Filter-paper Partition Chromatography of Sugars

2. AN EXAMINATION OF THE BLOOD GROUP A SPECIFIC SUBSTANCE FROM HOG GASTRIC MUCIN AND THE SPECIFIC POLYSACCHARIDE OF *BACTERIUM DYSENTERIAE* (SHIGA)

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The primary object of this investigation was to assess the value of the chromatographic technique described in the preceding paper (this vol. p. 238) as a qualitative method for the analysis of the hydrolysis products of complex polysaccharides. Interest especially centres in the general class of mucopolysaccharides—polysaccharides which contain hexosamines and which frequently show a special capacity for forming combinations with proteins—the reactions of which may be of considerable importance in the storage behaviour of foodstuffs. The specific polysaccharide prepared from the 'O' somatic antigen of *Bacterium dysenteriae* (Shiga) and the blood group 'A substance' from hog gastric mucin were chosen as being polysaccharides of this class which were available in a condition of presumed homogeneity and which had already been investigated by the classical methods.

The occurrence of complex polysaccharides having blood group A activity in certain mucinous secretions of man and animals has been dealt with in recent reviews by Wiener (1943) and Morgan (1945). A chemical investigation of the glycoproteins of hog gastric mucin was undertaken by Meyer, Smyth & Palmer (1937), who found the neutral carbohydrate portion of their product to consist of equimolecular proportions of galactose and *N*-acetylglucosamine. Later, Landsteiner & Hart (1940) showed that the material had blood group A activity and contained a component rich in amino-acids.

Morgan & King (1943) and Morgan (1944) extended these observations by preparing a highly purified and essentially undegraded specific substance from hog gastric mucin which contained some 15 amino-acids in addition to the sugar residues.

The constitution of the carbohydrate portion of the complex was further studied by Bray, Henry & Stacey (1946 *a, b*) who isolated material showing blood group A activity from a number of different sources. The group A specific substance from pepsin was studied by the method of methylation and hydrolysis, with subsequent isolation of the methylated sugars. Four sugar constituents were found, viz. glucosamine, galactose, fucose and mannose. A

parallel experiment was carried out with the blood group 'A substance' isolated by Morgan & King from hog gastric mucin, and from this substance also a fully methylated *L*-fucose derivative was obtained in 6% yield, but the remaining sugar constituents are not stated.

It has since been pointed out (Aminoff, Morgan & Watkins, 1946) that the apparently homogeneous specimens of 'A substance' isolated from commercial hog gastric mucin possess both A and O blood group specificity. Mucoids with A or O specificity only may be prepared from the stomachs of individual hogs and it is probable that commercial hog gastric mucin, which is derived from many animals, contains a mixture of two mucoids of very similar chemical and physical properties but of differing immunological specificity.

The sample of bacterial specific polysaccharide used in the present investigations was supplied by Dr W. T. J. Morgan. It was obtained from the somatic 'O' antigen of *Bact. dysenteriae* (Shiga) (Morgan, 1936, 1937) by gentle hydrolysis with acetic acid, and has been shown to contain *L*-rhamnose, *D*-galactose and an acetylhexosamine believed to be *N*-acetylglucosamine (Morgan, 1938).

EXPERIMENTAL

Blood group A substance

The sample of blood group A substance was kindly supplied by Dr W. T. J. Morgan. It had been obtained from commercial hog gastric mucin by extraction with phenol and was shown to be electrophoretically homogeneous at pH 4 and 8. On hydrolysis with 0.5*N*-HCl at 100° the reducing sugar reached its maximum after 4 hr., when the reducing value (expressed as glucose) was 50%.

The substance (30 mg.) was sealed with 1.5 ml. of 0.5*N*-H₂SO₄ in an ampoule and heated for 4 hr. at 100°. 1 ml. of the hydrolysate was brought to pH 4.5 with Ba(OH)₂ solution and the BaSO₄ removed by centrifugation. The clear solution, together with the washings, was concentrated to dryness under vacuum at room temperature and made up to 0.5 ml. in readiness for application to the chromatograms. Phenol-1% (w/v) NH₃, *s*-collidine and butanol-acetic acid mixture were used as solvents for the

chromatograms and glucose, D-glucosamine hydrochloride, chondrosamine hydrochloride, L-fucose, D-galactose and D-mannose were included as reference sugars.

s-Collidine. The chromatograms gave four spots corresponding to fucose, galactose, glucosamine and an unknown reducing substance giving a well defined circular spot at R_F 0.26. Mannose was absent.

Phenol-1% (w/v) NH₃. The chromatogram showed three well defined spots corresponding to fucose, glucosamine and galactose. Chondrosamine was absent. Mannose was not detectable since it would be obscured by galactose.

Butanol-acetic acid. The chromatogram gave clear indication of fucose, galactose and glucosamine. Mannose was absent, but an unknown reducing substance yielded a spot at R_F 0.055.

The principal sugars present were galactose and glucosamine, fucose appeared to be present in smaller amount. In case hydrolysis had not been completed under the conditions used for the above series, a further sample of the polysaccharide was hydrolyzed for 10 hr. with $N-H_2SO_4$, and the hydrolysate treated as before. The resulting chromatograms were similar to those previously obtained except that the spot given by fucose was relatively lighter, and that given by glucosamine was heavier.

The R_F value of the unknown reducing substance was below the range normally encountered for hexoses, pentoses and amino sugars, and the presence of a reducing disaccharide was considered unlikely since the unknown spot persisted after hydrolysis under more vigorous conditions.

Treatment with cation-exchange resin. The hydrolysate from 30 mg. of the polysaccharide (contained in 3 ml. solution) was shaken for 30 min. with an activated and washed sample of 'Zeo Karb 215' (0.5 g.) as described in the previous communication. The concentrated filtrate was then applied to further chromatograms. The treatment with ion-exchange resin resulted in removal of the unknown material together with glucosamine, and the chromatograms in all three solvents showed two spots only, corresponding to galactose and fucose (Pl. 4 a). It is probable, therefore, that the unknown substance is basic in character.

The experiments described above indicate clearly the presence of galactose, glucosamine and fucose, but give no indication of the presence of mannose in the sample of blood group A substance used for the investigation.

The specific polysaccharide of Bact. dysenteriae (Shiga)

The polysaccharide had N 1.6% and yielded 97% of reducing substances calculated as glucose after acid hydrolysis (Hagedorn & Jensen). A solution of the material (10 mg.) in 0.5N- H_2SO_4 (2 ml.) was sealed in an ampoule and heated at 100° for 5 hr. The hydrolysis mixture was clear and colourless and a measured volume (1 ml.) was neutralized to pH 4.5 with $Ba(OH)_2$ solution. After removal of the $BaSO_4$ the solution, together with the washings, was transferred to a small crucible and evaporated to dryness under vacuum at room temperature. The dry residue was dissolved in water (0.1 ml.) and the solution stored at +2° over chloroform. Where one-dimensional chromatograms were applied, the following sugars were included as reference substances; glucose, D-glucosamine hydrochloride, N-acetylglucosamine, chondrosamine hydrochloride, L-rhamnose and D-galactose. Collidine, phenol-1% (w/v) NH_3 and butanol-acetic acid mixture were used as solvents.

s-Collidine. The developed chromatogram showed two spots. One of them at R_F 0.59 corresponded to rhamnose, while the second spot at R_F 0.32-0.34 appeared to be multiple. The slower moving substance gave rise to a black coloration with $AgNO_3$ -ammonia as soon as the temperature of the oven reached 90° and later (at 105°) the spot extended in size with development of a brown colour.

It was considered that the multiple spot at R_F 0.32-0.34 was probably due to both glucosamine and galactose, and for this reason the experiment was repeated using a spraying reagent, based on the Morgan & Elson colour reaction for hexosamines. (Details are given on p. 240 of this volume.) In order to show the presence of N-acetylhexosamines, a chromatogram was sprayed with the modified Ehrlich's reagent alone, while to show both free hexosamine and the N-acetylhexosamines a second chromatogram was sprayed with the alkaline acetylacetone reagent, followed by the acid Ehrlich's reagent. Under these conditions the spot at R_F 0.33 showed the presence of free glucosamine in small quantity while a third spot appeared at R_F 0.50 corresponding to N-acetylglucosamine.

Phenol-1% (w/v) NH₃. With this solvent galactose was shown unambiguously, but the rhamnose spot was confused with that due to glucosamine (Pl. 4 b). As before both free glucosamine and N-acetylglucosamine could be detected as red-violet spots by means of the modified Morgan & Elson reagents.

Butanol-acetic acid. Like collidine this solvent mixture led to the unambiguous identification of rhamnose, but as in the case of collidine, the spot due to glucosamine was confused with that due to galactose.

Since N-acetylglucosamine was found it was evident that the conditions of hydrolysis were not sufficiently severe to complete the deacetylation of the amino sugar. For this reason the experiments described above were repeated, hydrolysis being carried out with $N-H_2SO_4$ for 10 hr. at 100°. The hydrolysate no longer showed the presence of N-acetylglucosamine, and the spots due to free glucosamine were correspondingly heavier. Galactose and rhamnose appeared in much the same concentration as before.

As confirmatory evidence, a two dimensional chromatogram was set up with a hydrolysis mixture prepared by heating the polysaccharide with $N-H_2SO_4$. The chromatogram was run first for 24 hr. with phenol-1% (w/v) NH_3 , and after drying turned through 90° and irrigated with collidine for a further period of 24 hr. The chromatogram showed two heavy spots, which could be identified from their co-ordinates as galactose and rhamnose. The glucosamine appeared as a diffuse trail which was unsuitable for the purpose of identification from co-ordinates.

DISCUSSION

As would be expected in the case of a homogeneous polysaccharide, the identification of the sugars present in the product of hydrolysis of the specific substance from *Bact. dysenteriae* (Shiga) was uncomplicated by the presence of interfering substances; galactose, glucosamine and rhamnose could readily be recognized by reference to the respective R_F values in each of three solvents. The presence of the hexosamine was confirmed by spraying the developed chromatograms with alkaline acetyl-

- (a) Hydrolysate of blood group A specific substance after treatment with ion-exchange resins; showing the presence of galactose and fucose. The hydrolysate after treatment with 'Zeo-Karb 215' was placed in the circle marked 'A'. B shows position of a hydrolysate treated with both 'Zeo-Karb' and 'Deacidite'. Chromatogram run with phenol for 20 hr.

- (b) Hydrolysate of polysaccharide from *Bact. dysenteriae* (Shiga); showing presence of galactose and a multiple spot due to rhamnose and glucosamine. The hydrolysate was placed in position marked 'C'. Chromatogram run with phenol for 16 hr.

acetone followed by an acid solution containing *p*-dimethylaminobenzaldehyde, and as further confirmation the presence of galactose and rhamnose was shown by carrying out a two-dimensional chromatogram according to the technique described for amino-acids by Consden, Gordon & Martin (1944).

With the blood group A substance, the analysis was complicated by the fact that only some 70 % of the material was carbohydrate in nature, and an interfering substance was found which gave rise to a spot at R_F 0.26 in collidine after spraying with the ammoniacal silver nitrate reagent. This substance was removed by treatment with a cation-exchange resin, and was therefore considered to be basic in nature. The analysis showed clearly the presence of glucosamine, galactose and fucose, but, in spite of indications in the literature of the presence of mannose in allied blood group A substances, this sugar was not found in the hydrolysis products of the polysaccharide under investigation.

The analysis of these two trial substances indicates clearly the importance of control of the hydrolysis conditions, and in assessing the results of such analyses it should be borne in mind that the hydrolysis of complex polysaccharides may proceed

in stages, some destruction of the less stable sugar residues taking place before complete liberation of residues constituting a resistant core.

SUMMARY

1. The method of separating reducing sugars on filter-paper partition chromatograms described in this volume p. 238 has been applied to the analysis of the products of hydrolysis of two carbohydrate-containing materials: (a) the specific polysaccharide of *Bact. dysenteriae* (Shiga), and (b) the blood group A substance from hog gastric mucin.

2. The results of the analysis of (a) agree with results in the literature in showing the presence of glucosamine, galactose and rhamnose.

3. In the hydrolysis products of (b) glucosamine, galactose and fucose were found. Mannose, which has been observed in active polysaccharide fractions from other sources, was not found in the specific substance from hog gastric mucin.

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Urinary Elimination of Synthetic Oestrogens and Stilboestrol Glucuronide in Animals

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Clinical experience has shown that oestrogen treatment of carcinoma of the prostate fails after a varying lapse of time in many cases. Although a similar delayed recurrence of symptoms also frequently follows treatment of prostatic carcinoma by castration (Angrist & Khoury, 1944; Nesbit, Pazzos & Cummings, 1944; Huggins, 1946) there is a slight possibility that the failure of continued

oestrogen treatment (in spite of increased dosage) is due to some bodily adaptation involving an altered metabolism of the drug. Hence it was thought worth while to study the metabolism of synthetic oestrogens in patients and animals; at the same time the opportunity has been taken to collect the mono-glucuronides of dienoestrol (4:4'-dihydroxy- γ : δ -diphenylhexa- β : δ -diene), hexoestrol (4:4'-dihydroxy-