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# A 70000-molecular-weight protein isolated from purified pig gastric mucus glycoprotein by reduction of disulphide bridges and its implication in the polymeric structure

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The glycoprotein of pig gastric mucus has been isolated free of non-covalently bound protein as judged by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and equilibrium density-gradient centrifugation. After reduction with 0.2 M-mercaptoethanol, protein was released from the glycoprotein, which consisted of a major 70000-mol.wt. component and a minor 60000-mol.wt. component. The 70000-mol.wt. protein fraction was separated from the reduced glycoprotein by either density-gradient centrifugation in CsCl or by gel filtration. Analysis of the 70000-mol.wt. protein fraction showed that, within the limits of the analysis, it was non-glycosylated, and its amino acid analysis was quite different from that of the reduced glycoprotein, which is high in serine, threonine and proline. There was a ratio of one 70000-mol.wt. protein per native glycoprotein molecule of $2 \times 10^6$ mol.wt. Dissociation of the native glycoprotein into glycoprotein subunits ($5 \times 10^5$ mol.wt.) by reduction or proteolysis results in the release or hydrolysis respectively of the 70000-mol.wt. protein. A similar 70000-mol.wt. protein is demonstrated in human gastric mucus glycoprotein. A structural role for the proteins in these mucus glycoproteins is proposed.

The glycoprotein of pig gastric mucus can be isolated free of non-covalently bound protein, and has a consistent protein content, which is 14% by weight of the total glycoprotein (Starkey *et al.*, 1974; Scawen & Allen, 1977). This glycoprotein, of mol.wt. $2 \times 10^6$, is a covalent polymer of an average of four glycoprotein subunits (mol.wt. $5.2 \times 10^5$) joined by disulphide bridges (Snary *et al.*, 1970). Reductive cleavage of disulphide bridges by 0.2 M-mercaptoethanol or proteolysis, e.g. pepsin or papain, dissociates the $2 \times 10^6$-mol.wt. glycoprotein into the subunits. The proteolytically degraded gastric glycoprotein, also of mol.wt. about $5 \times 10^5$, has lost, as protein, up to 5.6% by weight of the original glycoprotein without loss of carbohydrate (Scawen & Allen, 1977).

In the present paper we describe the isolation and analysis of a protein of mol.wt. 70000, which is joined to pure native pig gastric mucus glycoprotein by disulphide bridges (Pearson & Allen, 1980). On dissociation of the glycoprotein into subunits by reduction or proteolysis, this 70000-mol.wt. protein is either released in the reduced form or lost with proteolytic digestion. A 70000-mol.wt. protein is also released on reduction of the human gastric mucus glycoprotein. This human glycoprotein has previously been shown to have the same overall polymeric structure as the pig gastric mucus glycoprotein (Pearson *et al.*, 1980).

Abbreviation used: SDS, sodium dodecyl sulphate.

## Methods and materials

Mucus gel from pig gastric mucosal scrapings was solubilized in 0.2 M-NaCl by homogenization and the insoluble cell debris removed by centrifugation (Robson *et al.*, 1975). This soluble mucus was fractionated by gel filtration on Sepharose 4B into the excluded glycoprotein fraction and an included protein fraction. The glycoprotein fraction was purified from the remaining free protein by equilibrium density-gradient centrifugation in a CsCl gradient (Starkey *et al.*, 1974; Scawen & Allen, 1977).

Glycoprotein preparations were fractionated by gel filtration on Sepharose 2B columns (100 cm × 2.5 cm) in 0.075 M-sodium phosphate buffer, pH 6.5. The purified native glycoprotein was also fractionated by a second equilibrium density-gradient



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centrifugation in the presence or absence of 0.2M-mercaptoethanol in either (1) 60% (w/v) CsCl or (2) in 60% (w/v) CsCl containing 4M-guanidinium chloride.

Glycoprotein was measured by the modified periodic acid–Schiff method of Mantle & Allen (1978) and protein either by the $A_{280}$ or by the method of Lowry *et al.* (1951). Amino acids were analysed as described in Starkey *et al.* (1974).

Gel electrophoresis in 1% SDS was performed with 7.5% polyacrylamide disc gels (Weber *et al.*, 1972). The sample buffer used was 0.01M-phosphate with or without 0.2M-mercaptoethanol. Proteins were detected by staining with Coomassie Blue.

Exhaustive proteolytic digestion of the glycoprotein (5–10mg/ml) was with papain (Sigma) at 1% (w/w) enzyme:glycoprotein as described by Pearson *et al.* (1980).

Countercurrent immunoelectrophoresis was carried out in 1% agarose gels (type II; Sigma) and 0.1M-barbitone acetate buffer, pH8.6 (Milford-Ward, 1977). Samples (1$\mu$l) in 1mm wells cut 8mm apart were electrophoresed at a field strength of $6\,V \cdot cm^{-1}$ for 90min. After electrophoresis the plates were washed, dried and stained with Amido Black or Coomassie Blue for maximum sensitivity. The method was capable of detecting a human serum albumin standard to a concentration of 10$\mu$g/ml. Mucus fractions were analysed with a variety of rabbit antisera specific for human α-chain (Behring, Seward Diagnostics, and a gift from Mr. L. M. Prior, Colworth House, Unilever Research); human secretory component (Behring, Seward Diagnostics, and a gift from Mr. L. M. Prior); human serum albumin and bovine serum albumin (prepared by S. H. Parry) and whole pig serum (Seward Diagnostics). Bovine serum albumin (Sigma) and human serum albumin (Sigma) were used as standard antigens.

## Results

### *Identification of protein released by reduction of pig gastric mucus glycoprotein*

The native pig gastric mucus glycoprotein was purified from the solubilized mucus by the standard procedure of gel filtration on Sepharose 4B followed by fractionation of the excluded glycoprotein by equilibrium centrifugation in a CsCl density gradient (Starkey *et al.*, 1974). The resulting native glycoprotein was analysed for non-covalently bound protein by SDS/polyacrylamide-gel electrophoresis in the presence and absence of 0.2M-mercaptoethanol. No detectable protein bands were observed by polyacrylamide-gel electrophoresis of the glycoprotein in the absence of mercaptoethanol with a glycoprotein loading of 250–500$\mu$g/gel and staining for protein with Coomassie Blue (Fig. 1). All

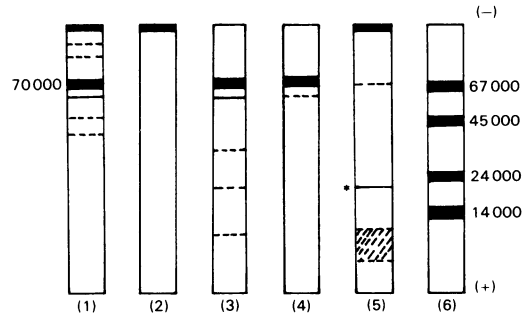


Fig. 1. *Gel electrophoresis of preparations from purified pig gastric mucus glycoprotein*

For details see the text. (1) Purified glycoprotein; reduced in 0.2M-mercaptoethanol. (2) Purified glycoprotein; non-reduced. (3) Protein separated from reduced glycoprotein by equilibrium density-gradient centrifugation in CsCl (fractions 1 and 2, Fig. 1 of Pearson & Allen, 1980). (4) Protein separated from reduced glycoprotein on Sepharose 2B chromatography (fractions 85–110, Fig. 3). (5) Proteolytic digest of glycoprotein with papain (position marked by *), after 24h incubation; reduced in 0.2M-mercaptoethanol. (6) Standard proteins (molecular weights shown).

glycoprotein preparations on polyacrylamide-gel electrophoresis gave a strongly staining band at the origin, where the high-molecular-weight glycoprotein had not entered the gel.

A protein band of 70000 mol.wt. was observed after polyacrylamide-gel electrophoresis of the pig gastric glycoprotein which had been reduced in 0.2M-mercaptoethanol (Fig. 1). Another much weaker-staining protein band of 60000 mol.wt. was also present, and in some preparations two very faint bands, of mol.wts. 30000–40000 and 90000–100000 respectively, were also seen. The 70000-mol.wt. and other protein bands were only seen after reduction of the glycoprotein in 0.2M-mercaptoethanol, and never in the non-reduced preparations. Densitiometric scans of the corresponding polyacrylamide gels from Fig. 1 are shown in Fig. 2. Although only approximate, estimation of the area under the peaks shows that the peak for the 70000-mol.wt. protein represents about 70% of the protein on the gel excluding the reduced glycoprotein band at the origin. This estimate assumes that the proteins stain equally strongly with Coomassie Blue.

Gel filtration of the pig gastric mucus glycoprotein in 0.2M-NaCl gave only a single periodic acid–Schiff-positive peak, which was excluded (fraction $V_0$), with no included material at all (Fig. 3). After reduction in 0.2M-mercaptoethanol, the

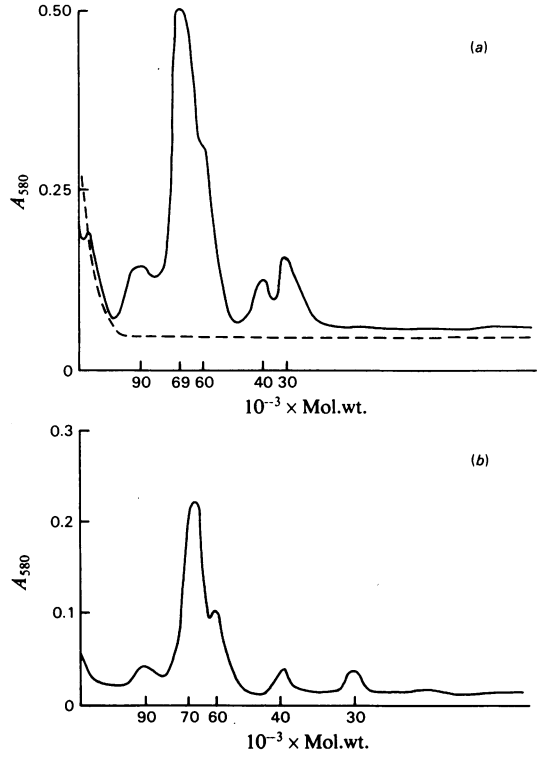


Fig. 2. *Spectrophotometric scans of preparations from purified pig gastric mucus after SDS/polyacrylamide-gel electrophoresis*

Scans are shown for: (*a*) purified pig gastric glycoprotein (——) reduced in 0.2 M-mercaptoethanol or (– – – –) non-reduced; (*b*) protein separated from reduced purified gastric glycoprotein by equilibrium centrifugation in a CsCl density gradient containing 0.2 M-mercaptoethanol. Electrophoresis is from left to right. Numbers on the abscissa show the apparent molecular weights of the main components.

reduced glycoprotein on gel filtration with Sepharose 2B was eluted as a single included glycoprotein peak, with no periodic acid–Schiff-positive material present in the excluded fraction $V_0$, or the totally included fraction, $V_t$. On the basis of previous studies (Mantle & Allen, 1978; Pearson *et al.*, 1980), these results showed that complete reduction of the glycoprotein to subunits had occurred. A protein peak was present in the totally included volume from gel filtration on Sepharose 2B of the reduced glycoprotein only (Fig. 3). These protein-containing fractions, which contained no detectable glycoprotein-positive material, were pooled, freeze-dried and examined by SDS/polyacrylamide-gel electrophoresis. The pattern of protein bands observed was the same as that for the unfractionated reduced glycoprotein (Fig. 1), except that the 70000-mol.wt. band was even more dominant, and the 60000-mol.wt. band was very faint. The glycoprotein digested with papain was eluted from Sepharose 2B as a single included glycoprotein peak in the same position as the reduced glycoprotein. Again, no excluded ($V_0$) or totally included ($V_t$) glycoprotein-positive material was eluted with the papain-digested glycoprotein. The glycoprotein that had been digested with papain and then reduced was found by polyacrylamide-gel electrophoresis to contain only a very faint 70000-mol.wt. protein band, together with some lower-molecular-weight material of about 10000–12000 mol.wt.

The pig gastric mucus glycoprotein was fractionated further by a second equilibrium centrifugation in a CsCl density gradient in the presence and absence of 0.2 M-mercaptoethanol (Pearson & Allen, 1980). Under reducing conditions only, a protein peak in the low-density fractions separated from the reduced glycoprotein. This protein peak contained no detectable glycoprotein-positive material as deter-

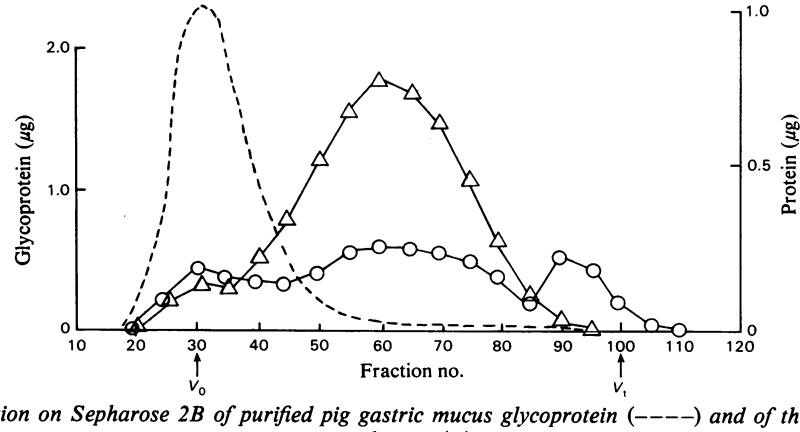


Fig. 3. *Gel filtration on Sepharose 2B of purified pig gastric mucus glycoprotein* (– – – –) *and of the glycoprotein after reduction* (△)

Glycoprotein was measured by the method of Mantle & Allen (1978) and (○) protein by the method of Lowry *et al.* (1951).

mined by the periodic acid–Schiff method. SDS/polyacrylamide-gel electrophoresis of this protein material showed a similar pattern to that seen in the original unfractionated glycoprotein, with the major band a protein, of mol.wt. 70000 (Fig. 1). Repetition of the above experiment with 4M-guanidinium chloride included in the CsCl gradient gave exactly the same results, and in particular no protein was released in 3·0M-CsCl/4M-guanidinium chloride without the presence of 0.2M-mercaptoethanol. The amount of the 70000-mol.wt. protein fraction was compared quantitatively with the amount of reduced glycoprotein from which it had separated in the CsCl/0.2M-mercaptoethanol gradient by the method of Lowry *et al.* (1951) and the periodic acid–Schiff method (Mantle & Allen, 1978) respectively. The 70000-mol.wt. protein fraction released on reduction was found to be between 3.2 and 4.2% by weight (average 3.7%; eight separate experiments) of the total glycoprotein present. This value was confirmed by a comparison of the freeze-dried weights of the protein with that of the total glycoprotein.

The glycoprotein from human gastric mucus was prepared by fractionation of the solubilized mucus by equilibrium centrifugation in a CsCl density gradient only (Pearson *et al.*, 1980). This preparative procedure was not as extensive as that used for pig gastric mucus glycoprotein, since, owing to scarcity of material, it omitted the initial fractionation of the solubilized mucus by gel filtration with Sepharose 4B. On polyacrylamide-gel electrophoresis the non-reduced human gastric mucus glycoprotein still gave protein bands, including a band of 68000 mol.wt. However, after reduction a new strongly staining protein band of 72000 mol.wt. appeared, and the 68000-mol.wt. protein band previously present gained in intensity (Fig. 4).

*Analysis of the protein released by reduction of pig gastric mucus glycoprotein*

The 70000-mol.wt. protein fraction separated from the reduced glycoprotein by equilibrium centrifugation (Pearson & Allen, 1980) was dialysed, freeze-dried and analysed for amino acids (Table 1). This fraction was particularly rich in glycine and glutamic acid, with substantial amounts of cysteine. No galactosamine or glucosamine was detected in this protein hydrolysate after being hydrolysed for 24h at 110°C in 6M-HCl. An analysis of pig serum albumin, included for comparison, showed a significantly different amino acid composition. The 70000-mol.wt. protein fraction differed from albumin particularly in its content of histidine, serine, glycine, valine and leucine.

The 70000-mol.wt. protein fraction released on reduction was analysed by immunoelectrophoresis (Table 2). None of the pig or human glycoprotein preparations or the isolated 70000-mol.wt. protein fractions reacted with antisera to human secretory component, human immunoglobulin A or human immunoglobulin. There was a reaction between

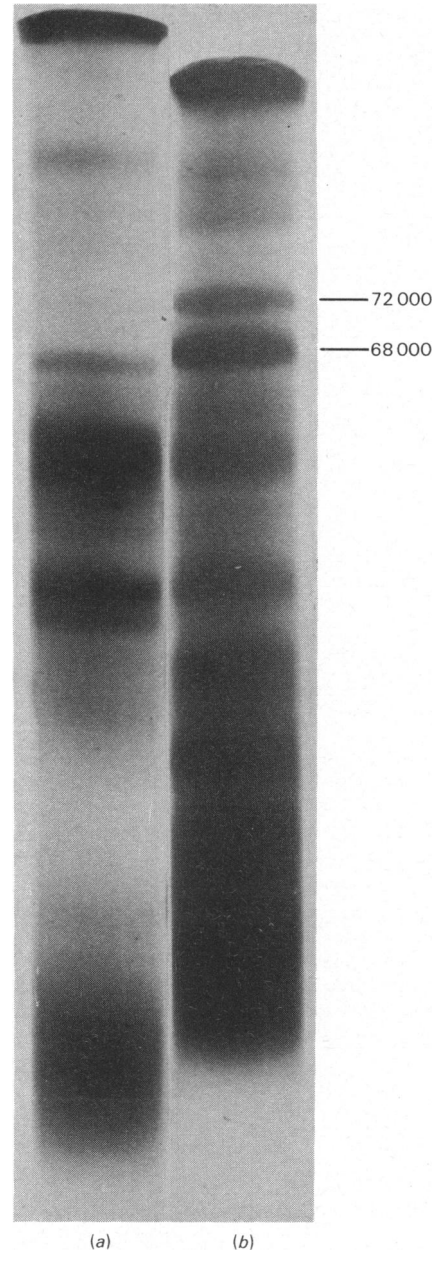


Fig. 4. *SDS/polyacrylamide-gel electrophoresis of partially purified human gastric mucus glycoprotein (a) in non-reducing conditions and (b) in reducing conditions*
The numbers refer to the apparent molecular weights of the components. The gels are aligned to show components of corresponding molecular weight.

Table 1. *Amino acid analysis of the 70000-mol.wt. protein fraction and the gastric mucus glycoprotein before and after reduction with 0.2 M-mercaptoethanol*

The reduced glycoprotein was prepared by equilibrium centrifugation in a CsCl gradient or by gel filtration on Sepharose 2B. The 70000-mol.wt. protein fraction was prepared by equilibrium centrifugation in a CsCl density gradient. The cysteine values are low because the protein was not carboxymethylated before analysis. Values in parentheses are the numbers of different samples on which duplicate analyses were performed.

| | | Reduced glycoprotein | | | | |
|---|---|---|---|---|---|---|
| | | CsCl | | | | |
| Amino acid | Native glycoprotein (μmol/mg) | (μmol/mg) | (% loss) | Sepharose 2B (μmol/mg) | 70000-Mol.wt. protein fraction (μmol/mg) | Pig serum albumin (μmol/mg) |
| His | 0.024 | 0.013 | 44 | 0.015 | 0.111 | 0.255 |
| Lys | 0.035 | 0.016 | 53 | 0.021 | 0.225 | 0.726 |
| Arg | 0.049 | 0.030 | 36 | 0.033 | 0.237 | 0.341 |
| Asp | 0.072 | 0.033 | 52 | 0.038 | 0.615 | 0.725 |
| Thr | 0.190 | 0.170 | 7 | 0.164 | 0.467 | 0.308 |
| Ser | 0.201 | 0.186 | 4 | 0.184 | 0.616 | 0.256 |
| Glu | 0.103 | 0.059 | 41 | 0.061 | 0.877 | 1.006 |
| Pro | 0.175 | 0.155 | 9 | 0.146 | 0.432 | 0.385 |
| Gly | 0.082 | 0.056 | 29 | 0.060 | 0.700 | 0.245 |
| Ala | 0.075 | 0.055 | 24 | 0.059 | 0.563 | 0.678 |
| Cys | 0.043 | 0.012 | 71 | | 0.253 | |
| Val | 0.094 | 0.063 | 31 | 0.065 | 0.600 | 0.377 |
| Met | 0.007 | Trace | — | Trace | 0.052 | Trace |
| Ile | 0.040 | 0.027 | 30 | 0.027 | 0.229 | 0.276 |
| Leu | 0.048 | 0.024 | 48 | 0.023 | 0.490 | 0.805 |
| Tyr | 0.026 | 0.009 | 64 | 0.012 | 0.265 | 0.276 |
| Phe | 0.026 | 0.014 | 44 | 0.015 | 0.254 | 0.400 |
| | (3) | (3) | | (3) | (2) | (4) |

Table 2. *Immunological analysis of glycoprotein preparations by countercurrent electrophoresis*

Glycoprotein preparations were: A, after gel filtration only; B, after two fractionations in CsCl; C, reduced glycoprotein purified free of protein. The human glycoprotein was after one fractionation in CsCl. For details of sources of antisera see the Methods and materials section. (+) and (−) show the presence and absence of stained precipitin lines respectively.

| | Pig | | | | Human | |
|---|---|---|---|---|---|---|
| | Glycoprotein preparations | | | | | |
| Antisera | A | B | C | Isolated reduced protein fraction | Glycoprotein | Isolated reduced protein fraction |
| Human secretory component | | | | | | |
| Behring | − | − | − | − | − | − |
| Colworth | − | − | − | − | − | − |
| Seward | − | − | − | − | − | − |
| Human immunoglobulin | | | | | | |
| Behring | − | − | − | − | − | − |
| Colworth | − | − | − | − | − | − |
| Seward | − | − | − | − | − | − |
| Human immunoglobulin | − | − | − | − | − | − |
| Bovine serum albumin | + | − | − | − | − | − |
| Human serum albumin | − | − | − | − | + | + |

anti-(bovine serum albumin) sera and the impure pig gastric glycoprotein, prepared by gel filtration only, without the CsCl-density-gradient step. There was no reaction, however, between this anti-(serum albumin) serum and the purified pig gastric glycoprotein or the 70000-mol.wt. protein fraction. Reduction of either pig or human serum albumin did not affect their reaction with antisera. Anti-(human serum albumin) sera reacted with both the native human gastric mucus glycoprotein and the protein

fraction isolated from the reduced glycoprotein by equilibrium density-gradient centrifugation in CsCl.

## Discussion

The pig gastric glycoprotein was shown to be free of non-covalently bound protein and in the covalent form of $2 \times 10^6$ mol.wt. by the following criteria: no protein could be detected in the glycoprotein on polyacrylamide-gel electrophoresis under non-reducing conditions (Fig. 1), and no protein was detected in the low-density fractions of a second equilibrium centrifugation in a density gradient of CsCl containing 4M-guanidinium chloride without mercaptoethanol (Pearson & Allen, 1980). On this basis the protein released on reduction of the glycoprotein was taken to be part of the covalent structure of the molecule and joined to it by disulphide bridges. On gel filtration on Sepharose 2B all the gastric glycoprotein was excluded, whereas after reduction or proteolysis all the glycoprotein was eluted as a single included peak (Fig. 3). This showed in these experiments that the starting purified glycoprotein preparation (all excluded) contained no degraded material, whereas reduction or proteolysis had completely dissociated the glycoprotein into subunits (included), as described in previous studies (see Pearson *et al.*, 1980). Previous work has shown that the glycoprotein is a single covalent entity and is not dissociated into subunits by a variety of non-covalent-bond-breaking treatments, including 4M-guanidinium chloride, 2.0M-NaCl, 3.5M-CsCl, or boiling in 1% SDS (Scawen & Allen, 1977).

On reduction of the glycoprotein, protein was released, the principal component of which was identified by polyacrylamide-gel electrophoresis as a protein of 70000 mol.wt. Minor amounts of a 60000-mol.wt. protein were also observed, and it is possible that this protein and some of the others sometimes seen in trace amounts were derived by proteolysis from the main 70000-mol.wt. protein. The 70000-mol.wt. protein band on polyacrylamide-gel electrophoresis represented over 70% of the protein fraction released by reduction, as measured from the densitometric gel scans and assuming that the protein bands all stain equally well with Coomassie Blue. The 70000-mol.wt. protein fraction was isolated from the reduced glycoprotein by two methods: gel filtration on Sepharose 2B (Fig. 3) and equilibrium centrifugation in a CsCl density gradient (Pearson & Allen, 1980). Analysis by polyacrylamide-gel electrophoresis of this isolated 70000-mol.wt. protein fraction showed that the 70000-mol.wt. protein band was the main component, with only trace amounts of the 60000-mol.wt. and other protein bands (Fig. 1).

The amino acid content of the isolated 70000-mol.wt. protein fraction in which the two most plentiful amino acids were glycine and glutamic acid (Table 1) contrasted with the amino acid content of the non-reduced glycoprotein, or even more so that of the purified reduced glycoprotein, which were characteristically high in serine, threonine and proline. The 70000-mol.wt. protein was non-glycosylated, as judged by (1) the absence of periodic acid–Schiff-positive material in the protein fraction when isolated by equilibrium centrifugation in CsCl or by gel filtration on Sepharose 2B (Fig. 3, and Pearson & Allen, 1980) and (2) the absence of galactosamine and glucosamine from the amino acid hydrolysate. The conditions used for amino acid hydrolysis will destroy some of the hexosamines, but, if there was significant glycosylation of the 70000-mol.wt. protein, enough should have survived to be detected.

Comparison of the amino acid analysis of the 70000-mol.wt. protein with that of pig serum albumin (Table 1) showed that it was not serum albumin, a protein of around 70000 mol.wt. that has frequently been associated with mucus secretions (Creeth, 1978). This was supported by the immunological analysis, when no positive reaction was obtained against antisera to bovine or human serum albumin with purified or reduced pig gastric glycoprotein or with the isolated (albeit reduced) 70000-mol.wt. protein fraction (Table 2). However, anti-(bovine serum albumin) serum did react with the impure gastric mucus glycoprotein from the first stage of purification on Sepharose 4B. This showed that pig serum albumin reacted under our conditions with anti-(bovine serum albumin) serum, and that serum albumin, although present in the impure pig gastric mucus glycoprotein, was removed by equilibrium centrifugation in a CsCl gradient and was therefore non-covalently associated with the glycoprotein. Another protein associated with the mucus secretions is secretory immunoglobulin (Clamp, 1977), the secretory component of which is also around 70000 mol.wt. (Tomasi & Bienenstock, 1968). The amino acid composition of the isolated 70000-mol.wt. protein fraction is close to that of secretory component from a variety of sources (Lamm & Greenberg, 1972). However, neither the 70000-mol.wt. protein nor the intact pig gastric glycoprotein cross-reacted with a variety of antisera to human secretory component or to human immunoglobulin A (Table 2). It is possible that pig secretory component will not cross-react with the anti-(human secretory component) serum, or the preparative procedures used for the glycoprotein (e.g. 3.5M-CsCl and reduction in 0.2M-mercaptoethanol) had modified the 70000-mol.wt. protein fraction such that it would not react with the antibody. Therefore the possible identity of the 70000-mol.wt. protein as secretory component cannot be disregarded.

Comparison of the amounts of individual amino acids lost from the glycoprotein on reduction with the amino acid content of the isolated 70000-mol.wt. protein fraction shows a good correlation (Table 1). The protein content, calculated from the amino acid analysis, was 13.3 and 9.5% by weight of the isolated non-reduced and reduced glycoproteins respectively. Therefore 3.7% ($3.8 \times 96.2/100$) by weight of protein is lost from the glycoprotein according to the respective amino acid contents in Table 1. Independent quantitative determination of the protein removed from the glycoprotein on the reducing CsCl density gradient (Pearson & Allen, 1980) was $3.7 \pm 0.5$% by weight of the non-reduced glycoprotein, in excellent agreement with the value calculated from Table 1. With removal of the 70000-mol.wt. protein fraction there is a loss from the glycoprotein of over 30% by weight of all the amino acids except threonine, serine and proline, where about 10% is lost (Table 1). This agrees with the results that have been discussed above, which show that protein and not carbohydrate is lost on reduction, whereas threonine, serine and proline, the amino acids characteristic of the glycosylated region of the protein, would be expected to be conserved in the reduced glycoprotein.

The amount of protein lost from the glycoprotein on reduction (3.7% by weight) is slightly more than 3.5% by weight, the amount that would be equivalent to one 70000-mol.wt. protein for every native glycoprotein molecule of $2 \times 10^6$ mol.wt. The 70000-mol.wt. protein fraction is also removed from the glycoprotein by exhaustive proteolysis. Thus polyacrylamide-gel electrophoresis under reducing conditions of the papain-digested glycoprotein showed that only a trace of the 70000-mol.wt. protein remained (Fig. 1) and the conditions used here for papain digestion were not exhaustive. Data from Scawen & Allen (1977) compiled from the values for the individual amino acids in their Table 3 indicates that protein equivalent to between 4.4 and 5.6% by weight can be removed from the glycoprotein by different proteolytic enzymes, and this is equivalent to a mol.wt. of 88000–112000. It follows that the 70000-mol.wt. protein is a major part of the non-glycosylated protein that is removed from the glycoprotein by proteolysis, although a significant amount of protein which is not removed from the glycoprotein by reduction is still susceptible to proteolysis. Removal of this 70000-mol.wt. protein by either reduction or proteolysis is accompanied by the breakdown of the glycoprotein ($2 \times 10^6$ mol.wt.) into its subunits (about $5 \times 10^5$ mol.wt.), and this was confirmed by gel filtration in these studies.

The 70000-mol.wt. protein in pig gastric mucus glycoprotein is an integral part of the covalent structure of the molecule, with which it is combined on a 1:1 basis. The above data are compatible with a structure where the native glycoprotein is formed by an average of four subunits, each one of which is joined by disulphide bridges to the central 70000-mol.wt. protein. Proteolysis cleaves peptide bonds between these interchain disulphide bridges and the glycosylated portion of each subunit, with digestion of the 70000-mol.wt. protein and release of glycoprotein subunits. A less likely model, where the glycoprotein subunits are joined to each other by disulphide bridges and the 70000-mol.wt. protein is separately attached, cannot be ruled out. In our previous model for the structure of pig gastric mucus glycoprotein, all the non-glycosylated protein was depicted as part of the peptide chains of the four subunits joined together by disulphide bridges (Allen, 1978; Scawen & Allen, 1977). The present results show that a large part of this non-glycosylated peptide is a separate peptide chain of 70000-mol.wt. joined to the subunit peptide chains by disulphide bridges (Allen *et al.*, 1980).

Owing to shortage of material, human gastric mucus glycoprotein, which has an overall polymeric structure like that from pig (Pearson *et al.*, 1980), was purified by a one-step procedure of equilibrium centrifugation in a CsCl gradient. This purified human gastric mucus glycoprotein still contained protein bands on polyacrylamide-gel electrophoresis in non-reducing conditions (Fig. 4), including a band at 68000 mol.wt. On reduction the 68000-mol.wt. protein band gained in intensity and a new protein band of 72000 mol.wt. appeared. The non-reduced glycoprotein and the protein released on reduction, and separated by equilibrium centrifugation in a CsCl gradient, both reacted with anti-(human serum albumin) serum, showing that serum albumin was associated with the human gastric mucus glycoprotein. The presence of serum proteins in mucus secretions has been previously reported (see Creeth, 1978). Neither human gastric glycoprotein nor the protein released on reduction reacted with the various antibody preparations to human secretory components. Although the original impurity of the human gastric mucus glycoprotein makes interpretation of exactly what is released uncertain, the results do show that a strongly staining protein band of about 72000 mol.wt. does appear after reduction. The 68000-mol.wt. band could well be the serum albumin, and if this is so then the greater intensity of this band after reduction suggests some of it could be covalently bound to the glycoprotein. On dissociation of pig small-intestinal glycoprotein into subunits by reduction of the disulphide bridges, a 90000-mol.wt. protein is released, and in amounts sufficient for one protein molecule for every native glycoprotein molecule (Allen *et al.*, 1980; Mantle *et al.*, 1981).

From the model proposed above for pig gastric mucus glycoprotein, these proteins of 70000 and

90000 mol.wt. would have a central structural role in gastrointestinal mucus glycoproteins. This may also have important implications for other mucus glycoproteins that depend on disulphide bridges for their structure. Such glycoproteins are known to occur elsewhere in the gastrointestinal tract (Allen, 1978), the respiratory tract (Roberts, 1976) and the cervical tract (Gibbons, 1978). In detailed studies on bronchial glycoproteins, purified by density-gradient centrifugation in CsBr and then CsCl, Creeth *et al.* (1977) observed the release of variable amounts of protein and a substantial decrease in molecular weight on thiol reduction. Other workers have reported the release of proteins from respiratory-mucus glycoproteins after reduction (Roberts, 1976; Rose *et al.*, 1979), but here the glycoproteins had not been purified in a CsCl density gradient and were not shown to be free of non-covalently-bound protein before reduction.

Connective-tissue proteoglycan aggregates have been shown to consist of an ionic complex of proteoglycan, hyaluronic acid and link protein (Hascall & Sajdera, 1969). Similarly, from studies on respiratory mucus, a cross-linking protein between the glycoprotein molecules within the gel has been proposed (Roberts, 1976). The structure described in the present paper is different from either of those structures in that it describes a single covalent entity, namely native pig gastric mucus glycoprotein of mol.wt. $2 \times 10^6$. The 70000-mol.wt. protein is part of this covalent structure and does not provide a link between the native glycoprotein molecules in the gel.

## References


Allen, A. (1978) *Br. Med. Bull.* **34**, 28–33

Allen, A., Mantle, M. & Pearson, J. P. (1980) in *Perspectives in Cystic Fibrosis* (Sturgess, J. ed.), pp. 102–112, Canadian Cystic Fibrosis Foundation, Toronto

Clamp, J. R. (1977) *Biochem. Soc. Trans.* **5**, 1579–1581

Creeth, J. M. (1978) *Br. Med. Bull.* **34**, 17–24

Creeth, J. M., Bhaskar, K. R., Horton, J. R., Das, I., Lopez-Vidriero, M. T. & Reid, L. (1977) *Biochem. J.* **167**, 557–569

Gibbons, R. A. (1978) *Br. Med. Bull.* **34**, 34–38

Hascall, V. C. & Sajdera, S. W. (1969) *J. Biol. Chem.* **244**, 2384–2396

Lamm, M. E. & Greenberg, J. (1972) *Biochemistry* **11**, 2744–2750

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Mantle, M. & Allen, A. (1978) *Biochem. Soc. Trans.* **6**, 607–609

Mantle, M., Mantle, D. & Allen, A. (1981) *Biochem. J.* **195**, 277–285

Milford-Ward, A. (1977) in *Techniques in Clinical Immunology* (Thompson, R. A., ed.), pp. 1–24, Blackwell Scientific Publications, Oxford

Pearson, J. P. & Allen, A. (1980) *Biochem. Soc. Trans.* **8**, 388–389

Pearson, J. P., Allen, A. & Venables, C. W. (1980) *Gastroenterology* **78**, 709–715

Roberts, G. P. (1976) *Arch. Biochem. Biophys.* **173**, 528–537

Rose, M. C., Lynn, W. S. & Kaufman, B. (1979) *Biochemistry* **18**, 4029–4037

Robson, T., Allen, A. & Pain, R. H. (1975) *Biochem. Soc. Trans.* **3**, 1105–1107

Scawen, M. & Allen, A. (1977) *Biochem. J.* **163**, 363–368

Snary, D., Allen, A. & Pain, R. H. (1970) *Biochem. Biophys. Res. Commun.* **40**, 844–851

Starkey, N. J., Snary, D. & Allen, A. (1974) *Biochem. J.* **141**, 633–639

Tomasi, T. B. and Bienenstock, J. (1968) *Adv. Immunol.* **9**, 1–96

Weber, K., Pringle, J. R. & Osborn, M. (1972) *Methods Enzymol.* **26**, 3–27