proteokeratan sulphates can be regarded as being on the borderline between glycoproteins and proteoglycans (Rodén, 1970), we may be attempting to make an artificial distinction. The problem should be resolved by further purification.

Glycoprotein A contained very little hydroxyproline, but glycoprotein B contained 1.2%, corresponding to a collagen content of 8%. This value emphasizes a major problem in the isolation of glycoproteins, which is the difficulty of obtaining them free of hydroxyproline, particularly when they are prepared from 3M-MgCl₂ extracts. At this stage it is not certain whether the hydroxyproline represents contamination with collagen or is a genuine component of the glycoprotein in which a small part of the polypeptide chain may be collagenous.

The complete absence of precipitin lines on Ouchterlony double diffusion showed that neither glycoprotein was significantly contaminated with serum proteins and hence the glycoproteins could be regarded as true components of tendon. However,
material eluted by 1M-NaCl from SE-Sephadex during chromatography of the 0.2M-NaCl–0.1% NaN3 extract did show a precipitin line. Thus any serum proteins originally present in the extracts were removed by SE-Sephadex.

Three facts became apparent on isoelectric focusing of glycoproteins A and B. First, each glycoprotein contained several components; secondly, most of these components were acidic, with isoelectric points of about pH 4, and thirdly, each had a minor component with an isoelectric point near neutrality. These properties resemble those of proteoglycans (Steven et al., 1969). Thus it seemed probable that these glycoproteins also exhibited micro-heterogeneity, and this could explain the single band observed for the glycoprotein–collagen complexes on isoelectric focusing. However, their acidic isoelectric points were also similar to those of the serum glycoproteins, fetuin and α1-acid glycoprotein, which have isoelectric points in the range pH 3–4 (Graham, 1966). Indeed, it has been shown that fetuin exhibits micro-heterogeneity on isoelectric focusing (Pagé, 1971).

The acidity of glycoproteins A and B could not be wholly due to a predominance of residues of aspartic acid and glutamic acid, but must also have been a function of their sulphate content, since their sialic acid and phosphate content was low. In contrast, the acidity of fetuin and α1-acid glycoprotein can be accounted for by their high content (8–10%) of sialic acid (Graham, 1966).

It was difficult to apply criteria of purity to these glycoproteins (other than to monitor for proteoglycan and collagen content), in much the same way that it is difficult to define the purity of a proteoglycan. It seemed very likely that both glycoproteins contained identical components. However, if this was so, an explanation must be found for the fact that glycoprotein B was not extracted with 0.2M-NaCl and initially required 3M-MgCl2 for extraction, but was subsequently extracted by 0.2M-NaCl from the precipitated collagen. Possibly glycoprotein B was extracted as a magnesium salt, which did not interact as strongly with collagen as a sodium salt. However, it is much more probable that more glycoprotein is exposed to the extracting solution in the 3M-MgCl2 dispersion (Gustavson, 1956) than in 0.2M-NaCl.

A strong association was demonstrated between each glycoprotein and calf skin tropocollagen by isoelectric focusing, and the association of crude glycoprotein B with collagen was used as a method of fractionation. These findings could support the idea that glycoproteins play some part in stabilizing the tissue, perhaps by maintaining the structural stability of the collagen fibrils that are above a certain diameter, as suggested by Jackson & Bentley (1968). However, it could be argued that glycoprotein–collagen binding might prevent normal interactions between collagen molecules and decrease the stability of the fibre.

The character of intermediate fractions in the isolation of glycoprotein B resembled a fraction isolated by Toole & Lowther (1968) from bovine heart valves, which they designated dermatan sulphate–protein. Toole & Lowther (1968), after preliminary exhaustive extraction of the tissue with water and 1M-NaCl, extracted it with 6M-urea at 60°C. The final fractionation step was CsCl density-gradient ultracentrifugation (but not in a dissociative solution), which gave two dermatan sulphate proteins. The lighter one had 70% protein and 30% dermatan sulphate, and contained an appreciable amount of collagen. This fraction can be compared to our top fractions from CsCl centrifugation in solution UC1 (see Scheme 2). However, the hexuronic acid was largely removed by density-gradient ultracentrifugation in solution UC2. The presence of dermatan sulphate was indicated by assay for hexuronic acid of the bottom fraction (42mg) from density-gradient centrifugation of the gel in solution UC2. The ratio of hexuronic acid measured by the method of Dische (1947) to that found by the Bitter & Muir (1962) method was 0.75. According to Bitter & Muir (1962), pure dermatan sulphate gave a value of 0.5, whereas chondroitin sulphate gave a ratio of 1.0. Thus this bottom fraction was probably 50% dermatan sulphate. The ratio was similarly determined for the proteoglycan isolated from NaCl extraction (see Scheme 1), and was found to be 1.0, showing that it contained little or no dermatan sulphate.

It can be concluded that in tendon there is a complex mixture of glycoproteins that are unrelated to those of serum. A proportion of these glycoproteins is easily extractable, but some appear to be firmly bound to the collagen component. The isolated glycoproteins were shown to associate strongly with collagen, and thus they may have a function in stabilizing collagen fibrils.

We are grateful to Mrs. S. Jackson for her excellent technical assistance.

References