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The Effect of Bovine Tendon Glycoprotein on the Formation of Fibrils from Collagen Solutions

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The formation of collagen fibrils under physiological conditions of ionic strength, pH and temperature was markedly affected by the presence of small amounts of bovine tendon glycoprotein. The absorbance of the gels at 400nm was decreased, and they took longer to form. Over the range of concentration tested, the negative specific absorbance, $-\Delta A_{sp.}$, and the specific retardation, $R_{sp.}$, both increased with the glycoprotein to collagen ratio. When added during the nucleation phase, glycoprotein was still able to exert its effect almost fully, and so must act to inhibit the later stages of fibril formation. Several pieces of evidence showed that glycoprotein acts via a weak binding to the collagen molecule. Electron microscopy established that fibrils formed in the presence of glycoprotein had a normal cross-striation pattern, but were significantly thinner than fibrils formed in control gels. The results suggest that glycoprotein could act in tissues to help regulate the diameter of collagen fibrils.

We have previously described the isolation and partial characterization of a soluble glycoprotein from bovine achilles tendon (Anderson, 1975, 1976). A proteodermatan sulphate of low molecular weight and high protein content was closely associated with this glycoprotein, but most of the glycoprotein was easily separated from proteoglycan by gel filtration. However, separation of proteodermatan sulphate from all glycoprotein could be accomplished only under dissociating conditions (Anderson, 1975). We have reported the isolation of an antigenically identical protein from bovine skin, together with a proteoglycan of molecular weight close to that of tendon proteodermatan sulphate (Anderson *et al.*, 1977).

There have been few detailed studies of interactions between glycoproteins and collagen. In our original experiments with crude tendon fractions (Anderson & Jackson, 1972), isoelectric focusing may well have demonstrated non-specific binding, and conditions were certainly not physiological. Highberger *et al.* (1951) found that, in the presence of α_1 acid glycoprotein, acetic acid-soluble collagen formed fibrous long-spacing fibres, of periodicity 200–300nm. This interaction has been the subject of a more detailed study by Franzblau *et al.* (1976). Brief references to the effect of glycoproteins on collagen-fibril formation have been made by Adelmann *et al.* (1966) and Rajamäki & Kulonen (1971), who reported that glycoproteins from rat skin and rat granuloma, respectively, accelerated collagen-fibril formation.

In the present paper our object was to examine any interaction between glycoprotein and collagen under physiological conditions of ionic strength, pH and temperature. The original procedure of Gross & Kirk (1958), as modified by Oegema *et al.* (1975), was used, which allowed study of the kinetics of collagen precipitation in the presence of other macromolecules. The results presented here show that tendon glycoprotein influences collagen fibrillogenesis significantly.

Materials and Methods

Materials

All reagents were of analytical grade. Bovine tendon glycoprotein was prepared as described by Anderson (1975). Proteinase-free bacterial collagenase was grade I collagenase (Boehringer Corp., London W5 2TZ, U.K.) or was prepared by the method of Lee-Own & Anderson (1975*a*). Pronase and Nigrosine were from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Noble agar was from Difco Laboratories, Detroit, MI, U.S.A. Bovine serum albumin (Cohn fraction V) was from Koch–Light Laboratories, Colnbrook, Bucks, U.K., and bovine immunoglobulin G from Miles Laboratories, Stoke Poges, Bucks., U.K.

Preparation of collagens

Bovine foetal skin and calf skin acid-soluble collagens were prepared by the method of Steven & Jackson (1967) and collagen was also prepared from calf skin by papain digestion as described by Lee-Own & Anderson (1976). Collagen samples were dissolved

in 0.5M-acetic acid and then dialysed extensively at 4°C against 0.005M-acetic acid. The solution was centrifuged at 100000*g* for 3h, and the residue was freeze-dried and weighed. This allowed the volume of the supernatant to be adjusted with 0.005M-acetic acid so that it had a collagen concentration of 2mg/ml.

Gelation of collagen

The procedure followed method A of Oegema *et al.* (1975). Before use, collagens were kept at room temperature (21°C) for 2h and were deaerated. For each experiment, a test sample (0.72ml) of deaerated 0.14M-NaCl/0.008M-Na_2HPO_4/KH_2PO_4, pH7.4 (containing the desired amount of glycoprotein), 0.03M-NaOH (0.167ml) and 1.4M-NaCl/0.08M-Na_2HPO_4/KH_2PO_4, pH6.9 (0.13ml), were mixed together in a clean cuvette. Collagen (1.0ml of 2mg/ml solution in 0.005M-acetic acid) was added, the solution was gently mixed in the cuvette and placed in a spectrophotometer exactly 20s after commencing addition of the collagen solution. A Pye–Unicam SP.800 spectrophotometer (Pye Instruments, Cambridge, U.K.), with a sample compartment thermostatically controlled at 37°C, was used. The change in A_{400} with time was recorded on a Servoscribe flat-bed recorder. After gelation, the pH of samples was checked as a routine and found to be 7.4. Before re-use, cuvettes were soaked in conc. HCl/conc. HNO_3 (3:1, v/v).

Extraction of gels

The contents of the cuvette were removed with a micro-spatula and were then extracted with 0.15M-NaCl/0.02M-NaH_2PO_4, pH7.4 (20ml), followed by 0.05M-Tris/HCl/0.005M-$CaCl_2$, pH7.4 (20ml). The extracts were combined, dialysed against water, and freeze-dried. The extracted gels were digested for 2 days at 37°C with proteinase-free collagenase (100μg/gel) in 0.05M-Tris/HCl/0.005M-$CaCl_2$, pH7.4 (5ml). After centrifugation (2000*g*, 15min), the residue was washed and submitted to a further digestion under the same conditions for 1 day. The supernatants from digestion were combined, dialysed against distilled water and freeze-dried. Material from both extraction and collagenase digestion was dissolved in 0.15M-NaCl/0.02M-NaH_2PO_4, pH7.4 (50μl/gel), for double diffusion.

Antiserum

Antiserum to tendon glycoprotein was raised in rabbits as described by Anderson *et al.* (1977).

Double diffusion

Double diffusion was performed in 1.5% agar gel at room temperature in a moist atmosphere for 3–5 days. Plates were washed in 0.9% NaCl for 7 days, stained with 0.5% (w/v) Nigrosine in 5% (v/v) acetic acid for 30min, and destained in 2% acetic acid.

Electron microscopy

Gels were formed in exactly the same manner as described above, except that the amount of 0.14M-NaCl/0.008M-Na_2HPO_4/KH_2PO_4, pH7.4, which contained either 200μg (test sample) or no glycoprotein (control), was increased to give a final collagen concentration of 0.2mg/ml instead of 1mg/ml. These gels were formed by incubation in an ordinary water bath maintained at 37°C for approx. 30min. Samples for electron microscopy were prepared by touching the surface of gels with carbon-coated Formvar films supported on copper grids. The grids were dried, negatively stained with 2% (w/v) phosphotungstic acid (adjusted to pH7.0 with 1M-NaOH) and examined with a Philips EM 301S electron microscope.

Assay for proteinase activity

This test was carried out by the method of Peterkofsky & Diegelmann (1971) by incubating glycoprotein (200μg) for 18h with [^{3}H]tryptophan-labelled chick-embryo protein as substrate.

Assay for collagenase activity

The viscometric assay of Gallop *et al.* (1957) was used, as described by Lee-Own & Anderson (1975*a*). The substrate was pepsin-treated collagen from guinea-pig skin (Miller, 1972), and a sample of 100μg of glycoprotein was tested.

Results and Discussion

Influence of glycoprotein on fibril formation

Tendon glycoprotein produced two effects on the precipitation of collagen fibrils at 37°C: (*a*) decrease in the final A_{400}, and (*b*) retardation of fibril formation. Typical results are shown in Fig. 1. To explore the effect further, the parameters used by Oegema *et al.* (1975), which were specific change in absorbance ($\Delta A_{sp.}$) and specific retardation ($R_{sp.}$), were used:

$$\Delta A_{sp.} = \frac{\Delta A - \Delta A^0}{\Delta A^0} \quad (1)$$

$$R_{sp.} = \frac{t_{\frac{1}{2}} - t^0_{\frac{1}{2}}}{t^0_{\frac{1}{2}}} \quad (2)$$

where ΔA is the net change of absorbance (or ΔA^0 for samples with no added glycoprotein) and $t_{\frac{1}{2}}$ is the time taken to reach one-half of ΔA (or $t^0_{\frac{1}{2}}$ the time to reach one-half of ΔA^0). These measurements are also indicated in Fig. 1.

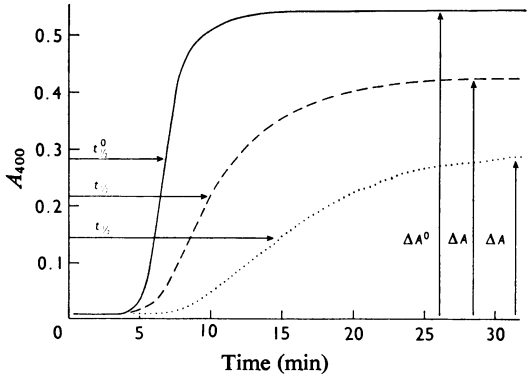


Fig. 1. *Effect of bovine tendon glycoprotein on the gelation of calf skin acid-soluble collagen*

——, Control run with collagen alone (no addition of glycoprotein); – – – –, addition of 80μg of glycoprotein/mg of collagen; · · · ·, addition of 160μg of glycoprotein/mg of collagen. The test sample was deaerated 0.14M-NaCl/0.008M-Na_2HPO_4/KH_2PO_4, pH7.4 (0.72ml), or the same amount of this buffer in which the required amount of glycoprotein was dissolved. To this was added 0.03M-NaOH (0.167ml) and 1.4M-NaCl/0.08M-phosphate, pH6.9 (0.13ml), in a clean cuvette. Deaerated collagen solution (1.0ml of 2mg/ml of solution in 0.005M-acetic acid) was added, the solution was gently mixed in the cuvette and placed in a spectrophotometer exactly 20s after commencing addition of the collagen solution. The sample compartment of the spectrophotometer was thermostatically controlled at 37°C. The change in A_{400} with time was recorded. The indicated parameters were calculated from each run.

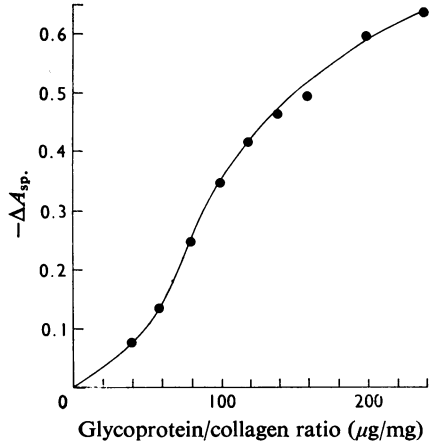


Fig. 2. *Relationship of specific change in absorbance ($\Delta A_{sp.}$) of collagen gels to the concentration of bovine tendon glycoprotein*

Individual runs were carried out as described for Fig. 1. $\Delta A_{sp.}$ was calculated for each run from ΔA and ΔA^0 values as shown by eqn. (1) given in the text.

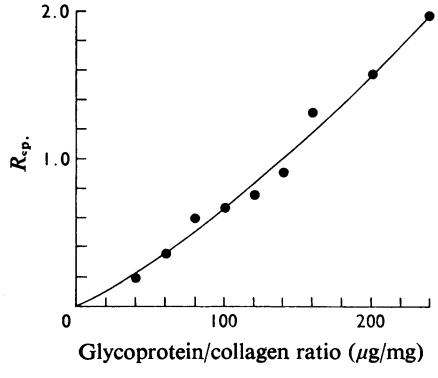


Fig. 3. *Relationship of specific retardation of collagen gelation ($R_{sp.}$) to the concentration of bovine tendon glycoprotein*

Individual runs were carried out as described for Fig. 1. $R_{sp.}$ was calculated for each run from $t_{\frac{1}{2}}$ and $t^0_{\frac{1}{2}}$ values as shown by eqn. (2) given in the text.

The result of measuring $\Delta A_{sp.}$ and $R_{sp.}$ with increasing concentration of glycoprotein gave the data shown in Figs. 2 and 3 respectively. The plots show that, over the range of concentration tested, $-\Delta A_{sp.}$ and $R_{sp.}$ both increased with the glycoprotein to collagen ratio. Both relationships appear to be sigmoidal; presumably the plots in both Figs. 2 and 3 would eventually become horizontal at sufficiently high ratios of glycoprotein to collagen, when the binding sites on the collagen molecule are saturated. It is thus likely that each plot represents the initial part of a sigmoidal curve. This behaviour contrasts with that of proteoglycan subunit (Oegema *et al.*, 1975), where $\Delta A_{sp.}$ and $R_{sp.}$ are both related hyperbolically to the concentration of proteoglycan subunit, and maximum values are reached at low ratios of proteoglycan to collagen. We think that the different behaviour reflects the difference in binding affinity, proteoglycan subunit binding strongly to collagen (Oegema *et al.*, 1975; Lee-Own & Anderson, 1975*b*), whereas tendon glycoprotein binds only weakly.

The presence of glycoprotein produces a more transparent gel, but it does not appear to affect the rigidity of the gels. Gels that contained as much as 240μg of glycoprotein/mg of collagen could still be withdrawn from the cuvette as a block that retained the shape of the cuvette.

Effect of adding glycoprotein to collagen solutions during the nucleation phase

The ability of glycoprotein to act when added at different times after the start of incubation is shown

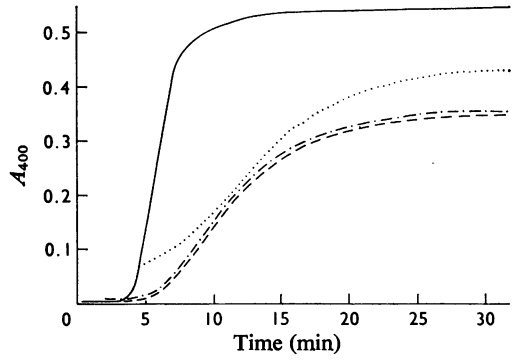


Fig. 4. *Effect of adding bovine tendon glycoprotein to solutions of collagen during the nucleation phase*
——, Control run with collagen alone (no glycoprotein addition); ····, addition of 100μg of glycoprotein/mg of collagen at 4.3min; –·–·, addition of 100μg of glycoprotein/mg of collagen at 2.0min; – – – –, addition of 100μg of glycoprotein/mg of collagen at zero time. After incubation at 37°C for 2 or 4.3min, 200μg of glycoprotein in 0.2ml of buffer was added to the incubation mixture prepared as described for Fig. 1, except that the initial amount of buffer was 0.52ml. The solutions were quickly mixed and returned to the spectrophotometer.

in Fig. 4. When added at one-third of $t_{\frac{1}{2}}^0$, glycoprotein still exerted its full effect on ΔA and $t_{\frac{1}{2}}$. However, addition of glycoprotein at two-thirds of $t_{\frac{1}{2}}^0$ resulted in only a partial decrease in absorbance, but full retardation. The studies by Trelstad (1975) indicated that collagen fibrillogenesis proceeds through a number of stages. Initially, distinct aggregates 5nm in diameter appear, which later form into tactoidal aggregates of diameter 15–17.5nm. The earliest fibrils are formed by linear aggregation of these tactoidal structures, and thicker fibrils are formed by twisting together of these early fibrils. The formation of the tactoidal aggregates and the subsequent fibril formation can be thought of approximately as the 'nucleation' and 'growth' phases respectively, proposed by Wood & Keech (1960). The behaviour of glycoprotein suggests that it exerts its most striking effects by interfering with the linear aggregation of tactoidal aggregates and the subsequent entwining of the early fibrils. When added late in the nucleation phase it is still able to exert its full retarding effect, but presumably some early fibrils have already formed by this time which dictate the eventual fibre morphology.

Specificity of glycoprotein effect

Bovine serum albumin and immunoglobulin G were tested under the same conditions as the tendon glycoprotein, and at a ratio of 200μg/mg of collagen. Neither altered ΔA or $t_{\frac{1}{2}}$ significantly when compared with the control. As far as we know at the moment, therefore, the properties of glycoprotein described above are specific. They are distinct from those of proteoglycan subunit, which, although retarding fibrillogenesis, leads to an increased absorbance (Lowther *et al.*, 1970; Lowther & Natarjan, 1972; Oegema *et al.*, 1975).

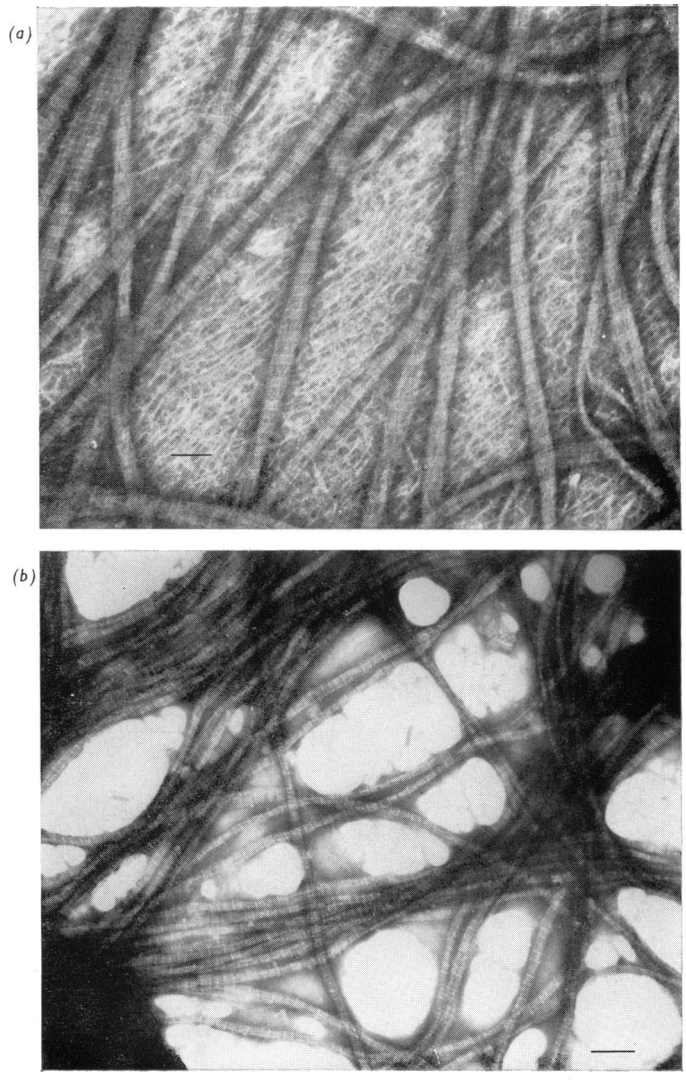
Binding of glycoprotein to collagen

In experiments to find out if glycoprotein was associated strongly with collagen, gels formed in the presence of 0, 60, 100 and 140μg of glycoprotein/mg of collagen were extracted and then digested with proteinase-free bacterial collagenase. Extracts and digests were tested for the presence of glycoprotein by double diffusion against antiserum to glycoprotein. Only the extracts from gels formed with 100 and 140μg of glycoprotein/mg of collagen gave precipitin lines of identity with glycoprotein (Fig. 5*b*). Although the double-diffusion method was sensitive enough to detect glycoprotein extracted from the gel formed in the presence of 60μg of glycoprotein/mg of collagen, the negative result is thought to be due to loss of material during the extraction, dialysis and freeze-drying procedure. The alternative explanation, that about 60μg of glycoprotein/mg of collagen is indeed strongly bound, seems unlikely, since the plots shown in Figs. 2 and 3 do not show steeper gradients up to this concentration ratio. Glycoprotein was not detected in the collagenase digests (Fig. 5*c*). This evidence supports the conclusion drawn above from Figs. 2 and 3 that the glycoprotein only binds weakly to collagen. Weak binding of glycoprotein to collagen was also suggested by the original isolation procedure, in which glycoprotein was extracted by 0.2M-NaCl from the collagenous precipitate formed on dialysis of the 3M-$MgCl_2$ extract of tendon (Anderson, 1975).

The results described above were obtained by using solutions of acid-soluble calf skin collagen. It was observed that glycoprotein also decreased ΔA and increased $t_{\frac{1}{2}}$ of solutions of bovine foetal skin collagen and collagen prepared from calf skin by papain digestion.

Electron microscopy of collagen fibrils

Electron micrographs of collagen fibrils formed in the absence and presence of bovine tendon glycoprotein are shown in Plate 1. It is concluded that the presence of glycoprotein does not alter the periodicity, but significantly decreases the fibril diameter. Detailed measurements of fibril width were made in two sets of experiments. The average of approximately ten measurements on single collagen fibrils was calculated. The average fibril widths (nm) for a number of different fibrils were averaged and the



EXPLANATION OF PLATE 1

Electron micrographs of collagen gels formed in the absence and presence of bovine tendon glycoprotein

(*a*) Collagen alone (no glycoprotein addition); (*b*) addition of 100μg of glycoprotein/mg of collagen. Bovine foetal skin acid-soluble collagen was used. The results shown are from Expt. 2 (see Table 1). Gels were formed as described for Fig. 1, except that the amount of 0.14M-NaCl/0.008M-Na_2HPO_4/KH_2PO_4, pH 7.4, was increased to lower the final collagen concentration to 0.2mg/ml. The mixtures were incubated at 37°C for approx. 30min, and samples for electron microscopy were prepared by touching the surface of gels with carbon-coated Formvar films supported on copper grids. The grids were dried, negatively stained with 2% phosphotungstic acid and examined with a Philips EM 301S electron microscope. Magnification ×57000; the bar represents 100nm.

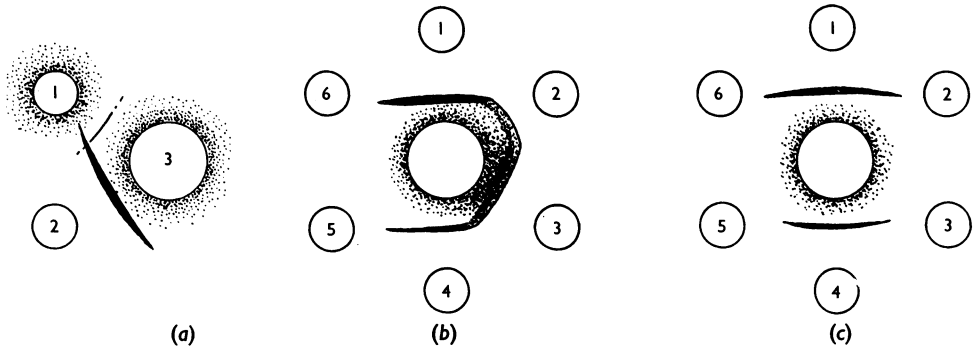


Fig. 5. *Double-diffusion experiments*
(*a*) Well 1, undiluted bovine serum; well 2, glycoprotein (0.3mg/ml); well 3, antiserum to tendon glycoprotein. (*b*) Wells 1 and 4, glycoprotein at 0.3mg/ml and 0.15mg/ml respectively; wells 2, 3, 5 and 6, extracts of gels formed in the presence of 100, 140, 60 and 0μg of glycoprotein/mg of collagen respectively; central well, antiserum to tendon glycoprotein. (*c*) Wells 1 and 4, glycoprotein at 0.3mg/ml and 0.15mg/ml respectively; wells 2, 3, 5 and 6, collagenase digests from gels formed in the presence of 60, 100, 140 and 0μg of glycoprotein/mg of collagen respectively; central well, antiserum to tendon glycoprotein. Double diffusion was carried out in 1.5% agar in a moist atmosphere at room temperature for 3–5 days. Plates were then washed for 7 days with 0.9% NaCl, stained with 0.5% Nigrosine in 5% acetic acid, and destained in 2% acetic acid.

Table 1. *Average fibril diameters for collagen fibrils formed in the absence and presence of bovine tendon glycoprotein*
The average fibril diameter of each collagen fibril was calculated from approximately ten measurements at different places along the fibril. For a number (given in parentheses) of different fibrils, these average fibril diameters were themselves averaged and the standard deviations were calculated.

	Fibril diameter (nm)	
	Collagen	100μg of glycoprotein/mg of collagen
Expt. 1	53.9±10.1 (28)	32.5±7.0 (18)
Expt. 2	33.5± 5.5 (17)	26.6±3.3 (20)

standard deviations were calculated. The results are shown in Table 1. The probability, P, that these means could arise from identical fibril populations was calculated by Student's t test, and it was found that $P<0.001$. An identical result was obtained when the data were treated non-parametrically by using the Mann–Whitney U test (Siegel, 1956).

Control and test samples for each experiment were made up by using the same solutions and both tubes were incubated side by side under identical conditions. However, experiments 1 and 2 were done at different times with different batches of solutions, and different preparations of foetal collagen solution. There may also have been differences in other parameters, e.g. time taken to reach 37°C, and precise length of incubation time. We think that these factors probably account for the different fibril diameters observed in the two experiments. Indeed, Wood & Keech (1960) observed that the final fibril width was sensitive to changes in ionic strength, pH and temperature.

It is therefore apparent that, in the presence of tendon glycoprotein, normal fibrils are formed, but that these are significantly thinner than those formed in the absence of glycoprotein. Wood & Keech (1960) showed conclusively that there was a direct relationship between the final absorbance of the collagen gel and the fibril width as seen by electron microscopy. It seems that a similar situation might exist *in vivo*, since collagen fibrils of cornea are known to be thinner than those of sclera or tendon (Grant *et al.*, 1969). The smaller diameter of collagen fibrils formed in the presence of glycoprotein is therefore presumably responsible for the decreased final absorbance.

Assay of proteinase and collagenase activity

The proteinase activity of glycoprotein was very low. It released only 2% of the activity released by Pronase (200μg). Collagenase activity was completely absent from the glycoprotein preparation, which failed to decrease the flow time of the collagen solution, even after a 60min incubation. We therefore conclude that glycoprotein cannot be influencing fibril formation by acting as a proteinase or collagenase.

Test for serum proteins

Previous evidence indicated that the glycoprotein preparation was free of serum protein (Anderson

et al., 1977). However, more recent studies on double diffusion of antiserum to glycoprotein against undiluted bovine serum, in which precipitin lines were stained by a sensitive method, have detected a weak precipitin line of non-identity with the major antigenic component of glycoprotein (Fig. 5*a*). Thus the tendon glycoprotein preparation contains as a very minor component a protein that occurs in serum. Its concentration in the glycoprotein sample was not high enough to give a precipitin line (Fig. 5*a*). On the other hand, it is clear that the major antigen is not present in serum. Other evidence arguing against a serum origin for tendon glycoprotein is its ability to influence collagen-fibril formation and to aggregate with proteodermatan sulphate, although Anderson (1963) has shown that some serum proteins are able to bind to proteochondroitin sulphate.

General Discussion

The evidence presented here shows that bovine tendon glycoprotein molecules have two effects on fibrillogenesis: they retard the assembly of collagen molecules and they decrease the final fibril diameter if present early enough in the aggregation process. These effects are brought about by a low-affinity interaction between glycoprotein and collagen. We have no evidence to suggest that glycoprotein has such functions *in vivo*. However, it is well known that, in different tissues, fibres of type I collagen are characterized by different fibre diameters (Grant *et al.*, 1969). A possible mechanism for achieving a fibre diameter correct for the function of the tissue would be for the fibroblast to secrete a molecule capable of regulating the width of collagen fibres. It may well be that some glycoproteins fill this type of role, and so might proteoglycans.

The influence of tendon glycoprotein on fibrillogenesis becomes apparent when the ratio of glycoprotein to collagen is quite low. At a higher ratio, 137 μg of glycoprotein/mg of collagen, $t_{\frac{1}{4}}$ is increased to $2t^0_{\frac{1}{4}}$ ($R_{sp.} = 1$), and at 150 μg of glycoprotein/mg of collagen ΔA is decreased to half ΔA_0 ($\Delta A_{sp.} = -0.5$). Although these ratios correspond to 0.65 and 0.71 molecule of glycoprotein (approx. mol.wt. 60000) per molecule of collagen monomer (mol.wt. 285000), it should be emphasized that, because of the low binding affinity, the ratio (glycoprotein bound to collagen)/collagen will be considerably less than this. Possibly the use of a weakly binding regulator molecule would be of advantage to the cell, because molecules would not be continually removed by strong binding to new collagen.

The low binding affinity of tendon glycoprotein for collagen is established beyond doubt by the evidence presented here, and also by the detection of significant amounts of glycoprotein in the initial saline extracts of tendon (Anderson & Labedz, 1977). The fact that part of the total glycoprotein in the tendon can only be released by treatment with 3 M-$MgCl_2$ or by digestion with proteinase-free collagenase (Anderson & Labedz, 1977) suggests that some glycoprotein is sequestered by occlusion within small interfibrillar spaces.

The importance of using a buffer of physiological ionic strength becomes apparent as it is known that tendon glycoprotein binds quite firmly to collagen at zero ionic strength (Anderson, 1975). A similar situation exists with studies on the interaction of α_1 acid glycoprotein and collagen. The binding that leads to the formation of fibrous long-spacing fibres and incorporation of glycoprotein into the fibre (Highberger *et al.*, 1951; Franzblau *et al.*, 1976) occurs at zero ionic strength. It has been reported (Lowther *et al.*, 1970) that only native fibres are formed under physiological conditions. Thus, although it is well known that α_1 acid glycoprotein is present in many tissues (Anderson, 1976), whether it can influence collagen morphology *in vivo* is unknown.

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