Purification and Characterization of Sulfated Glycoproteins and Hyaluronidase-Resistant Mucopolysaccharides from Dog Gastric Mucosa*

Treva Pamer, George B. Jerzy Glass, and Martin I. Horowitz

ABSTRACT: Sulfated glycoproteins were purified from papain-digested mucosal scrapings of the dog fundic stomach. The acidic fraction, isolated by ethanol and cetylpyridinium chloride precipitations, was freed of nucleic acids by precipitation at pH 1.5 and of digestible mucopolysaccharides by treatment with testicular hyaluronidase. After gel filtration on Bio-Gel P-30 and Sephadex G-100, final separation of three sulfated glycoprotein fractions was achieved by elution from DEAE-Sephadex with NaCl. Each fraction exhibited a single band on cellulose acetate electrophoresis at pH 3.0 and 9.0. The three fractions were similar in having equimolar ratios of hexosamine and galactose, the major carbohydrate components, and in having glucosamine and galactosamine in equimolar ratios. Sulfate was present in each fraction with a molar ratio to hexosamine ranging from 0.11 to 0.45; this ratio increased with increasing molarity of eluting salt. Fucose and sialic acid content decreased with increasing molarity of eluting salt. Total protein ranged from 10.9 to 12%. Threonine, serine, proline, and valine constituted 88% of the total amino acid residues while aromatic and sulfur amino acids were present only in trace quantities. Mild acid hydrolysis produced a rapid release of fucose and sialic acid, indicating their presence as end groups. Hexosamine was the most slowly released carbohydrate, suggesting its involvement in linkage to the peptide core. Sulfate was stable to 0.04 N HCl, indicating the absence of *N*-sulfate groups. Two hyaluronidase-resistant mucopolysaccharides were partially purified by copper precipitation and tentatively identified as chondroitin sulfate-B and heparitin sulfate on the basis of their reactions in the carbazole and orcinol assays for uronic acids.

For many years studies of the sulfated mucopolysaccharides of the gastric mucosa centered around mucoitin sulfate, first isolated by Levene and Lopez-Suarez (1916) from hog gastric mucin and later from dog gastric juice (Komarov, 1935). Doubts concerning the existence of mucoitin sulfate arose when Masamune (1949) and Smith and Gallop (1953) were unable to isolate the compound. The latter authors concluded that heparin, and not mucoitin sulfate, was the glucosamine-containing mucopolysaccharide of hog gastric mucosa.

Recent interest in the sulfated products of the gastrointestinal tract has been stimulated by the isolation of sulfated glycoproteins from the colonic mucosa of sheep (Kent and Marsden, 1963) and pig (Inoue and Yosizowa, 1966), dog gastric mucosa (Hakkinen *et al.*, 1965b), and the gastric juice of dog (De Graef and Glass, 1967) and man (Hakkinen *et al.*, 1965a; Martin *et al.*, 1967, 1968). These and the glycoproteins found in human bronchial mucus (Havez *et al.*, 1965) and dog submaxillary mucin (Bignardi *et al.*, 1964) are all similar in being free of uronic acids and in containing hexosamine, hexose, fucose, sialic acid, and sulfate. Thus, they appear to constitute a class of compounds which is common to many mucous secretions.

Histochemical and secretory studies (Gerard *et al.*, 1967a,b, 1968; De Graef and Glass, 1968a,b; Woussen-Colle and De Graef, 1968) have indicated that CHS-A[1] forms the major sulfated component of the dog gastric juice and is derived from the chief peptic cell, while the sulfated glycoprotein predominates in the mucous gel and is a product of the crypt and surface epithelial cells.

Preliminary studies in this laboratory indicated that the purification methods previously used by other authors (Hakkinen *et al.*, 1965b; Inoue and Yosizowa, 1966) gave incomplete separation of the sulfated glycoproteins of dog gastric mucosa from sulfated mucopolysaccharides and nucleic acids.

The present communication deals with an improved method for the purification of sulfated glycoproteins from the dog gastric mucosa and their characterization as well as that of two hyaluronidase-resistant mucopolysaccharides from the same source.

Experimental Section

Analytical. Hexoses, hexosamines, fucose, and protein

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: CHS, chondroitin sulfate; CPC, cetylpyridinium chloride; PAS, periodic acid–Schiff.

were quantitatively determined by the methods employed previously (Glass *et al.*, 1967). The ratio of glucosamine to galactosamine was determined by the colorimetric method of Tracey (1955). Hydrolysis with 4 N HCl at 100° for 4 hr was utilized for both total hexosamine and glucosamine:galactosamine ratio determinations. Sialic acids were determined by the thiobarbituric acid method of Warren (1959) with *N*-acetylneuraminic acid (General Biochemicals, Chagrin Falls, Ohio) as the standard. Uronic acids were determined with the carbazole–borate–sulfamate method of Galambos (1967). Sulfate was determined with a modification of the benzidine method of Spencer (1960) employing hydrolysis in 5 N formic acid at 100° for 18 hr. Hydrolysate (2 ml) and 0.1% benzidine (5 ml) in ethanol were added to each tube. Amino acids were assayed on the Beckman–Spinco amino acid analyzer after hydrolysis with 6 N HCl for 18 hr in evacuated, sealed vials. HCl was removed by repeated evaporation *in vacuo* over solid NaOH and P_2O_5. Susceptibility to digestion with testicular hyaluronidase was determined by CPC turbidity before and after incubation with the enzyme as described by De Graef and Glass (1968b). Nucleic acids were determined by ultraviolet absorption at 260 mμ using yeast RNA (Worthington Biochemical, Freehold, N. J.) as a reference material.

After hydrolysis in 2 N HCl at 100° for 5 hr and removal of excess HCl as described for amino acid analysis, hexoses and 6-deoxyhexose were qualitatively identified by the thin-layer chromatographic system of Lamkin *et al.* (1966) modified here by employing Avicel plates; separations similar to those reported for cellulose MN plates were obtained. After hydrolysis as described for amino acid assays, hexosamines were identified by paper chromatography using a benzene–1-butanol–pyridine–H_2O (1:5:3:3) solvent system. Spots were developed with ninhydrin. Glucuronic and iduronic acids were identified by their differential reactions with orcinol–HCl (Brown, 1946) and carbazole–H_2SO_4 (Dische, 1947) as reported by Hoffman *et al.* (1956).

Electrophoresis. Zone electrophoresis was performed on Sepraphore III (Gelman Instrument Co., Ann Arbor, Mich.) cellulose acetate strips (1 × 6.75 in.) in the Gelman Rapid Electrophoresis Chamber. Samples (usually 200 μg in 10 μl) were applied at the cathodic supporting bridge and run for 40 min at 250 V. Sodium borate (pH 9.0, *I* 0.2) and pyridine formate (pH 3.0) (16 ml of 90% formic acid and 8 ml of pyridine per l.) buffers were used. Strips were run in duplicate and stained with 1% alcian blue at pH 1.5 (De Graef, 1964) and with PAS stain (Köiw and Grönwall, 1952). CHS-C and heparin (gifts of Dr. I. Danishefsky) were used as reference standards.

Immunological. Rabbit antiserum to canine gastric mucus was prepared as described by Horowitz (1967). Purified sulfated glycoprotein fractions were tested for reaction with this antiserum by the Ouchterlony immunodiffusion technique using glycoprotein concentrations of 4 and 8 mg per ml.

Preparation of Mucosa and Homogenate. The fundic portions of 23 dog stomachs were separated on the basis of anatomical landmarks, washed with 0.9% NaCl, and stored frozen until processed. The entire mucosal layer was separated by scraping with glass slides, yielding 1 kg of wet mucosa. The scrapings were homogenized with three volumes of 0.2 M sodium acetate buffer (pH 5.6) (containing 0.79 g of cysteine hydrochloride and 1.26 g of EDTA per l.).

Papain digestion was employed for destruction of proteins. Batches (100 g) of the mucosal homogenate were incubated at 65° for 36 hr with 1 g of purified papain powder (Mann Laboratories, New York, N. Y.). The small amount of brown residue remaining was removed by filtration through glass wool. Three volumes of cold ethanol were added to the filtrate and the resulting suspension was kept at 4° overnight. The precipitate was collected by centrifugation at 30,000*g* in a Sorvall refrigerated centrifuge, washed with cold ethanol, and dissolved in distilled water. An aliquot of the ethanol supernatant was concentrated in a rotary evaporator, dialyzed, and lyophilized. Examination of this material on cellulose acetate electrophoresis showed only traces of alcian blue positive components, indicating that acidic mucopolysaccharides and sulfated glycoproteins were essentially absent from the ethanol supernatant.

CPC precipitation was employed to isolate acidic materials from the dissolved ethanol precipitate. A 5% solution of CPC was added dropwise until coagulation of the precipitate occurred. The precipitate was collected by centrifugation at 10,000*g* at room temperature, washed with distilled water, and dissolved in 2 N $MgCl_2$ at 40°. The acidic components were precipitated two times with three volumes of ethanol. The final ethanol precipitate was dissolved in distilled water, dialyzed, and lyophilized. The material from all batches was pooled, yielding 6.2 g (fraction I). An aliquot of the CPC supernatant was treated with three volumes of ethanol. The resulting precipitate was dissolved in distilled water, dialyzed, lyophilized, and examined on cellulose acetate electrophoresis. No alcian blue positive material was found, indicating complete precipitation of both acidic mucopolysaccharides and sulfated glycoproteins with CPC. Two PAS-positive bands of slight anodic mobility were shown by the CPC supernatant material.

Removal of nucleic acids from fraction I was achieved by precipitation at pH 1.5. Fraction I was dissolved in distilled water and adjusted to pH 1.5 with dilute HCl in the cold, and the resulting precipitate of nucleic acids (fraction I:A) was removed by centrifugation at 40,000*g* for 1 hr. The precipitate was dissolved in distilled water, dialyzed, and lyophilized, yielding 0.8 g. The supernatant (fraction I:B) was neutralized with dilute NaOH, dialyzed, and lyophilized, yielding 5.4 g. Nucleic acids and sulfate were determined in fractions I:A and I:B. Fraction I:A contained 75% of the total recovered nucleic acids and 3% of the total recovered sulfate.

Testicular hyaluronidase digestion was performed to remove CHS-A and C and hyaluronic acid from fraction I:B. Fraction I:B (5.4 g) and 250 mg of testicular hyaluronidase (Worthington, Freehold, N. J.) were dissolved in 150 ml of 0.1 M sodium acetate buffer (pH 5.0) containing 1.3 g of NaCl and incubated at 37° for 24 hr. Digestion was performed in a dialysis bag with simultaneous dialysis against 1.5 l. of the same buffer under

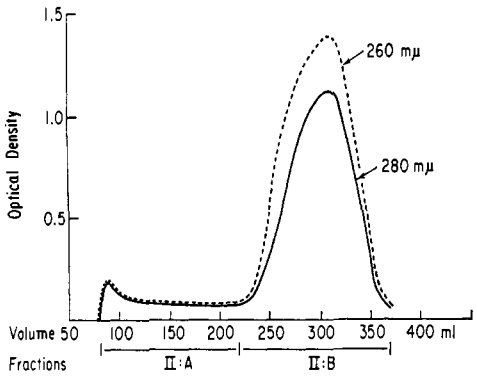

FIGURE 1: Gel filtration on Bio-Gel P-30 (200–400 mesh) of fraction II. An 800-mg sample was applied to the column (2.9 × 45 cm) and eluted with distilled H_2O at a flow rate of 30 ml/hr.

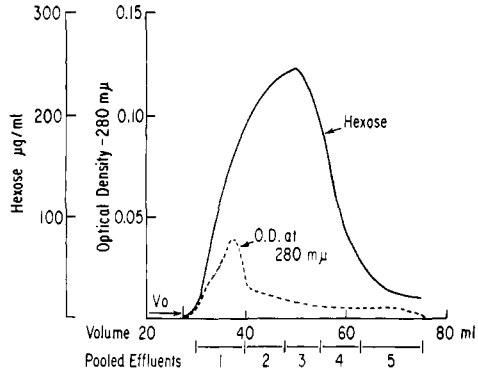

FIGURE 2: Gel filtration on Sephadex G-100 of fraction II:A. A 74-mg sample was applied to a column (1.45 × 61 cm) and eluted with distilled H_2O at a flow rate of 12 ml/hr.

toluene. The bag contents were then dialyzed against 10 l. of distilled water for 4 hr and precipitated with CPC and ethanol as described above, yielding 1.6 g (fraction II). Fraction II was assayed for susceptibility to digestion with hyaluronidase and found to contain no digestible components.

Gel filtration on Bio-Gel P-30 of fraction II was employed to separate the bulk of sulfated glycoprotein from the remaining nucleic acid fragments and hyaluronidase-resistant mucopolysaccharides. Bio-Gel P-30 (200–400 mesh) (Bio-Rad Laboratories, Richmond, Calif.) was allowed to swell in distilled water, washed repeatedly with distilled water at 80°, cooled to room temperature, and packed into a column (2.9 × 45 cm). Fraction II was applied to the column in two aliquots of 800 mg each, in 10 ml of distilled water, and eluted with distilled water at a flow rate of 30 ml/hr. Fractions (7 ml) were collected, and their optical density at 260 and 280 mμ was determined. The elution diagram of one of the columns is shown in Figure 1. The eluted materials were divided into two fractions, as indicated in Figure 1, and the corresponding fractions from the two columns pooled together and lyophilized. The initial effluent having low optical density at both 260 and 280 mμ yielded 511 mg (fraction II:A), and the retarded peak of high optical density at 260 and 280 mμ yielded 664 mg (fraction II:B).

Gel filtration on Sephadex G-100 was employed to study the molecular size and homogeneity of fraction II:A and to isolate the carbohydrate-rich components. Sephadex G-100 (Pharmacia, Upsala, Sweden) was treated as described for Bio-Gel P-30 and packed into a column (1.45 × 61 cm). Fraction II:A (74 mg) in 2 ml of distilled water was applied to the column and eluted at a flow rate of 12 ml/hr. Fractions of 2.5 ml were collected, and the optical density at 280 mμ and hexose content of each were determined. The void volume of the column was determined using human serum as a marker. The elution diagram of the fractionation is shown in Figure 2. The effluent was arbitrarily divided into five pools which were assayed for carbohydrate components, protein, and sulfate.

A similar fractionation was performed on a preparative scale to isolate the carbohydrate-rich components (313 mg of fraction II:A on a column 2.9 × 60 cm). The effluent material corresponding to pools 2–4 of Figure 2 was pooled and lyophilized, yielding 180 mg (fraction III).

Chromatography on DEAE-Sephadex was utilized for further fractionation of fraction III. DEAE-Sephadex A-50 (Pharmacia, Upsala, Sweden) was recycled twice with dilute NaOH and HCl, equilibrated with 0.2 N NaCl, and packed into a column (0.9 × 21 cm). Fraction III (180 mg) dissolved in 5 ml of 0.2 N NaCl was applied to the column, and the column was washed with the same salt solution. Stepwise elution with 0.4, 0.6, 0.8, and 2.0 N NaCl was performed at a flow rate of 20 ml/hr. Fractions (10 ml) were collected and their optical density at 215 mμ was determined. Fractionation on DEAE-Sephadex is shown in Figure 3. The material eluted at each salt step was pooled, concentrated in a rotary evaporator, dialyzed, and lyophilized. Effluent at 0.2 and 2.0 N NaCl yielded 1 and 4 mg, respectively. These materials were not studied further. Effluent at 0.4 (fraction III:A), 0.6 (fraction III:B), and 0.8 N NaCl (fraction III:C) yielded 72, 65, and 19 mg, respectively, with a total recovery from the column of 90%. These three major fractions were assayed for carbohydrate components, protein, and sulfate. Carbohydrate components were identified by chromatography, and the electrophoretic behavior of the fractions was studied on

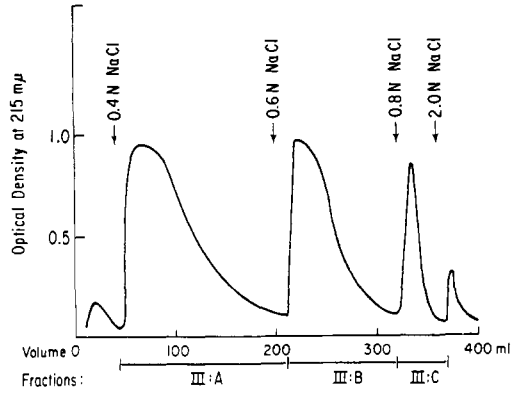

FIGURE 3: DEAE-Sephadex (A-50) chromatography of fraction III. A 180-mg sample was applied to a column (0.9 × 21 cm) and eluted stepwise with NaCl as indicated.

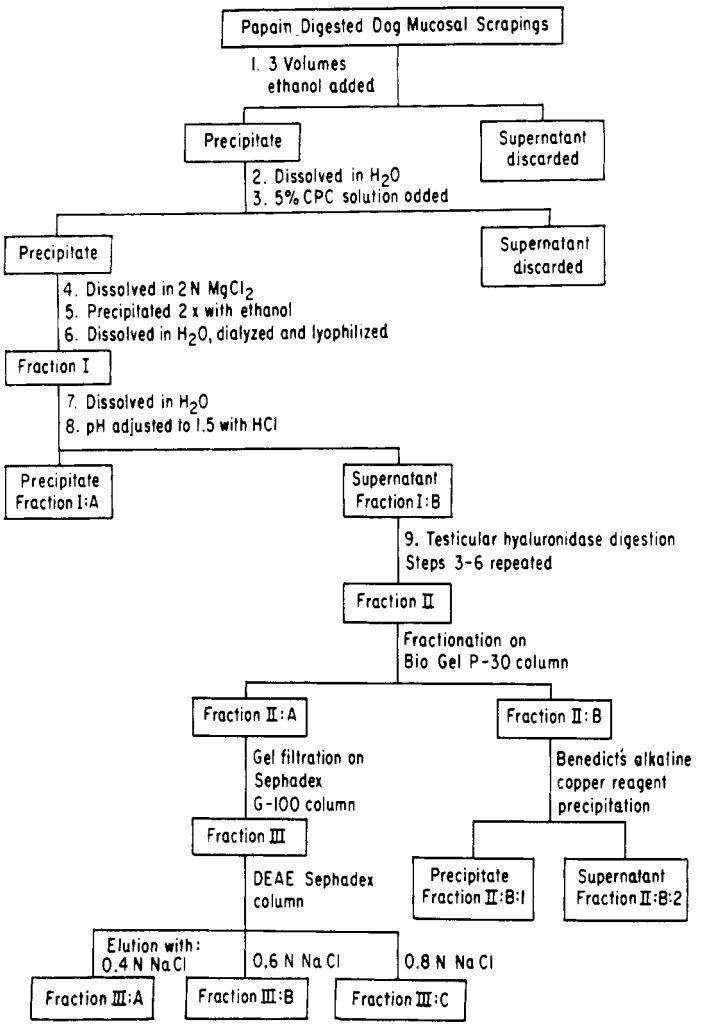

FIGURE 4: Flow diagram of the fractionation of the acidic components of dog fundic mucosa.

cellulose acetate. Amino acid compositions were determined for fractions III:A and III:B.

Further fractionation of fraction II:B was achieved by the copper precipitation method of Cifonelli *et al.* (1958) which selectively precipitates CHS-B. After separate passage through Dowex 50 (H^+), both the copper precipitate (fraction II:B:1) and the supernatant (fraction II:B:2) were dialyzed and lyophilized, yielding 198 and 300 mg, respectively. The two fractions were assayed for uronic acids by both the carbazole–H_2SO_4 and orcinol–HCl methods. The scheme of the entire fractionation is shown as a flow diagram in Figure 4.

Partial Acid Hydrolysis of Fraction II:A. Aliquots (3 mg) of fraction II:A were hydrolyzed at 100° for 1–2 hr in 5 ml of 0.04 or 0.1 N HCl. The hydrolysates were neutralized with dilute NaOH and dialyzed; the bag contents were diluted to 10 ml and assayed for carbohydrates and sulfate. Per cent loss of these components was calculated by comparison with an untreated sample in the same dilution.

Results

Electrophoretic Characterization of Fractions I and II:A. Cellulose acetate electropherograms of fractions I and II:A at pH 3.0 and 9.0 stained with alcian blue are shown in Figure 5. Fraction I exhibited three bands at pH 3.0 (strip 1) which have been identified by comparison with purified materials. The most rapidly migrating band moved like the heparin standard and represents only a trace component since heparin stains much more intensely than CHS. The intermediate band migrated like the CHS-C standard. The slow band corresponded to the sulfated glycoprotein (fraction II:A) which is shown on strip 2.

At pH 9.0, fraction I (strip 3) had only two bands. The fast band migrated like the heparin and CHS-C standards which were unresolved at this pH. The slow band corresponded to the sulfated glycoprotein (fraction II:A) shown on strip 4. Duplicates of each of the four strips stained with PAS showed only one band corresponding to that of the sulfated glycoprotein.

Composition of Fraction I. The per cent composition of fraction I has been calculated from the weight of the subfractions obtained at various steps of the fractionation procedure. The scheme for evaluation of each component and the results of the calculations are shown in Table I. The hyaluronidase-digestible components

TABLE I: Composition of Fraction I (6200 mg).[a]

Component	Fraction	Wt (mg)	%
Nucleic acids	I:A	1070[b]	17
CHS-A and C and hyaluronic acid	I:B minus II	3800[c]	61
CHS-B	II:B:1	64[d]	1.1
Heparitin sulfate	II:B:2	101[d]	1.6
Sulfated glycoprotein	II:A	511	8.2

[a] Each component was estimated from the weight of the subfraction in which it was isolated. [b] Corrected for 75% recovery. [c] Weight loss on hyaluronidase digestion. [d] Uronic acid (mg) by orcinol–HCl × 2.8 = mg of sulfated mucopolysaccharide.

(CHS-A and -C and hyaluronic acid) constituted 61% of fraction I. No attempt was made in this study to identify or quantitate the individual members of this group. Nucleic acid fragments constituted 17% of fraction I, after correction for the recovery of 75% noted in the Experimental Section. Hyaluronidase-resistant mucopolysaccharides, tentatively identified as CHS-B and heparitin sulfate, constituted 1.1 and 1.6%, respectively, based on uronic acid content. Characterization of these two components will be discussed below. Fraction II:A composed of a mixture of sulfated glycoprotein species constituted 8.2% of fraction I. This tabulation includes only 88.9% of fraction I since no correction has been made for incomplete recoveries from Bio-Gel P-30 and from the copper precipitation step.

Bio-Gel P-30 Chromatography of Fraction II. Fraction II:A, the sulfated glycoproteins, had no detectable uronic acids. This fraction, along with standards of CHS-C and galactose, was assayed for uronic acid by the method of Galambos (1967). This method has been reported to suppress the interfering reaction of galactose while giving nearly equal extinctions for glucuronic, galacturonic, and iduronic acids. The concentrations and optical densities at 525 mμ, respectively, of the samples tested were: fraction II:A (2.0 mg/ml, 0.250), galactose (0.6 mg/ml, 0.270), CHS-C (0.02 mg/ml, 0.121), and fraction II:A (2.0 mg/ml) plus CHS-C (0.02 mg/ml, 0.352). The total absorbancy of fraction II:A could be attributed to its galactose content of 29%. CHS-C could easily be detected at a concentration representing a 1% contamination and when added to fraction II:A at this level gave 84% recovery.

The absence of nucleic acids from fraction II:A was confirmed by ultraviolet spectrum, where neither a 260-mμ maximum nor a 230–240-mμ minimum was found, and by the spectrum of the thiobarbituric acid chromogen, where 549-mμ adsorption characteristic of 2-deoxyribose (Warren, 1959) was not found. Fraction II:A showed a single PAS and alcian blue positive band on cellulose acetate electrophoresis at both pH 3.0 and 9.0 (see Figure 5, strips 2 and 4).

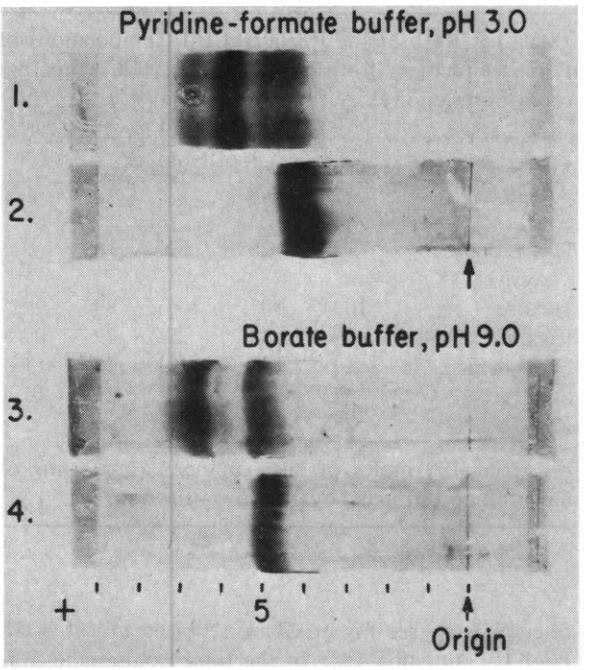

FIGURE 5: Electrophoresis on cellulose acetate of fraction I (strips 1 and 3) and fraction II:A (strips 2 and 4) at 250 V for 45 min.

Fraction II:B consisted primarily of hyaluronidase-resistant mucopolysaccharides and nucleic acids. Electrophoretic analysis showed a minor band with a mobility similar to that of the sulfated glycoproteins.

Partial Acid Hydrolysis of Fraction II:A. The results are shown in Table II where they are expressed as per cent loss compared with the untreated original material. Fucose and sialic acid were the most rapidly released components, with 74 and 92% loss, respectively, after 1-hr hydrolysis in 0.04 N HCl, during which time no sulfate or hexosamine was released. Hexose was released more rapidly than hexosamine under each of the three hydrolysis conditions, and the loss of sulfate closely paralleled that of hexosamine.

Sephadex G-100 Chromatography of Fraction II:A. Hexosamine recovery data and the composition of the materials eluted in Sephadex G-100 chromatography of

TABLE II: Results of Partial Acid Hydrolysis of Fraction II:A.[a]

		Component (% loss)				
HCl (N)	Time (hr)	Hexosamine	Hexose	Fucose	Sialic Acid	SO_4^{2-}
0.04	1	0	28	74	92	0
0.04	2	19	53	87	95	20
0.1	2	31	54	89	92	28

[a] Expressed as per cent loss on dialysis after hydrolysis at 100°.

TABLE III: Hexosamine Recovery and Compositions of Pooled Fractions from Sephadex G-100 Chromatography of Fraction II:A.

	Pool Number				
	1	2	3	4	5
Hexosamine (% recovery)	15	26	32	18	9
Hexose[a]	103	107	106	99	99
Fucose[a]	27	32	29	29	29
Sialic acid[a]	10	17	19	20	17
SO_4^{2-}[a]	37	23	18	21	15
Protein[b]	28	6	5	6	

[a] Moles/100 moles of hexosamine. [b] Mg/100 mg of hexosamine, estimated by the Lowry method.

fraction II:A (see Figure 2) are reported in Table III. Pool 1, containing 15% of the total hexosamine, was excluded from the column and exhibited a relatively high content of both sulfate and protein with a relatively low sialic acid content. This material would seem to represent a protein-rich component resulting from incomplete papain digestion. The bulk of the carbohydrate-rich material was retarded on the column and eluted over a large volume of the column effluent. Pools 2–4 (fraction III), containing 76% of the total hexosamine, showed very similar composition and low protein content.

Fraction III gave no precipitin reaction with antiserum to dog gastric mucous secretion. This antiserum showed eight precipitin bands in immunoelectrophoretic tests against pooled dog gastric secretion (Horowitz, 1967). Thus fraction III was free of any intact protein and glycoprotein components of the dog gastric mucus which react with this antiserum.

Pool 5, containing 9% of the total hexosamine, differed from pools 2–4 primarily by having lower sulfate content.

TABLE IV: Compositions of Fractions Obtained from DEAE-Sephadex Chromatography of Fraction III.[a]

Component	Fraction		
	III:A	III:B	III:C
Hexosamine	27.9	26.6	25.8
Galactose	25.8	26.6	25.1
Fucose	9.3	7.6	4.9
Sialic acid	8.6	5.1	3.9
SO_4^{2-}	1.6	3.5	6.2
Protein (amino acid analysis)	12.0	10.9	
Protein (Lowry)	1.7	1.3	2.3

[a] Expressed as per cent of dry weight of lyophilized material.

TABLE V: Compositions of Fractions Obtained from DEAE-Sephadex Chromatography of Fraction III.[a]

Component	Fraction		
	III:A	III:B	III:C
Glucosamine	50	50	50
Galactosamine	50	50	50
Galactose	92	99	97
Fucose	36	31	21
Sialic acid	18	11	9
SO_4^{2-}	11	25	45

[a] Expressed as moles/100 moles of total hexosamine.

DEAE-Sephadex Chromatography and Characterization of Fraction III and Its Subfractions. Quantitative data on carbohydrate components, sulfate, and protein of the three major fractions obtained from DEAE-Sephadex chromatography of fraction III are reported in Table IV as per cent by weight and in Table V as molar ratios. The three fractions were similar in having nearly equimolar ratios of galactose to hexosamine and of glucosamine to galactosamine. With increasing molarity of eluting salt, the molar ratio of sulfate to hexosamine increased, as would be expected, ranging from 0.11 to 0.45. Fucose and sialic acid content decreased with increasing molarity of the eluent. The protein content as estimated from amino acid composition was about ten times greater than that estimated by the Lowry method with serum albumin as the standard.

TABLE VI: Amino Acid Composition of Fractions III:A and III:B.

Amino Acid	μg/mg		Residues/1000 Residues	
	III:A	III:B	III:A	III:B
Aspartic acid	1.5	1.1	9	7
Threonine	51.3	48.2	335	346
Serine	33.1	30.4	245	248
Glutamic acid	2.6	2.4	14	14
Proline	29.2	25.3	197	188
Glycine	3.9	3.8	41	43
Alanine	3.1	3.1	27	30
Valine	15.2	12.8	101	93
Methionine	0.8	0.8	4	4
Isoleucine	1.5	1.5	9	10
Leucine	1.8	1.7	10	11
Tyrosine[a]	0.9	0.9	4	4
Phenylalanine[a]	0.8	0.7	4	3

[a] Estimated from peak height.

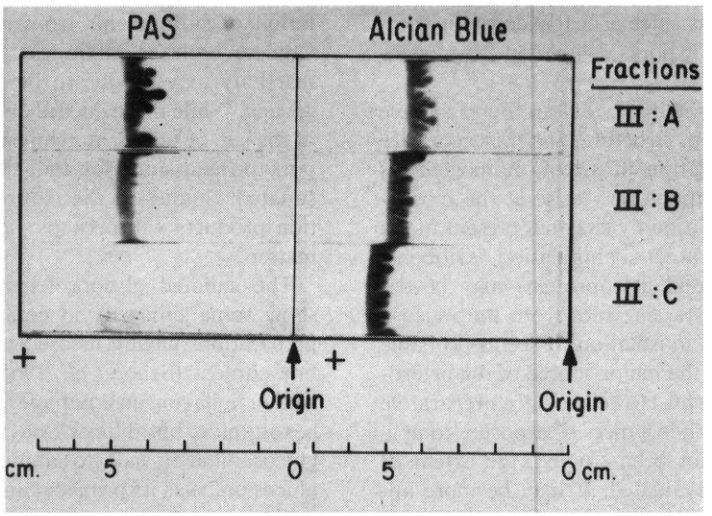

FIGURE 6: Electrophoresis on cellulose acetate of fractions III:A, III:B, and III:C in pyridinium formate buffer at 250 V for 45 min.

Qualitative analysis showed the presence of galactose, fucose, glucosamine, and galactosamine. Sugars resolved by the systems employed and found to be absent were glucose, mannose, rhamnose, and mannosamine.

The amino acid composition of fractions III:A and III:B are reported in Table VI. The two fractions showed similar amino acid composition, with serine, threonine, proline, and valine constituting 88% of the total amino acid residues. Methionine and aromatic amino acids were present only in low concentrations while half-cystine was not detected. The low concentration of aromatic amino acids and high concentration of proline explain the low values for protein found by the Lowry method.

Cellulose acetate electropherograms of fractions III:A, III:B, and III:C at pH 3.0 stained with alcian blue and PAS are shown in Figure 6. Each fraction showed a single band. The mobility of the sulfated glycoprotein increased slightly as sulfate content increased. The three DEAE-Sephadex fractions exhibited narrower bands than that of fraction II:A (see Figure 5, strip 2), and a composite of the three could be superimposed on the broader band of fraction II:A. The three fractions showed identical migrations at pH 9.0. The irregular trailing edge of the bands disappeared upon a decrease of the amount of sample applied and was unchanged by both increase (two and four times) and decrease (one-half times) of ionic strength.

The intensity of staining with PAS was strongly dependent upon sulfate content. Fraction III:A having the lowest sulfate content stained strongly with PAS, while fraction III:C having highest sulfate stained only faintly at the same concentraion, and fraction III:B was intermediate in both properties.

Characterization of Fractions II:B:1 and II:B:2. Both fractions were found to be completely resistant to hyaluronidase. Each had a major electrophoretic band of mobility of the CHS-C standard and a minor band of sulfated glycoprotein. No bands of heparinlike mobility were detected in either fraction. Fraction II:B:1 had a carbazole:orcinol ratio of 0.35 while fraction II:B:2 had a ratio of 0.95.

Discussion

The sulfated glycoproteins of dog gastric mucosa are readily separated from the neutral glycoproteins by CPC precipitation; however, the separation of the sulfated glycoproteins from acidic mucopolysaccharides and nucleic acids which are the major components of the CPC precipitate is more difficult to achieve. The sulfated glycoprotein fractions obtained from DEAE-Sephadex by the method described here are devoid of acidic mucopolysaccharides, nucleic acids, and neutral glycoproteins.

The three sulfated glycoprotein fractions from DEAE-Sephadex appear to be closely related with similar amino acid composition and similar percentages of galactose, glucosamine, and galactosamine. The three fractions differ from each other primarily in their degree of sulfation with lesser variations in fucose and sialic acid content. The sulfated glycoproteins of dog gastric mucosa would seem to comprise a continuous spectrum of variation of sulfate content rather than a small number of discrete components since fraction II:A moves as a broad diffuse band on electrophoresis at pH 3.0, where sulfate is the principal anionic group. If a small number of discrete components were present, a pattern of at least partially resolved bands would be expected. The three DEAE-Sephadex fractions show increasing electrophoretic mobility as sulfation increases. Each fraction moves as a narrow band, and the composite of the three bands spans the width of the broader band of fraction II:A, indicating that each consists of a narrow range of compositions.

Since the DEAE-Sephadex fractions are derived from materials excluded on Bio-Gel P-30 and retarded on Sephadex G-100, they may be approximated to have a molecular weight range of 30,000 to near 100,000. The variations of composition found on DEAE-Sephadex fractionation of fraction III would seem to be indepen-

dent of molecular weight since a nearly constant composition was observed for pools 2–4 eluted from Sephadex G-100.

Many types of glycoproteins have been found to have variations of composition, structure, and molecular size which preclude the isolation of a truly homogeneous preparation. These variations may arise at the biosynthetic level, from degradation by enzymes present in the tissues, or from preparative manipulation (Gibbons, 1966). The gastric sulfated glycoproteins may be degraded both by proteolytic enzymes from the mucosa and by papain employed in isolation. It seems probable that such degradation is the major source of the heterogeneity in molecular weight. However, since preparative methods which avoid both instances of exposure to proteolytic enzymes have not been studied, the extent of degradation cannot be evaluated. It may be more appropriate to employ the name glycopolypeptide (Schultze, 1958) for the materials studied here since the protein moiety of the native secretory product may have been fragmented.

However, the variations in sulfate and sialic acid contents would seem to arise at the biosynthetic level. Histochemical studies of the dog fundus have demonstrated a highly sulfated glycoprotein in the crypt cells while the sialic acid rich surface epithelial cells have lesser amounts of sulfate (Gerard *et al.*, 1967b). These results suggest that the cellular site of synthesis may determine the degree of sulfation and the relative amounts of sulfated and nonsulfated glycoproteins.

The sulfated glycoproteins of dog gastric mucosa are clearly quite distinct from the acidic mucopolysaccharides since they have no uronic acid component. The amino acid composition of the sulfated glycoproteins resembles that of many glycoproteins of mucous secretions in having a predominance of serine, threonine, and proline with only trace amounts of aromatic and sulfur-containing amino acids (Buddecke, 1966). However, the high content of valine relative to other amino acids found here seems to be unique.

A structure having glycosidic linkages between the hexosamine of the oligosaccharide side chains and serine and threonine of the peptide core is suggested by the resistance of this sugar to loss on mild acid hydrolysis and the predominance of these two amino acids. The low content of aspartic and glutamic acids eliminates these as possible linkage sites of importance. Fucose and sialic acid appear primarily as nonreducing end groups, rapidly lost on hydrolysis. The close correlation between the rates of loss of sulfate and hexosamine suggests that this sugar may be sulfated. However, no definite conclusion may be drawn in this regard since the molar ratio of sulfate to hexosamine is less than one. The retention of all the sulfate during 1-hr hydrolysis in 0.04 N HCl indicates the absence of highly acid labile *N*-sulfate linkages such as have been found in heparin (Jorpes *et al.*, 1950).

The inverse relationship between the intensity of PAS staining and the degree of sulfation found here has been reported previously by Mowry (1954) who found that dextran sulfate was PAS negative while dextran was PAS positive. Spicer (1965) produced PAS reactivity by desulfation of sulfomucins from rabbit glossal glands and Chinese hamster sublingual glands. The loss of PAS reactivity may be due to blocking of vicinal hydroxyl groups. While this evidence does not allow identification of the site of sulfate attachment it does offer strong support to the assumption that the carbohydrate moiety is sulfated. Studies of the nature of the periodate oxidation products will perhaps yield more definitive information.

The sulfated glycoproteins of dog gastric mucosa show some similarity in composition to keratosulfate preparations having fucose and sialic acid and low sulfate content (Seno *et al.*, 1965). They resemble keratosulfate in having an equimolar ratio of galactose to total hexosamine, but differ by having an equimolar ratio of glucosamine to galactosamine while keratosulfate has glucosamine as its principal amino sugar. The rapid hydrolysis of galactose, as compared with that of hexosamine, is inconsistent with the keratosulfate structure which is known to consist of a disaccharide repeating unit (Hirano *et al.*, 1961).

Hyaluronidase-digestible components constituted 61% of the CPC precipitate obtained from papain digested dog gastric mucosa while the sulfated glycoproteins constituted 8.2%. This distribution is quite different from that expected on the basis of histochemical studies of the dog fundus where the crypt cells, containing sulfated glycoprotein, stain more intensely with alcian blue or high iron diamine than the chief peptic cells, containing CHS-A (Gerard *et al.*, 1967b). The histochemical evidence may give a low estimate of the contribution of CHS due to washing out during fixing and partial blocking of acidic groups by protein components, while the scrapings employed in the present study may contain CHS of connective tissue origin leading to a high estimate.

In addition to the sulfated glycoproteins and CHS-A, the dog gastric mucosa contains two components which are resistant to hyaluronidase digestion and contain uronic acid. These constitute 2.7% of the total CPC precipitate. The two mucopolysaccharides were separated by copper precipitation. Fraction II:B:1 may be tentatively identified as CHS-B on the basis of its formation of a copper precipitate (Cifonelli *et al.*, 1958) and its low carbazole to orcinol ratio (Hoffman *et al.*, 1956). Fraction II:B:2 remaining in the copper supernatant and having a carbazole to orcinol ratio near that of glucuronic acid may represent heparitin sulfate (Brown, 1957). Since both fractions contained sulfate glycoprotein, further purification is necessary to permit the identification of hexosamines and more definite characterization of these two materials.

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