Glycoconjugate Journal (1991) **8**:330–339

Characterization of the major and minor mucus glycoproteins from bovine submandibular gland

ANTHONY P. CORFIELD[1]*, CLARICE DO AMARAL CORFIELD[1], RÜDIGER W. VEH[2], SUSAN A. WAGNER[1], JOHN R. CLAMP[1] and ROLAND SCHAUER[3]

[1] *Department of Medicine, Bristol Royal Infirmary, Bristol BS2 8HW, UK*

[2] *Abteilung für Neuroanatomie, Ruhr-Universität Bochum, Universitätsstr. 150, D-4630 Bochum, Germany*

[3] *Biochemisches Institut, Christian-Albrechts-Universität, Olshausenstr. 40, D-2300 Kiel, Germany*

Received 21 February 1991

Two mucins were isolated from bovine submandibular glands and termed major and minor on a quantitative basis. The major mucin representing over 80% of the total glycoprotein fraction contained 37% of its dry weight as protein in contrast to 62% for the minor mucin. Differences in the amino acid composition reflected the higher proportion of typically non-glycosylated peptide in the minor mucin. The molar ratio of *N*-acetylgalactosamine to serine plus threonine was 0.82 in major and 0.65 in minor mucins, indicating a lower degree of substitution of potential glycosylation sites in the minor mucin.

Differences in the carbohydrate composition were found largely related to the sialic acids, with higher relative amounts of *N*-glycoloylneuraminic acid in the minor mucin. In addition, the proportion of di-*O*-acetylated sialic acids was higher in the major mucin. The rate of sialidase action on the two mucins could be correlated with the content of *N*-glycoloylneuraminic acid in each glycoprotein. There was no difference in the type of oligosaccharide found in each mucin and the differences in relative proportions reflected the monosaccharide composition for the two mucins. Gel filtration on Sepharose CL 2B showed a lower molecular weight distribution for the minor in contrast to the major mucin which was partially excluded. Density gradient centrifugation reflected this variation. SDS-PAGE demonstrated a regular banding pattern for the major mucin with a lowest subunit size of 1.8×10^5 Da and aggregates in excess of 10^6 Da, while the minor mucin ranged from 3.0×10^5 to 10^6 Da. The chemical composition of the isolated mucins was compared with previous histochemical analysis of mucin distribution in bovine submandibular glands and indicates a possible cellular location for each mucin.

Keywords: mucin, sialic acids, submandibular gland, glycoproteins, oligosaccharides, sialidases

Abbreviations: PBS, 0.01 M sodium phosphate buffer, pH 7.3, containing 0.15 M NaCl; Neu5Ac, *N*-acetylneuraminic acid; Neu5Gc, *N*-glycoloylneuraminic acid; GalNAc-ol, *N*-acetylgalactosaminitol.

The histochemical analysis of mucus glycoproteins (mucins) in the bovine submandibular gland has suggested the occurrence of two glycoproteins each located in a different cell type, either the mucous or serous cells in this tissue [1, 2]. Little biochemical data are available on the distribution of the mucins within these cells. However, the isolation of a mixed mucin fraction obtained from whole glands has been reported [3] and this could be separated into two components, the major and minor mucins, based on the relative proportion of each. The detection of oligosaccharides released by alkaline-borohydride treatment [4–6] confirmed the mucin nature of the glycoproteins, but no comparison has been made between the major and minor components. Similarly, a large number of sialic acid species have been detected in the total gland glycoprotein fraction [7]. More detailed information on the major mucin has been reported by Bhavanandan and Hegarty [8] suggesting a polypeptide backbone of 60 000 Da and a corresponding glycosylated monomer subunit of 170 000 Da. Cloning and cDNA analysis of the major mucin has shown the presence of two domains, one corresponding to the glycosylated subunit polypeptide, rich in serine and threonine, and a second unexpected cysteine-rich domain [9]. The native mucin consists of several self-associating subunits to give the characteristically high molecular weight complexes (>500 000 Da) reported earlier [3]. The present report compares the biochemical properties of the major and minor mucin fractions which show correlations with the histochemical data presented before [1].

* To whom correspondence should be addressed.

0282-0080/91 $03.00+.12

Materials and methods

MATERIALS

Dowex 2 × 8 (200–400 mesh), Dowex 50W, hydroxyapatite (Bio-Gel HTP), Bio-Gel P4 (200–400 mesh) and piperazine diacrylamide (PDA) were from Bio-Rad (Watford, UK), and Sepharose CL 2B from Pharmacia (Hemel Hempstead, UK). Pronase, colominic acid and fetuin were supplied by the Sigma Chemical Co. (Poole, UK), sialidase from *Vibrio cholerae* was a product of Behringwerke (Marburg, Germany) and *Clostridium perfringens* and Newcastle disease virus sialidases were obtained as described [10]. Sodium boro[^{3}H]hydride (22.0 GBq mmol^{-1}), *N*-acetyl-D-[4,5,6,7,8,9-^{14}C]neuraminic acid (8.95 GBq mmol^{-1}) and [^{3}H] Hyperfilm were from Amersham International (Amersham, UK). Cetylpyridinium ammonium bromide, precoated silica gel 60, and cellulsoe thin-layer plates were obtained from Merck (Darmstadt, Germany). Other chemicals of analytical grade were also products of Merck.

Oligosaccharides used as reference compounds were Neu5Acα(2-3)Galβ(1-3)-GalNAc-ol and Neu5Acα(2-3)Gal β(1-3)[Neu5Acα(2-6)]GalNAc-ol from fetuin [11], Neu5Acα(2-6)GalNAc-ol from porcine seminal gel glycoprotein [12] and Neu5Acα(2-3)lactose from bovine colostrum [13]. Galβ(1-3)GalNAc-ol and *N*-acetylgalactosaminitol were prepared from desialylated fetuin or seminal gel oligosaccharides, respectively.

METHODS

Preparation of submandibular gland major and minor mucins

The mucin was prepared from fresh bovine submandibular glands by the method of Tettamanti and Pigman [3]. Briefly, homogenization of the sliced glands in 0.01 M NaCl with an Ultraturrax; centrifugation at 2000 × *g*; adjustment of the supernatant to pH 4.7 with acetic acid; removal of the precipitate; addition of 10% (w/v) cetylpyridinium bromide to the supernatant; collection of precipitated mucin and solubilization in 50% (w/v) $CaCl_2$; precipitation with 50–75% ethanol and solubilization of the mixed mucin in distilled water and lyophilization. The major and minor mucins were prepared from the mixed product as described [3] using hydroxyapatite. For this purpose, the mixed mucin was dissolved in 0.01 M sodium phosphate buffer, pH 6.8 (5 mg ml^{-1}) and hydroxyapatite added at 50 mg dry weight hydroxyapatite per mg mucin protein. The suspension was stirred for 2 h and centrifuged at 10000 × *g* for 20 min. The pellet was washed with the 0.01 M sodium phosphate buffer and recentrifuged as before. The pooled supernatants contained the major mucin, and the minor mucin was eluted from the hydroxyapatite with 10 volumes (w/v) of 0.15 M sodium phosphate buffer, pH 6.8, after stirring for 2 h and centrifugation at 20000 × *g* for 20 min Fractions were lyophilized after extensive dialysis against distilled water. Five preparations were made, and these showed only minor differences in yield and composition; each preparation was analysed and used individually.

Saponification of *O*-acetyl groups of the mucin samples was carried out in 50 mM NaOH at 4°C for 60 min, followed by neutralization of the solution with 2 M HCl and dialysis against 100 vol of water for 10 h. Less than 0.2% of total sialic acid was lost in any preparation during saponification. The samples were lyophilized and stored at 4°C. A glycopeptide mixture was prepared by exhaustive pronase digestion of the major mucin [14], and the products were separated by gel filtration on Sephadex G-50. The excluded material was collected.

Radioactive labelling of major and minor mucins was carried out after saponification as described before [15] by periodate oxidation and borotritide reduction. The tritium-labelled glycoproteins were repurified by chromatography on Sepharose CL 2B as described below.

Preparation and analysis of sialic acids

The sialic acids of oligosaccharides and glycoproteins were quantified by gas chromatography after methanolysis and trimethyl sialylation in routine analysis of the total monosaccharide composition [16]. This procedure measures *N*-acetylneuraminic acid (Neu5Ac), as *O*- and *N*-substituents are removed and re-*N*-acetylation is included in the work up.

The quantitation of different *N*- and *O*-acyl neuraminic acids was achieved after release of bound sialic acids by formic acid hydrolysis, and purification over Dowex 50W and 2 × 8 ion-exchange resins as described by Schauer [17, 18]. Total sialic acids were measured colorimetrically by the Fe^{3+}/orcinol or periodate/thiobarbituric acid methods or by gas chromatography [17, 18]. The *O*-acetyl esters present in mucin and sialic acid fractions were quantified by the hydroxamate method [17]. Individual sialic acids were analysed by gas chromatography/mass spectrometry under standard conditions as described [7]. The presence of disialyl linkages in mucin fractions was tested by methylation analysis using colominic acid as a standard [19]. Thin layer chromatography of sialic acids was carried out on cellulose plates as described before [17]. Two-dimensional thin layer chromatography was performed on 10 × 10 cm cellulose plates as detailed previously [13, 17], applying the sample at one corner of the plate before development with butan-1-ol:propan-1-ol:0.1 M HCl, 1:2:1 by vol in the first dimension. After drying and with or without exposure to ammonia, the plate was turned through 90° and run in the second dimension in the same solvent. Standards were applied for each dimension. The spots were visualized using the Fe^{3+}/orcinol reagent [17].

Preparation and separation of oligosaccharides from major and minor mucins

The alkali-catalysed β-elimination reaction was carried out in the presence of sodium borohydride on the high molecular mass major and minor mucins [20]. The mucins were

dissolved in 50 mM NaOH, 1 M $NaBH_4$ at 2.5 mg ml^{-1} and incubated at 45°C for 16 h. After this time the excess borohydride was destroyed with acetic acid, the solution evaporated to dryness and the residue dissolved in water and passed through Dowex 50W H^+. Finally, boric acid was removed by repeated evaporation from methanol. The products of β-elimination were dissolved in 0.1 M pyridinium acetate, pH 5.0, and fractionated on a column of Bio-Gel P4 (200–400 mesh, 150 × 2.2 cm) at room temperature.

Tritium-labelled oligosaccharides were prepared under similar conditions containing 740–925 MBq $NaB[^3H_4]$ (32–42 μmol) in 10 ml incubations. Sialic acid and radioactivity were determined in individual fractions and analysed by thin layer chromatography on silica gel 60 plates developed with ethanol:butan-1-ol:pyridine:water:acetic acid, 100:10:10:30:3 by vol, as before [13]. Fractions were pooled according to the pattern observed on thin layer chromatography, and repeated chromatography on Bio-Gel P4 was used to obtain individual oligosaccharide fractions. The total carbohydrate composition of the oligosaccharide fractions was analysed by gas/liquid chromatography after methanolysis [16]. The nature of the parent sialic acids (either Neu5Ac or Neu5Gc) was determined after mild acid hydrolysis of the oligosaccharides (0.1 M HCl at 80°C for 60 min) and thin layer chromatography of the hydrolysate on silica gel 60 plates in the presence of Neu5Ac and Neu5Gc standards. Quantitative analysis of Neu5Ac and Neu5Gc was made by gas chromatography of the sialic acids in the hydrolysates after ion-exchange purification as described above.

Incubation of mucins with sialidases

Sialidases from *Vibrio cholerae*, *Clostridium perfringens* and Newcastle disease virus were tested with mucins, glycopeptides and oligosaccharides. The initial rate of reaction was measured as detailed before [10, 21] for the different substrates under comparable incubation conditions containing 2 mM substrate sialic acids. Incubations (100 μl) contained *V. cholerae* enzyme (50 mU) in 50 mM sodium acetate, 9 mM $CaCl_2$ and 150 mM NaCl buffer, pH 5.5; *C. perfringens* enzyme (50 mU) in 50 mM sodium acetate, pH 5.1, or Newcastle disease virus enzyme (3 mU) in 50 mM sodium acetate, pH 5.5. In all incubations containing *O*-acetylated sialic acids a preliminary saponification step was included in the colorimetric assay to avoid underestimation of released sialic acid [22].

Gel filtration

Major and minor mucins were submitted to chromatography on columns (30 × 1 cm) of Sepharose CL 2B in 0.01 M sodium cacodylate, 0.5 M NaCl buffer, pH 6.0. The columns were eluted under gravity at a flow rate of 0.7 ml min^{-1} and fractions of 2 ml were collected and analysed for sialic acid and OD at 280 nm.

Density gradient centrifugation

Major and minor mucin samples were submitted to density gradient centrifugation as described by Carlstedt *et al.* [23]. The mucins were dissolved in 4 M guanidine hydrochloride in PBS at a concentration of 0.5 mg ml^{-1} in a total volume of 8 ml. Solid CsCl was added to give a starting density of 1.43 g ml^{-1} and samples were centrifuged for 48 h at 40 000 rev min^{-1} (100 000 × g in a MSE Europa model ultracentrifuge at 10°C. Aliquots of 0.7 ml were aspirated from the tubes and the density determined by weight. The mucins were detected by slot blotting onto nitrocellulose sheets and staining by the periodic acid-Schiff method [24]. Carbohydrate (see below) and OD_{280} measurements were also made. The pooled fractions after centrifugation were dialysed exhaustively against water and lyophilized.

Polyacrylamide gel electrophoresis

Major and minor mucin fractions were analysed by electrophoresis in the presence of sodium dodecyl sulphate (SDS) under conditions described by Laemmli [25] with the following modifications for 3% gels. The samples were run on 1.5 mm, 3% polyacrylamide gels with 2.5% stacking gels at a constant voltage of 70 V per gel for approximately 4 h. The polyacrylamide gels were prepared using piperazine diacrylamide (PDA) as cross-linker in place of methylene bisacrylamide (Bis) according to the manufacturers instructions (Bio-Rad). Staining for carbohydrate was by the periodic acid-Schiff method with prior saponification to remove *O*-acetyl groups on the sialic acids [26]. Proteins were visualized using the silver stain (Bio-Rad Silver Stain kit). Standards of myosin (205 000 Da), and IgM (990 000 Da) were used as molecular weight markers.

Other analytical methods

Total carbohydrate was determined colorimetrically by the periodic acid-Schiff method of Mantle and Allen [27]. Amino acids were measured using an autoanalyser method [28] by Dr R. Mentlein, Biochemisches Institut, Christian-Albrechts-Universität Kiel, Germany. Sulfate was determined by the method of Clarke and Denborough [29] and organic phosphate as described by Ames [30]. Autoradiography of dried polyacrylamide gels was carried out with $[^3H]$ Hyperfilm as recommended by the manufacturers.

Results and discussion

Characterization of the major and minor glycoprotein fractions isolated from whole bovine submandibular glands was carried out to allow a comparison with the histochemical results reported earlier [1, 2].

Preparation

The purification of major and minor mucins from bovine submandibular glands is summarized in Table 1. The yield

Table 1. Purification of bovine submandibular gland major and minor mucins. The purification steps 1–5 are indicated and the recovery of mucin expressed as total sialic acid, measured by the orcinol-ferric chloride assay. The molar ratio of *O*-acetyl ester to sialic acid is given as the *O*-acetyl index (*).

Step	*Sialic acid*		
	Total (g)	*O-Acetyl-index**	*Yield* (%)
1 Gland extract	4.09	1.2	100
2 Precipitation at pH 4.7	3.60	1.5	84
3 CPB precipitate[a]	2.99	1.7	73
4 Ethanol fractionation	2.25	1.9	55
5 Hydroxyapatite			
Non-adsorbed (major mucin)	1.60	2.2	39
Adsorbed (minor mucin)	0.25	1.1	6

[a] CPB, cetylpyridinium bromide.

of the major mucin was six-fold greater than that for the minor mucin. The final products isolated after hydroxyapatite treatment had protein contents of $37 \pm 4\%$ for the non-adsorbed (major) mucin and $62 \pm 5\%$ for the minor, adsorbed component. This is in good agreement with results previously reported for bovine [3, 8] and ovine [3] submandibular mucins. Assays for sulfate and phosphate were negative for both major and minor mucins.

Amino acid and carbohydrate composition of major and minor mucin fractions

The amino acid and monosaccharide composition of the purified mucins together with those for a glycopeptide prepared from the major mucin are shown in Table 2. The major mucin contains approximately 30% of the total amino acids as serine and threonine while the minor mucin contains 23% as these two amino acids. Glycine, alanine and proline make up >40% of the major mucin amino acids. Cysteine and tyrosine were absent from the major mucin and only found associated with the minor mucin. The low amounts of methionine, phenylalanine, histidine and lysine in the major mucin were lost in the preparation of the glycopeptide suggesting that they were present in a protease-accessible peptide fraction.

The pattern of amino acid composition for the major mucin was very similar to the results of Tettamanti and Pigman [3] with the exception of a lower serine content. The minor mucin was clearly different to the major mucin with higher aspartic and glutamic acids, cysteine, methionine, hydrophobic and aromatic amino acids. The minor mucin also varied from that reported by Tettamanti and Pigman [3] in the content of aspartic acid, alanine, cysteine and tyrosine, although the pattern was otherwise similar. A

Table 2. Amino acid and carbohydrate composition of major and minor mucins. The amino acid and monosaccharide composition of the two mucins and the major mucin glycopeptide is given as residues per 100 residues. The ratio of *N*-acetylgalactosamine to serine and threonine and *N*-acetylneuraminic acid is calculated from these data.

	Mucin		*Major mucin glycopeptide*
	Major	*Minor*	
Amino acid	Residues per 100 residues		
Asp	2.2	7.2	2.6
Thr	16.5	10.1	15.7
Ser	13.6	12.8	13.6
Glu	6.9	9.0	6.5
Pro	12.4	7.1	12.0
Gly	18.7	12.9	18.9
Ala	11.0	9.3	10.8
Val	5.1	7.1	7.3
Cys	0	0.4	0
Met	0.1	0.9	0.2
Isoleu	1.7	3.4	2.0
Leu	4.6	6.2	5.6
Tyr	0.4	1.9	0
Phe	0.7	2.5	0.3
His	0.3	1.1	0
Lys	1.0	3.9	0
Arg	4.9	3.9	4.2
Carbohydrate	Residues per 100 amino acid residues		
Fucose	1.6	0.7	0.5
Mannose	0	0.3	0
Galactose	7.8	2.7	7.0
N-Acetylglucosamine	7.1	4.7	6.5
N-Acetylgalactosamine	24.7	15.1	30.2
N-Acetylneuraminic acid	24.0	11.3	27.8
N-Acetylgalactosamine: serine + threonine	0.82	0.65	0.97
N-Acetylneuraminic acid: *N*-Acetylgalactosamine	0.97	0.75	0.92

cDNA sequence recently reported for the major mucin [9] indicates the presence of an extra 224 amino acid C-terminal domain not present in the purified major mucin. The amino acid analysis of this product contains cysteine in contrast to the major mucin isolated in this study and 10-fold higher than that detected in the minor mucin (Table 2). This makes it unlikely that the minor mucin represents the complete mucin defined by the cDNA sequence.

The monosaccharide composition of the major and minor mucins from stage 5 (Table 1) of the preparation showed high amounts of hexosamine and sialic acid in similar proportions to earlier work [3] in addition to galactose and fucose (Table 2). Levels of galactose (>twofold higher) and fucose (>twofold lower) differed to those reported before

[3] and no mannose was detected for the major mucin. The minor mucin had a galactose content three times lower and that of fucose approximately twice as low as shown before [3], in addition some mannose was found.

The molar ratio of *N*-acetylgalactosamine to serine plus threonine (Table 2) was 0.82 for the major mucin indicating that nearly all of the potential glycosylation sites were occupied. In the minor mucin this ratio was significantly lower indicating a reduced level of glycosylation. In addition, the ratio of sialic acid to *N*-acetylgalactosamine was near to 1.0 for the major mucin, while a lower result was found for the minor mucin (Table 2). The glycopeptide derived from the major mucin had an amino acid and monosaccharide composition consistent with the removal of approximately 7% of susceptible protein after the protease digestion (Table 2). The potential glycosylation sites were almost completely substituted and the sialic acid to *N*-acetylgalactosamine ratio remained the same as for the native major mucin.

Sialic acid pattern

The total sialic acid content measured by gas chromatography after methanolysis [16] does not give any indication of the *N*- and *O*-acyl substitution pattern of these monosaccharides. The measurement of Neu5Ac and Neu5Gc in major and minor mucins was carried out using an alternative gas chromatographic method [7, 17] after saponification and mild acid hydrolysis. The recovery of sialic acids in this procedure was 91 ± 4% for three separate determinations. A predominance of Neu5Ac over Neu5Gc was found in both major and minor mucins (Table 3), but the ratio was nearly twice as high for the major compared with the minor mucin.

The presence of *O*-acyl groups in the sialic acids was analysed by direct measurement of *O*-acyl esters in the isolated mucins (Table 3), showing a molar ratio of *O*-acetyl ester to sialic acid twice as high for the major over the minor mucin. The pattern of sialic acid *O*-acylation determined after mild acid hydrolysis monitored by thin layer chromatography in two dimensions (Fig. 1) revealed similar sialic acid species in both mucins. However, the total amount of mono- and di-*O*-acetyl sialic acids varied. Less di-*O*-acetyl sialic acid was observed for the minor mucin on two-dimensional thin layer chromatography (Fig. 1), and gas chromatographic/mass spectrometric analysis of pooled samples from the ion-exchange purification confirmed this result (Table 3). The introduction of a saponification step after thin layer chromatography in the first dimension showed the distribution of the parent *N*-acylneuraminic acids in the *O*-acetylated derivatives for each mucin. The sialic acids containing two *O*-acetyl groups appeared to be largely *N*-acetylneuraminic acids, only mono-*O*-acetylated *N*-glycoloylneuraminic acid was seen for both major and minor mucin sialic acids (Fig. 1).

The presence of α(2-8) disialyl linkages was not found. Methylation analysis of major and minor mucins gave products compatible with only mono-sialyl linkages and none corresponding to those detected with colominic acid as a positive control [19].

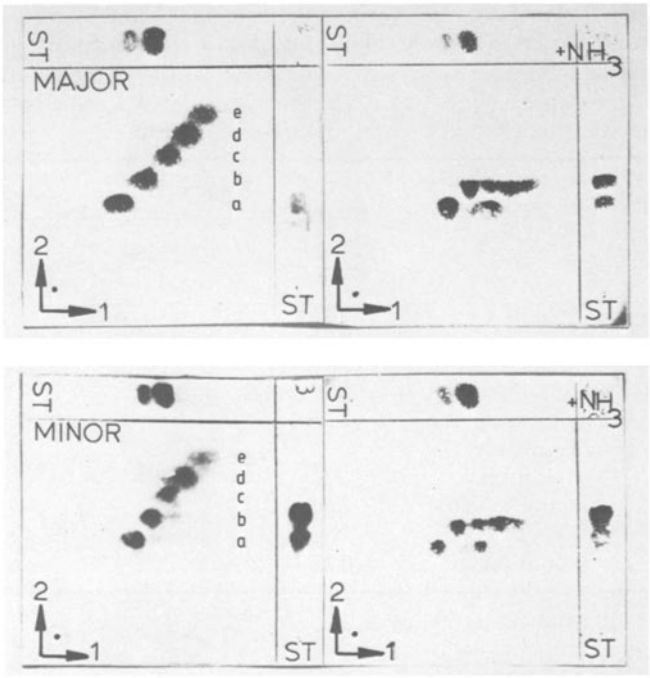


Figure 1. Two dimensional thin layer chromatography of the total sialic acid pool from major and minor mucins after mild acid hydrolysis and Dowex ion-exchange purification. The chromatograms were run without and with ($+NH_3$) ammonia treatment after the first development. The first (1) and second (2) chromatographic migrations are shown and Neu5Ac (upper spot) and Neu5Gc (lower spot) standards (St) run in each dimension. a, Neu5Gc; b, Neu5Ac; c, *N*-acetyl-7-*O*-acetylneuraminic acid and *N*-glycoloyl-9-*O*-acetylneuraminic acid; d, *N*-acetyl-9-*O*-acetylneuraminic acid; e, di-*O*-acetylated Neu5Ac.

Table 3. The pattern of sialic acid substitution in bovine submandibular gland mucins. Chemical analysis of sialic acid content was by gas chromatography (1), colorimetric analysis (2) and gas chromatography/mass spectrometry (3).

Molar ratio	*Major*	*Minor*
1 $\frac{N\text{-acetylneuraminic acid}}{N\text{-glycoloylneuraminic acid}}$	3.90:1	2.00:1
2 $\frac{O\text{-acetyl esters}}{\text{Sialic acid}}$	2.02:1	1.01:1
3 $\frac{\text{Mono-}O\text{-acetyl esters}}{\text{Di-}O\text{-acetyl esters}}$	0.83:1	2.20:1

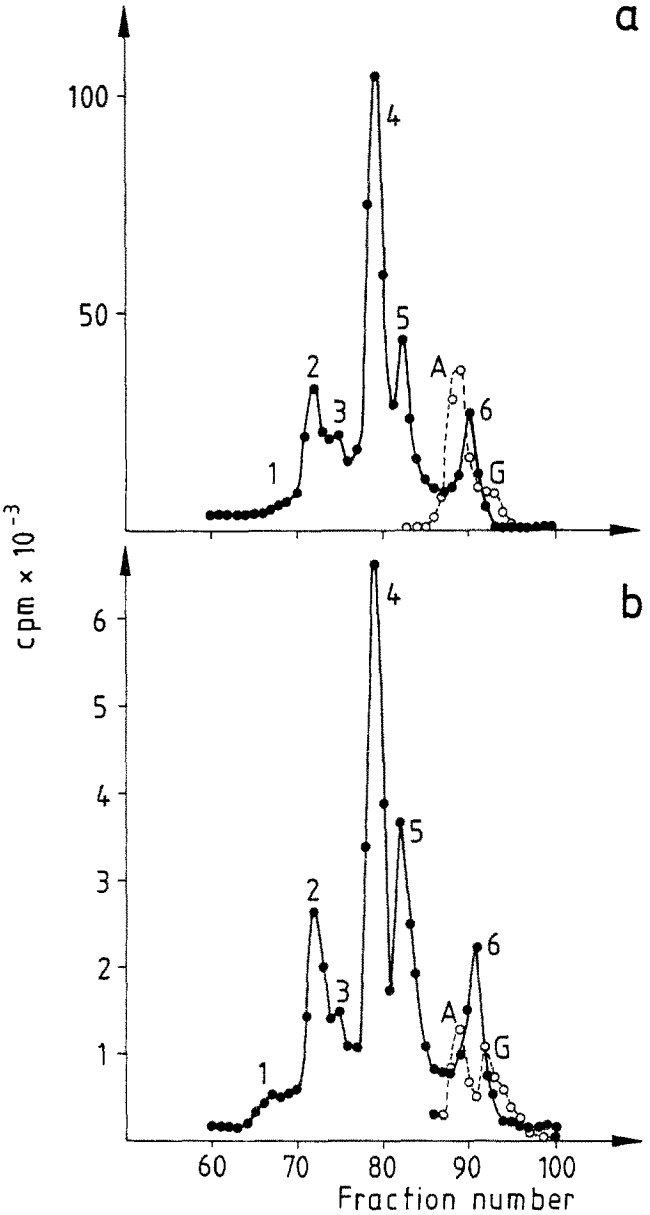


Figure 2. Separation of borotritide-labelled oligosaccharides on Bio-Gel P-4. The total oligosaccharide pool for major (a) and minor (b) mucins was run after β-elimination (●). The position and elution profile of the free sialic acids Neu5Ac (A) and Neu5Gc (G) liberated from oligosaccharides during the procedure is indicated and was determined by the orcinol/ferric chloride colorimetric method for each fraction (○).

Oligosaccharide pattern

The elution profile of the *O*-linked [^{3}H]-oligosaccharides from major and minor mucins released by β-elimination and fractionated on a column of Bio-Gel P-4 are shown in Fig. 2. Larger scale nonradioactive preparations gave similar fractionation profiles. The pattern for major and minor mucins were essentially the same and only small variations in the six components were detected. The oligosaccharide

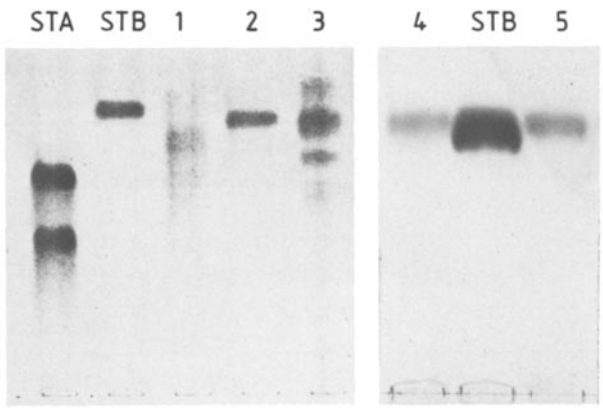


Figure 3. Thin layer chromatography of oligosaccharides isolated after β-elimination of mixed mucin (step 4, Table 1). Fractions 1–5 correspond to the peaks in Fig. 2 and fractions in Table 4. Fractions 4 and 5 contain Neu5Ac and Neu5Gc respectively. Standards of Neu5Ac (upper spot) and Neu5Gc (STA) and Neu5Acα(2-6)GalNAc-ol (STB) were run for comparison.

composition of fractions 1–5 was investigated on thin layer chromatography (Fig. 3). Fractions 1 and 3 contained oligosaccharide mixtures while fractions 2, 4 and 5 contained single bands. The sialic acids present in the fractions were identified on thin layer chromatography after mild acid hydrolysis and the monosaccharide composition determined by gas chromatography (Table 4). Fraction 2 contained equimolar amounts of Neu5Ac, *N*-acetylglucosamine and *N*-acetylgalactosaminitol, while fractions 4 and 5 showed equimolar sialic acid and *N*-acetylgalactosaminitol. Fractions 4 and 5 showed the same mobility as authentic Neu5Acα(2-6)GalNc-ol isolated from porcine seminal gel glycoprotein. They could be distinguished from each other by the nature of their parent sialic acids (Table 4). Fraction 6 was *N*-acetylgalactosaminitol, and the positions of free Neu5Ac (A) and Neu5Gc (G), detected colorimetrically, are also shown. Major and minor mucins showed differences in the proportion of the oligosaccharide peaks 4, 5 and 6 reflecting a variation in Neu5Ac and Neu5Gc disaccharide content and free *N*-acetylgalactosaminitol. The amount and ratio of free sialic acids found was different between the two mucins, and this was assumed to have been lost from the disaccharide during β-elimination leading to the free *N*-acetylgalactosaminitol and sialic acids. The ratio of Neu5Ac and Neu5Gc in the total recovered fractions matches well with the values found for the native mucins in Table 3 (3.9:1 and 2.0:1, respectively, for Neu5Ac:Neu5Gc). The results did not indicate any qualitative difference in the pattern of oligosaccharides found for each of the mucins.

Molecular size

The molecular composition of the major and minor mucins isolated after hydroxyapatite treatment was followed by gel filtration (Fig. 4), density gradient centrifugation (Fig. 5) and SDS-polyacrylamide gel electrophoresis (Fig. 6).

Table 4. Nature of oligosaccharides isolated on Bio-Gel P-4 chromatography. Oligosaccharides were isolated from mucin fractions at step 4 (A) or step 5 (B) as described in Table 1. The oligosaccharide numbers correspond to those shown in Figs 2 and 3. Analysis of A was by gas/liquid chromatography after methanolysis and re-*N*-acetylation, and the data in B derived from the radiolabelling experiments shown in Fig. 2.

No	*A (Ethanol precipitation) Monosaccharides (molar ratio)*					*% total carbohydrate*		*B (Hydroxyapatite-purified) % total sialic acid*			
	Fuc	*Gal*	*GlcNAc*	*Neu5Ac*	*GalNAc-ol*	*Major*	*Minor*	*Major*		*Minor*	
								Neu5Ac	*Neu5Gc*	*Neu5Ac*	*Neu5Gc*
1	0.2	0.4	0.8	1.4	1.0	4.0	5.0	3.0	1.0	3.0	2.0
2	–	–	1.1	1.1	1.0	16.7	16.8	16.7	0	16.8	0
3	–	0.2	0.6	1.1	1.0	5.7	5.4	3.6	2.1	3.4	2.0
4	–	–	–	1.2	1.0	46.0	34.9	46.0	0	34.9	0
5	–	–	–	1.1	1.0	16.9	22.6	0	16.9	0	22.6
6	–	–	–	–	+	10.7	15.3	0	0	0	0
A	–	–	–	+	–	–	–	8.7	0	8.6	0
G	–	–	–	+	–	–	–	0	2.0	0	6.7

Separation of the mucins on Sepharose CL 2B revealed marked differences between the major and minor mucins. The major mucin showed an excluded peak (V_0) and material eluting as a broad included peak, whereas a single, regular peak at a lower molecular weight range was found for the minor mucin.

The major and minor mucins behaved differently on density gradient centrifugation when studied at a concentration of 0.5 mg ml^{-1} (Fig. 5). The minor mucin sedimented at lower densities reflecting the lower carbohydrate to protein ratio of this fraction. A distribution at a slightly higher density was observed with the major mucin, although this overlapped with part of the minor mucin profile. Samples run at lower concentrations (approximately 0.1 mg ml^{-1}) showed similar profiles and equivalent concentrations of radiolabelled, saponified mucins gave the same pattern as those shown in Fig. 5. The concentration of the sample analysed was important, as gel formation was observed above concentrations of 1.5 mg ml^{-1} resulting in a pellet at the bottom of the tube.

Analysis of the mucins by SDS-polyacrylamide gel electrophoresis was followed using the periodate-Schiff carbohydrate stain method due to the poor staining by Coomassie Blue and silver stain methods for protein [8]. Improved detection of the mucins on the gels was achieved using saponification prior to the staining procedure [26] in order to remove *O*-acetyl groups present on the sialic acids blocking oxidation by periodic acid.

The minor mucin showed a series of bands in the range 10^6 to 300 000 Da. In contrast, the major mucin showed material at very high molecular weight in excess of 10^6 and bands at regularly decreasing molecular weights down to around 180 000. The major and minor mucins showed only minimal amounts of material with a molecular weight below 100 000.

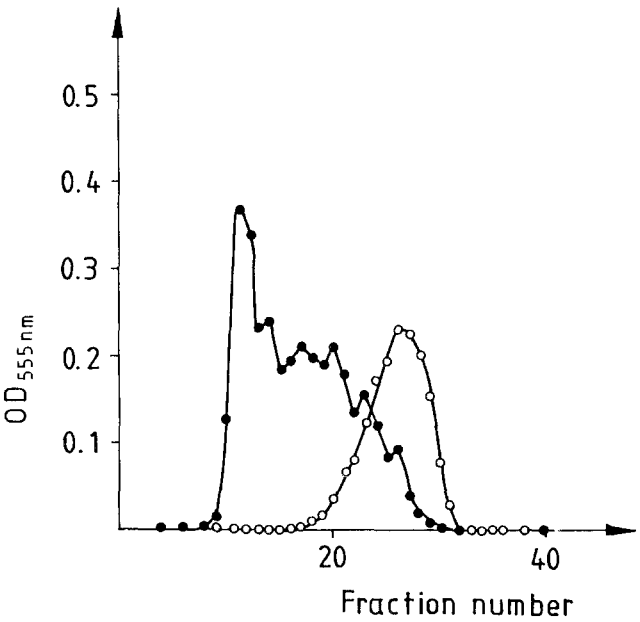


Figure 4. Chromatography of major (●) and minor (○) mucins in separate runs was on Sepharose CL 2B. Detection of sialic acid by the orcinol/ferric chloride method is shown.

These results demonstrate the formation of aggregates at lower molecular weight for the minor mucin, this is apparent on the gel filtration on Sepharose CL 2B (Fig. 4) and SDS-PAGE analysis. The SDS-PAGE results show a ladder of regularly increasing molecular weight units leading to the

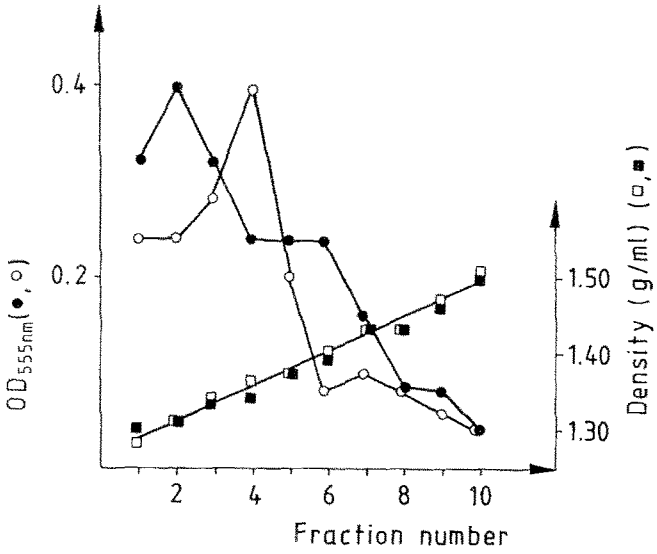


Figure 5. Separation of major (○, □) and minor (●, ■) mucins at 0.5 mg ml^{-1} on separate CsCl gradients in 4 M guanidine hydrochloride. Fractions were dialysed extensively against water and the carbohydrate content measured (○, ●). The density of each fraction is indicated (□, ■). Fractions of 0.7 ml were collected.

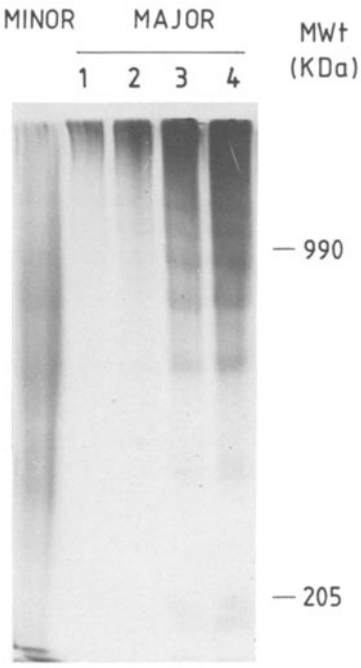


Figure 6. SDS-Polyacrylamide gel electrophoresis of major and minor mucins. Pooled fractions from Sepharose CL 2B chromatography were run on 3% polyacrylamide gels. Fractions were pooled as follows; Minor, 20–32; Major 1, 9–11; 2, 12–15; 3, 16–22 and 4, 23–30 (see Fig. 4). The position of the molecular weight markers myosin (205 kDa) and IgM (990 kDa) are indicated. Gels were stained by the periodic acid-Schiff method [26].

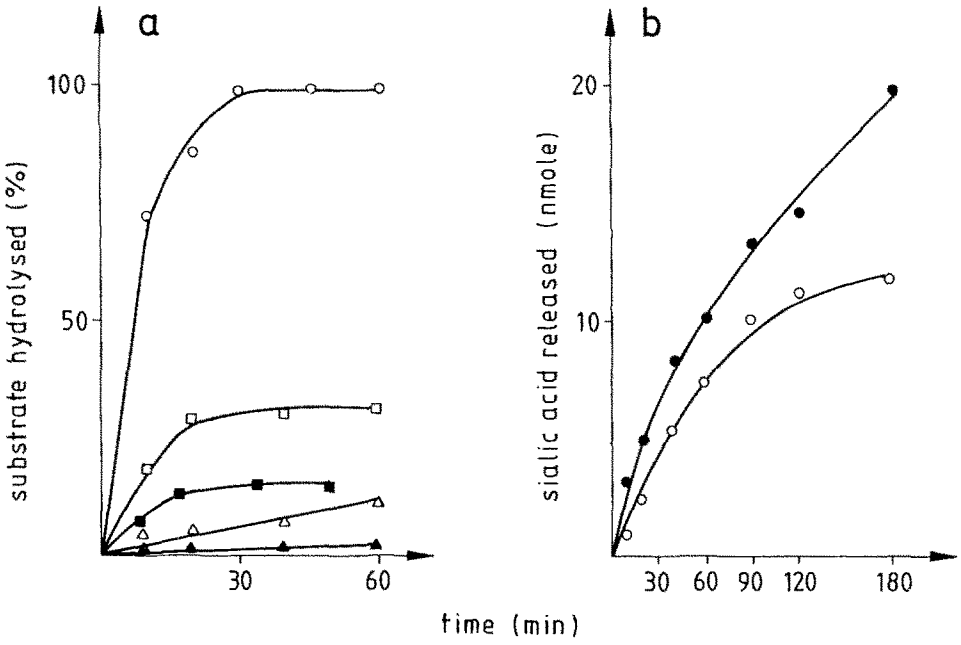
formation of aggregates at a maximum of approximately 10^6 for the minor and of several million for the major mucin.

Action of sialidases

The action of different sialidase preparations with major and minor mucins, the glycopeptide and disaccharides reflected the sialic acid composition and molecular organization of these compounds. Using *C. perfringens* sialidase, the glycopeptide preparation from the major mucin was completely desialylated within 30 min while only 30% of saponified major or minor mucin desialylation and less than 10% of sialyl disaccharide desialylation occurred (Fig. 7a). The influence of the *O*-acetyl substitution could be shown clearly with both bacterial and viral sialidases in agreement with previous work [22, 31]. An increase in desialylation was observed on saponification in all cases studied, indicating the blocking effect of *O*-acetylation on sialidase action. The influence of the Neu5Gc content on sialidase action could be demonstrated for both major and minor mucins by the rate of desialylation (Fig. 7b) together with the thin layer chromatographic demonstration of the released sialic acids (Fig. 7c). Initial rates of enzyme action were associated with release of Neu5Ac, while the reduction in rate correlated with the appearance of Neu5Gc in the incubation. The difference in desialylation rates by sialidase between major and minor mucins may reflect higher relative content of Neu5Gc in minor mucin (Table 3) and the slower release of this sialic acid by the enzymes. This is in good agreement with earlier studies [21] showing a more rapid release of Neu5Ac from the mucin disaccharide Neu5Acα(2-6)GalNAc relative to the Neu5Gc-containing disaccharide.

Relation of results to histochemical data

The data reported here indicate the existence of two bovine submandibular gland mucins. The mucins differ essentially in their carbohydrate moieties and in particular with respect to their sialic acid composition. Other workers have focused attention on the major mucin from bovine submandibular glands [5, 6, 8] and apart from the original work of Tettamanti and Pigman [3] there is no report on the minor mucin. We have previously reported a histochemical analysis of the bovine submandibular gland relating the morphological and morphometric data of the different secretory cells to their mucin type and sialic acid staining pattern [1, and unpublished data]. The main secretory areas within the glands were found to be mucous cells (72%) while seromucous cells comprised 18%. The proportion of stored material based on these data is similar to that found for the major (87%) and minor (13%) mucins isolated from the whole glands (Table 1). Histochemical analysis of the sialic acid in the two different secretory cells [1] by the mild-PAS method [32, 33] together with sequential methods including saponification, periodate oxidation and borohydride reduction led to the conclusion that the mucous cells contain



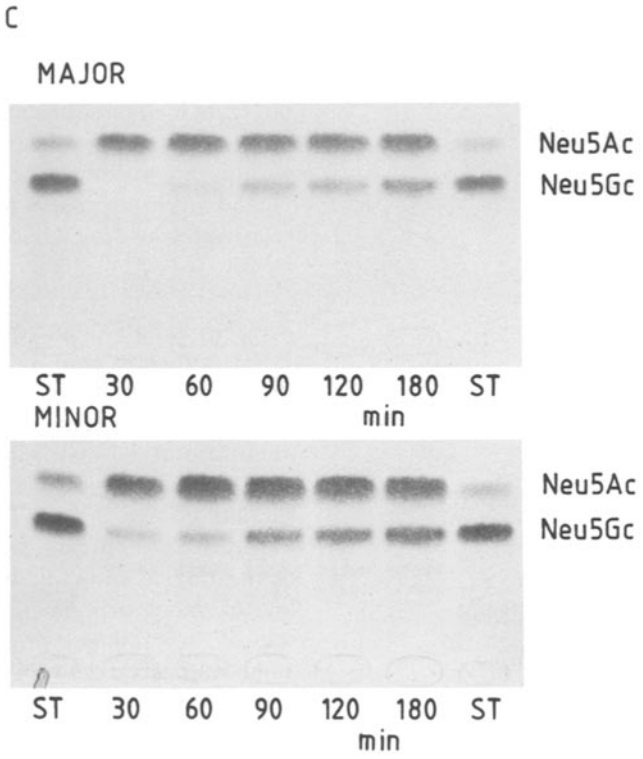


Figure 7. Action of sialidases on submandibular gland mucins and the glycopeptide. (a) Rate of reaction of *Clostridium perfringens* sialidase with the minor mucin (■), the major mucin (□), the major mucin glycopeptide (○) and the Neu5Ac(△)- and Neu5Gc(▲)-containing disaccharides derived from the glycoprotein. The percentage of total sialic acid released is shown. (b) The rate of sialic acid release from saponified major (●) and minor (○) mucins by *Vibrio cholerae* sialidase. The concentration of sialic acid in each incubation was the same. At the times indicated the total sialic acids released were collected. These pools were separated from the mucin by dialysis and approximately 35 μg applied to cellulose thin-layer plates (c) ST, Neu5Ac and Neu5Gc standards.

material with a high amount of di-*O*-acetylated sialic acids in contrast to the seromucous cells where these derivatives were detected only in very low amounts. The data presented in Table 3 provide indirect evidence that the major mucin may correspond to the material detected in the mucous cells and the minor to that in the seromucous cells. Direct demonstration of the cellular distribution of these two mucins must await analysis with specific antibodies.

Conclusion

The major and minor mucin fractions isolated from bovine submandibular glands differ essentially in the nature of their protein content and sialic acid patterns. The minor mucin fraction adsorbed onto hydroxylapatite has a higher relative protein content than the major mucin. Differences in the sialic acid contents are reflected in the relative proportions of *N*-acetyl to *N*-glycoloyl forms found in the main disaccharide and in the level of *O*-acetylation, most di-*O*-acetyl-*N*-acetylneuraminic acid being found in the major mucin. Recent molecular biological approaches to the study of the bovine salivary mucins have indicated a minimum subunit of about 170 000 Da for the major mucin [8], which is in good agreement with the results presented here. The cDNA sequence indicates a larger subunit [9], which was not detected in this work and may be cleaved to yield the subunit found here and by others [3, 8]. No information is yet available for the minor mucin, and more work is required to deduce the polypeptide structure for comparison with the major mucin sequence.

Acknowledgements

Financial support from the Fonds der Chemischen Industrie, the Medical Research Council and the Department of Medicine, University of Bristol, is gratefully acknowledged. We wish to thank Dr R. Mentlein (Anatomisches Institut, Universität Kiel) for amino acid analyses, Dr Gerd Reuter for GC/MS analyses and M. El Madani for technical assistance (Biochemisches Institut, Universität Kiel).

References

1. Veh RW, Corfield AP, Schauer R, Andres KH (1979) In *Proc Vth Int Symp Glycoconjugates* (Schauer R, Boer P, Buddecke E, Kramer MF, Vliegenthart JFG, Wiegandt H, eds) pp 191–94. Stuttgart: Thieme.
2. Schauer R, Casals-Stenzel J, Corfield AP, Veh RW (1988) *Glycoconjugate J* **5**:257–70.
3. Tettamanti G, Pigman W (1968) *Arch Biochem Biophys* **124**:41–50.
4. Bertolini M, Pigman W (1970) *Carbohydr Res* **14**:53–63.
5. Tsuji T, Osawa T (1986) *Carbohydr Res* **151**:391–402.
6. D'Arcy SM, Donoghue CM, Koeleman CAM, van den Eijnden DH, Savage AV (1989) *Biochem J* **260**:389–93.
7. Reuter G. Pfeil R, Stoll S, Schauer R, Kamerling JP, Versluis C, Vliegenthart JFG (1983) *Eur J Biochem* **134**:139–43.
8. Bhavanandan VP, Hegarty JD (1987) *J Biol Chem* **262**:5913–17.
9. Bhargava AK, Woitach JT, Davidson EA, Bhavanandan VP (1990) *Proc Natl Acad Sci USA* **87**:6798–802.
10. Corfield AP, Wember M, Schauer R, Rott R (1982) *Eur. J. Biochem.* **124**:521–25.
11. Spiro RG, Bhoyroo VD (1974) *J Biol Chem* **249**:5704–17.
12. Rana SS, Chandrasekaran EV, Kennedy J, Mendicino J (1984) *J Biol Chem* **259**:12899–907.
13. Bayard B, Kerckaert JP, Roux D, Strecker G (1979) In *Protides of Biological Fluids* (Peeters H, ed.) pp 153–56. Oxford: Pergamon Press.
14. Clamp JR, Fraser G, Read AE (1981) *Clin Sci* **61**:229–34.
15. Veh RW, Corfield AP, Sander M, Schauer R (1977) *Biochim Biophys Acta* **486**:145–60.
16. Clamp JR, Bhatti T, Chambers RE (1971) *Methods Biochem Anal* **19**:229–344.
17. Schauer R (1978) *Methods Enzymol* **50**:64–89.
18. Schauer R (1987) *Methods Enzymol* **138**:132–61.
19. Haverkamp J, Kamerling JP, Vliegenthart JFG, Veh RW, Schauer R (1977) *FEBS Lett* **73**: 215–19.
20. Clamp JR, Cooper B, Creeth JM, Ene D, Barrett J, Gough M (1983) *Biochem J* **215**:421–23.
21. Corfield AP, Veh RW, Wember M, Michalski J-C, Schauer R (1981) *Biochem J* **197**:293–99.
22. Corfield AP, Sander-Wewer M, Veh RW, Wember M, Schauer R (1986) *Biol Chem Hoppe-Seyler* **367**:433–39.
23. Carlstedt I, Lindgren H, Sheehan JK, Ulmsten U, Wingerup L (1983) *Biochem J* **211**:13–22.
24. Thornton DJ, Holmes DF, Sheehan JK, Carlstedt I (1989) *Anal Biochem* **182**:160–4.
25. Laemmli UK (1970) *Nature* **227**:680–5.
26. Sarris AH, Palade GE (1979) *J Biol Chem* **254**:6724–31.
27. Mantle M, Allen A (1978) *Biochem Soc Trans* **6**:607–9.
28. Mentlein R, Heiland S, Heymann E (1980) *Arch Biochem Biophys* **200**:547–59.
29. Clarke AE, Denborough MA (1972) *Biochem J* **121**:891–16.
30. Ames BN (1966) *Methods Enzymol* **8**:115–18.
31. Schauer R, Faillard H (1968) *Hoppe-Seylers Z Physiol Chem* **349**:961–8.
32. Veh RW, Meesen D, Kunz HD, May B (1982) In *Colonic Carcinogenesis* (Malt RA, Williamson RCN, eds), pp 355–65. Lancaster: MTP Press.
33. Roe R, Corfield AP, Williamson RCN (1989) *Histochem J* **21**:216–22.