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A Micro Method for the Separation and Determination of Polysaccharides by Zone Electrophoresis

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The separation and determination of the individual polysaccharides present in a mixture is a difficult and tedious process which often can only be carried out on the macro-scale. Northcote (1954) has used electrophoresis in borate buffer in the Tiselius apparatus as a method for determining the minimum number of constituents of a mixture, but the recovery of the materials behind each boundary, which is necessary if the monosaccharide constituents of each component are to be studied, is difficult. This difficulty might be overcome if the electrophoresis could be carried out on some form of strip support, and the present work describes such a technique.

Geldmacher-Mallinckrodt & Wienland (1953) have attempted to separate glycogen and galactan by electrophoresis in veronal-acetate buffer with strip supports of silk and of paper. Whilst the work to be described below was in progress, the use of filter paper as a support was investigated by Bertrand & Laszt (1956) using various buffers, and by Foster, Newton-Hearn & Stacey (1956) using borate buffer.

EXPERIMENTAL

Materials

Monosaccharides. All monosaccharides were run chromatographically and electrophoretically to establish the presence of only one reducing sugar. Concentrations of solutions were checked by polarimetry and the solutions diluted to 0.5 g./100 ml.

Polysaccharides. Those not listed below were identical with those used by Northcote (1954). Elecampane inulin, kindly given by Dr D. J. Bell, was prepared by Bell & Palmer (1952), snail galactan as by May (1934), wheat-straw xylan as by Doré (1947) and lucerne mannogalactan as by Andrews, Hough & Jones (1952). Details of dextran A (mol.wt. 10 000) and dextran B (mol.wt. 150 000) are given

by Northcote (1953). Apple pectin was a commercial sample (British Drug Houses Ltd., London).

The polysaccharides were applied as 1% aqueous solutions, except for inulin (0.5%) and ivory-nut mannan A (0.1% in 0.05M tetraborate solution). Concentrations of standard solutions of polysaccharides were checked by polarimetry. The mixed solution of polysaccharides contained 0.5 g. of each/100 ml., of yeast mannan, yeast glycogen, snail galactan and inulin, previously dried to constant weight over P_2O_5 at 100° and 0.01 mm. Hg pressure.

Methods

Apparatus. The strip support was suspended horizontally in a tank as shown in Fig. 1. The two electrode compartments each held approx. 250 ml. of buffer and the cooling fluid was tap water. The voltage applied from anode to cathode was 2000 v and since the effective length of the strip was about 40 cm. the field strength was approx. 50 v/cm. For strips 15 cm. wide, and 0.1M borate buffer, pH 9.3, typical current values were, with silk 25 mA, with paper 100 mA and with glass paper 140 mA (at 33 v/cm.). All electrophoreses were run for 90 min., except where otherwise stated. Colorimetric determinations were made in the Unicam SP. 350 colorimeter.

Buffer solutions. pH measurements were made with the glass electrode. Borate buffer was prepared from AnalaR sodium borate ('borax') and molarities were expressed in terms of sodium tetraborate ($Na_2B_4O_7 \cdot 10H_2O$). Veronal

Fig. 1. Electrophoresis apparatus. Further details are given in the Experimental section.

buffer (0.1M, pH 9.3) was prepared according to Geldmacher-Mallinckrodt & Wienland (1953).

Strip supports. (a) Whatman no. 54 filter paper. This was washed by elution with water and then dried. This process was repeated four times in an effort to remove soluble carbohydrate (Huffman, Rebers, Spriestersbach & Smith, 1955).

(b) Bleached pure silk (wt. approx. 9 mg./cm.²) was washed thoroughly in hot water and pure soap flakes and then in several changes of water. This process was repeated three times, but on occasions further washings were necessary to reduce the background absorption (see below) to a suitable low level.

(c) Glass-fibre filter paper (glass paper) was supplied by H. Reeve Angel and Co. Ltd., London.

Application of carbohydrate. With silk and paper, the polysaccharides were applied to the strip support before it was sprayed with buffer solution, but with the glass paper the reverse procedure was adopted as this gave more compact spots. For the quantitative experiments the polysaccharides were applied as a line, not greater than 5 cm. in length, at right angles to the electric field. The mixture was run in duplicate on the same strip.

Detection of polysaccharides. (a) Spray reagents. Although reagents for the detection of certain polysaccharides are available, e.g. iodine vapour for starch and glycogen or urea-HCl for inulin (Dedonder, 1952), none of the spray reagents tested proved suitable for the general detection of polysaccharides on paper. The α -naphthol spray reagent devised by Geldmacher-Mallinckrodt & Wienland (1953) was used on silk and glass paper. Polysaccharide spots on glass paper were also detected by a reagent consisting of 1 g. of *p*-anisidine and 2 ml. of conc. H₂SO₄ in 100 ml. of moist butanol. The sprayed paper was heated at 100° for about 15 min.

(b) Elution and colorimetric determination. Where the spray reagents could not be used or if quantitative data were required, each 5 cm. strip corresponding to an applied polysaccharide was cut into 1 cm. segments numbered consecutively from the anode compartment and the polysaccharide on each segment was eluted by water into a correspondingly numbered graduated tube. The eluates were made up to 1.5 ml. and to each tube was added 3.0 ml. of randomly sulphonated α -naphthol in conc. H₂SO₄ (Devor,

1950), the tubes being cooled during the mixing and then heated in a boiling-water bath for 10 min. The solutions were cooled and their absorption at 555 m μ . was determined. A graph showing the plot of absorption against distance from the anode (i.e. tube number) thus indicates the position of polysaccharide zones as peaks (Figs. 3-7).

It was found that segments not bearing the applied polysaccharide yielded an eluate which gave some absorption at 555 m μ . and this is referred to as background absorption.

Measurement of movement. Movements were measured by the distance between the centre of the polysaccharide zone and the centre of the 2:3:6-tri-*O*-methyl-D-glucose spot run in parallel with it. The trimethylglucose is referred to as the stationary marker since it does not acquire a charge in borate buffer (Foster, 1953) and therefore its movement is due to factors other than electrophoresis, e.g. electroendosmosis.

Removal of the borate buffer and hydrolysis of the polysaccharides. After detection of the positions of the polysaccharide zones, the bands carrying each separate zone were cut out of the duplicate strip, eluted with 2-3 ml. of methanol to remove some of the borate, dried and the polysaccharides eluted from them by 0.5 ml. of water. The eluate was collected in a glass tube (0.5 cm. in diameter, 15 cm. long), 0.25 ml. of 9% (w/v) HNO₃ added, the tube sealed and heated at about 95° for 60 min. This gave maximum reducing power for the mixture (Fig. 2). After hydrolysis the solutions were dried over P₂O₅ and NaOH flakes *in vacuo*. The residues, dissolved in 5-10 μ l. of water, together with two washings each of 5-10 μ l. of water, were transferred to Whatman no. 1 filter paper.

Separation of monosaccharides. The monosaccharides present in the hydrolysates were separated electrophoretically in 0.05M Na₂B₄O₇·10H₂O buffer, pH 9.3 (Consden & Stanier, 1952).

Detection and determination of reducing sugars. The sugars were detected with *p*-anisidine reagent (Hough, Jones & Wadman, 1950), and determined by Nelson's method (1944), so modified that the final dilution was 10 ml., and the absorption was measured at 660 m μ .

Boundary electrophoresis. This was carried out in 0.05M Na₂B₄O₇·10H₂O buffer, pH 9.3, as described by Northcote (1954).

RESULTS

Filter paper

All electrophoreses on filter paper were performed in borate-buffer solution.

Yeast mannan (150 μ g.) was run on unwashed and washed filter paper and very little difference was observed (Fig. 3*a, b*). The background absorption (expressed in terms of log I_0/I) on unwashed paper was, however, sometimes as high as 0.250 and varied from run to run, and on washed paper it was usually between 0.100 and 0.150. A figure of 0.200 for log I_0/I represents the equivalent of approx. 10 μ g. of glucose. Snail galactan (150 μ g.) did not give a definite peak, but the material slowly streaked towards the anode (Fig. 4). Amylopectin (100 μ g.) formed a zone which moved towards the cathode, but which tailed back to the origin. Amylose (20 μ g.), on the other hand, tended to stick

Fig. 2. Reducing power of the polysaccharide mixture, containing 25 μ g. each of yeast mannan, yeast glycogen, snail galactan and elecampane inulin, after hydrolysis with 3% (w/v) nitric acid in sealed tubes. Determined by Nelson's (1944) method.

Fig. 3. Electrophoresis of 100 $\mu\text{g.}$ of yeast mannan in 0.1 M borate buffer, pH 9.3, at approx. 50 v/cm. for 90 min., and determination of eluates from 1 cm. segments with the α -naphthol reagent (a) on Whatman no. 54 filter paper (unwashed); (b) on Whatman no. 54 filter paper (washed).

Fig. 4. Electrophoresis of 100 $\mu\text{g.}$ of snail galactan in 0.1 M borate buffer, pH 9.3, at approx. 50 v/cm. for 90 min. Support was Whatman no. 54 filter paper (washed). Determination of eluates from 1 cm. segments with the α -naphthol reagent.

Fig. 5. Electrophoresis on washed silk and in 0.1 M borate buffer, pH 9.3, at approx. 50 v/cm. for 90 min. Determination of eluates from 1 cm. segments with the α -naphthol reagent. (a) Yeast mannan (150 $\mu\text{g.}$); (b) snail galactan (150 $\mu\text{g.}$).

firmly at the origin with some material streaking towards the cathode. The ratios of the movements of some polysaccharides to the movement of yeast mannan are given in Table 1. Actual distances moved by yeast mannan on paper (Fig. 3), on silk (Fig. 5) and on glass paper (Fig. 8) are given so that the distances moved for the other polysaccharides can be calculated.

Silk

Borate buffer. The zone for yeast mannan (150 $\mu\text{g.}$) (Fig. 5a) is as clearly defined as on filter paper, and the background absorption much lower and more constant. On this support, the snail galactan (150 $\mu\text{g.}$) moved as a distinct zone (Fig. 5b) which was broader than that of yeast mannan. The ratios of movements of polysaccharides to movement of yeast mannan for a number of polysaccharides are listed in Table 1.

The mixture of yeast mannan (100 $\mu\text{g.}$), yeast glycogen (100 $\mu\text{g.}$), inulin (100 $\mu\text{g.}$) and snail galactan (100 $\mu\text{g.}$) was subjected to electrophoresis and showed four distinct zones (Fig. 7b). These correspond, approximately, with the positions of the components when run separately under comparable conditions.

The absorption/ $\mu\text{g.}$ of polysaccharides with the α -naphthol reagent (Devor, 1950) varies according to the monosaccharides present in the polymer, and hence before the recoveries can be assessed in terms of $\mu\text{g.}$ of sugar it is necessary to know the constituents of each polysaccharide zone. If the isolated polysaccharides are available and their constituent monosaccharides known, markers can be run in parallel to establish the nature of the zones. With the mixture, this procedure suggested that zone 1 was galactan, zone 2 mannan, zone 3 glycogen and zone 4 inulin (Fig. 7b). The presence of inulin in zone 4 was confirmed by the use of the urea-HCl reagent (Dedonder, 1952). A more general method, however, is to remove the borate, hydrolyse the polysaccharide and identify the constituent monosaccharides by electrophoresis. If the monosaccharides are eluted after electrophoresis, some quantitative data regarding the constituents of each zone can be obtained. By this method 40 % of zone 1

Fig. 6. Electrophoresis of 150 $\mu\text{g.}$ of yeast mannan on washed silk and in 0.1 M veronal-acetate buffer, pH 9.3, at approx. 50 v/cm. for 90 min. Determination of eluates from 1 cm. segments with the α -naphthol reagent.

Table 1. *Relative electrophoretic mobilities of polysaccharides*

	Ratio of movement of polysaccharide to movement of yeast mannan			Ratio of mobility of polysaccharide to mobility of yeast mannan as measured by boundary electrophoresis
	Filter paper	Silk	Glass paper	
Yeast mannan	1.00	1.00	1.00	1.00
Yeast glycogen	0.49	0.58	0.45	0.48
Snail galactan	1.11	1.33	1.25	1.33
	(measured to front of streak)			
Elecampane inulin	0.25	0.25	0.28	—
Amylose	Origin	Origin	0.38	0.36
Amylopectin	0.27	0.42	0.45	0.41
Wheat-straw xylan	—	Origin	0.45	0.40
Lucerne mannogalactan	—	Origin	1.17	—
Ivory-nut mannan	—	—	0.73	0.64
Apple pectin	—	—	1.53	—
Dextran A	—	0.33	0.33	—
Dextran B	—	0.50	0.33	—

Fig. 7. Electrophoresis of the polysaccharide mixture, containing 100 $\mu\text{g.}$ each of yeast mannan, snail galactan, yeast glycogen and elecampane inulin, on washed silk at 50 v/cm. for 90 min. Determination of eluates from 1 cm. segments with the α -naphthol reagent. (a) In 0.1 M veronal-acetate buffer, pH 9.3; (b) in 0.1 M borate buffer, pH 9.3. Compare with Figs. 8 and 9.

was recovered as galactose, 30% of zone 2 as mannose and 50% of zone 3 as glucose. Quantitative information concerning zone 4 was not obtained. No indications of other monosaccharides were found except in zone 3 where both glucose and fructose were detected. Determination indicated that as much as 25% of the inulin was present in this zone. In view of the incomplete separation of zones 3 and 4 (Fig. 7b), this result is not unexpected.

The amount of polysaccharide corresponding to each zone can be calculated by dividing the total colour value for each peak by the absorption/ $\mu\text{g.}$ of

the appropriate polysaccharide. This gives 90 $\mu\text{g.}$ of galactan in zone 1, 93 $\mu\text{g.}$ of mannan in zone 2 and 168 $\mu\text{g.}$ of glycogen plus inulin in the combined zones 3 and 4; that is, a total recovery of 87% of the polysaccharides applied.

An alternative method of calculating the overall recovery is to sum the colorimeter readings and compare them directly with a value for the whole mixture. This gives 83%, which compares favourably with the previous figure. The latter method takes no account of differences in the absorption/ $\mu\text{g.}$ of the polysaccharides, and if any selective losses occur the calculated recovery may not correspond with that on a weight basis.

Veronal buffer. Yeast mannan (Fig. 6) gave a broad double-humped band which moved towards the cathode to approximately the same extent as trimethylglucose, the stationary marker.

The mixture (Fig. 7a) showed no clear separation into its components, but formed a peak with a shoulder, the whole moving back to a position slightly nearer the cathode than the trimethylglucose run in parallel with it.

Glass paper

The α -naphthol spray reagent was used in an attempt to locate polysaccharide material on this support, but the definition was not good and a considerable amount of background colour was present. The modified *p*-anisidine spray provided a reagent of increased sensitivity and gave a very low background colour.

The mixture (10 $\mu\text{l.}$) and 50 $\mu\text{g.}$ of each of the polysaccharides (yeast mannan, yeast glycogen, inulin and snail galactan) were subjected to electrophoresis in borate buffer at a field strength of about 33 v/cm. for 45 min. The spots were developed with the *p*-anisidine reagent and the separation obtained

is shown in Fig. 8. The mixture separated into its four components, the actual distance moved by the yeast mannan being 8.8 cm. towards the cathode. It was possible to wash the paper for re-use by eluting with 96 % ethanol and then with water.

Boundary electrophoresis

The mixture was diluted to half its original concentration and subjected to electrophoresis in borate buffer for 30 min. It separated into four distinct boundaries (Fig. 9). The ratios of the mobilities in the Tiselius apparatus (see Table 1) are calculated from the values given by Northcote (1954). Snail galactan and wheat-straw xylan gave

single peaks in the Tiselius apparatus with mobilities of $11.7 \times 10^{-5} \text{ cm.}^2 \text{ v}^{-1} \text{ sec.}^{-1}$ and $3.5 \times 10^{-5} \text{ cm.}^2 \text{ v}^{-1} \text{ sec.}^{-1}$ respectively.

DISCUSSION

Greenway, Kent & Whitehouse (1953) describe a technique for the detection of polysaccharides after separation by electrophoresis on paper, but give no details of the electrophoretic method or the results obtained. The separation of polysaccharide fractions from extracts of wheat by electrophoresis in acetate and borate buffers was claimed by Preece & Hobkirk (1955), but the use of aniline oxalate to detect the positions of the bands suggests that the polysaccharides were not of very great molecular weight. Also the fact that no characterized polysaccharides were used makes it difficult to interpret their results. More recently, Foster *et al.* (1956) have subjected amylosaccharides to electrophoresis in borate buffer on filter paper and both their results and those of the present work suggest that the movements of amylose and amylopectin on paper are unsatisfactory. The work described here shows also that snail galactan does not move as a discrete band on this material and that the carbohydrate eluted from the paper itself is sufficient to make quantitative work difficult. These disadvantages can be overcome by the use of washed silk, which also permits the use of the α -naphthol spray reagent for the qualitative detection of polysaccharides.

Fig. 8. Electrophoresis of the polysaccharide mixture containing $50 \mu\text{g.}$ each of yeast mannan, snail galactan, yeast glycogen and elecampane inulin, in 0.1M borate buffer, pH 9.3, on glass paper. Run for 45 min. at 33 v/cm. Distance between the yeast mannan and the trimethylglucose spot is 8.8 cm. Spots were detected by *p*-anisidine-sulphuric acid reagent. Compare with Figs. 7 and 9.

Fig. 9. Boundary electrophoresis in 0.05M borate buffer, pH 9.3, for 30 min., of the mixture containing $50 \mu\text{g.}$ each of snail galactan, yeast mannan, yeast glycogen and inulin. (a) Ascending limb; (b) descending limb. Compare with Figs. 7 and 8.

However, this reagent is unsatisfactory since it is not very sensitive (about 50 μ g. of polysaccharide is required), the background obtained is not clear, and the silk tends to disintegrate. With the glass paper the modified *p*-anisidine spray reagent gives better results, and since the glass paper can be washed and used again, this provides a quick, easy and economical qualitative method. For quantitative work the use of washed silk and elution and determination of the polysaccharides is a suitable method, recoveries of about 85% being obtained. The resolution of the polysaccharide zones is, however, somewhat limited, but the technique does provide for the identification of the constituent monosaccharides, although the recoveries of these sugars are not quantitative. Because of the number and nature of the operations involved in these determinations, high recoveries can hardly be expected.

The hydrolysis results presented for the mixture do not conflict with the idea that each of the zones in Fig. 7*b* represents a distinct polysaccharide; their movements are comparable with the movements of the polysaccharides by themselves and are consistent with the degree of complexing with borate which might be expected from their structures.

The time for hydrolysis is short (60 min.) compared with that for the complete hydrolysis of 1 g. of yeast mannan in 100 ml. of *N* hydrochloric acid, which would require at least 6 hr. However, the maximum value for the reducing sugar, obtained after 60 min. (Fig. 2), was 90% of the calculated value, and electrophoresis of the hydrolysate at this time indicated that all the reducing sugars were present as monosaccharides. The low concentration of the polysaccharide used, about 0.01 g./100 ml., might well account for this short hydrolysis time.

The results with yeast mannan (Fig. 6) and the mixture (Fig. 7*a*) in veronal buffer on silk suggest that this buffer is not suitable for the electrophoretic separation of neutral polysaccharides. It is, however, difficult to compare our results directly with those of Geldmacher-Mallinckrodt & Wienland (1953) since different conditions were used and the separation they obtained was of a two-component mixture. Bertrand & Laszt (1956) observed no essential difference between veronal and borate buffers in the electrophoresis of glycogens, and state that the best buffer for this purpose is a triethanolamine phosphate buffer at pH 7.0 in gelatin to reduce diffusion effects. Since these workers separated no mixtures and did not measure the electroendosmosis it is difficult to know whether any electrophoretic movement occurred. Northcote (1954) has shown that borate is essential for satisfactory electrophoresis of neutral polysaccharides in

the Tiselius apparatus, and the present work indicates that this is true for electrophoresis on strip supports. With acidic polysaccharides Rienits (1953) has obtained separation on filter paper by using phosphate and acetate buffers.

The ratios given in Table 1 for movement on silk, glass paper and in the Tiselius apparatus correspond very well, and therefore the separation on both silk and glass paper is electrophoretic and generally absorption plays little or no part. However, with silk three of the polysaccharides, amylose, straw xylan and lucerne mannogalactan remain at the point of application and presumably these polysaccharides are strongly absorbed. Since the borate complex has a negative charge the polysaccharides would be expected to move towards the anode as in the Tiselius apparatus, but on the strip support at pH 9.3 the electroendosmosis is great, which results in a net movement towards the cathode. Geldmacher-Mallinckrodt & Wienland (1953) describe their technique as electrochromatography, that is, movement in the buffer flow and separation of the components because of differing absorptions, and this is a possible explanation of their results since they used veronal buffer at pH 9.3, when it is difficult to see how the glycogen and galactan used in their experiments could acquire a charge.

Electrophoresis on strip supports has several advantages, the most important being: (1) Only about 50 μ g./component of the mixture is required. (2) The monosaccharide constituents of each polysaccharide zone can be identified. (3) If proteins are present they will not be detected by the techniques used, but if information regarding these is required it could be obtained, on the glass paper, by the use of the appropriate reagent. In the Tiselius apparatus any difference in refractive index will be detected as a boundary and thus no difference between polysaccharides and proteins would be shown.

SUMMARY

1. A zone-electrophoretic method for the quantitative separation of neutral polysaccharides is described. The strip support used is silk and the method gives 85% recovery of a four-component mixture containing 100 μ g. of each component.

2. A qualitative zone-electrophoretic method with glass paper as a support and *p*-anisidine as a general spray reagent is described.

3. The electrophoretic movement of the neutral polysaccharides used is dependent upon the use of borate buffer.

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Helicorubin and Cytochrome *h*

PURIFICATION AND PHYSICAL PROPERTIES

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Helicorubin is a naturally occurring haemochromogen found in the crop and intestinal tract of land snails and some other invertebrates. It is the only known extracellular haemoprotein apart from haemoglobin and chlorocruorin, and in many of its properties it resembles some of the components of cytochrome. Although helicorubin was the first haemoprotein of the haemochromogen type to be described, it was never purified, and all the properties so far attributed to it have been those of the crude material.

In 1876 Sorby found that the crop and intestine of the garden snail contain a red-brown fluid which has an absorption spectrum very like that of a haemochromogen; he also found that this type of absorption spectrum could be seen in the digestive gland ('liver' or hepatopancreas) and other organs. When exposed to air, the pigment underwent oxidation and its strong absorption bands disappeared, but they reappeared when the fluid was made alkaline and the pigment became reduced. Sorby's observations were confirmed by Krukenberg (1882), who named the pigment 'helicorubin', and by MacMunn (1883, 1885, 1886*a, b*), who named it 'enterohaematin'. The name 'helicorubin' given by Krukenberg has now been generally adopted.

The first attempt at the purification of helicorubin was made by Dhéré & Vegezzi (1917*a, b*) and Dhéré (1919), who ultrafiltered snail 'bile' and

separated a red solution containing helicorubin, which passed through the collodion membrane, from a brown pigment which they named 'helico-fuscine'. They found that alkaline helicorubin has a haemochromogen type of absorption spectrum consisting of a sharp α -band at 562.5 m μ ., a weaker β -band at 529.5 m μ ., and a γ -(Sorét) band at 427.5 m μ . The absorption bands in the visible region can be seen when snail 'bile' is examined *in situ* by transilluminating a loop of the intestine, and their positions agree with those recorded by Sorby (1876).

On acidification of the 'bile', these sharp bands are replaced in the visible region of the spectrum by the two diffuse bands of oxidized helicorubin; of these the β -band is stronger than the α -band, and the γ -band now lies at 415 m μ . The oxidized, acid helicorubin may be changed to reduced helicorubin either by the addition of alkali or by treating the acid solution with a reducing agent, e.g. sodium dithionite. In the latter case the α -band of the haemochromogen is asymmetrical near its peak, and on direct spectroscopic examination appears to be double. Dhéré & Vegezzi concluded that helicorubin is a protein with the same prosthetic group as haemoglobin.

Anson & Mirsky (1925) claimed, from work with crudesnail 'bile', that helicorubin is a haemochromogen capable of undergoing oxygenation as well as oxidation, but this claim has not been confirmed.

Roche & Morena (1936*a, b*), using material which had been partly cleared of other pigments

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