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The Fractionation and Characterisation of the Acid Polysaccharides of the Gastric Wall of the Dog

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Acid polysaccharides of the gastric wall of the dog have been isolated after papain digestion and fractionated by precipitation with cetylpyridinium chloride. Six fractions were obtained of which one yielded two subfractions on passage through Sephadex G-200. The homogeneity of each fraction was confirmed by several methods and made structural characterisation possible. A substance, very similar to chondroitin sulphuric acid B was found in two of the fractions, which differed only in the content of sulphate. One of the subfractions (Sephadex G-200) yielded hyaluronic acid-like material. The other four fractions contained sulphate in increasing amounts (fractionation into the four components, perhaps depended on this difference); other conducted analyses yielded very similar results for these fractions. When the results were compared with those obtained for human gastric juice, the four fractions were concluded to contain epithelial, *i.e.* secreted, polysaccharides.

The polysaccharides in the gastrointestinal tract have been analysed in many well-known classical studies but rarely has a clear distinction been drawn between secreted polysaccharides and those derived from tissues.[1–4]

In those studies the polysaccharides were usually fractionated with alcohol. In a later study the polysaccharides of the large intestine were fractionated by a new method based on precipitation with Rivanol after digestion with papain.[5] In the same work, precipitation with cetylpyridinium chloride was used to effect fractionation.

The precipitation and fractionation with cetylpyridinium chloride has been used mainly for studying the polyanions in cartilage and connective tissue.[6,7] This method is considered as being suitable for the separation of the polyanions on both macro and micro scales.

The fractionation of unknown polyanions in the gastric epithelium by any method is beset with difficulties. In order to confirm the homogeneity of the fractions, fractionation alone is not sufficient. Repeated fractionation by different methods was not found to be more effective, probably because the polyanions were separated on the basis of the same properties.[8,9]

When the method of fractionation used in a modified paper-electrophoretic method [10] was applied to the native mucoproteins of the gastric juice the macromolecules containing carbohydrates closely followed the proteins.

The splitting of proteins from the protein-carbohydrate complexes makes the separation and fractionation of the carbohydrate residues possible using the above methods. Biological methods can be used for the characterisation of the fractions and are of importance when the homogeneity of the fractions is being judged.

In the present work the acid polysaccharides in the gastric wall of the dog have been fractionated and their characterisation has been attempted to serve as a basis for a biological study of the early changes occurring in the gastric wall when peptic ulcer is developing.

MATERIAL AND METHODS

The examined material consisted of the stomachs of dogs from which the fat had been removed. The stomachs were homogenised in a 0.2 M acetate buffer, which buffer was used also as the medium in the digestion with papain. Twice crystallised papain [11] was used in the ratio of 6 mg per gram of wet tissue in a 0.2 M acetate buffer of pH 5.6 which contained 0.079 g of cysteine (puriss., Fluka AG) and 0.126 g of EDTA (E. Merck AG) per 100 ml. The tissue homogenates were digested at 65°C for 1½ days. The undigested tissue residue was washed with a small volume of 4 N sodium chloride to dissolve any secondary protein-polysaccharide complexes.

One per cent cetylpyridinium chloride (CPC) (Recip, Stockholm) was added to the combined filtrates to precipitate the acid polysaccharides. The precipitate was dissolved in 2.5 N magnesium chloride and reprecipitated with 4 volumes of alcohol. The precipitate was dissolved in a small volume of water and added to a cellulose column.

For the removal of the nucleic acids there were two methods to choose from. They could be removed by lowering the pH to 1.5 with 1 N HCl or they could be left in the sample and removed as their cetylpyridinium complexes by elution with 0.5 N $MgCl_2$. The fractionation was performed by the method described earlier [6] on cellulose columns of various sizes impregnated with a 1 % aqueous solution of CPC in 0.05 N NaCl. Using small columns (0.2 × 6 cm) and a small sample containing less than 500 μg of the polysaccharides, one fractionation was found sufficient. If the sample was larger or the fractions were to be characterised by several methods, a refractionation was carried out on columns containing cellulose treated with cetylpyridinium chloride. The polyanions precipitated in the columns as their cetylpyridinium complexes and were obtained in 6 fractions by stepwise elution with salt solutions. The salt concentrations chosen after preliminary experiments were 0.3 N NaCl, 0.5 N, 0.6 N, 0.8 N, 1.0 N, and 1.2 N $MgCl_2$. For control, a final elution with 3 N $MgCl_2$ was usually performed.

The fractions were precipitated as their cetylpyridinium complexes by lowering the salt concentration below the critical point of precipitation. The precipitates were dissolved in 2.5 N magnesium chloride and reprecipitated with alcohol as in the beginning of the method. The precipitates were then washed with alcohol several times.

The fraction precipitated by 0.3 N sodium chloride was put through a column of Sephadex G-200 (Pharmacia, Uppsala), the size of the column usually being 1 × 40 cm, and eluted with distilled water at a rate of 5 ml per hour, collecting 1-ml fractions in a fraction collector.

Both the unfractionated and fractionated polysaccharides were subjected to electrophoresis on cellulose acetate using a veronal buffer (0.05 M sodium diethylbarbiturate and 0.01 M diethylbarbituric acid) of pH 8, a gradient of 115 V/cm, and a running time of 25 min. Staining was carried out with alcian blue.

The infra-red spectrum of each fraction was recorded with a Perkin Elmer 237 infrared spectrophotometer. One milligram of each polysaccharide fraction was mixed with 150 mg of potassium bromide when preparing the tablet.

The polysaccharides were analysed as their sodium or magnesium salts. Hyaluronic acid (Sigma, grade 1) was used as a reference compound.

The chromatographic analyses. A sample was hydrolysed with Dowex-50 and hydrochloric acid and the hydrolysates were fractionated by ion exchange chromatography to identify the monosaccharides in the polysaccharides. Using this method, the neutral sugars, hexosamines and uronic acids were separated.[12]

The individual neutral sugars and hexosamines were separated by ascending chromatography on Whatman No. 1 paper (20 × 30 cm) with a water:butanol:pyridine:acetone (225:450:300:325) mixture. The run was performed twice, allowing the paper to dry between the runs; the running time was 5 h. The neutral sugars were stained with aniline phthalate [13] or benzidine trichloroacetate.[14] The hexosamines were stained with 0.2 % ninhydrin in butanol.

The uronic acids were isolated using a butanol:acetic:acid:water (4:1:2) mixture [15] and staining with benzidine trichloroacetate.

To isolate the sialic acids, the polysaccharides were hydrolysed with 0.1 N sulphuric acid [16] and the liberated sialic acids were separated by ion exchange chromatography.[12] The hydrolysate was resolved also by paper chromatography on Whatman No. 3 mm paper using a sec.butanol:acetic acid:water (4:1:5) solvent mixture.[17] The spots were developed by the periodate-thiobarbituric acid method.[18]

Quantitative colorimetric analyses. To liberate the hexosamines, the polysaccharides were hydrolysed in 6 N hydrochloric acid at 100°C for 8 h. The Elson-Morgan color reaction was carried out on the residue remaining after evaporation of the solution to dryness.[19] Glucosamine and galactosamine were separated by ion exchange chromatography as described by Gardell.[20]

The uronic acids were analysed by applying the carbazole [21] and orcinol [22] reactions.

The galactose determinations were made by the cysteine-mannose-H_2SO_4 method.[23]

The sulphate contents were determined by the benzidine method.[24]

Nitrogen was analysed by a modification of the micro-Kjeldahl technique.

The sialic acids were estimated by the thiobarbituric acid method [25] after mild hydrolysis and ion exchange chromatography as in the paper-chromatographic analyses.

Fucose was determined by a colorimetric method for methylpentoses.[26]

RESULTS AND DISCUSSION

Every salt concentration eluted a polysaccharide fraction. On refractionation these fractions were eluted in the same sequence as in the first fractionation. It soon became evident that the original fractions were not quite pure, for small amounts of fractions other than the main fractions were found in the first refractionation. After the second fractionation, the fractions were found to be fairly pure. The error arising from the impurities was not found to be of any importance in micro-scale analyses.

All the carbohydrate were eluted from the columns by 1.2 N magnesium chloride. Fig. 1 presents the relative amounts of carbohydrates in each fraction calculated from the aminosugar analyses.

The fraction eluted with 0.3 N sodium chloride formed two bands on electrophoresis, whereas the other fractions were electrophoretically homogeneous.

The fraction eluted by 0.3 N sodium chloride was divided into two subfractions A and B (Fig. 2) on Sephadex G-200.

The fraction eluted with 0.5 N magnesium chloride contained nucleic acids because these substances were not removed before the fractionation. After precipitation with hydrochloric acid a macro-molecular fraction containing aminosugars and precipitable with alcohol remained in the supernatant.

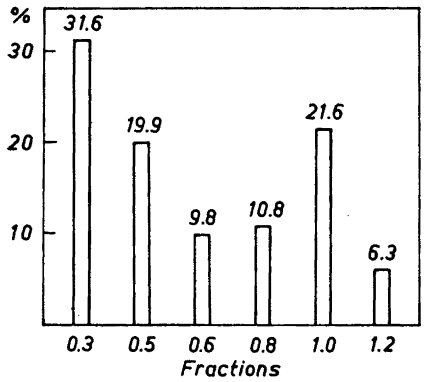

Fig. 1. Acid polysaccharide fractions separated by cetylpyridinium chloride precipitation and stepwise salt elution. Ratios of individual fractions in per cent calculated from the aminosugar contents.

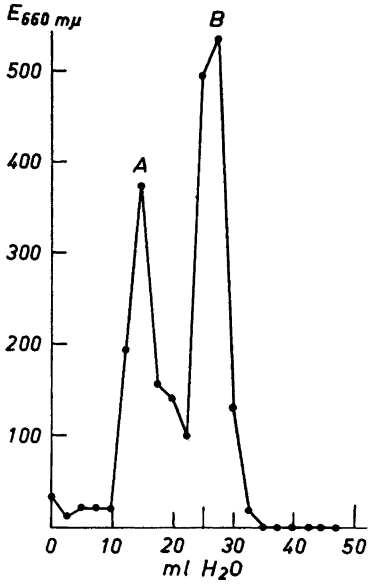

Fig. 2. Separation of fraction precipitated by cetylpyridinium chloride and 0.3 N sodium chloride into two subfractions by elution from a 1 × 40 cm Sephadex G-200 column, with distilled water at a rate of 5 ml per hour. The polysaccharides were colorimetrically determined after reaction with orcinol.

The infra-red spectra of the fractions eluted by 1.0 N and 1.2 N magnesium chloride were identical with the spectrum of chondroitin sulphuric acid B.[27] The spectrum of the sodium salts of the fraction eluted by 0.3 N sodium chloride part B was similar to the spectrum of the sodium hyaluronidate used as the reference substance. The spectrum of the subfraction A of the fraction eluted by 0.3 N sodium chloride showed a very low sulphate peak, but in other respects the spectrum was similar to the spectra of the fractions eluted by 0.5, 0.6, and 0.8 N sodium chloride. The fractions differed only in the sulphate content (1240 cm^{-1}), which increased with the eluting salt concentration.

By paper chromatography every fraction was found to contain hexosamines. Paper chromatography alone was insufficient to identify them as glucosamine and galactosamine, and hence the identification was performed as described below.

The problem of estimating uronic acids in some of the fractions was difficult. The fractions eluted by 1.2 and 1.0 N magnesium chloride were found to contain uronic acids. No other sugars were found in these two fractions, except for a trace of galactose. Similar to these fractions was the subfraction B of the

Table 1. The ratios of glucosamine to galactosamine in polysaccharide fractions calculated from aminosugar levels after ion exchange chromatographic separation.

Fraction	Glucosamine	Galactosamine
0.3A	0.5	0.5
B	1.0	0
0.5	0.5	0.5
0.6	0.5	0.5
0.8	0.5	0.5
1.0	0	1.0
1.2	0	1.0

fraction eluted by 0.3 N sodium chloride. Uronic acid was found in the subfraction A of the fraction eluted by 0.3 N sodium chloride and in the fractions eluted by 0.5, 0.6, and 0.8 N magnesium chloride fractions when a relatively large amount (many milligrams) of the polysaccharides was taken for analyses. The last observation is qualitative, but we think the amounts of uronic acid to be small compared to the amounts of aminosugars. These fractions always contained also sialic acid, galactose, and fucose, which make a quantitative determination of uronic acids by known methods impossible. We think that the uronic acid found came from very small amounts of chondroitin sulphuric

Table 2. Gastric acid polysaccharide fractions. Levels of aminosugars, uronic acids, galactose, fucose, sialic acids, sulphate, and nitrogen (a) expressed in μg per μg of aminosugars and (b) molar ratios.

a)					Uronic acid			
Fraction	Fucose	Galactose	Sialic acid	Aminosugar	Carbazole	Orcinol	$-SO_4$	N
0.3 A	36.7	96.0	4.7	100.0			0.88	
B				100.0	102.0			
0.5	25.3	90.0	5.1	100.0			11.4	
0.6	17.0	64.0	5.3	100.0			30.0	36.4
0.8	10.7	62.0	4.0	100.0			35.0	34.0
1.0	—	trace	—	100.0	54.5	117.0	49.2	23.2
1.2	—	—	—	100.0	80.5	110.0	66.0	23.8

b)					Uronic acid		
Fraction	Fucose	Galactose	Sialic acid	Aminosugar	Carbazole	Orcinol	$-SO_4$
0.3 A	0.835	1.990	0.057	2.09			0.034
B	—	—	—	1.030	0.970		—
0.5	0.575	1.850	0.061	2.070			0.440
0.6	0.390	1.320	0.060	2.070			1.160
0.8	0.250	1.280	0.050	2.070			1.350
1.0	—	—	—	1.000		1.080	0.930
1.2	—	—	—	0.930		0.940	1.130

acid still present as an impurity in the fractions. We have, as reported in another paper,[29] isolated from human gastric juice four acid polysaccharides similar to those in the fractions eluted by 0.3 A, 0.5, 0.6, and 0.8 N salt solution except for the uronic acids, which were not found in the former. This observation supports the above opinions.

The ratios of glucosamine to galactosamine calculated from the aminosugar levels are shown in Table 1. The fractions eluted with 1.0 N and 1.2 N magnesium chloride contained only galactosamine and subfraction B of the fraction eluted by 0.3 N sodium chloride only glucosamine. The ratio in the other fractions was very nearly 1:1.

The results for aminosugars, galactose, fucose, sialic acids, sulphate, nitrogen, and uronic acids are shown, expressed in μg and in molar ratios, in Table 2. The results of the analyses are correlated with the amount of aminosugars in every fraction. We found the results of the carbazole-orcinol analyses for the fractions eluted by 1.0 and 1.2 N magnesium chloride to be dependent on the iduronic acid content.[22] The fraction eluted by 1.0 N magnesium chloride contained very close to the theoretical amount of sulphate corresponding to chondroitin sulphuric acid, while the fraction eluted with 1.2 N magnesium chloride contained a slightly greater amount. This may be due to elution of chondroitin sulphuric acid as a separate fraction. All the results indicate that these two fractions are composed of substances similar to chondroitin sulphuric acid B.

The relation between the uronic acid and the aminosugar levels and the lack of sulphate in subfraction B of the fraction eluted by 0.3 N sodium chloride point to the presence of hyaluronic acid (the presence of glucosamine only in this fraction supported this opinion).

Sialic acid was found in quite similar amounts, about 4—5 % of the amounts of aminosugars in the subfraction A of the fraction eluted by 0.3 N sodium chloride and in the fractions eluted by 0.5, 0.6, and 0.8 N magnesium chloride. As sialic acid is readily split off from the macromolecule, these results are perhaps a little too low. The sulphate content increased from the subfraction A of the fraction eluted by 0.3 N sodium chloride to the fraction eluted by 0.8 N magnesium chloride. We think this to be the most important difference between these fractions. The difference was evident also in the infra-red spectra.

We will again stress our opinion that the strongest argument in favor of the assumption that these fractions (eluted by 0.3 A, 0.5, 0.6, and 0.8 N salt solution) each contain mainly one polysaccharide entity is that similar fractions were found in human gastric juice and that these gave similar analytical data.[29] The examined material did not contain any connective tissue polysaccharides as contaminants.

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REFERENCES

1. Levene, P. A. *Hexosamines and Mucoproteins*, Longmans, London 1925.
2. Meyer, K. *Advan. Protein Chem.* **2** (1945) 249.
3. Smith, H. and Gallop, R. C. *Biochem. J.* **53** (1953) 666.
4. Werner, I. *Acta Soc. Med. Upsalien.* **58** (1953) 1.
5. Draper, P. and Kent, P. W. *Biochem. J.* **86** (1963) 248.
6. Antonopoulos, C. A., Borelius, E., Gardell, S., Hamnström, B. and Scott, J. E. *Biochim. Biophys. Acta* **54** (1961) 213.
7. Antonopoulos, C. A., Gardell, S., Szirmai, J. A. and De Tyssonsk, E. R. *Biochim. Biophys. Acta* **83** (1964) 1.
8. Ringertz, N. R. and Reichard, P. *Acta Chem. Scand.* **14** (1960) 303.
9. Schmidt, M. *Biochim. Biophys. Acta* **63** (1962) 346.
10. Glass, G. B. J. *Gastroenterology* **23** (1963) 636.
11. Kimmer, J. R. and Smith, E. L. *J. Biol. Chem.* **207** (1954) 515.
12. Simkin, J. L., Skinner, E. R. and Seshadri, H. S. *Biochem. J.* **90** (1964) 316.
13. Partridge, S. M. *Nature* **164** (1949) 443.
14. Chan, B. and Chain, J. *Nature* **192** (1961) 69.
15. Radhakrishnamurthy, B. and Berenson, G. S. *Arch. Biochem. Biophys.* **101** (1963) 360.
16. Svennerholm, L. *Acta Chem. Scand.* **12** (1958) 547.
17. Gottshalk, A. In *The Chemistry and Biology of Sialic Acids and Related Substances*, Cambridge University Press 1960.
18. Warren, C. *Nature* **186** (1960) 237.
19. Gardell, S. In Glick, D. *Methods of Biochemical Analysis*, Interscience Publishers, New York 1958, vol. 6, p. 289.
20. Gardell, S. *Acta Chem. Scand.* **7** (1953) 207.
21. Dishe, Z. *J. Biol. Chem.* **167** (1947) 189.
22. Hoffman, P., Linker, A. and Meyer, K. *Science* **124** (1956) 1252.
23. Dishe, Z., Shettles, L. B. and Osmos, M. *Arch. Biochem. Biophys.* **22** (1949) 167.
24. Häkkinen, I. *Acta Physiol. Scand.* **51** (1961) *Suppl.* **177**.
25. Warren, L. *J. Biol. Chem.* **234** (1959) 1971.
26. Dishe, Z. and Shettles, L. B. *J. Biol. Chem.* **175** (1948) 595.
27. Mathews, M. B. *Nature* **181** (1958) 421.
28. Orr, S. F. D. *Biochim. Biophys. Acta* **14** (1954) 173.
29. Häkkinen, I., Hartiala, K. and Terho, T. *Acta Chem. Scand.* **19** (1965) 797.

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