Electrophoresis of some Neutral Polysaccharides

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Criteria by which the homogeneity of any neutral polysaccharide material can be defined are difficult to obtain. The elucidation of the structure of starch depended upon the chemical and biochemical proof that it is composed of two distinct molecular species of polysaccharide; thus any analytical method which differentiates between components of the amylose and amylopectin type whilst having a more general application is of use for the investigation of polysaccharides. In particular, the study of a complete cellular structure in which several constituent polysaccharides occur, as for example in a plant cell wall, is primarily dependent upon an analytical method which will determine and differentiate these constituents. The work described below uses the Tiselius electrophoresis apparatus in an experimental approach to this problem; the application depends on the use of borate buffer solutions in which the various neutral polysaccharides acquire a negative charge and consequently move in an electric field towards the anode.

EXPERIMENTAL

Materials

The methods of preparation and the characteristics of the polysaccharides are those given by the authors shown in Table 1. In each instance the sample was analysed for total N and P. The commercial starch preparations were treated with aqueous methanol to remove fatty material (Schoch, 1942a).

Methods

Analyses. Nitrogen and phosphorus analyses were made as described by Northcote & Horne (1952).

Electrophoreses. The polysaccharides were examined as 1% solutions in 0.05M borate buffer, pH 9.2, and in certain instances as 1% solutions in glycine buffer (0.1M glycine-0.1M-NaOH), pH 9.2, or 0.1M veronal buffer, pH 9.2. The ivory nut mannan A and the potato amylose were only partially soluble in the buffer solutions; in these cases saturated solutions were used. Each solution was dialysed for 48 hr. against two changes of buffer and finally centrifuged before placing the clear supernatant in the 2 ml. electrophoretic cell. Electrophoresis was conducted at 0° in a Tiselius electrophoretic apparatus (Perkin Elmer and Co., U.S.A.; Model 38). The conductivity of the polysaccharide material in the buffer was measured at 0°. pH Measurements were made with the glass electrode at room temperature.

RESULTS

The mobilities were calculated from the electrophoretic data and are given in Fig. 1 and Tables 2 and 3.

Borate buffer

Field strength, 5.0 v/cm.; current, 5.2 ma

All the boundaries using this buffer were clearly defined and were maintained throughout the course of the experiments which lasted about 4-5 hr. or until the boundaries had passed out of the field of view. The ascending and descending boundaries in all cases were mirror images (Fig. 6a, b).

Table 1. *Polysaccharides used in electrophoresis experiments*

Polysaccharide	N (%)	P (%)	Description
Yeast glycogen	0.06	0.07	Prepared according to Northcote (1953)
Rabbit liver glycogen	0.01	0.003	Kindly given by Dr D. J. Bell (Bell, 1948; Bell & Manners, 1952)
Yeast mannan	0.20	0.30	Prepared according to Haworth, Heath & Peat (1941)
Ivory nut mannan	0.07	0.001	Prepared according to Aspinall, Hirst, Percival & Williamson (1953)
Amylose	0.01	0.007	Prepared from the potato starch according to Haworth, Peat & Sagrott (1946)
Amylopectin	0.06	0.075	
Waxy maize starch	0.01	0.005	Kindly given by Dr W. Whelan as part of a sample from Dr T. J. Schoch. Derived from single cross waxy seed corn and containing less than 0.03% amylose (Schoch, 1942b)
Potato starch	0.09	0.06	Commercial samples obtained from British Drug Houses Ltd., London
Wheat starch	0.02	0.02	
Rice starch	0.06	0.004

Fig. 1. Mobilities of polysaccharides in 0.05 M borate buffer, pH 9.2, 0°.

Starches (Figs. 2 and 8). Each starch showed two boundaries except that from waxy maize, which gave a single very sharp peak (Fig. 8a). The electrophoretic analysis of the separated amylose and amylopectin components showed that the amylose was the slower moving fraction in the whole starch. Separately, amylose and amylopectin gave single symmetrical peaks (Fig. 2c, d), but a mixture behaved very similarly to the original starch on electrophoresis (Fig. 2a, b).

Glycogens (Fig. 4a, b). Rabbit liver glycogen moved as a single boundary, but yeast glycogen showed two components. Small amounts of a slower-moving fraction were indicated and the general electrophoretic pattern of the yeast glycogen resembled that of starch (Fig. 4b).

Mannans (Figs. 6 and 9). Both yeast mannan and ivory nut mannan A moved more quickly than either the starches or the glycogens and in each case a single symmetrical peak was observed.

Yeast mannan and glycogen mixture (Fig. 10b). This mixture separated clearly into two separate boundaries.

Yeast glycogen and rabbit liver glycogen mixture. These components had very similar mobilities and failed to indicate any separation (Fig. 10c).

Yeast mannan and ivory nut mannan mixture (Fig. 10a). These components separated into two distinct boundaries.

Table 2. *Mobilities of polysaccharides in 0.1 M glycine buffer, pH 9.2, 0°*

Polysaccharides	Mobilities of boundaries (cm. ² v. ⁻¹ sec. ⁻¹ × 10 ⁻⁵)
Yeast glycogen	0.7, 0.0
Rabbit liver glycogen	1.0
Potato starch	2.2, 0.0
Potato amylopectin	1.7, 0.0

Table 3. *Mobilities of polysaccharides in 0.1 M veronal buffer, pH 9.2, 0°*

Polysaccharides	Mobilities of boundaries (cm. ² v. ⁻¹ sec. ⁻¹ × 10 ⁻⁵)
Potato starch	1.6
Potato amylopectin	1.2

Glycine buffer

Field strength, 6.3 v/cm.; current, 1.3 mA

In all cases, when mobilities could be measured, these were much lower than in borate (Table 2). Boundaries were ill defined and disintegrated during the course of the experiment over a period of 3-4 hr. The ascending and descending boundaries were not mirror images (Fig. 7).

Potato starch (Fig. 3). A stationary and a fast-moving peak were apparent in the initial stages of the experiment, although the moving boundary rapidly dispersed. Analysis of the individual components of the starch showed that amylopectin gave a pattern almost identical with that of whole

Fig. 2. 0.05 M Borate, pH 9.2. (a) Potato starch, (b) amylopectin + amylose, (c) amylopectin, (d) amylose.

Fig. 3. 0.1 M Glycine, pH 9.2. (a) Potato starch, (b) amylopectin.

Fig. 4. 0.05 M Borate, pH 9.2. (a) Rabbit liver glycogen, (b) yeast glycogen.

Fig. 5. 0.1 M Glycine, pH 9.2. (a) Rabbit liver glycogen, (b) yeast glycogen.

Fig. 6. 0.05 M Borate, pH 9.2. Yeast mannan: (a) desc., (b) asc.

Fig. 7. 0.1 M Glycine, pH 9.2. Yeast mannan: (a) and (c) desc., (b) and (d) asc.

Fig. 8. 0.05 M Borate, pH 9.2. (a) Waxy maize starch, (b) wheat starch, (c) rice starch.

Fig. 9. 0.05 M Borate, pH 9.2. Ivory nut mannan A.

Fig. 10. 0.05 M Borate, pH 9.2. (a) Ivory nut + yeast mannans, (b) yeast glycogen + yeast mannan, (c) rabbit liver glycogen + yeast glycogen.

Fig. 11. 0.1 M Glycine, pH 9.2. Yeast glycogen + yeast mannan: (a) asc., (b) desc.

Fig. 12. 0.1 M Veronal, pH 9.2. Yeast mannan: (a) asc., (b) desc.

Fig. 13. 0.1 M Veronal, pH 9.2. (a) Potato starch, (b) amylopectin.

starch (Fig. 3b) whilst no definite boundary could be obtained with amylose.

Glycogens (Fig. 5). Slow-moving boundaries were obtained with both rabbit liver and yeast

glycogens, but the stability and shape of the diagrams were not satisfactory for analysis.

Yeast mannan (Fig. 7). No single boundary was obtained but material passed towards the anode in a continuous flow made up of a gradation of fast- to slow-moving substance.

Yeast glycogen and mannan mixture. The mannan passed towards the anode in the manner described above, whilst the glycogen remained almost stationary. Fig. 11 shows the pattern after 143 min. when the movement of the mannan was no longer apparent.

Veronal buffer

Field strength, 4.2 v/cm.; current, 5.1 ma

The boundaries were more stable than those in glycine buffer and the mobilities were again much lower than in borate (Table 3). The ascending and descending boundaries were generally not mirror images (Fig. 12).

Potato starch (Fig. 13a). The boundary was asymmetric, and after 290 min. separated into two small, almost equal, peaks. Amylopectin gave a very similar pattern (Fig. 13b).

Yeast mannan (Fig. 12). The movement resembled that obtained with glycine buffer.

DISCUSSION

The movement of polysaccharide material in an electrical field has already been used in certain analytical and preparative methods, but this has usually been confined to separations in which one of the polysaccharide components at least carries a charge due to esterification or other combination with a polar group. Samec, Nucic & Pirkmaier (1941) obtained fractions from potato starch by such a method owing to the phosphate ester groups on the amylopectin component, and the method has been applied to the analysis of various blood group polysaccharides (Krejci, Sweeny & Zittle, 1949) and 'acid polysaccharides' of gastric mucosa (Smith & Gallop, 1953). The influence of borate on certain polysaccharides has been investigated with relation to solubility, viscosity and gel formation (Deuel, Neukom & Weber, 1948; Deuel & Neukom, 1949), but no systematic study of the movement of such polysaccharide-borate compounds in an electrophoresis apparatus has been made. The present work shows that such an electrophoretic method using borate buffer has proved successful in the analyses of potato and cereal starches, mannans and glycogens. The cereal starches are known to contain no esterified phosphate on the amylopectin and thus the presence of such an ester group is not a necessary factor for electrophoresis under these conditions. Waxy maize starch, which gives a well-defined boundary with a mobility comparable with the other amylopectin components (Fig. 1), contains

very small amounts of fatty substances and therefore it is unlikely that lipid sometimes associated with starches is the factor contributing to the electrophoretic separations described here.

The starch components amylose and amylopectin, isolated by a standard procedure, gave single boundaries in the apparatus; whereas an artificial mixture of these two amylosaccharides gave an electrophoretic picture very similar to that of the original starch (Fig. 2). Thus polysaccharides different in shape, size and structure, but composed of the same sugar, can be differentiated using the Tiselius apparatus. This conclusion is also indicated by the separation of yeast and ivory nut mannans (Fig. 10a). As with other applications of electrophoresis, a single peak only implies the presence of species homogeneous with respect to charge per unit area of surface for the large molecules used in this investigation; interpretations indicating homogeneity of any other type, such as of molecular weight, must have due regard to this restriction.

The two principal soluble polysaccharides obtained from yeast are glycogen and mannan and these can be clearly separated by electrophoretic analysis (Fig. 10b). Thus the method should prove useful in a preliminary study of a mixture of polysaccharide material such as that obtained from many plant products and from cell structures such as cell walls. Furthermore, the chemical fractionation of such a mixture can be followed by this method. Isherwood (1949) has already hinted that an electrophoretic method applied to polysaccharides would be a useful tool for investigations of this type and he has tentatively proposed a method using 4% sodium hydroxide solution as a solvent for the substance.

Although at the pH used some of the polysaccharides investigated do move in the electric field in the absence of borate, the boundaries are not suitable for analytical study for one or more of three reasons.

(1) The movement is disorganized, and no definite boundary is formed. This is apparent with yeast mannan both in glycine and veronal buffers. It is of interest to note that Lindquist (1953) obtained a mobility of -1.38×10^{-5} cm.² v⁻¹ sec.⁻¹ for yeast mannan at pH 9.3 in veronal buffer although no information about this movement or its cause is given; a secondary cathodic migration was observed in some of his experiments at this pH.

(2) The boundaries which separate do not seem to coincide with the known chemically different molecular species present in the mixture. This can be seen very clearly in the case of potato starch analysed both in glycine and veronal buffer. Separation of two boundaries occurred (Figs. 3a and 13a); but amylopectin in each case showed a

very similar electrophoretic pattern to that of the whole starch (Figs. 3b and 13b).

(3) The boundaries obtained disintegrated fairly rapidly, more so in glycine than in veronal buffer, and since the movement is so much slower than in borate buffer, satisfactory separations of material could not be obtained.

Borate would thus seem essential in such investigations and contributes to the origin of the charge on the polysaccharide. The reaction of borate with polysaccharides containing monosaccharide units which possess free vicinal *cis*-hydroxyl groups, such as occur in mannan, is well known (Deuel *et al.* 1948; Deuel & Neukom, 1949), but with starches and glycogens it is more difficult to visualize. There is already some indication from other studies that a reaction can occur with these latter polysaccharides which contain only *trans*-vicinal hydroxyl groups (Zittle, 1951) and a comparison of electrophoretic mobilities in the absence and presence of borate provides additional evidence for this.

SUMMARY

1. An electrophoretic method for the analysis of neutral polysaccharides has been described. It is suggested that the method has a general application to soluble polysaccharide material. Borate buffer at pH 9.2 is used and the polysaccharides in this solution move towards the anode.

2. Borate buffer is essential for satisfactory electrophoretic analysis even with polysaccharides containing only *trans*-vicinal hydroxyl groups. This indicates a combination of borate with this type of polysaccharide.

3. Yeast mannan, ivory nut mannan and rabbit liver glycogen show no indication of any other molecular species when analysed electrophoretically. Yeast glycogen and starches show the presence of at least two components.

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The Branched-chain Fatty Acids of Butterfat

5. THE ISOLATION OF 12-METHYLTRIDECANOIC ACID

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Detailed analyses of a butterfat sample (G/43) recently yielded trace quantities of two isomeric C_{15} branched-chain fatty acids, (+)-12-methyltetradecanoic acid and 13-methyltetradecanoic acid (Hansen, Shorland & Cooke, 1954). In this present communication is reported the isolation from the same sample of a C_{14} iso-acid, 12-methyltridecanoic acid.

EXPERIMENTAL

As reported earlier (Hansen *et al.* 1954) the acids from hydrogenated butterfat after removal of part of the steam-volatile acids, were crystallized from 10 vol. of acetone at -33° , the 'liquids' converted into methyl esters (wt. 1876 g., sap. equiv. 223.8, iod. val. 4.7) and fractionated *in vacuo* in a 490×3.8 cm. stainless steel column packed with glass helices. Thirty-three fractions (KL1 to KL33) and a large residue resulted. For the purpose of this present investigation the following fractions were combined and denoted K27: KL24, wt. 35.24 g., sap. equiv. 232.5, iod. val. 2.0; KL25, wt. 38.38 g., sap. equiv. 238.6, iod. val. 2.5; KL26, wt. 34.78 g., sap. equiv. 240.2, iod. val. 3.5; KL27, wt. 35.99 g., sap. equiv. 241.4, iod. val. 2.9.

Fraction K27 was hydrolysed, unsaponifiable matter removed and the acids reconverted into methyl esters (sap. equiv. 235.6, iod. val. 0.9); 134 g. of these were refractionated in a 122×3.3 cm. column (column B, Shorland, 1952). Of the twenty-four fractions distilled over, those relevant to this paper are: K27L7, wt. 3.31 g., sap. equiv. 229.0, iod. val. 1.5, m.p. ester -5.2 to -4.8° , m.p. acid 38.8 – 39.7° ; K27L8, wt. 10.78 g., sap. equiv. 238.6, iod. val. 0.6, m.p. ester 3.8 – 4.5° , m.p. acid 50.5 – 50.8° ; K27L9, wt. 11.55 g., sap. equiv. 241.4, iod. val. 0.5, m.p. ester 4.0 – 4.8° , m.p. acid 43.8 – 45.0° . Fraction K27L8 (denoted K34) was refractionated in column E (Shorland, 1952) to yield eight fractions (K34L1 to K34L8) and a residue K34LR. The following fractions, K34L4, wt. 1.52 g., sap. equiv. 238.6, m.p. ester 4.2 – 5.1° ; K34L5, wt. 0.56 g., sap. equiv. 242.2, m.p. ester 4.2 – 5.1° ; K34L6, wt. 1.10 g., sap. equiv. 241.5, m.p. ester 4.2 – 5.1° ; K34L7, wt. 0.83 g., sap. equiv. 241.0, m.p. ester 4.5 – 5.3° ; were combined (K36) and converted

into fatty acids (m.p. 51.9 – 52.6° ; mixed m.p. with pure myristic acid gave m.p. 39.8 – 40.3°). Purification of K36 was then effected by repeated low-temperature crystallization from 40 vol. of the following solvents, the soluble fraction being removed after each crystallization: Light petroleum (b.p. 50 – 60°) at -40° , 5 crystallizations; methanol at -40° , 10 crystallizations; acetone at -70° , 1 crystallization; acetone at -60° , 1 crystallization; acetone at -50° , 1 crystallization. After the final crystallization the soluble fraction K36S17L (0.03 g.) had m.p. 50.0 – 51.1° while the insoluble fraction K36S17S (2.01 g.) had m.p. 53.7 – 54.0° .

The properties of fraction K36S17S were as follows: wt. 2.01 g., m.p. acid 53.7 – 54.0° , m.p. methyl ester 5.0 – 6.0° , sap. equiv. 227.8 (calc. for $C_{14}H_{28}O_2$, 228.4), iod. val. 0.0; X-ray long spacing $26.7 \text{ \AA}$. (Found: C, 73.9; H, 12.4%. Calc. for $C_{14}H_{28}O_2$: C, 73.6; H, 12.4%). C-methyl, 10.5% (equiv. to 1.6 mols. of acetic acid); n_D^{25} (acid) 1.4250; n_D^{25} (methyl ester) 1.4360; $[\alpha]_D^{25}$ 0.0° in CHCl_3 (c, 13.3). When mixed in equal proportions with pure myristic acid (m.p. 54.0 – 54.4°) it gave a mixed m.p. of 40.8 – 41.8° , and when mixed with pure synthetic 12-methyltridecanoic acid kindly supplied by Professor E. Stenhagen of Uppsala University (m.p. 53.3 – 53.6°) it gave a mixed m.p. of 53.5 – 53.8° .

Fraction K36S17S, as described above, was derived from fraction K27L8. Investigation of two adjacent fractions K27L7 and K27L9 by the same techniques yielded a number of fractions with corresponding characteristics. These were bulked together, denoted K56, and recrystallized from light petroleum (2 crystallizations), methanol (3 crystallizations), and acetone (6 crystallizations) respectively, using in each case 40 vol. of solvent at -40° . The resulting fraction K56S10S possessed the following chemical and physical properties: wt. 4.39 g., m.p. 53.5 – 54.0° , sap. equiv. 227.9, iod. val. 0.0; X-ray long spacing $26.7 \text{ \AA}$. Found C, 73.7; H, 12.2%; C-methyl, 10.3% (equiv. to 1.6 mols. acetic acid). When mixed with an equal quantity of pure myristic acid (m.p. 54.0 – 54.4°) it gave a mixed m.p. of 40.2 – 41.0° .

X-ray diffraction measurements recorded in this work were made with a Philips Geiger X-ray spectrometer using