Tamm–Horsfall Urinary Glycoprotein

THE SUBUNIT STRUCTURE

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1. Subunit molecular weights of 76000–82000 were obtained for native and alkylated Tamm–Horsfall glycoprotein by gel filtration on Sephadex G-200 in the presence of sodium dodecyl sulphate. 2. A further estimate of the subunit molecular weight of 79000 ± 4000 was obtained by disc gel electrophoresis in sodium dodecyl sulphate. 3. A minimum value of the chemical molecular weight of 79000 ± 6000 was obtained from the number of *N*-terminal amino acids released by cyanogen bromide cleavage of the glycoprotein. 4. Similar values were obtained for the subunit molecular weight of Tamm–Horsfall glycoprotein from patients with cystic fibrosis. 5. On ultracentrifugation both in 1.0% sodium dodecyl sulphate and in 70% formic acid, Tamm–Horsfall glycoprotein sedimented as a single component, slightly faster than serum albumin. 6. On reduction of the disulphide bonds the same subunit molecular weight was obtained, which suggested that these bonds are intrachain.

The urinary glycoprotein first characterized by Tamm & Horsfall (1950, 1952) is a filamentous molecule with a molecular weight of several million, possessing a marked tendency to aggregate further (Maxfield, 1961). Viscosity, solubility and light-scattering studies on T–H glycoprotein* have shown that the degree of intermolecular aggregation is influenced by pH, ionic strength and the type of cation, and especially certain bivalent cations (Curtain, 1953; McQueen & Engel, 1966). In several recent studies the subunit structure of the glycoprotein has been investigated in the presence of disaggregating agents. Thus in 50% acetic acid Friedmann & Johnson (1966*a,b*) obtained a single species of molecular weight 94000, and the number of peptides released on tryptic digestion related to the amino acid composition of the glycoprotein also suggested that the molecular weight was of this order. Since ultracentrifugation of the performic acid-oxidized glycoprotein gave several components of molecular weight 32000, they concluded that the subunit of molecular weight 94000 was composed of three or four different polypeptide chains linked by interchain disulphide bonds. On the basis of calculations from the amino acid composition of the glycoprotein, Maxfield & Stefanye (1962) claimed that the ultimate subunit was a single polypeptide chain of molecular weight 28000. A more recent study of the native and the *S*-alkylated glycoprotein in 6*M*-guanidine hydrochloride (Stevenson & Kent,

1970) has suggested that the subunit of T–H glycoprotein is a single polypeptide chain of molecular weight approx. 100000. Electron microscopy of T–H glycoprotein extracted from urinary casts in the presence of sodium dodecyl sulphate has shown disaggregation of the characteristic fibrillar macromolecule and the appearance of small discrete particles (Fletcher, McLaughlin, Ratcliffe & Woods, 1970*a*).

The present paper describes the effect of various chemical modifications on the state of aggregation of the glycoprotein. The subunit molecular weight of the glycoprotein was measured by two methods in the presence of sodium dodecyl sulphate and a further estimate of the molecular weight was obtained by relating the number of *N*-terminal groups liberated by cyanogen bromide cleavage to the methionine content. T–H glycoprotein prepared from patients with cystic fibrosis, claimed by Maxfield & Wolins (1962) to possess a molecular abnormality, was included in these studies.

MATERIALS

Trypsin (bovine pancreas), β -lactoglobulin (bovine) and cyanogen bromide were obtained from Koch–Light Laboratories Ltd. (Colnbrook, Bucks., U.K.), catalase (ox liver), aldolase (rabbit muscle) and creatine kinase (rabbit muscle) were from C.F. Boehringer und Soehne G.m.b.H. (Mannheim, Germany), ovomucoid (hen's egg-white) was from Worthington Biochemical Corp. (Freehold, N.J., U.S.A.), cytochrome *c* (horse heart), sodium

* Abbreviation: T–H glycoprotein, Tamm–Horsfall glycoprotein.

dodecyl sulphate (specially pure) and 2,4,6-trinitrobenzenesulphonic acid were from BDH Chemicals Ltd. (Poole, Dorset, U.K.), ribonuclease (bovine pancreas) and serum albumin (bovine) were from Armour Pharmaceutical Co. Ltd. (Eastbourne, Sussex, U.K.), Eriochrome Black T and Amido Black 10B were from Edward Gurr Ltd. (London S.W.14, U.K.) and Sephadex G-25, G-75 and G-200 and Blue Dextran 2000 were from Pharmacia (G.B.) Ltd. (London W.5, U.K.). α_1 -Acid glycoprotein (human), transferrin (human) and γ -globulin L chain (rabbit) were kindly given by Dr R. W. Jeanloz, Dr D. J. Perkins and Dr J. W. Prahl. Dr R. D. Marshall prepared the ovalbumin (hen's egg-white) and γ -globulin (rabbit).

METHODS

T-H glycoprotein. Both the analytical and molecular-weight studies were carried out on normal T-H glycoprotein II and T-H glycoprotein II from fibrocystic subjects, and their preparation was described in the preceding paper (Fletcher, Neuberger & Ratcliffe, 1970b). Included in the disc gel electrophoresis studies are T-H glycoprotein I, and also T-H glycoprotein III, from a fibrocystic subject, which was prepared from 5400 ml of urine from a fibrocystic adult. (The roman numerals refer to material prepared at different times, but by the same method.) Sodium azide (0.02%) was included as a preservative throughout.

Reduction and alkylation and amino acid analysis. The methods were described in the preceding paper (Fletcher *et al.* 1970b).

Succinylation. The method was based on that of Riordan & Vallee (1964). The molar ratio of succinic anhydride to glycoprotein (where the molecular weight was taken as 100000) was 125 and the pH was maintained at 8.0. The reaction solution was dialysed against 50 mM-KCl in 125 mM-tris-HCl buffer, pH 8.0, followed by water, and recovered by freeze-drying. The extent of amino-group modification was determined with 2,4,6-trinitrobenzenesulphonic acid (Mokrasch, 1967). Non-acylated tyrosine was determined by the spectrophotometric method of Edelhoch (1967) as described by Fletcher *et al.* (1970b). The contribution of *O*-succinyltyrosine to the extinction of the succinylated glycoprotein at 280 and 288 nm is relatively small.

Gel filtration in sodium dodecyl sulphate. Sephadex G-200, swollen in a large excess of water, was equilibrated with 0.5% (w/v) sodium dodecyl sulphate in 50 mM-tris-acetic acid buffer, pH 8.0, for 2 days and packed in an acrylic column of 4 cm² cross-section obtained from Wright Scientific Ltd. (Kenley, Surrey, U.K.). The eluent was pumped upwards through the gel at 5 ml/h by a Chroma-pump (Baird and Tatlock Ltd., Chadwell Heath, Essex, U.K.) and fractions of constant volume in the range 2.0–2.5 ml were collected on a Chromafac fraction collector (Baird and Tatlock Ltd.). The eluent leaving the column was monitored at 256 nm with an L.K.B. Uvicord and had a base-line extinction of 0.1 at 280 nm. Experiments were carried out at room temperature. The void volume, V_0 , was determined from the elution volume of Blue Dextran 2000 and the column was calibrated with the following proteins and glycoproteins of known molecular weight applied separately at a concentration of 3–5 mg/ml: ribonuclease (mol.wt. 13700) and trypsin (mol.wt. 23300)

(both calculated from the amino acid sequence given by Dayhoff & Eck, 1967–68); γ -globulin L chain (mol.wt. 23500) (Rutishauser, Cunningham, Bennett, Konigsberg & Edelman, 1968); ovomucoid (mol.wt. 31500) (Deutsch & Morton, 1961); creatine kinase (mol.wt. 40000) (Dawson, Eppenberger & Kaplan, 1967); ovalbumin (mol.wt. 45000) (Warner, 1954); catalase (mol.wt. 60000) (Sund, Weber & Molbert, 1967); serum albumin (mol.wt. 68000) (Tanford, Kawahara & Lapanje, 1967); transferrin (mol.wt. 90000) (Koechlin, 1952); γ -globulin (mol.wt. 137000) (Cammack, 1962). Provided that the columns were continuously pumped, many chromatographic runs could be obtained. Samples of T-H glycoprotein were either applied directly to the column at a concentration of approx. 3 mg/ml or were extensively dialysed against the buffer before application to the column. When a protein was eluted as more than one peak, the relative peak areas were calculated from the extinction of the eluent at 280 nm.

Disc gel electrophoresis in sodium dodecyl sulphate. Procedure I. The disc gel electrophoresis method of Davis (1964) was modified by increasing the acrylamide concentration from 7.5 to 9.4% and including 0.1% sodium dodecyl sulphate in the gel and electrode buffer (Marshall & Zamecnik, 1969). The protein samples (40 μ g) were equilibrated in the electrode buffer for 2 h before application to the gel, and Bromophenol Blue was added as a tracking dye. Electrophoresis was performed at a constant current of 5 mA per gel. The gels were stained by 1% Amido Black 10B in 7% acetic acid and destained in 7% acetic acid. The following proteins of known molecular weights were chosen to standardize the method: β -lactoglobulin (mol.wt. 18400) (Tanford *et al.* 1967); ovalbumin (mol.wt. 45000); serum albumin (mol.wt. 68000); transferrin (mol.wt. 90000); γ -globulin (mol.wt. 137000). Procedure II was repeated with the sodium dodecyl sulphate concentration increased from 0.1 to 0.5%. It was found necessary to increase the protein applied per gel to 75 μ g.

Procedure II. This method was essentially that of Weber & Osborn (1969), except that the molarity of the gel buffer was halved to decrease the time taken for electrophoresis. The gel consisted of 10% acrylamide and 2.7% cross-linker. Pretreatment of the protein samples involved incubation for 2 h at 37°C in 10 mM-sodium phosphate buffer, pH 7.0, containing (a) 1% sodium dodecyl sulphate, (b) 1% sodium dodecyl sulphate and 8 M-urea or (c) 1% sodium dodecyl sulphate and 1% 2-mercaptoethanol. The samples were then dialysed for several hours against 10 mM-sodium phosphate buffer, pH 7.0, containing 0.1% sodium dodecyl sulphate (and 0.1% 2-mercaptoethanol after pretreatment c). The following standard proteins were pretreated in the same way as the proteins for molecular-weight determinations: trypsin (mol.wt. 23300); aldolase (mol.wt. 40000) (Kawahara & Tanford, 1966); ovalbumin (mol.wt. 45000); catalase (mol.wt. 60000); serum albumin (mol.wt. 68000); transferrin (mol.wt. 90000). The standard proteins (each 20 μ g) and T-H glycoprotein (80 μ g) were clearly visible when stained with a fresh saturated solution of Eriochrome Black T in methanol-water-acetic acid (5:5:1, by vol.). The gels were destained in this solvent. The behaviour of the glycoproteins ovomucoid (mol.wt. 31500) and α_1 -acid glycoprotein (mol.wt. 44000) (Smith,

Brown, Weimer & Winzler, 1950) on gel electrophoresis in sodium dodecyl sulphate was studied. Ovomucoid (80 μ g) stained poorly with Amido Black 10B, but was easily visible when stained with Eriochrome Black T.

Although the two systems of gel electrophoresis in sodium dodecyl sulphate differed in the composition and pH of their buffers and the acrylamide content of the gels, the main difference was in the pretreatment of the protein samples before their application to the gel. In Procedure I, the proteins were pretreated with 0.1 or 0.5% sodium dodecyl sulphate, whereas in Procedure II proteins were pretreated with 1% sodium dodecyl sulphate and also either 8 M-urea or 2-mercaptoethanol. The electrophoretic mobilities of the various proteins studied in both procedures were related to the mobility of Bromophenol Blue. Measurements of a protein band were made from the leading edge of the band, which was always well defined.

Cyanogen bromide cleavage. Iodoacetamide-alkylated T-H glycoprotein from normal and fibrocystic subjects and cytochrome *c* were treated with cyanogen bromide in 70% (w/v) formic acid as described by Steers, Craven, Anfinsen & Bethune (1965) and the peptides were recovered by freeze-drying.

N-Terminal analysis. The peptides produced by cyanogen bromide cleavage were dansylated in 0.5 M-NaHCO₃ (Gray, 1967) and then hydrolysed in 6 M-HCl at 110°C for 10–16 h. In one experiment 8 M-urea was included in the dansylation reaction and the peptides were desalted by gel chromatography on Sephadex G-15 in 0.1% sodium dodecyl sulphate. DNS-amino acids were identified by the two-dimensional chromatographic method described by Woods & Wang (1967) on polyamide layers in their solvents 1, 2 and 3. The combination of solvents 1 and 2 separated leucine and isoleucine, and valine and proline, whereas solvents 1 and 3 were required to separate aspartic acid and glutamic acid. DNS-amino acids in the hydrolysates were identified from their relative positions and by comparison with a standard mixture of DNS-amino acids. These identifications were confirmed by including the authentic DNS-amino acid in a repeat of the chromatography. Clearer chromatograms were obtained if the DNS-amino acids were extracted in ethyl acetate to remove the relatively large amount of 1-dimethylaminonaphthalene-5-sulphonic acid present (Gray, 1967). Those DNS-amino acids not present in the extract were identified in the hydrolysate before extraction or in the residue after extraction.

Ultracentrifugation studies. These studies were carried out with a MSE analytical ultracentrifuge. The sedimentation rates of native and alkylated T-H glycoprotein and albumin were compared in 0.1% sodium dodecyl sulphate, 1.0% sodium dodecyl sulphate and 70% (w/v) formic acid. The protein was dissolved in the solvent immediately before each experiment. The sedimentation coefficients have not been corrected to water at 20°C, nor have corrections been applied for the amount of sodium dodecyl sulphate bound to the protein. Expts. 1–4 (see Table 3) were carried out at 60 000 rev./min and at temperatures of 20.4, 20.0, 23.0 and 22.0°C respectively.

RESULTS AND DISCUSSION

Effect of S-alkylation and succinylation. It was shown by Fletcher *et al.* (1970b) that T-H glyco-

Fig. 1. Plot of elution volume, V_e , against molecular weight for proteins of known molecular weight on a Sephadex G-200 column (4 cm $\times$ 74 cm) in 0.5% sodium dodecyl sulphate in 50 mM-tris-acetic acid buffer, pH 8.0. For experimental details see the Methods section. Glycoproteins are represented by open circles.

protein has a high half-cystine content of approx. 1 per 11 amino acid residues. T-H glycoprotein that had been alkylated with iodoacetate and iodoacetamide dissolved completely in water and various buffers at neutral pH, whereas the aminoethylated glycoprotein was soluble in 0.5 M-acetic acid and almost insoluble in water and buffers at neutral pH. The molecular weight of the alkylated glycoproteins was determined by gel filtration on Sephadex G-200 in either 50 mM-tris-HCl buffer, pH 8.0, or 0.5 M-acetic acid. The alkylated glycoproteins emerged at the void volume of the columns, indicating that their molecular weight was at least 500 000, the nominal exclusion limit of the gel. Succinylation of the native and the iodoacetamide-alkylated glycoprotein greatly increased their solubility in water and buffers at neutral pH. Succinic anhydride reacted with 73% of the lysine residues and 40% of the tyrosine residues of the iodoacetamide-alkylated glycoprotein. This modified protein also emerged at the void volume of a column of Sephadex G-200 in 50 mM-tris-HCl buffer, pH 8.0, indicating that the combined effects of alkylation and succinylation, under the conditions used, fail to produce disaggregation of the glycoprotein.

Molecular-weight determination by gel filtration in sodium dodecyl sulphate. The subunit molecular weights of several proteins have been obtained by gel filtration on dextran gels in the presence of sodium dodecyl sulphate (Kretschner, 1967; Pagé & Godin, 1969a). Sodium dodecyl sulphate is an effective disaggregating agent at low concentrations, but unlike guanidine hydrochloride and urea is readily incorporated in the eluting solvent and appears not to affect greatly the fractionating range

of the gel. The curve obtained by plotting the elution volume, V_e , of the standard proteins and glycoproteins against the logarithm of the molecular weight (Fig. 1) is similar to that obtained for a column of Sephadex G-200 in the absence of the detergent (Andrews, 1965). The volumes V_0 and V_e include a small volume derived from the tubing connecting the pump to the column and the column to the fraction collector. The variations of V_e/V_0 and V_e obtained for a given protein from one column to another are probably due to differences in the extent of swelling of the gel (Andrews, 1965). It was noted that values of V_e/V_0 for a given molecular weight were slightly lower for a Sephadex G-200 column run in sodium dodecyl sulphate. This could be explained by the apparent increase in the Stokes radii of proteins in the presence of sodium dodecyl sulphate (Pagé & Godin, 1969b), possibly due to an increase in the effective size of the proteins caused by adsorption of detergent molecules. Neither creatine kinase, which may require the presence of a reducing agent (Dawson *et al.* 1967), nor catalase was disaggregated to its ultimate subunit in this system. Catalase was eluted as two peaks (10 and 90% of the total peak area), corresponding to the molecular weights of the trimer and dimer of the ultimate subunit. However, catalase was completely disaggregated in the system for disc gel electrophoresis in sodium dodecyl sulphate (procedure II) after pretreatment with 1% 2-mercaptoethanol. The glycoproteins ovomucoid, ovalbumin, transferrin and γ -globulin conformed to the relationship between V_e and logarithm of the molecular weight found for proteins. In the absence of the detergent certain glycoproteins have been found to deviate from this relationship by behaving as if they were larger than their true molecular weight (Andrews, 1965). It was suggested that this was partly due to the shape of the glycoproteins and their elution volumes were more closely related to their diffusion coefficients than to their molecular weights. It is therefore possible that in the presence of sodium dodecyl sulphate the denatured glycoproteins were behaving more closely to the ideal of a globular protein.

Molecular weights obtained for T-H glycoprotein and its various modifications by gel chromatography in the presence of 0.5% sodium dodecyl sulphate are summarized in Table 1. Samples of glycoprotein that had been applied directly to the column dissolved in the eluent gave the same elution profile and molecular weights as samples that had been extensively dialysed against the eluent. Native T-H glycoprotein, however, occasionally gave no peak corresponding to molecular weight 300 000, but only a single peak of molecular weight 76 000. Several modifications of T-H glycoprotein gave more than one peak, and it seemed likely that these

Table 1. *Molecular weights of T-H glycoprotein and its derivatives obtained by gel filtration on Sephadex G-200 in the presence of 0.5% sodium dodecyl sulphate*

	Peak area (%)	Molecular weight
Native T-H glycoprotein	10	300 000
	90	76 000
Native T-H glycoprotein from fibrocystic subjects	20	500 000
	80	77 000
Ethyleneimine-alkylated T-H glycoprotein	50	300 000
	50	137 000
Iodoacetamide-alkylated T-H glycoprotein	100	140 000
Iodoacetate-alkylated T-H glycoprotein	100	81 000
Succinylated T-H glycoprotein	10	500 000
	90	107 000
Succinylated iodoacetamide-alkylated T-H glycoprotein	13	>1000 000
	87	82 000
Succinylated iodoacetate-alkylated T-H glycoprotein	100	210 000

different molecular-weight forms were not in equilibrium but represented relatively stable, partially disaggregated, forms of the glycoprotein. The native glycoprotein, iodoacetate-alkylated glycoprotein and succinylated iodoacetamide-alkylated glycoprotein always gave approximately the same molecular weight. This suggests that the subunit of T-H glycoprotein is a single polypeptide chain with a molecular weight of approx. 76 000–82 000. However, it was noted that T-H glycoprotein alkylated with ethyleneimine or iodoacetamide without subsequent succinylation gave high molecular weights of at least 140 000, suggesting that these modified proteins were incompletely disaggregated. The aminoethylated glycoprotein was only partially soluble in 0.5% sodium dodecyl sulphate and only the soluble portion was applied to the column. Alkylations producing negatively charged substitutions appear to have resulted in complete disaggregation, whereas alkylations producing neutral and positively charged substitutions were only partially disaggregated. Although succinylation of the iodoacetamide-alkylated glycoprotein decreased the molecular weight obtained to 82 000, it increased the molecular weight of the native glycoprotein and iodoacetate-alkylated glycoprotein. The state of aggregation of the glycoprotein in the presence of sodium dodecyl sulphate is therefore not only dependent on the net negative or positive charge of the glycoprotein. Pitt-Rivers & Impiombato (1968) found that the amount of sodium dodecyl sulphate bound to glycoproteins at

equilibrium was related to the polypeptide content and to accessibility of the polypeptide chain since the amount bound increased on reduction and alkylation. Highly charged proteins bound less sodium dodecyl sulphate, and succinylation of serum albumin decreased the amount bound by one-third. These experiments suggest that the highly negatively charged modifications of T-H glycoprotein, namely the succinylated glycoprotein and the succinylated iodoacetate-alkylated glycoprotein, might be expected to bind less sodium dodecyl sulphate, which could be insufficient to produce complete disaggregation.

Since complete reduction and alkylation of disulphide bonds does not decrease the molecular weight, those bonds must be intrachain and therefore not directly involved in the bonding between subunits in the native glycoprotein. Stevenson & Kent (1970) have also concluded that the disulphide bonds are intrachain, obtaining similar subunit molecular weights for the native and the alkylated T-H glycoprotein in the presence of 6M-guanidine hydrochloride.

Native T-H glycoprotein in 0.5% sodium dodecyl sulphate was extensively dialysed to remove sodium dodecyl sulphate (Pitt-Rivers & Impiombato, 1968) and desalted by passage through a column of Sephadex G-25 in 0.2M-acetic acid. On further gel filtration on Sephadex G-200 in the same solvent the glycoprotein was found to emerge at the void volume, indicating that removal of the detergent results in reaggregation to a molecular weight in excess of 500 000.

Molecular-weight determination by disc gel electrophoresis in sodium dodecyl sulphate. In procedure I each standard protein was run on a different gel and in procedure II up to three proteins were run on each gel. All the protein standards in the presence of sodium dodecyl sulphate conformed to the linear relationship between mobility and logarithm of the molecular weight. A series of standard proteins was run for each molecular-weight determination. The results of the subunit molecular-weight determinations are summarized in Table 2. By procedure I the T-H glycoproteins from normal and fibrocystic subjects each gave one main band of molecular weight approx. 105 000. Several minor bands of higher molecular weight than the main band were visible and these were assumed to represent incompletely disaggregated T-H glycoprotein. There were no components present with molecular weights less than that of the main band. Alkylated glycoprotein was of higher molecular weight than the native glycoprotein and several minor components other than the main band were present. The effect of increasing the sodium dodecyl sulphate concentration in the electrode buffer and gel to 0.5% made all protein bands more diffuse, but the molecular

Table 2. *Molecular weights of T-H glycoprotein and other glycoproteins obtained by disc gel electrophoresis in sodium dodecyl sulphate*

For details of procedures I and II see the Methods section.

Procedure I	Native T-H glycoprotein	104 000
	Native T-H glycoprotein from fibrocystic subjects	107 000
	Iodoacetamide-alkylated T-H glycoprotein	110 000
	Iodoacetate-alkylated T-H glycoprotein	140 000
	Ethyleneimine-alkylated T-H glycoprotein	125 000
Procedure II	Pretreatment (a) (1% sodium dodecyl sulphate)	
	Native T-H glycoprotein	98 000
	Iodoacetamide-alkylated T-H glycoprotein	113 000
	Ovomucoid	47 000–58 000
	α_1 -Acid glycoprotein	45 000
	Pretreatment (b) (1% sodium dodecyl sulphate and 8 M-urea)	
	Native T-H glycoprotein	90 000
	Iodoacetamide-alkylated T-H glycoprotein	110 000
	Pretreatment (c) (1% sodium dodecyl sulphate and 1% 2-mercaptoethanol)	
	Native T-H glycoprotein	79 000
	Iodoacetamide-alkylated T-H glycoprotein	98 000
	Succinylated T-H glycoprotein	87 000
	Ovomucoid	32 000–42 000
	α_1 -Acid glycoprotein	45 000

weight of the native T-H glycoprotein was unchanged.

When the native glycoprotein and alkylated glycoprotein were pretreated with 1% sodium dodecyl sulphate, the molecular weights obtained by procedure II were very similar to those obtained by procedure I. Thus in two different electrophoretic systems of different pH similar molecular weights were obtained for the native and the alkylated glycoprotein treated with sodium dodecyl sulphate (1, 0.5 and 0.1%). Pretreatment with 1% sodium dodecyl sulphate and 8M-urea only slightly decreased the molecular weight of the native glycoprotein. However, pretreatment with 1% sodium dodecyl sulphate and 1% 2-mercaptoethanol considerably decreased the molecular weight of the native glycoprotein, but only slightly decreased that of the alkylated glycoprotein. The apparent molecular weight of the native glycoprotein after

this treatment was $79\,000 \pm 4\,000$ from five different gels run on separate occasions. The mobility of the standard proteins after pretreatment with 1% sodium dodecyl sulphate was not significantly altered by pretreatment with 1% sodium dodecyl sulphate and 1% 2-mercaptoethanol. A higher molecular weight was obtained for the iodoacetamide-alkylated glycoprotein compared with the native glycoprotein, which is consistent with the results of gel filtration in 0.5% sodium dodecyl sulphate and is probably due to incomplete disaggregation. Occasionally a minor protein component migrated slightly ahead of the main protein band of the native glycoprotein.

When α_1 -acid glycoprotein, which contains approx. 40% carbohydrate, was pretreated with 1% sodium dodecyl sulphate or 1% sodium dodecyl sulphate and 1% 2-mercaptoethanol, the molecular weights obtained by procedure II were very close to the known value of 44 000 (Table 2). Ovomucoid, which has a molecular weight of 31 500 and a carbohydrate content of approx. 25%, has a high disulphide-bond content similar to T-H glycoprotein. After reduction of the disulphide bonds, the lowest molecular weight obtained was very similar to its known molecular weight. A range of molecular weights is given for ovomucoid in Table 2, since it behaved as a diffuse band on electrophoresis. It should be mentioned that preparations of ovomucoid may also contain ovoglycoprotein (Monsigny, Adam-Chosson & Montreuil, 1968).

A large number of proteins were shown to be separated by gel electrophoresis in sodium dodecyl sulphate according to the molecular weights of their polypeptide chains (Weber & Osborn, 1969). It must be concluded, from the good values obtained for the molecular weight of ovomucoid and α_1 -acid glycoprotein, that the presence of carbohydrate in these glycoproteins does not produce a deviation from the behaviour of polypeptide material alone. Reduction of disulphide bonds by 1% 2-mercaptoethanol would be expected to result in an increase in the sodium dodecyl sulphate bound to a protein (Pitt-Rivers & Impiombato, 1968) and it was noted that a reasonable molecular-weight value for ovomucoid was obtained only after reduction of the disulphide bonds. The lowest molecular weight of human T-H glycoprotein (79 000) was also obtained after the reduction of the disulphide bonds and pretreatment with 1% sodium dodecyl sulphate and is therefore taken as the subunit molecular weight of T-H glycoprotein by this method.

Cyanogen bromide cleavage of T-H glycoprotein. Amino acid analysis of the cyanogen bromide peptides of T-H glycoprotein indicated an almost quantitative (94%) loss of methionine. Attempts to separate the peptides by gel filtration on Sephadex G-75 in either 0.2M-acetic acid or 0.2M-ammonium

hydrogen carbonate indicated that the peptides were highly aggregated, since most of the peptide material emerged at the void volume. On gel filtration on Sephadex G-75 in 6M-urea in 50 mM-tris-HCl buffer, pH 9.6, the peptides appeared to be partially disaggregated, but again most of the peptide material emerged at the void volume. The exclusion limit of Sephadex G-75, nominally 75 000 in aqueous solution, is decreased in the presence of 6M-urea, but even in this solution a peptide must have a molecular weight of at least 20 000 if it is to emerge at the void volume. This suggested that the separation of the cyanogen bromide peptides of T-H glycoprotein might be achieved only in the presence of a dissociating agent. The determination of the molecular weight from the number of cyanogen bromide peptides was therefore abandoned in favour of the following method.

The number of *N*-terminal amino acids released by cyanogen bromide cleavage was measured by identification of their dansyl derivatives. The same number of *N*-terminal amino acids were identified in T-H glycoprotein peptides dansylated in the presence and absence of 8M-urea. The following dansylated *N*-termini were identified in the peptides of T-H glycoprotein from both normal and fibrocystic subjects: arginine, aspartic acid, *S*-carboxymethylcysteine, lysine, tyrosine, phenylalanine, leucine, valine, alanine and threonine. The fluorescent area identified as DNS-threonine was considerably less intense than those of the other DNS-amino acids. Since we were unable to detect an *N*-terminal amino acid in T-H glycoprotein (see Fletcher *et al.* 1970b), the release of ten different *N*-termini by cyanogen bromide cleavage indicates that there are at least ten methionyl peptide bonds/molecule of protein. From the methionine content obtained by amino acid analysis, minimum molecular weights of $79\,000 \pm 6\,000$ and $83\,000 \pm 3\,000$ were calculated for T-H glycoproteins from normal and fibrocystic subjects respectively. Glutamic acid and isoleucine were identified as the *N*-termini of the peptides produced by cyanogen bromide cleavage of cytochrome *c*. These are the amino acids predicted from the amino acid sequence of cytochrome *c* (horse) by Margoliash, Smith, Kreil & Tuppy (1961).

The values of molecular weight obtained by this method could be underestimates, since different peptides with the same *N*-terminal are not distinguished. The dansyl method fails to detect *N*-terminal tryptophan, and DNS-homoserine, derived from a methionylmethionyl dipeptide, is not resolved from DNS-threonine.

Ultracentrifugation studies. In all solvents studied T-H glycoprotein and the *S*-alkylated glycoprotein sedimented as a single component. The sedimentation-velocity studies on T-H glycoprotein are

Table 3. *Comparative ultracentrifugation studies on T-H glycoprotein and serum albumin in sodium dodecyl sulphate and formic acid*

The sedimentation coefficients have not been corrected to water at 20°C, nor have corrections been applied for the amount of sodium dodecyl sulphate bound by the proteins. Abbreviation: SDS, sodium dodecyl sulphate.

Expt. no.	Protein	Concn. of protein (mg/ml)	Solvent	SDS/protein ratio (w/w)	$s_{\text{obs.}}$ (S)
1	Iodoacetate-alkylated T-H glycoprotein	0.6	0.1% SDS	0.17:1	17.9
2	Native T-H glycoprotein	0.43	1.0% SDS	2.3 :1	3.1
	Iodoacetate-alkylated T-H glycoprotein	0.43	1.0% SDS	2.3 :1	2.4
3	Native T-H glycoprotein	0.64	1.0% SDS	1.6 :1	3.5
	Serum albumin	0.63	1.0% SDS	1.6 :1	2.3
4	Iodoacetamide-alkylated T-H glycoprotein	0.6	70% formic acid	—	0.92
	Serum albumin	0.6	70% formic acid	—	0.60

summarized in Table 3. At a sodium dodecyl sulphate/protein ratio (w/w) 0.17:1 the iodoacetate-alkylated glycoprotein gave an observed sedimentation-coefficient value of 17.9S, suggesting that it was still highly aggregated. A sodium dodecyl sulphate concentration of 0.1% produced almost complete disaggregation of the protein in the system for disc gel electrophoresis in sodium dodecyl sulphate for the determination of molecular-weight (procedure I). However, the sodium dodecyl sulphate/protein ratio in the sample applied to the gel was at least 1:1. The observed sedimentation-coefficient value of the iodoacetate-alkylated glycoprotein was decreased to 2.4S in 1.0% sodium dodecyl sulphate at a sodium dodecyl sulphate/protein ratio 2.3:1. Pitt-Rivers & Impiombato (1968) found that native T-H glycoprotein bound 0.44mg of sodium dodecyl sulphate/mg of glycoprotein, and therefore at a sodium dodecyl sulphate/protein ratio less than 0.44:1 optimum binding of the detergent could not occur. The exact relationship, however, between the sodium dodecyl sulphate/protein ratio and the extent of disaggregation of T-H glycoprotein has not been investigated.

The low observed sedimentation-coefficient values obtained in 70% formic acid were probably due to the high density and viscosity of this solvent. Martin (1960) showed that serum albumin was not degraded in 88% (w/v) formic acid since it had a molecular weight of 77 000. In both 1% sodium dodecyl sulphate and 70% formic acid native T-H glycoprotein and the iodoacetamide-alkylated glycoprotein sedimented 50% faster than albumin. These experiments therefore suggest that T-H glycoprotein consists of a single polypeptide chain that is unlikely to have a molecular weight less than that of albumin (68 000).

Gel-filtration studies and disc gel electrophoresis in the presence of sodium dodecyl sulphate have

indicated that the subunit of T-H glycoprotein is a single polypeptide chain of molecular weight approx. 80 000, and a similar value was obtained by a chemical method involving specific cleavage of the protein by cyanogen bromide. On ultracentrifugation of the performic acid-oxidized glycoprotein, Friedmann & Johnson (1966b) obtained several components of molecular weight 32 000 that may have resulted from peptide-bond cleavage during performic acid oxidation (Martin, 1960). Maxfield (1966) claimed that treatment of T-H glycoprotein with formic acid produced a species of molecular weight 27 000; however, the details of this treatment have not been published. Peptide bonds, however, are known to be stable for up to a week in high concentrations of formic acid (Martin, 1960; Steers *et al.* 1965). The subunit molecular weight of approx. 100 000 obtained by Stevenson & Kent (1970) by ultracentrifugation in 6M-guanidine hydrochloride is somewhat higher than the value indicated by the present studies. A value of the subunit molecular weight of 106 100 was also obtained by Stevenson & Kent (1970) from the intrinsic viscosity of the glycoprotein in 6M-guanidine hydrochloride. It was assumed, however, that the carbohydrate prosthetic groups did not contribute to the intrinsic viscosity, which would seem unlikely.

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