HUMAN GASTRIC MUCOSA

PART I. THE PREPARATION OF A GLYCOPOLYPEPTIDE AND SOME ASPECTS OF ITS STRUCTURE

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ABSTRACT

Different methods for extracting mucin components from human gastric mucosa have been evaluated. An extensive fractionation study of a product solubilised by pronase digestion is described. By chromatography of the crude extract on Bio-Gel P-30, a neutral glycopolypeptide fraction (*DP-1*) of molecular weight >300,000 was obtained which had a composition similar to that of blood-group substance and contained most of the original carbohydrate. A second fraction, which was a mixture of glycopeptides, peptides, and nucleic acid, was further separated into neutral and acidic components. Another preparation of the glycopolypeptide was examined in greater detail. There was no evidence that this glycopolypeptide contained sulphate, contradicting the chemical investigations of other workers, but in agreement with known histochemical results. Evidence is presented that it consists of a polypeptide core composed preponderantly of threonine, serine, proline, alanine, and glycine, in which approximately 75% of the threonine and serine residues were involved in *O*-glycosidic linkages to oligosaccharide chains of variable length, through 2-acetamido-2-deoxygalactose residues. Structural investigations by periodate-oxidation studies and by gel chromatography of alkaline degradation products indicated that all of the 2-acetamido-2-deoxygalactose residues were substituted at O-6 and more than 85% at O-3.

INTRODUCTION

The underlying cause in the genesis of peptic ulceration is the breakdown of agencies normally responsible for protecting the mucosa of the gastro-duodenal area from being attacked by the intraluminal contents[1], although other factors are important. The most important protective agency is the ability of the mucosa to secrete a "mucous barrier" — a thin layer of mucus, the viscosity, adhesiveness, and cohesiveness of which allows it to form a continuous layer protecting the epithelial mucosa cells from the proteolytic and hydrolytic contents of the stomach. This mucus is continually renewed as its superficial layers are eroded by the passage of the intraluminal contents.

Rationalisation of the biochemical mechanism of the protective role of gastric mucus, in terms of its detailed chemical structure, would lead to a better understanding of the aetiology of gastric ulcer.

Owing to their greater availability, mammalian stomachs other than human have been the subjects of most studies, where gastric mucin has been isolated from both gastric juice and gastric mucosa. The more recent work has been directed towards mucin isolated from the mucosa of various animals, including dogs[2–4], pigs[5,6], and rabbits[7]. However, direct parallels cannot be drawn with human gastric mucin, in view of the important evidence of species variations[8]. Studies of human gastric mucin have been limited almost exclusively to aspirated, gastric juice[9–12], apart from the preparation of blood-group substances from human stomachs, both normal and carcinomatous[13–17].

Analytical data indicate that gastric mucin is composed of 2-amino-2-deoxyglucose, 2-amino-2-deoxygalactose, galactose, fucose, and sialic acid, together with some mannose and glucose, sulphate, and various amino acids in unusual, but characteristic, proportions[12]. The purity and homogeneity of the materials analysed were frequently in question, and some of the methods of analysis were unreliable. Therefore, our attention was drawn first of all to an investigation of methods of extraction and fractionation of mucin from the mucosa, followed by preliminary structural studies of a major glycopolypeptide component of human gastric mucin.

EXPERIMENTAL

Materials. — Surgical specimens of human stomach were kindly supplied by Dr. Goodier, St. Helier Hospital, Carshalton, and by Mr. Cox, Hammersmith Hospital, Du Cane Road, London, W.12. Samples were placed in aqueous ethanol (70%, v/v) immediately following surgery, and stored at −20° until required. Pooling of samples from different individuals was avoided because of the variations in composition that have been observed.

Preparation of defatted mucosa. — The mucosa was carefully dissected from the *muscularis mucosae*. Allowing the samples to stand in acetone for a few minutes facilitated the operation. A suspension of the chopped mucosa in acetone was macerated in a homogeniser (Measuring and Scientific Equipment Ltd., London) at −50°; several bursts of 0.5-min duration were sufficient. After filtration (Whatman No. 54), the powdered mucosa was treated with acetone (5 times) to extract water and fats, and, following air-drying on the filter, was stored *in vacuo* over phosphorus pentoxide and sodium hydroxide pellets.

Evaluation of different procedures for the extraction of gastric mucin from defatted mucosa. — *(a) Extraction of mucosa with aqueous sodium chloride at 4°, treating with ultrasonic waves.* Gastric mucosa (71 mg) was suspended in aqueous sodium chloride (0.9% w/v; 10 ml) at 4° and treated with ultrasonic waves (100-watt Ultrasonic Disintegrator, Measuring and Scientific Equipment, Ltd.) in ten 0.5-min bursts, allowing 5 min between each burst. The suspension was stirred for 18 h at 4°

and then centrifuged. Less than 1 mg of carbohydrate material was present in the supernatant, as ascertained by the phenol–sulphuric acid test[18].

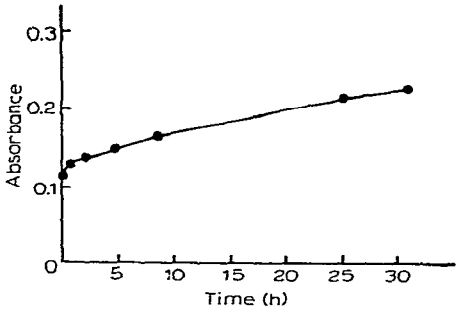


Fig. 1. Release of carbohydrate from gastric mucosa by treatment with 8M urea at 20°, as determined by the phenol–sulphuric acid assay. Absorbances were determined at 490 nm.

(*b*) *Extraction of mucosa with* 8M *urea.* Gastric mucosa (100 mg) was shaken with 8M urea (25 ml) for 30 h at 20°. The release of carbohydrate into solution (Fig. 1) was monitored by the phenol–sulphuric acid test[18]. Following centrifugation, the supernatant was fractionated on a column (60 × 1.3 cm) of Bio-Gel P-30 by eluting with water (Fig. 2). In a more-vigorous treatment, involving ultrasonic disintegration and maceration at 10°, large amounts of material were solubilised in 8M urea. However, the highly viscous solution precipitated when the urea was removed by dialysis, but was only partially soluble in the usual buffers. Moreover, the soluble fraction could not be removed from DEAE-Sephadex gel when a fractionation was attempted on the gel, even under regeneration conditions.

(*c*) *Digestion of mucosa by pepsin.* Gastric mucosa (40 mg) was treated[19] with pepsin (14 mg) in 0.2M sodium acetate buffer (pH 5.6; 3 ml; containing 5mM cysteine hydrochloride and 5mM EDTA) for 24 h at 50°. Following centrifugation, the supernatant was fractionated on a column (60 × 1.3 cm) of Bio-Gel P-300 by elution with M sodium chloride (Fig. 3).

(*d*) *Extraction of mucosa with water at 50°.* Experiment (*c*) was repeated, except that water (3 ml) replaced the solution of enzyme in buffer, etc. The corresponding fractionation on Bio Gel P-300 is given in Fig. 4.

(*e*) *Digestion of mucosa by pronase.* Gastric mucosa (180 mg) was shaken with a solution of pronase (1.4 g/l; 25 ml) in 0.1*M*-tris(hydroxymethyl)methylamine hydrochloride [Tris] buffer (pH 8.3) containing 5mM calcium chloride for 25.5 h at 37°. The release of carbohydrate into solution (Fig. 5) was monitored as before[18]. Aliquots of the supernatant solution after 3, 7.5, and 25.5 h were fractionated on a column (60 × 1.3 cm) of Bio-Gel P-30 by elution with water (Fig. 6).

Pronase treatment of gastric mucosa and fractionation of the products. — Defatted gastric mucosa (1 g) was shaken with pronase solution (1.03 g/l; 80 ml) for 7.5 h as described above. Following centrifugation, the supernatant solution was concen-

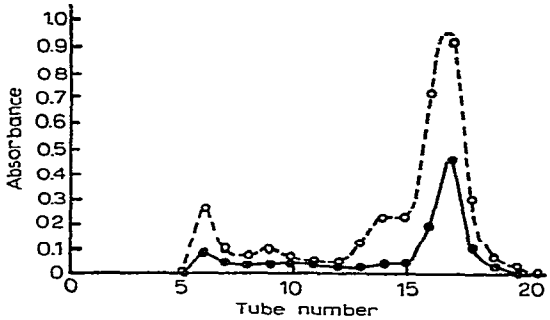


Fig. 2. Fractionation on Bio-Gel P-30 of gastric mucin released from mucosa by treatment with 8M urea for 30.4 h at 20°. Absorbances were determined at 260 nm O--O--O, and at 490 nm after reaction in the phenol–sulphuric acid assay ●—●—●.

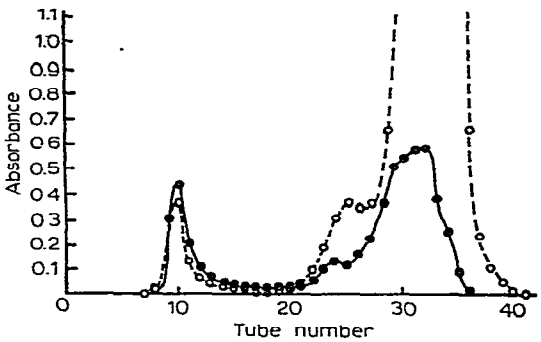


Fig. 3. Fractionation on Bio-Gel P-300 of gastric mucin released from mucosa by treatment with pepsin (at pH 5.6) for 24 h at 50°. Absorbances were determined at 260 nm O--O--O, and at 490 nm after reaction in the phenol–sulphuric acid assay ●—●—●.

trated to 40 ml and fractionated on a column (80 × 3.5 cm) of Bio-Gel P-30 by elution with water (Fig. 7). The three fractions, *DP-I*, *DP-II*, and *DP-III*, were obtained by combining the appropriate tubes as indicated (Fig. 7). These fractions were each fractionated on a column (60 × 1.2 cm) of A-50 DEAE-Sephadex (Cl^- form) in 0.1*I*-Tris buffer (pH 8) by elution with (*i*) buffer (50 ml); (*ii*) 0→2M sodium chloride gradient in buffer (450 ml); (*iii*) 4M sodium chloride in buffer (50 ml). For *DP-I* and *DP-II*, the carbohydrate material appeared in the neutral zone as single peaks, well separated from the u.v.-absorbing material associated with these fractions (see Fig. 7), which was eluted by the salt gradient. For *DP-III*, the carbohydrate material appeared as a single peak in the neutral zone, and a broad, heterogeneous peak in the salt-gradient zone, both associated with strong u.v.-absorbance.

DP-III was separated into a neutral fraction (*DP-III-A*) and an acidic fraction (*DP-III-B*) by chromatography on a column (103 × 2.5 cm) of A-50 DEAE-Sephadex (Cl^- form), with elution by buffer and M sodium chloride in buffer, respectively. *DP-I* and *DP-II* were not purified at this stage, and were recovered by lyophilisation

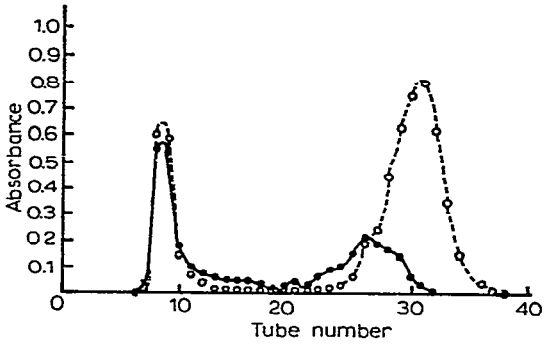


Fig. 4

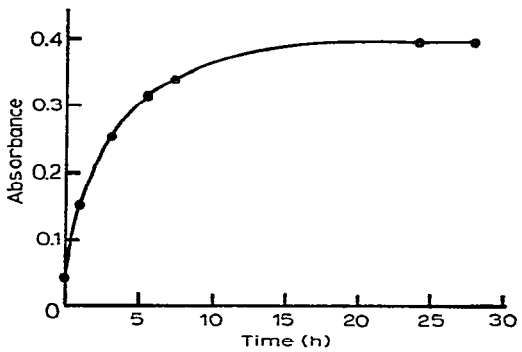


Fig. 5

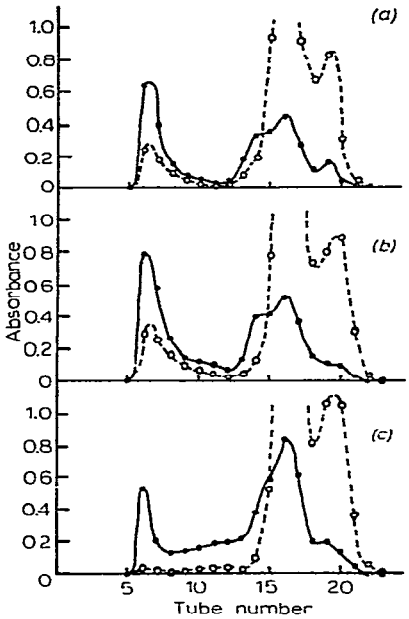


Fig. 6

Fig. 4. Fractionation on Bio-Gel P-300 of gastric mucin released from mucosa by treatment with water at 50° for 24 h. Absorbances were determined at 260 nm O--O--O, and at 490 nm after reaction in the phenol–sulphuric acid assay ●—●—●.

Fig. 5. Release of carbohydrate from gastric mucosa by treatment with pronase in 0.1*I*-Tris buffer (pH 8) at 37°, as determined by the phenol–sulphuric acid assay. Absorbances were determined at 490 nm.

Fig. 6. Fractionation on Bio-Gel P-30 of gastric mucin released from mucosa by treatment with pronase in 0.1*I*-Tris buffer (pH 8) at 37° for (*a*) 3 h; (*b*) 7.5 h; (*c*) 25.5 h. Absorbances were determined at 260 nm O--O--O, and at 490 nm after reaction in the phenol–sulphuric acid assay ●—●—●.

of the combined fractions from the Bio-Gel P-30 column (Fig. 7). *DP-III-A* and *DP-III-B* were recovered by lyophilisation, following removal of material of low molecular weight (including 80–90% of the u.v.-absorbing material) on Bio-Gel P-6.

Analysis of the various fractions for amino acids, amino sugars, and neutral sugars was carried out on the Technicon Autoanalyzer. The standard method for amino acid analysis was used following hydrolysis with 6M hydrochloric acid for 24 h at 111°. Amino sugars were analysed[20] following hydrolysis with 2M hydrochloric acid for 18 h at 100°. Neutral sugars were analysed[21] after hydrolysis with M sulphuric acid for 6 h at 100°. Each hydrolysis was carried out in a sealed ampoule under nitrogen. Sialic acid was determined[22] after hydrolysis with 50mM sulphuric acid for 1 h at 80°. The analytical results are given in Table I. The reaction of the four fractions in the Dische[23] carbazole assay for uronic acids[24] was examined; all of the

colour at 530 nm could be accounted for in terms of the neutral hexose content, with the exception of *DP-III-B*; the latter gave a colour with an absorption maximum at 472 nm.

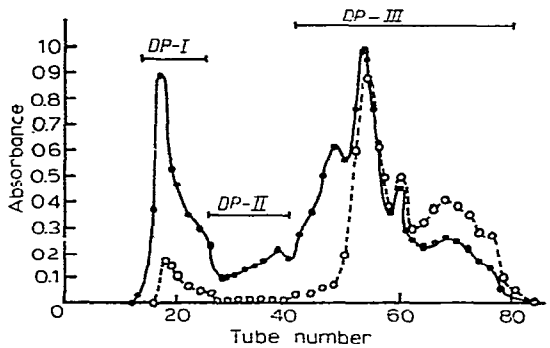


Fig. 7. Large-scale fractionation on Bio-Gel P-30 of gastric mucin released from mucosa by treatment with pronase in 0.1*I*-Tris buffer (pH 8) for 7.5 h at 37°. Absorbances were determined at 260 nm O--O--O, and at 490 nm after reaction in the phenol–sulphuric acid assay ●—●—●. Fractions were combined as indicated.

TABLE I

ANALYSIS OF THE VARIOUS FRACTIONS EXTRACTED FROM GASTRIC MUCOSA BY PRONASE DIGESTION

Sugars (nmoles/mg)	DP-I	DP-II	DP-III-A	DP-III-B	CHP-I
Gal	1140	984	137	0	1470
GlcN	784	670	235	33	1190
GalN	427	217	72	22	894
Fuc	652	665	70	0	263
Man	0	96	219	0	0
Glc	0	43	58	0	0
Rib	0	0	0	25	46
Amino acids (nmoles/mg)					
Asp	73	202	568	734	40
Thr	350	289	332	154	720
Ser	233	230	262	188	436
Glu	81	157	694	924	58
Pro	188	244	1110	234	300
Gly	188	324	979	382	114
Ala	[a]	[a]	406	163	196
Val	84	128	250	107	70
Cys	10	33	114	149	0
Met	5	13	38	30	0
Ile	44	65	96	43	42
Leu	61	107	177	68	36
Tyr	27	54	[a]	15	10
Phe	23	39	[a]	22	22
Lys	44	89	[a]	63	32
His	37	48	[a]	52	30
Arg	36	74	[a]	29	38

[a]Not determined.

The preparation of a glycopolypeptide from gastric mucosa. — Defatted gastric mucosa (14 g) was treated with pronase solution (1 g/l; 150 ml) for 7.5 h as described above. Following centrifugation and removal on G-10 Sephadex of all material of small molecular weight from the supernatant solution, the crude gastric mucin was fractionated on a column (90 × 4.5 cm) of A-25 DEAE-Sephadex (Cl^- form) by elution with (*i*) 0.1*I*-Tris buffer (pH 8, 2 l); (*ii*) 0.25M sodium chloride in buffer (2 l); (*iii*) M sodium chloride in buffer (2 l); (*iv*) 2M sodium chloride in buffer (2 l); (*v*) 4M sodium chloride in buffer (2 l). Fig. 8 shows the elution profile for (*i*)–(*iii*); there was no evidence of any eluted material in the 2M and 4M sodium chloride eluates. The appropriately combined fractions were designated *CHP-I*, *CHP-II*, *CHP-III*, and *CHP-IV*, in order of elution, as indicated in Fig. 8. Chromatography of *CHP-I* on Bio-Gel P-30 removed salt and the remainder of the u.v.-absorbing material. The other fractions were desalted by fractionation on G-25 Sephadex, and recovered by freeze-drying to give *CHP-I*, 120 mg; *CHP-II*, 25 mg; *CHP-III*, ~1 mg; *CHP-IV*, 30 mg.

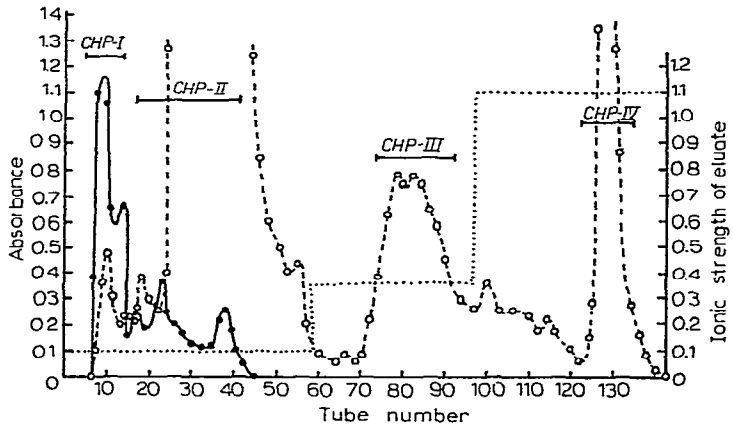
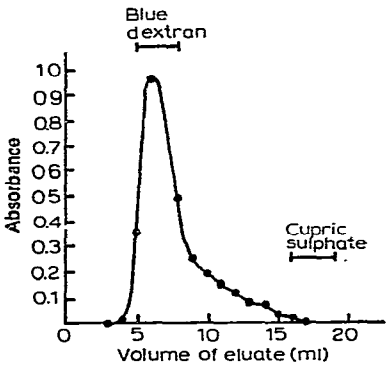


Fig. 8. Fractionation on A-25 DEAE-Sephadex of gastric mucin released from mucosa by treatment with pronase in 0.1*I*-Tris buffer (pH 8) for 7.5 h at 37°. Absorbances were determined at 260 nm O--O--O, and at 490 nm after reaction in the phenol–sulphuric acid assay ●—●—●. The column was eluted with 0.1*I*-Tris buffer (pH 8, stepwise increase in ionic strength ····· as described in text). Fractions were combined as indicated.

CHP-I (2 mg) was fractionated on a column (50 × 1 cm) of Bio-Gel P-300 by elution with M sodium chloride (Fig. 9). Most of the material appeared to be of molecular weight >300,000.

CHP-I was analysed by methods described, the results being given in Table I.

Structural investigations of the glycopolypeptide (CHP-I). — (*a*) *Infrared analysis.* The i.r. spectrum of *CHP-I* (1 mg) was determined on a Perkin–Elmer Model 325 spectrometer by using a potassium bromide disc. The spectrum showed absorption bands at 3400 (broad, O–H stretch), and at 1640 and 1540 cm^{-1} (acetamido group). No absorption at 1240 cm^{-1} (S=O stretch in sulphate group) was observed.



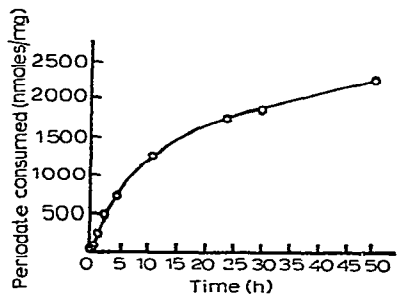


Fig. 9. Fractionation on Bio-Gel P-300 of gastric mucin fraction *CHP-I*. Absorbances were determined at 490 nm after reaction in the phenol–sulphuric acid assays.

Fig. 10. The reaction of the gastric mucin fraction *CHP-I* with periodate.

(*b*) *Oxidation with periodate.* A solution of *CHP-I* (1.71 mg) in 0.2mM sodium periodate (40 ml) was kept at 4° in the dark for 60 h. At intervals, aliquots (1 ml) in duplicate were added to a solution of ferrous 2,4,6-tri-2-pyridyl-*s*-triazine, according to the method of Avigad[25], and the absorbances at 593 nm were determined (Fig. 10). A control experiment showed no appreciable loss of periodate.

(*c*) *Analysis following oxidation with periodate.* An aqueous solution of *CHP-I* (1.71 mg/ml; 0.5 ml) was allowed to react with 0.2mM sodium periodate (20 ml) in the dark for 20 h at 4°. The reaction was terminated by addition of aqueous glycerol (0.5 ml; 10% v/v). Sodium borohydride (9 mg) was added and, after 2 h at room temperature, the excess of borohydride was decomposed by dropwise addition of acetic acid until efferverscence ceased. An aliquot (0.1 ml) of a standard, aqueous solution of L-rhamnose (567 nmoles/ml) was then added as internal standard, and the product was analysed for neutral sugars.

An identical experiment was carried out to examine the extent of oxidation of amino sugars, except that norleucine was used as the internal standard. The results, are given in Table II.

TABLE II

CARBOHYDRATE ANALYSIS OF *CHP-I* BEFORE AND AETER PERIODATE OXIDATION[a]

Sugar	*Content (nmoles/mg)*		
	Before	*After*	*Difference*
Gal	1470	719	753
GlcN	1190	1190	0
GalN	894	710	184
Fuc	263	41	184

[a]Oxidation for 20 h.

(*d*) *Treatment with alkali.* Samples of *CHP-I* (850 μg) were treated with 0.5M sodium hydroxide (5 ml) and 0.1M sodium hydroxide (5 ml) for 70 h at 20° under nitrogen. Absorbances at 241 nm were determined at intervals (Fig. 11).

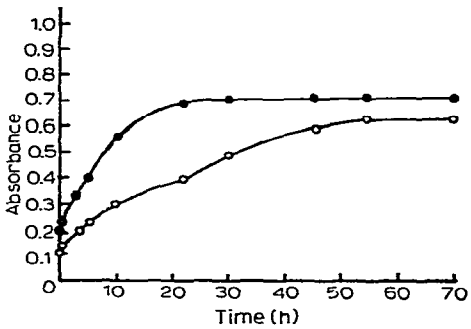


Fig. 11. The formation of α,β-unsaturated α-amino acids (as measured by the determination of absorbances at 241 nm) by the effect of (*i*) 0.5M sodium hydroxide, ●—●—● and (*ii*) 0.1M sodium hydroxide ○—○—○ on gastric mucin fraction *CHP-I* at 20° under nitrogen.

In a second experiment, *CHP-I* (1.71 mg) was allowed to react with 0.5M sodium hydroxide (4 ml), containing norleucine (400 nmoles) as internal standard, for 72 h at 20° under nitrogen. Following neutralisation (hydrochloric acid), aliquots of the reaction mixtures were hydrolysed with 2M hydrochloric acid (13 h) and 6M hydrochloric acid (24 h) and analysed for amino sugars and amino acids, respectively. The results, together with those of untreated *CHP-I*, are given in Table III.

In a third experiment, *CHP-I* (8.2 mg) was allowed to react with 0.5M sodium hydroxide (5 ml) for 72 h at 20° under nitrogen. An aliquot (0.25 ml) was analysed for the Morgan–Elson chromogen by a modification of the method of Reissig *et al.*[26]

TABLE III

AMINO SUGAR AND AMINO ACID ANALYSIS OF *CHP-I* BEFORE AND AFTER TREATMENT WITH SODIUM HYDROXIDE[a]

Amino sugar and amino acid	*Before (nmoles/mg)*	*After (nmoles/mg)*
GlcN	1190	1185
GalN	894	370
Asp	40	25
Thr	720	291
Ser	436	157
Glu	58	50
Pro	300	315
Gly	114	114
Ala	196	[b]
Val	70	70
Ile	42	48
Leu	36	25

[a]Treatment with 0.5M NaOH for 72 h. [b]Not determined.

(in which the heating step with the potassium tetraborate reagent was omitted), where the characteristic chromophore with *p*-dimethylaminobenzaldehyde was observed. Following neutralisation in a column (8 × 1 cm) of Dowex-50W, and concentration, the reaction mixture was fractionated on a column (90 × 2.5 cm) of Bio-Gel P-2. The fractions were scanned at 241 nm, by the phenol–sulphuric acid assay[18], and by Morgan–Elson assay[26] with and without the potassium tetraborate reagent. The fractionation pattern is given in Fig. 12, together with a similar fractionation of Blue Dextran 2000, raffinose, and D-galactose.

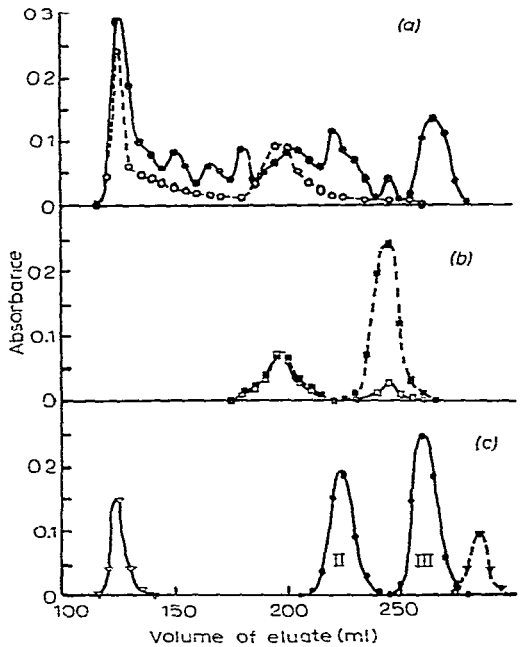


Fig. 12. Fractionation on Bio-Gel P-2 of gastric mucin fraction *CHP-I* after treatment with 0.5M sodium hydroxide for 72 h at 20° under nitrogen; (*a*) scan of fractions at 241 nm O—O—O, and at 490 nm ●—●—● after reaction in the phenol–sulphuric acid assay; (*b*) scan of fractions at 585 nm after reaction in the assay for 2-acetamido-2-deoxyhexoses □···□···□···□, and for Morgan–Elson chromogen □—□—□; (*c*) elution points of Blue Dextran ▽—▽—▽, II raffinose, and III galactose, as determined by the phenol–sulphuric acid assay at 490 nm ●—●—●, and IV cupric sulphate ▼--▼--▼, as determined in a separate fractionation experiment.

DISCUSSION

Investigations on gastric mucin from various mammalian sources have usually incorporated a proteolytic digestion step. Thus, gastric mucosa has been incubated with pepsin[3] (pH 5.6, 65°) or trypsin[7] (pH 7, 37°) and pronase[7] (pH 8), and gastric juice has been subjected to autoproteolysis[10,11] (*i.e.* pepsin digestion, pH 1.5, 37°). Ideally, an extraction procedure avoiding the use of proteolytic enzymes is to be preferred, in order to avoid chemical modification of the native mucin macromolecule.

Thus, bombardment of a suspension of mucosa in either 0.9% saline or 8M urea,

under carefully controlled conditions, was investigated as a means of solubilising the gastric mucin. Carbohydrate-containing material, mainly of low molecular weight (Fig. 2), was slowly released into solution by the latter treatment (Fig. 1), presumably by breaking down intermolecular attractions due to hydrogen bonding. The more-vigorous treatment in 8M urea gave rise to various manipulative difficulties concerning high viscosity, and precipitation when the urea was removed by dialysis; when a fractionation of the soluble components was attempted on DEAE-Sephadex, an irreversible interaction with the gel took place. Waldron-Edward and Skoryna[27] have isolated a glycoprotein (mol. wt. 2×10^6) from gastric juice by dissolution in 8M urea, storage for 3–7 days at 37°, and gel filtration on Sephadex G-200.

In view of these difficulties, methods based on the use of enzymes were investigated. Pepsin has been reported[12] to reduce the molecular weight of mucin from $\sim 2 \times 10^6$ to $1.6–3.3 \times 10^5$, and this is attributed to enzymic hydrolysis at points where the polypeptide core is bare of carbohydrate side-chains, thereby facilitating the separation of peptides from the partially degraded mucin, the solubility of which then permits an accurate study.

The choice of pepsin would eliminate the advantages, in the present work, of utilizing mucin which has not been in direct contact with the other secretory products of the stomach. Moreover, the conditions of pepsin hydrolysis, or autoproteolysis[10,11] at pH 1.5 are sufficient to hydrolyse[28] certain glycosidic linkages, such as those of sialic acid, fucose, and galactose. The extraction of the mucosa with water at 50° (Fig. 4) appeared to invalidate the use of papain at elevated temperatures; indeed, the yield of carbohydrate-containing material of high molecular weight was greater in the absence of enzyme (see Figs. 3 and 4). The release of carbohydrate-containing material into solution by the pronase digestion of mucosa at 37° was maximal after 18 h (Fig. 5). However, fractionation of the solubilised material on Bio-Gel P-30 at various intervals of time (Fig. 6) revealed that the maximal yield of glycopeptide of high molecular weight was obtained after ~7.5 h.

Thus, the treatment of the mucosa with pronase for 7.5 h was selected as the optimal extraction procedure for the degradation of contaminating protein and substitution of the mucin. Fractionation of the pronase-digest on Bio-Gel P-30 revealed a considerable degree of heterogeneity in the material of low molecular weight (Fig. 7).

Fractionation of the components *DP-I*, *DP-II*, and *DP-III* on DEAE-Sephadex revealed that most of the carbohydrate-containing material was neutral. This was unexpected in view of reports[9–12] that human gastric mucin is sulphated. Recent work[29] on a glycopolypeptide having a varied degree of sulphation showed that the sulphate content must exceed 1% for retention on DEAE-Sephadex to occur. However, histochemical studies of human gastric mucosa showed no evidence of sulphation[8,30]; this material differed in this respect from the mucosa of other mammalian sources. However, Schrager and Oates[11] found that glycoprotein from human gastric aspirates are polydisperse with respect to sulphate content, the sulphate–2-amino-2-deoxyglucose ratio varying between zero and 2:3.

The absorption maximum of the colour obtained when *DP-III-B* (the acidic component of *DP-III*) reacted in the phenol–sulphuric acid test[18] was at 480 nm (characteristic of aldopentoses), and was attributed to ribonucleic acid and confirmed in the neutral sugar analysis (Table I).

Analysis of *DP-I*, *DP-II*, *DP-III-A*, and *DP-III-B* (Table I) showed that most of the carbohydrate was concentrated in the component of larger molecular weight (*DP-I*), which resembled the blood-group substances in its composition[31]. Although some mannose and glucose appeared in *DP-II*, most occurred in *DP-III-A*, which had a lowered content of galactose, fucose, and 2-amino-2-deoxygalactose. The data suggest that *DP-II* is probably a mixture of *DP-I* and *DP-III-A*. During prolonged pronase treatment (Fig. 6), the carbohydrate material of low molecular weight increased at the expense of that of high molecular weight, presumably due to the slow proteolysis of *DP-I*. Schrager and Oates[11] have reported the removal of mannose-containing material (which was attributed to contamination by plasma glycoproteins) by molecular-sieve chromatography of gastric mucin.

The amino acid composition of *DP-I* and, to a lesser extent, of *DP-II* showed the unusual, but characteristic, proportions observed for the blood-group substances[31] and gastric mucin[12], *i.e.* a preponderance of threonine, serine, proline, glycine, and alanine (Table I). *DP-III-A* and *DP-III-B* contained much larger quantities of peptide. It was interesting to note that aspartic acid and glutamic acid accounted for nearly 60% of the composition of *DP-III-B* (Table I), and more than 50% of *DP-III-A* consisted of aspartic acid, glutamic acid, proline, and glycine. Since *DP-III-A* and *DP-III-B* were excluded from Bio-Gel P-6, the molecular weight exceeded 6,000. The significance of this result is unknown at present.

None of the fractions appeared to contain uronic acid, but the absorption maximum of 472 nm observed for *DP-III-B* in the carbazole reaction could not be explained. The relatively small quantities of sialic acid were spread fairly evenly over the three neutral fractions (Table I).

A more-detailed investigation of the glycopolypeptide (*DP-I*) of high molecular weight was carried out on a new sample (*CHP-I*). In the revised purification procedure, which removed material of molecular weight $<30{,}000$ (*i.e.* the acidic and u.v.-absorbing material), *CHP-I* appeared as a single component, equivalent to *DP-I*. The fractionation on Bio-Gel P-300 (Fig. 9) revealed a degree of polydispersity with respect to molecular weight, probably arising from the initial treatment with pronase. The analysis of *CHP-I* closely resembled that of *DP-I* (Table I), although *CHP-I* contained considerably less fucose and contained a small proportion of ribose. A considerable variation with respect to fucose has been observed in a separate investigation, in which biopsy samples from 200 different humans were analysed for carbohydrate (results to be published). The absence of sulphate was confirmed by the i.r. spectrum.

Further studies of the glycopolypeptide (*CHP-I*) revealed a much greater degree of complexity than that suggested by Schrager and Oates[11], who proposed the

basic structure **1**, on which was superimposed the terminal oligosaccharide units that endow the molecule with blood-group activity

Gal–GlcNAc–Gal–GlcNAc–Gal–GlcNAc–Gal–GalNAc–(serine or threonine)

1

Periodate oxidation of *CHP-I* was hampered by the occurrence of "over-oxidation" (Fig. 10) which precluded an accurate estimation of periodate consumed by Malapradian oxidation. Units of fucose, galactose, and 2-amino-2-deoxygalactose, but not 2-amino-2-deoxyglucose, were partially destroyed by the periodate after 18 h (Table II).

Considerable evidence has accumulated that, for the blood-group substances and gastric mucin, the carbohydrate side-chains are glycosidically linked through 2-amino-2-deoxygalactose to hydroxyl groups of threonine and/or serine residues[12,31–33]. The lability[34] of such glycosides to alkali has been used to provide important structural information on many glycoproteins in which this type of linkage is suspected to occur[32–36]. Characteristic of such a reaction is the large increase in absorbance at 241 nm due to the formation of α,β-unsaturated α-amino acids, arising[34] from the β-elimination of the *O*-glycoside, which was observed for *CHP-I* (Fig. 11). Analysis of *CHP-I*, before and after treatment with alkali (Table III), revealed that a large proportion of the threonine and serine units had been destroyed. When hexosamine is involved in the linkage to threonine or serine, the appearance of Morgan–Elson chromogen is a characteristic feature[35,36] of alkaline treatment, accompanied by destruction of 2-amino-2-deoxygalactose. Both features were observed in the alkaline treatment of *CHP-I*.

Certain generalised observations can now be made to rationalise the data obtained in the alkaline degradation of *CHP-I*. Since the serine and threonine content exceeded that of 2-amino-2-deoxygalactose (Table I), and not all of the serine and threonine was destroyed by alkali (Table III), it was evident that some of these amino acid residues were not involved in *O*-glycosidic linkages. Also, the amounts of serine and threonine destroyed (708 nmoles/mg) were less than the total amount of 2-amino-2-deoxygalactose in *CHP-I* (894 nmoles/mg), suggesting that not all of this amino sugar was linked *O*-glycosidically to the hydroxyamino acids. However, alkaline-degradation studies[37] of synthetic serine *O*-glycosides have shown that, for β-elimination to occur, *both* the amino and carboxyl groups of the amino acid must be substituted. Hence *CHP-I*, obtained by proteolytic digestion, might contain a proportion of *terminal* hydroxyamino acid residues linked *O*-glycosidically.

The destruction of the 2-amino-2-deoxygalactose residues liberated by the β-elimination reaction to form 3-acetamido-5-(1,2-dihydroxyethyl)furan and 2-acetamido-2,3-dideoxy-hex-2-enofuranose (Morgan–Elson chromogen) represents a more-complicated reaction[38]. Substitution of the amino sugar at O-4 precludes chromogen formation, whereas substituents at O-3 are eliminated to give chromogen. Indeed, under the conditions used, chromogen formation is negligible unless the amino sugar residue is substituted at O-3, and even here the reaction may not proceed

to completion[35]. Since nearly 60% of the 2-amino-2-deoxygalactose residues were destroyed when *CHP-I* was treated with alkali (Table III), it may be concluded that the major proportion is substituted at O-3. It is noteworthy that the periodate-oxidation studies (Table II) show that ~20% of the 2-amino-2-deoxygalactose residues were unsubstituted at O-3 and O-4.

Fractionation of the alkaline-degradation products of *CHP-I* (Fig. 12) revealed that all of the chromogen formed was substituted, since the elution points were in the disaccharide and pentasaccharide regions, thus indicating that the original 2-amino-2-deoxygalactose residue was linked at O-6 (since substitution at O-4 precludes chromogen formation, and substitutents at O-3 would be eliminated). Unsubstituted chromogen is eluted at the expected monosaccharide region[36], thus eliminating the possibility of nonspecific interaction with the gel. 2-Amino-2-deoxyglucose was not the source of chromogen, since none was destroyed by the alkaline treatment. The presence of reducing 2-acetamido-2-deoxyhexose, which had not undergone chromogen formation by the alkaline treatment, was demonstrated (Fig. 12*a*). It was shown, using the Technicon Autoanalyser, that the amino sugar involved was 2-acetamido-2-deoxygalactose, but the elution at the disaccharide position (Fig. 12) revealed that it was substituted by a single sugar residue. The substituent is probably located at O-6, since a substituent at O-3 would have been eliminated to form chromogen by the alkali treatment, and a substituent at O-4 would have precluded chromogen formation even on heating with tetraborate.

The scan at 214 nm (Fig. 12*a*) showed two peaks, one in the excluded region attributable to the α,β-unsaturated α-amino acids formed during the β-elimination process, and another, coincident with the major Morgan–Elson chromogen peak at about 200 ml, which also absorbed at this wavelength. The multiple peaks observed in the phenol–sulphuric acid scan (Fig. 12*a*) appeared to coincide with different degrees of polymerisation, and presumably represented various oligosaccharide units originating from the elimination or "peeling" of these units from the O-3 position of the 2-amino-2-deoxygalactose residue. The extent of alkaline "peeling" action was limited by the presence of alkali-stable 2-amino-2-deoxyglucose residues in these oligosaccharide units. Further studies are in progress on these fragments.

Summarising, the preliminary structural investigations indicate that the glycopolypeptide (*CHP-I*) is composed of a polypeptide chain consisting principally of threonine, serine, proline, glycine, and alanine, substituted by oligosaccharide chains having different degrees of polymerisation, linked *O*-glycosidically through 2-acetamido-2-deoxygalactosyl-threonine or -serine bonds; all of this amino sugar appeared to be substituted at O-6, and a major proportion also at O-3. The glycopolypeptide of human gastric mucosa shows a strong resemblance to the structure of blood-group substances[31–33], supporting the reports[10–12] of other studies on gastric mucin, in spite of findings that human gastric mucin is sulphated. Indeed, Schrager[12] and Schrager and Oates[11] have detected a correlation between the carbohydrate composition and the blood-group activity of their mucin specimens, corresponding to the oligosaccharide units expected for the particular blood-group activities[39]. However,

a major difference was observed between the glycopolypeptide of mucosa and the blood-group substances, in that the former lacked alkali-labile, O-3 substituted, 2-amino-2-deoxyglucose residues. *CHP-I* originated from a patient of Blood Group B, but, unfortunately, the secretor status was unknown. However, the absence of terminal, non-reducing 2-amino-2-deoxygalactose residues is compatible with *CHP-I* possessing either blood-group B activity or Le^a activity (Non-secretor)[39].

The glycopeptides produced by the proteolytic treatment of gastric mucin were of similar molecular weight, indicating that the enzymic cleavage was not random and that such glycopeptides were probably linked together by peptide in the native gastric mucin. This would give a macromolecule of extremely high molecular weight, which would endow the mucin with the necessary properties of high viscosity, somewhat diminished by 2-mercaptoethanol by the breaking of disulphide bridges[40]. These observations suggest an important insight into the total structure of the native mucin macromolecules. Those lengths of peptide that are free of oligosaccharide prosthetic groups would be susceptible to proteolysis by the contents of gastric juice. Such a breakdown of gastric mucin *in vivo* is of considerable biochemical importance[27,28], whereby the digestion of spent mucin would facilitate its passage through the gastrointestinal tract. Finally, the peptide component rich in aspartic and glutamic acids (*DP-III*) might well be an integral part of the native mucin macromolecule, possibly fulfilling the function of the sulphate groups found in other mammalian gastric mucins.

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