Biochem. J. (1970) **116**, 791–796
Printed in Great Britain

Subunits of Tamm–Horsfall Glycoprotein

By F. K. STEVENSON and P. W. KENT
Department of Biochemistry, University of Oxford, Oxford OX1 3QU, U.K.

(*Received* 22 *September* 1969)

1. The effect of 6M-guanidine hydrochloride on the urinary glycoprotein described by Tamm & Horsfall (1952) is to produce a homogeneous subunit of molecular weight approx. 100000. 2. Complete reduction of the disulphide bonds of this subunit does not decrease the molecular weight, suggesting that all disulphide bonds are intrachain. 3. Comparison of sedimentation and viscometric behaviour of unreduced and reduced material in 6M-guanidine hydrochloride is consistent with reduction causing an opening-up of intrachain disulphide bonds to give a more asymmetric molecule.

The principal glycoprotein of urine has been extensively studied since its isolation in a purified state (Tamm & Horsfall, 1952). Its properties have been reviewed (Maxfield, 1966), although the molecular weight derived from viscosity measurements may need revision in view of a recent demonstration of non-Newtonian behaviour (Stevenson, 1968). It appears to be a filamentous molecule (Porter & Tamm, 1955) of very high molecular weight that can aggregate further in the presence of low concentrations of salts, e.g. 0.2M-sodium chloride (McQueen & Engel, 1966). Interest in this glycoprotein was aroused as the result of the report of an increase in viscosity shown by material obtained from patients with cystic fibrosis. Investigations with a low-shear viscometer, however, did not substantiate this (Stevenson, 1969).

Some investigations of the quaternary structure of this glycoprotein have been made. Friedmann & Johnson (1966*a*,*b*) produced an apparently homogeneous subunit of molecular weight approx. 100000 by the action of 50% (v/v) acetic acid. Oxidative cleavage of the disulphide bonds of this subunit produced heterogeneous fragments of molecular weight approx. 32000. It was proposed therefore that the subunit was composed of three or four peptide chains connected by disulphide bonds.

It was decided to reinvestigate the problem by using another denaturant, 6M-guanidine hydrochloride, and also, as the glycoprotein is extensively disulphide-bonded (approx. 50mol of half-cystine/100000g) to ascertain further the role of these disulphide bonds in maintaining structure.

EXPERIMENTAL

Preparation of glycoprotein. Tamm–Horsfall glycoprotein was prepared from pooled urine of normal male adults as described by Stevenson (1968). Samples were stored at 4°C as solutions of approx. 3mg/ml in water with 0.005% thiomersal [Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.] as preservative. Concentrations of thoroughly dialysed samples were determined in duplicate from dry weights.

Analyses. Sialic acid was determined after hydrolysis for 1h at 80°C in 50mM-H_2SO_4 by the thiobarbituric acid assay (Warren, 1959). The component sugars after methanolysis were determined by g.l.c., kindly carried out by Dr J. R. Clamp. For determination of sulphur-containing amino acids, cystine was oxidized to cysteic acid by the method of Moore (1963), with performic acid. Cysteic acid, half-cystine and carboxymethylcysteine were determined with a Locarte Mini Amino Acid Analyser after hydrolysis of samples in 5.7M-HCl in evacuated tubes at 102°C for 15h.

Guanidine hydrochloride was prepared as a 6M solution by the addition of a calculated amount of chilled conc. HCl to A.R.-grade guanidine carbonate (British Drug Houses Ltd., Poole, Dorset, U.K.). The pH was adjusted to 5.4 and the solution shaken with activated charcoal, filtered and the volume adjusted with water. When small amounts of guanidine hydrochloride were required, the biochemical reagent made by British Drug Houses Ltd. was used. The urea used was A.R. grade and solutions were deionized by passage through a filter funnel containing a mixed-bed resin (Amberlite Monobed resin MB-3; British Drug Houses Ltd.).

Viscometry. Viscosity measurements were carried out in a rotating-cylinder viscometer similar to that designed by Zimm & Crothers (1962) and described by Stevenson (1968). To measure relative viscosities in guanidine hydrochloride solutions it was necessary to use a specially prepared set of rotors that had been weighted with extra plasticine. All measurements were carried out at 25°C.

Ultracentrifugation. All ultracentrifugal analyses were in a Spinco model E ultracentrifuge at 25°C. Sedimentation velocities in guanidine hydrochloride solutions were measured by using a synthetic-boundary cell and a rotor speed of 42040 rev./min as described by Tanford, Kawahara & Lapanje (1967). Measurements at sedimentation equilibrium were carried out as described by Yphantis (1964), with a relatively high rotor speed of 23150 rev./min, which ensures that the concentration of

material at the meniscus of the solution column is essentially zero. Rayleigh interference optics and low protein concentrations (0.2–1.0mg/ml) were used. The time required to reach equilibrium was estimated from photographs taken at intervals up to 3½ days. The partial specific volume, $\bar{v}$, of proteins in 6M-guanidine hydrochloride has been found to be about 0.01ml/g less than that in aqueous buffers (Tanford *et al.* 1967). Accordingly, the value used for this glycoprotein was 0.707ml/g (Curtain, 1953) minus 0.01ml/g, i.e. 0.697ml/g. Densities of guanidine hydrochloride solutions were obtained from Kawahara & Tanford (1966*a*).

Polyacrylamide-gel electrophoresis. This was carried out as described by Stevenson & Straus (1968) with slabs of 7% (w/v) gel prepared in 8M-urea. Buffers used were as follows: for pH8, 10mM-tris–1mM-EDTA buffer containing 8M-urea in the inner buffer tanks and 0.2M-tris–10mM-EDTA buffer, pH8.0, in the electrode vessels; for pH5, 10mM-sodium acetate buffer containing 8M-urea in the inner buffer tanks and 10mM-sodium acetate–0.1M-NaCl buffer, pH5.0, in the electrode vessels. Running conditions were 15V/cm for 16h. The gels were stained with Amido Black and cleared electrophoretically (Ferris, Easterling & Budd, 1962). It was necessary for electrophoretic examination of samples that had been treated with 6M-guanidine hydrochloride to remove the denaturant first. This was achieved by dialysis into the appropriate urea-containing buffer for approx. 12h.

Exposure of glycoprotein to 6M-guanidine hydrochloride. When the glycoprotein was to be exposed to guanidine and subsequently examined electrophoretically a sample at approx. 3mg/ml was dialysed into 10mM-tris–1mM-EDTA buffer, pH8.0. A sufficient quantity of solid guanidine hydrochloride was then added, with stirring, to give a final concentration of 6M. The solution was left at room temperature for 4h and was then dialysed into 8M-urea containing the appropriate buffer. If required it could be concentrated by ultrafiltration through 8/32in dialysis casing (Visking) before electrophoresis.

For viscometric and ultracentrifugal analyses, an aqueous solution of glycoprotein (approx. 3mg/ml) was dialysed into freshly prepared 6M-guanidine hydrochloride, pH5.4, at 4°C. The material inside the sac was then concentrated by ultrafiltration to approx. 10mg/ml and allowed to dialyse for a further 2 days with no change of external solution. This prolonged dialysis was found to be necessary to ensure complete equilibration for the subsequent physical measurements. The concentration of material was estimated from the extinction at 277nm. The specific extinction coefficient $E^{1\%}_{1cm}$ in 6M-guanidine hydrochloride had been measured (J. Cleave, personal communication). A dilution series was then prepared by diluting portions of the concentrated material in the sac with external solution, and these were kept at 25°C for 16h before either sedimentation or viscometric analysis.

Reduction of disulphide bonds. For electrophoretic analysis, reduced samples were alkylated with iodoacetamide. Reduction was carried out by adding sufficient dithiothreitol (Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K.) to the glycoprotein in 6M-guanidine hydrochloride, pH8, to give a final concentration of 50mM. Glycoprotein concentration was usually approx. 1.5mg/ml. This solution was left at room temperature for 4h. It was then cooled to 4°C and sufficient iodoacetamide (recrystallized from hot ethanol) added as a 0.525M solution in 0.5M-tris buffer, pH8, to alkylate all the thiol groups and provide a 10% excess. The solution was kept at 4°C for 30min and then thoroughly dialysed with rapid stirring into 8M-urea at the appropriate pH. It could then be concentrated by ultrafiltration and subjected to electrophoresis.

Viscometric and ultracentrifugal analysis was carried out in the presence of reducing agent, and therefore alkylation was not necessary. Reduction in this case was by the addition of β-mercaptoethanol (Koch–Light Laboratories Ltd.) to the glycoprotein in 6M-guanidine hydrochloride. The guanidine solution was first adjusted to pH6.5 with 3M-NaOH and, after dialysis, the glycoprotein concentration was increased to approx. 10mg/ml by ultrafiltration. After estimation of concentration from the extinction, the internal and external solutions were brought to the appropriate concentration with respect to β-mercaptoethanol by the addition of a 2M solution in 6M-guanidine hydrochloride. Dialysis was then continued for 16h at 25°C before preparation of the dilution series as above.

Preparation of an antiserum to Tamm–Horsfall glycoprotein. Three rabbits were each immunized by subcutaneous injections of 6mg of glycoprotein emulsified in complete Freund's adjuvant (Difco Laboratories, Detroit, Mich., U.S.A.). The initial injection was followed 6 weeks later by three subcutaneous booster injections of 2mg of glycoprotein per rabbit, again in adjuvant. Bleeding from an ear vein was 5 days after the final booster injection. Antibody response was assessed by Ouchterlony analysis with 0.5% agar.

RESULTS

Table 1 shows the results of treating native glycoprotein with 6M-guanidine hydrochloride (*a*) before (G T–H) and (*b*) after reduction and alkylation in 6M-guanidine hydrochloride (RAG T–H). Before analysis the treated samples were thoroughly dialysed against water. The yields of material obtained by the procedures used show that there was no significant loss of diffusible components. The carbohydrate analyses also demonstrate, first, that overall carbohydrate composition of native material was similar to that found by Stevenson (1969), and, secondly, that there was no selective loss of sugars by the treatments used.

Fig. 1 shows an electrophoretic analysis of the treated samples on a 7% polyacrylamide gel in 8M-urea, pH8. Both G T–H and RAG T–H appear as single spots, the former running faster than the reduced and alkylated material. The sample in the central slot of the gel is native glycoprotein that has simply been dialysed into 8M-urea, pH8, for 12h before the run. It is rather more concentrated than the material in the other slots, but fails to stain as well. However, slight staining can be seen spreading from the origin to the position of G T–H.

Fig. 2 shows a similar electrophoretic run, conducted in this case in 8M-urea, pH5. All mobilities

Table 1. *Effects of treatment of native glycoprotein with 6M-guanidine hydrochloride and of reduction and alkylation of disulphide bonds in this solvent on carbohydrate composition*

	Yield from native glycoprotein (%)	Carbohydrate composition (g/100g of glycoprotein)					
		Fuc	Man	Gal	GlcNAc	GalNAc	Sialic acid
Native glycoprotein		1.1	6.5	6.5	9.6	1.0	6.1
Glycoprotein treated with 6M-guanidine hydrochloride	90	1.0	6.3	6.1	9.4	0.8	6.2
Glycoprotein reduced and alkylated in 6M-guanidine hydrochloride	86	1.2	7.5	7.3	11.0	1.0	6.0

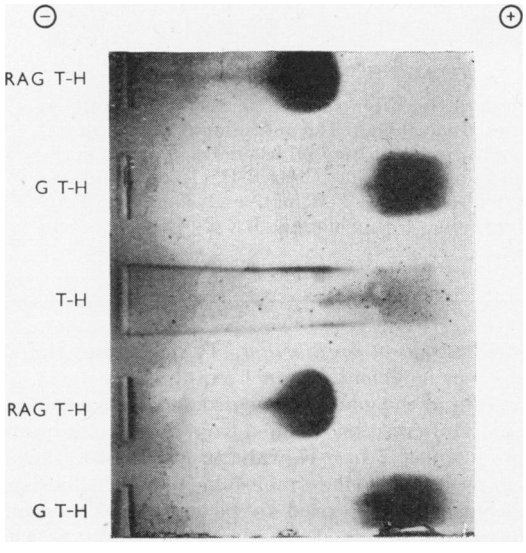

Fig. 1. Electrophoretic pattern given by Tamm–Horsfall glycoprotein, treated with various reagents, in polyacrylamide gel containing 8M-urea–10mM-tris–1mM-EDTA buffer, pH8. RAG T–H, glycoprotein after reduction and alkylation in 6M-guanidine hydrochloride; G T–H, glycoprotein after treatment with 6M-guanidine hydrochloride; T–H, native glycoprotein. Each sample was dialysed into the 8M-urea buffer, pH8, before the run, and concentrations were approx. 3mg/ml.

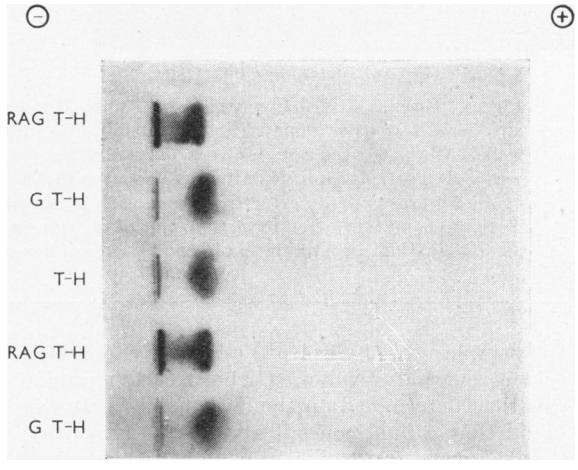

Fig. 2. Electrophoretic pattern given by Tamm–Horsfall glycoprotein, treated with various reagents, in polyacrylamide gel containing 8M-urea–10mM-sodium acetate buffer, pH5. RAG T–H, glycoprotein after reduction and alkylation in 6M-guanidine hydrochloride; G T–H, glycoprotein after treatment with 6M-guanidine hydrochloride; T–H, native glycoprotein. Each sample was dialysed into the 8M-urea buffer, pH5, before the run, and concentrations were approx. 3mg/ml.

were decreased at this pH but single spots were again seen for G T–H and RAG T–H, the former still running very slightly ahead. The native glycoprotein that had been exposed to 8M-urea, pH5, for 12h before the run this time gave a discrete spot indistinguishable from G T–H.

Extent of reduction of disulphide bonds. The total number of residues of half-cystine was determined as cysteic acid after performic acid oxidation of the native glycoprotein. The values obtained from two samples were 49.0 and 51.0mol/100000g of glycoprotein, in good agreement with Friedmann & Johnson (1966*b*). When a sample of native glycoprotein was hydrolysed and analysed directly for liberated cystine on the autoanalyser, a value of 46.5mol of half-cystine/100000g was obtained, thus showing that very little, if any, loss had occurred by direct analysis.

To investigate the extent of reduction by dithiothreitol in 6M-guanidine hydrochloride, final concentrations of 10mM, 50mM and 0.1M were used. The reduced material was alkylated with the appropriate amount of iodoacetamide, its concentration was estimated from the extinction at 277nm,

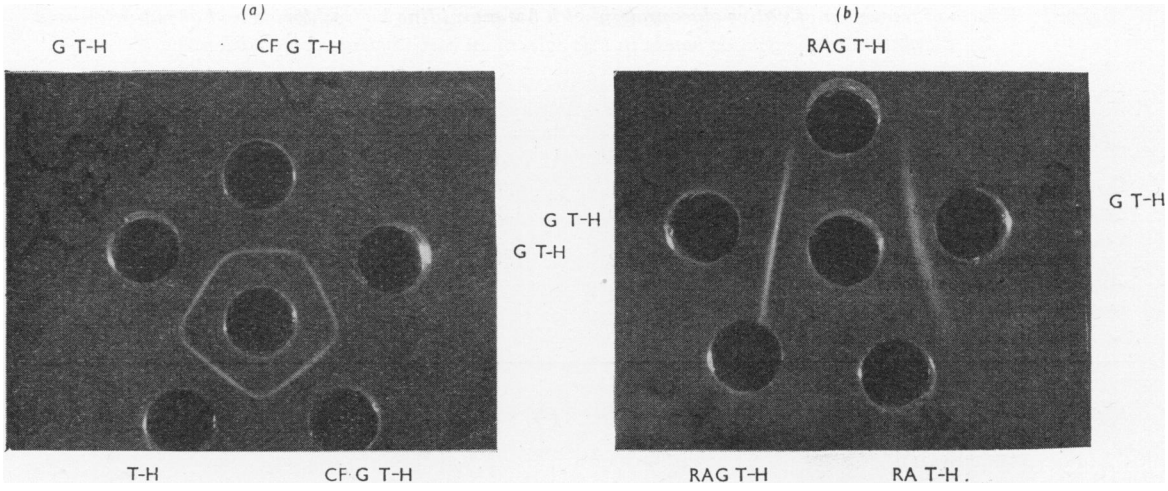

Fig. 3. Immunodiffusion patterns of Tamm–Horsfall glycoprotein, after various treatments, against an antiserum to native human Tamm–Horsfall glycoprotein raised in rabbits. The antiserum is in the central wells. All the treated samples were dialysed against water and adjusted to 1 mg/ml before being placed in the wells. G T–H, glycoprotein after treatment with 6 M-guanidine hydrochloride; CF G T–H, glycoprotein from cystic-fibrosis patients after treatment with 6 M-guanidine hydrochloride; T–H, native glycoprotein; RAG T–H, glycoprotein after reduction and alkylation in 6 M-guanidine hydrochloride; RA T–H, glycoprotein after reduction and alkylation in the absence of denaturant.

and samples were hydrolysed in 5.7 M-hydrochloric acid in evacuated tubes. Carboxymethylcysteine was then determined on the autoanalyser. It was found that a final concentration of 10 mM-dithiothreitol gave 80% reduction, but 50 mM- and 0.1 M-dithiothreitol both gave 100%. Varying the dithiothreitol concentration from 10 mM to 0.1 M did not affect the electrophoretic mobility of the RAG T–H spot; the lowest concentration resulted in a small amount of stainable material remaining near the slot. When reduction was carried out with 10 mM-dithiothreitol in the absence of 6 M-guanidine hydrochloride, only 35% reduction occurred.

Ouchterlony analysis. The G T–H and RAG T–H samples were dialysed into water and used as aqueous solutions of approx. 1 mg/ml. Native glycoprotein was used at the same concentration; it diffused much more slowly and was usually put in the sample hole 24 h before the antiserum and other samples. As shown in Fig. 3(*a*) there is a reaction of identity between native glycoprotein and G T–H. A noteworthy point is that the G T–H prepared from urine of patients with cystic fibrosis also gives a reaction of identity. Fig. 3(*b*) shows that reaction with the antiserum has been largely and possibly completely abolished by reduction and alkylation. Even the 35% reduction that occurs in buffer is seen to be sufficient to prevent formation of a precipitin line at this glycoprotein concentration.

Sedimentation equilibrium. It was found that 3 days was sufficient to reach equilibrium at 23 150 rev./min in the guanidine hydrochloride concentrations. Only results obtained from linear relations of log c against r^2 (see Kawahara & Tanford, 1966*b*) were used to calculate molecular weight. The best concentration appeared to be approx. 0.6 mg/ml. The results obtained are shown in Table 2. The value of approx. 100 000 is in good agreement with that obtained by Friedmann & Johnson (1966*a*) on the 50%-acetic acid subunit. However, reduction by the presence of 0.1 M- or 0.3 M-β-mercaptoethanol clearly caused no diminution in molecular weight.

Measurement of s^0. Sedimentation coefficients at various concentrations between 0 and 9 mg/ml were determined in 6 M-guanidine hydrochloride with and without 0.3 M-β-mercaptoethanol. Single peaks only were observed at all concentrations examined. Fig. 4 shows the values obtained. There is some degree of dependence of s on concentration for both solvents, but clearly the s values are higher for the unreduced material at all concentrations. The extrapolations to zero concentration are hazardous, especially as the curvature seems to increase as the y axis is approached.

Viscometry. The rotating-cylinder viscometer proved suitable for measuring relative viscosities of guanidine hydrochloride solutions. About 1 h was required for the time of rotation to become

Table 2. *Molecular-weight determinations on samples of Tamm–Horsfall glycoprotein in solutions of* 6M-*guanidine hydrochloride and* 6M-*guanidine hydrochloride containing β-mercaptoethanol by the method of sedimentation equilibrium*

Tamm–Horsfall glycoprotein preparation	Solvent	Molecular weight
1	6M-Guanidine hydrochloride	99500
2	6M-Guanidine hydrochloride	115000
1	6M-Guanidine hydrochloride–0.1M-β-mercaptoethanol	101000
2	6M-Guanidine hydrochloride–0.1M-β-mercaptoethanol	99500
3	6M-Guanidine hydrochloride–0.3M-β-mercaptoethanol	107000

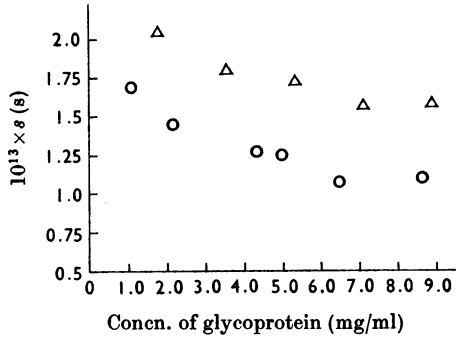

Fig. 4. Ultracentrifugal studies on Tamm–Horsfall glycoprotein in 6M-guanidine hydrochloride, both with and without reduction of disulphide bonds. All sedimentation velocities were determined at 25°C in a synthetic-boundary cell at a rotor speed of 42040 rev./min. △, Glycoprotein in 6M-guanidine hydrochloride; ○, glycoprotein in 6M-guanidine hydrochloride containing 0.3M-β-mercaptoethanol.

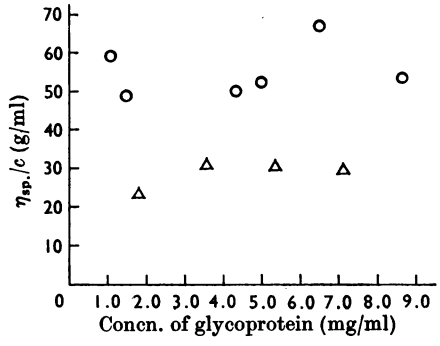

Fig. 5. Viscometric studies on Tamm–Horsfall glycoprotein in 6M-guanidine hydrochloride both with and without reduction of disulphide bonds. All viscosities were determined at 25°C in a rotating-cylinder viscometer. △, Glycoprotein in 6M-guanidine hydrochloride; ○, glycoprotein in 6M-guanidine hydrochloride containing 0.3M-β-mercaptoethanol.

steady after the solution had been put in the viscometer. Fig. 5 shows the plot of reduced viscosity $\eta_{sp.}/c_{(ml/g)}$ against concentration for solutions of glycoprotein in 6M-guanidine hydrochloride with and without 0.3M-β-mercaptoethanol. It is clear that the viscosity of the reduced material is higher than that of the unreduced material at all concentrations examined. The intrinsic viscosity $[\eta]$, i.e. $\lim_{c\to 0} \eta_{sp.}/c$, of the reduced material, estimated by extrapolation to zero concentration of the line of best fit calculated by the least-squares method, was found to be 52.9ml/g. By using the equation relating $[\eta]$ and n (the number of monomer units per polypeptide chain) for random-coil polypeptides (Tanford *et al.* 1967), i.e. $[\eta] = 0.716n^{0.66}$, the molecular weight of the polypeptide chain is estimated as 79550. This only represents 75% of the molecule as there is 25% carbohydrate, which brings the total molecular weight to 106100, i.e. almost exactly that obtained from sedimentation equilibrium.

DISCUSSION

Guanidine hydrochloride is known to be among the most powerful denaturants (Tanford, 1968) and it was decided to examine its effect on the urinary glycoprotein described by Tamm & Horsfall (1952), and also to compare the products obtained with those previouusly obtained after treatment with 50% acetic acid. Polyacrylamide-gel electrophoresis cannot be carried out in the presence of guanidine hydrochloride, but by transferring the unfolded material to 8M-urea it was hoped that significant refolding would not occur. Electrophoresis of such unfolded material showed a single compact spot at pH 8 and at pH 5, suggesting that a homogeneous product had been obtained. Treatment of native glycoprotein with 8M-urea at pH 8 gave an obviously heterogeneous product (Fig. 1), suggesting incomplete denaturation. However, treatment of native glycoprotein with 8M-urea at pH 5 gave a single compact spot with a mobility the same as that of the sample first unfolded in the 6M-guanidine. This suggests that the two methods of denaturation have produced the same product and is further evidence for its being a real subunit.

Determination of the molecular weight of the glycoprotein in 6M-guanidine hydrochloride by sedimentation equilibrium gave a value of approx. 100000, which is almost the same as that obtained by Friedmann & Johnson (1966*a*) in 50% acetic acid and again strongly suggests that this is a subunit. A similar value for the molecular weight of material obtained after treatment of native Tamm–Horsfall glycoprotein with sodium dodecyl sulphate has been recently obtained by A. P. Fletcher and co-workers (personal communication).

This glycoprotein is particularly rich in half-cystine residues (approx. 50mol/100000g), and therefore it was important to clarify their role, especially as Friedmann & Johnson (1966*b*) had suggested that they may be from interchain bridges. Reduction by 50mM-dithiothreitol in 6M-guanidine hydrochloride followed by alkylation with iodoacetamide accounted for all the half-cystine residues as carboxymethylcysteine. The reduced and alkylated product again showed a well-defined spot on polyacrylamide-gel electrophoresis in the presence of 8M-urea, both at pH8 and at pH5. However, the mobility at both these pH values was less than that of the guanidine-treated sample. This could be accounted for by an extensive unfolding of the molecule on reduction of the disulphide bonds, thereby slowing down the rate of movement through the gel.

It is noteworthy that reduction in buffer only, i.e. in the absence of denaturant, caused only 35% of the bonds to be reduced, showing that most are not readily accessible in the native state. However, reduction of even that number was sufficient to abolish antigenic reactivity as judged by the Ouchterlony technique.

Estimation of the molecular weight of the reduced glycoprotein in 6M-guanidine hydrochloride showed again a value of approx. 100000, and this was not decreased by increasing the concentration of β-mercaptoethanol from 0.1 to 0.3M. Thus, unless there is some other covalent bond holding chains together, the disulphide bonds must all be intrachain. Viscosity results confirmed the ultracentrifugal findings. First, viscosities in 6M-guanidine hydrochloride were greater for the reduced than for the unreduced glycoprotein, consistent with the opening-up of the molecule as intrachain bonds are reduced. Secondly, the value for the intrinsic viscosity $[\eta]$ of 52.9ml/g, when substituted in the equation relating viscosities of random-coil polypeptides to number of residues, gives a molecular weight of again approx. 100000. The actual value for the polypeptide chain is about 80000; it is assumed that the carbohydrate side chains are projecting outwards from this polypeptide backbone and do not restrict freedom of rotation.

Thus, if the reduced subunit exists as a random coil in 6M-guanidine hydrochloride, then it appears that the subunit obtained by guanidine treatment is more bead-like, owing to extensive intrachain disulphide bonding. In the native glycoprotein it is conceivable that these bead-like subunits are linked by non-covalent bonds to give the filamentous chains. Indeed, this subunit could correspond to the 'beads' seen in preparations of native glycoprotein in the electron microscope by Porter & Tamm (1955). The number of subunits linked in this way to produce the native material is difficult to estimate owing to inevitable inaccuracies in measuring the molecular weight of such a large macromolecule. However, by using the values obtained previously (Stevenson, 1969) for s^0 and $[\eta]$, and assuming a value of 3.60×10^6 for β, solution of the Scheraga–Mandelkern (1953) equation gives a value of 23×10^6 for molecular weight of the native glycoprotein. This would mean that there are approx. 230 subunits of molecular weight 100000 per macromolecule.

We thank Dr G. T. Stevenson for advice and Dr J. R. Clamp for carrying out the sugar analyses. This work was supported by the Cystic Fibrosis Research Foundation Trust.

REFERENCES

Curtain, C. C. (1953). *Aust. J. exp. Biol. med. Sci.* **31**, 255.
Ferris, T. G., Easterling, R. E. & Budd, R. E. (1962). *Am. J. clin. Path.* **38**, 383.
Friedmann, T. & Johnson, P. (1966*a*). *Biochim. biophys. Acta*, **121**, 292.
Friedmann, T. & Johnson, P. (1966*b*). *Biochim. biophys. Acta*, **130**, 355.
Kawahara, K. & Tanford, C. (1966*a*). *J. biol. Chem.* **241**, 3228.
Kawahara, K. & Tanford, C. (1966*b*). *Biochemistry, Easton*, **5**, 1578.
McQueen, E. G. & Engel, G. B. (1966). *J. clin. Path.* **19**, 392.
Maxfield, M. (1966). In *Glycoproteins*, vol. 5, p. 446. Ed. by Gottschalk, A. Amsterdam: Elsevier Publishing Co.
Moore, S. (1963). *J. biol. Chem.* **238**, 235.
Porter, K. R. & Tamm, I. (1955). *J. biol. Chem.* **212**, 135.
Scheraga, H. A. & Mandelkern, L. (1953). *J. Am. chem. Soc.* **75**, 179.
Stevenson, F. K. (1968). *Biochim. biophys. Acta*, **160**, 296.
Stevenson, F. K. (1969). *Clinica chim. Acta*, **23**, 441.
Stevenson, G. T. & Straus, D. (1968). *Biochem. J.* **108**, 375.
Tamm, I. & Horsfall, F. L., jun. (1952). *J. exp. Med.* **95**, 71.
Tanford, C. (1968). *Adv. Protein Chem.* **23**, 121.
Tanford, C., Kawahara, K. & Lapanje, S. (1967). *J. Am. chem. Soc.* **89**, 729.
Warren, L. (1959). *J. biol. Chem.* **234**, 1971.
Yphantis, D. A. (1964). *Biochemistry, Easton*, **3**, 297.
Zimm, B. H. & Crothers, D. M. (1962). *Proc. natn. Acad. Sci. U.S.A.* **48**, 905.