The Isolation of a High-Molecular-Weight Glycoprotein from Pig Colonic Mucus

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The principal components of a variety of mucous secretions have been found to be glycoproteins, many of mol. wt. 500,000 or more (Gottschalk, 1972). We have isolated the glycoprotein from pig colonic mucus and examined its structure in order to compare it with that of the well-characterized glycoprotein of the gastric mucus from the same species (Snary & Allen, 1971; Allen, 1977).

Mucosal scrapings were prepared from the washed colons of freshly slaughtered pigs. After exhaustive dialysis of the scrapings against water containing 0.02% azide, the water-soluble mucus was separated from the insoluble mucous gel and cell debris by squeezing through muslin followed by centrifugation (Fig. 1). The water-soluble mucus, which represented 70% dry wt. of the non-diffusible mucus, contained 72% by wt. protein, 10% by wt. nucleic acid and 4.6% by wt. glycoprotein. Nucleic acid was completely removed by digestion of the water-soluble mucus (concentration 4.7 mg/ml) with proteinase-free bovine pancreatic ribonuclease (EC 3.1.4.22; final concentration 15 μg/ml) and ox pancreatic deoxyribonuclease (EC 3.1.4.5; final concentration 2.5 μg/ml). Incubation was for 17 h at 37°C, after which all the nucleic acid was diffusible.

The non-covalently bound protein was separated from the glycoprotein by equilibrium density-gradient centrifugation in CsCl (Starkey et al., 1974). Owing to the high ratio of non-covalently bound protein to glycoprotein, it was necessary to repeat this step (on the recovered glycoprotein) in order to achieve complete separation of the two (Fig. 1). The purified glycoprotein was subsequently fractionated into a high-molecular-weight glycoprotein A (excluded) and a low-molecular-weight glycoprotein fraction B (included) by gel filtration on Sepharose 4B. The high-molecular-weight glycoprotein A was the principal component, accounting for 84% of the glycoprotein recovered from the Sepharose column. The low-molecular-weight minor component, the glycoprotein fraction B, has a similar ratio of sugars to glycoprotein A (Table 1) but a higher protein content (40% by wt.).

The high-molecular-weight glycoprotein A had a sugar analysis characteristic of glycoproteins from other mucous secretions (Gottschalk, 1972) with both sulphate and sialic acid present (Table 1). The glycoprotein possessed A blood-group activity (1.25 μg/0.1 ml; assayed by the method of Kabat & Bezer, 1945) which was doubled (0.63 μg/0.1 ml) by removal of the sialic acid residues with neuraminidase. A full complement of amino acids was present, although threonine, serine and proline comprised 54% of the total residues. It is noteworthy that the blood-group activity and both the sugar and the amino acid composition of the colonic glycoprotein are similar to that of the glycoprotein from pig gastric mucus (Starkey et al., 1974), except that the colonic glycoprotein has more sialic acid and less fucose and galactose. Further similarities between the glycoproteins from the two mucous secretions were apparent after Pronase digestion. The Pronase-digested glycoprotein from colonic mucus was separated from the peptides and added Pronase by equilibrium density-gradient centrifugation in CsCl. Analysis of the Pronase-digested glycoprotein showed that carbohydrate had been conserved, but 29.8% of the peptide was lost. Threonine, serine and proline comprised 75% of the remaining amino acids, showing a proportionately bigger loss in the other amino acids. This result points to the presence of non-glycosylated and glycosylated regions of the peptide core in the colonic glycoprotein similar to that found for glycoproteins from other mucous secretions (Donald, 1973; Scawen & Allen, 1975). The presence of sialic acid and sulphate together in the same glycoprotein has been shown for other glycoproteins from mucous secretions, for example sheep colonic mucus (Kent & Marsden, 1963) and pig gastric mucus (Starkey et al., 1974).
Pig colon: wash away contents, scrape mucus from surface, dialyse against water (+ 0.02% azide).
Centrifuge (2.3 × 10^4 g, 10 min)

Water-soluble mucus

Water-insoluble mucus

Nuclease digestion

Equilibrium density-gradient centrifugation in CsCl

Glycoprotein (bottom of tube) Free protein (top of tube)

Equilibrium density-gradient centrifugation in CsCl

Glycoprotein (bottom of tube) Residual free protein (top of tube)

Sepharose 4B in 0.2 M-NaCl

Glycoprotein A Glycoprotein fraction B

Fig. 1. Isolation and purification of the glycoproteins from pig colonic mucus

On sedimentation-velocity analysis, the colonic glycoprotein A ran as a single peak, hypersharpt at high concentrations and polydisperse at low concentrations. By combination of s_{20, w} and D_{20, w} (Table 1) the molecular weight was estimated as 15.1 × 10^6 (Table 1). The 0.2 M-mercaptoethanol-reduced and Pronase-digested glycoproteins each ran as single peaks on sedimentation-velocity analysis with mol.wts.
Table 1. Chemical and physical properties of the high-molecular-weight glycoprotein from pig colonic mucus

The results are average values from at least two quite separate preparations of glycoprotein, each formed by pooling material from three different batches of colons. Methods of chemical and physical analysis were those of Starkey et al. (1974). The sugar analyses, except sialic acid, were by g.l.c.

### Chemical properties

| Carbohydrate          | Fucose, 10.4% | galactose, 20.8% | glucosamine, 23.9% | galactosamine, 8.3% | sialic acid, 3.2% |

### Physical properties

<table>
<thead>
<tr>
<th>Solvent</th>
<th>$S_{25,w}$ (S)</th>
<th>$10^{-6} \times \text{Mol.wt.}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native glycoprotein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.18M-KCl/0.02M-potassium acetate, pH 5.5</td>
<td>86.9</td>
<td>15.1</td>
</tr>
<tr>
<td>0.18M-KCl/0.2M-sodium veronal/0.2M-mercaptoethanol, pH 8.5</td>
<td>33.7</td>
<td>6.0</td>
</tr>
<tr>
<td>6M-Guanidinium chloride</td>
<td>52.3</td>
<td>-</td>
</tr>
<tr>
<td>6M-Guanidinium chloride/0.2M-mercaptoethanol</td>
<td>33.0</td>
<td>-</td>
</tr>
</tbody>
</table>

| Pronase-digested glycoprotein    |                |                             |
| 0.18M-KCl/0.02M-potassium acetate, pH 5.5 | 20.2           | 1.51                        |
| 0.18M-KCl/0.02M-sodium veronal/0.2M-mercaptoethanol, pH 8.5 | 13.5           | 0.76                        |

of 6.0×10⁶ and 1.5×10⁶ respectively. However, the lowest-mol.wt. subunit (0.76×10⁶) was obtained only after digestion with Pronase followed by treatment with 0.2M-mercaptoethanol. Thus the glycoprotein from pig colonic mucus is a polymer of smaller glycoprotein units. The present data do not show the extent by which these glycoprotein subunits are held together by covalent bonds in contrast with non-covalent interactions. The high $S_{25,w}$ value for the glycoprotein in 6M-guanidinium chloride compared with that in 0.2M-mercaptoethanol/6M-guanidinium chloride (Table 1) suggests that, whatever the nature of the bonds holding the subunits together, they are not easily disrupted by solvents known to break non-covalent interactions. Pronase digestion of the insoluble mucous gel, followed by a similar purification procedure to that outlined for glycoprotein A (Fig. 1), also yielded a single glycoprotein with a mol.wt. of 0.7×10⁶ in the presence of 0.2M-mercaptoethanol.

Thus polymerization in the glycoprotein purified from colonic mucus is more complicated than that found in the glycoprotein from pig gastric mucus. In the latter, the native glycoprotein has a mol.wt. of 2×10⁶ and is comprised of four subunits joined by disulphide bridges (Starkey et al., 1974; Allen, 1977). It is notable that the lowest-molecular-weight unit obtained from proteolytic digestion and/or reduction of the glycoprotein from human ovarian-cyst mucus (Donald, 1973), bovine cervical mucus (Gibbons & Sellwood, 1972), pig gastric mucus and pig colonic mucus all have a mol.wt. of about 500000. This may reflect some pattern in the polymerization of these glycoproteins from different mucous secretions.


1977
Simultaneous Incorporation of N-Acetylglucosamine and N-Fluoroacetylglucosamine into Hyaluronic Acid by Mammalian Cells

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Double-labelling experiments have been carried out with rabbit tracheal explants maintained under culture conditions (Gallagher & Kent, 1975) in the presence of N-fluoroacetyl[1-14C]glucosamine (GlcNAcF, synthesized by Dr. C. G. Butchard of this laboratory) and N-acetyl[1-3H]glucosamine (GlcNAc) at similar concentrations but different specific radioactivities.

After 96h, the soluble macromolecular products in the culture fluid were treated with papain and were separated on DEAE-cellulose. The hyaluronic acid fraction (HA) was examined by zone electrophoresis on cellulose acetate strips at pH8.6 and found to be a single component. This fraction (HA) was doubly labelled, the 14C/3H ratio being 1:0.088 (±0.007, mean of four experiments±s.e.m.), compared with the ratio of 1:0.117±0.001 in the original culture fluid.

Labelling evidence indicated that both monosaccharides had been incorporated at similar rates into the same hyaluronic acid chains. Incubation with a mixture of testicular hyaluronidase, β-glucuronidase and N-acetyl-β-glucosaminidase (Linker et al., 1955) resulted initially in the preferential release of GlcNAc (30% of the 3H radioactivity in the incubation mixture). No free GlcNAcF was detected, the 14C radioactivity being retained in the undegraded oligosaccharide (14C/3H ratio, 1:0.094, average of two experiments).

Repetition of the hydrolysis of the oligosaccharide fraction with the mixture of the same enzymes resulted in further release of GlcNAc (48% of 3H radioactivity in the second incubation mixture) and a small amount of GlcNAcF (4% of the 14C radioactivity in the incubation mixture). The two monosaccharides were clearly separated by paper chromatography. Most of the 14C radioactivity (GlcNAcF) was retained in the remaining oligosaccharide fraction, which had a 14C/3H ratio still further enriched in 14C (14C/3H 1:0.113). Evidence was sought for possible deacylation or transacylation. The conversion of GlcNAcF by metabolic transformation into GlcNAc in the HA fraction was of the order of 20%. Further studies have been made with N-fluoro[3H]-acetyl-b-glucosamine (side-chain-labelled) to elucidate these transformations. Chemical hydrolysis was consistent with the structure inferred, i.e. a hyaluronic acid analogue in which N-acetyl and N-fluoroacetyl substituents are present on glucosamine residues. It is estimated that about one glucosaminyl residue in four carries a fluoroacetyl residue.

Kent & Mora (1973a,b) showed GlcNAcF to be an inhibitor of glucosamine utilization in transformed mouse cell lines. Later it appeared (Schultz & Mora, 1975) that the fluoro analogue might inhibit the intracellular synthesis with a number of sugar nucleotides, including UDP-N-acetylglucosamine and CMP-N-acetylneuraminic acid from labelled glucosamine. Further studies in the course of the present work show that this is due to isotopic dilution. The effects of the fluoro analogue are explicable if it undergoes phosphorylation and is converted into fluorosugar nucleotide analogues. Such a hypothesis is in keeping with the present finding that fluoroacetylglucosamine can be incorporated into a polysaccharide chain such as hyaluronic acid.