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The use of polyacrylamide-gel electrophoresis for the high-resolution separation of reducing saccharides labelled with the fluorophore 8-aminonaphthalene-1,3,6-trisulphonic acid

Detection of picomolar quantities by an imaging system based on a cooled charge-coupled device

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Various monosaccharides, oligosaccharides and small polysaccharides were labelled covalently at their reducing end groups with the fluorophore 8-aminonaphthalene-1,3,6-trisulphonic acid (ANTS), and the resulting fluorescent derivatives were separated by high-resolution PAGE. The electrophoretic mobilities of the labelled saccharides are related largely to the compounds' M_r values, but they are also influenced by the individual chemical structures of the saccharides. Various positional isomers and some epimers, for instance galactose and glucose, were resolved. Oligosaccharide and small polysaccharide derivatives, prepared from an enzymic digest of starch, each differing in size by a single hexose residue and with a range of degrees of polymerization from 2 to 26, were all resolved in a single gel. The method was relatively rapid and simple to perform. It enabled multiple samples to be analysed in parallel with high sensitivity. The fluorescent-labelling procedure was virtually quantitative. As little as 1 pmol of ANTS-labelled saccharide was detected photographically when the gels were illuminated by u.v. light. When the gels were viewed using an imaging system based on a cooled charge-coupled device, as little as 0.2 pmol was detected. The method may be useful for the structural analysis of the carbohydrate moieties of glycoconjugates and other naturally occurring oligosaccharides.

INTRODUCTION

It has become apparent that the carbohydrate moieties of glycoconjugates are involved in numerous important biological processes [1–3]. As a result, there has been considerable research into their chemical structures. Much of this work has depended on purification techniques such as h.p.l.c. and other chromatographic methods. Subsequent structural analysis has been performed by a combination of classical derivatization and degradation procedures, m.s. and n.m.r. [4–7]. Although these are powerful methods, they have significant limitations. In particular, m.s. gives little or no information about the anomeric configuration of glycosidic linkages, and the quantities of material required for some analyses can be relatively large compared with that which is available from many specific biological sources. In addition the equipment required for these methods is expensive and requires considerably expertise and technical support, which tends to restrict their use to few laboratories.

In order to overcome some of these disadvantages, several workers have used specific glycosidases to degrade complex oligosaccharides and have deduced their structures after separating the degradation products by various chromatographic and electrophoretic techniques [7–13]. This type of analysis can be used with picomolar quantities of material. In order to enable the sensitive detection of such quantities, a number of methods have been described in which saccharides and glycopeptides have been labelled with either ^3H, chromophores or fluorophores and the derivatives separated either chromatographically or electrophoretically [8,9,11,14–24]. A related, but novel, procedure is described here in which saccharides were labelled at their reducing ends with the fluorophore 8-aminonaphthalene-1,3,6-trisulphonic acid (ANTS). The fluorescent derivatives were separated with high resolution by PAGE. The fluorescent electrophoretograms showing the band pattern of the resolved saccharide derivatives were recorded both photographically in low picomolar quantities and in subpicomolar quantities by using a new imaging system based on a cooled charge-coupled device.

EXPERIMENTAL

Materials

ANTS was obtained from Molecular Probes (Eugene, OR, U.S.A.) as its disodium salt. Heat-hydrolysed wheat starch and the saccharides shown in Table 1 were obtained either from Sigma Chemical Co. or from Aldrich Chemical Co. The oligosaccharides I and II shown in Fig. 1 were the gift of Dr J. C. Klock, Glycomed, Alameda, CA, U.S.A. Electran or analytical-grade reagents were used for the electrophoretic and derivatization procedures and were obtained from BDH or from Sigma. α-Amylase (EC 3.2.1.1) from *Bacillus subtilis* was obtained from Boehringer-Mannheim, and β-galactosidase (EC 3.2.1.23) from *Escherichia coli* was obtained from Sigma. [U-^{14}C]glucose (10.0 GBq/mmol) was obtained from Amersham International.

Fluorescent labelling

The standard method for reacting saccharides with ANTS was as follows. Suitable volumes, usually 5 or 10 μl, of 1 mM-saccharide solutions in water were placed in microcentrifuge tubes and freeze-dried using a centrifugal vacuum evaporator (c.v.e.) (Gyrovap; V. A. Howe, Banbury, Oxfordshire, U.K.).

Abbreviations used: ANTS, 8-aminonaphthalene-1,3,6-trisulphonic acid; c.c.d., charge-coupled device; c.v.e., centrifugal vacuum evaporator; DMSO, dimethyl sulphoxide; $NaCNBH_3$, sodium cyanoborohydride; pixel, picture element; SM, standard mixture.

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Table 1. Saccharides analysed

No.	Abbreviated formula	Trivial name
1	2-Deoxy-D-Gal	2-Deoxygalactose
2	6-Deoxy-L-Gal	L-Fucose
3	2-Deoxy-D-Glc	2-Deoxyglucose
4	6-Deoxy-D-Glc	6-Deoxyglucose
5	D-Gal	Galactose
6	α-D-Glc	Glucose
7	D-Man	Mannose
8	3-*O*-Methyl-α-D-Glc	3-*O*-Methylglucose
9	D-GalNac	*N*-Acetylgalactosamine
10	α-D-GlcNac	*N*-Acetylglucosamine
11	D-GlcNac6SO_3	*N*-Acetylglucosamine 6-sulphate
12	α-D-Gal-(1→4)-D-Gal	Galactosylgalactose
13	β-D-Gal-(1→6)-D-Gal	Galactobiose
14	β-D-Gal-(1→4)-D-Glc	Lactose
15	α-D-Gal-(1→6)-D-Glc	Mellibiose
16	β-D-Gal-(1→4)-D-Man	Galactosylmannose
17	α-D-Glc-(1→3)-D-Glc	Nigerose
18	β-D-Glc-(1→3)-D-Glc	Laminaribiose
19	α-D-Glc-(1→4)-D-Glc	Maltose
20	β-D-Glc-(1→4)-D-Glc	Cellobiose
21	α-D-Glc-(1→6)-D-Glc	Isomaltose
22	β-D-Glc-(1→6)-D-Glc	Gentiobiose
23	α-D-Man-(1→3)-D-Man	Mannobiose
24	β-D-Gal-(1→4)-D-GlcNac	*N*-Acetyl-lactosamine
25	β-D-GlcNac-(1→4)-D-GlcNac	Diacetylchitobiose
26	α-D-Glc-(1→4)-α-D-Glc-(1→4)-α-D-Glc	Maltotriose
27	β-D-Glc-(1→4)-β-D-Glc-(1→4)-β-D-Glc	Cellotriose
28	α-D-Glc-(1→6)-α-D-Glc-(1→4)-α-D-Glc	Panose
29	α-D-Glc-(1→6)-α-D-Glc-(1→6)-α-D-Glc	Isomaltotriose
30	B-D-Gal3Neu5Ac-(1→4)-D-Glc	*N*-Acetylneuramin-lactose
31	[α-D-Glc-(1→4)-]$_3$α-D-Glc	Maltotetraose
32	α-D-Glc-(1→6)-[α-D-Glc-(1→4)-]$_2$α-D-Glc	
33	[α-D-Glc-(1→4)-]$_4$α-D-Glc	Maltopentaose
34	[α-D-Glc-(1→4)-]$_5$α-D-Glc	Maltohexaose
35	[α-D-Glc-(1→4)-]$_6$α-D-Glc	Maltoheptaose

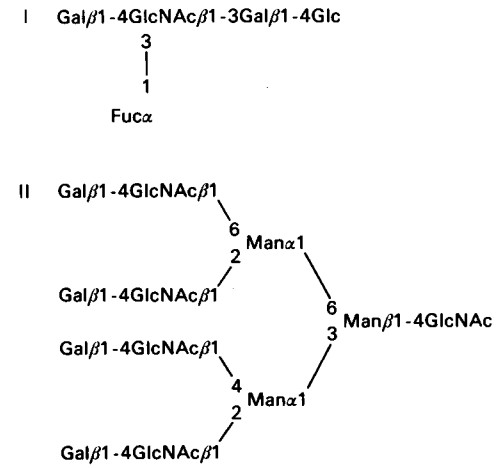

Fig. 1. Abbreviated structural formulae for the complex oligosaccharides I and II

To each dry sample was added 5 μl of 0.2 M-ANTS solution in acetic acid/water (3:17, v/v) and 5 μl of 1.0 M-$NaCNBH_3$ solution in dimethyl sulphoxide (DMSO). The solution was vortex-mixed, centrifuged briefly at 10000 *g* to ensure all the reactants were in the tips of the tubes, and incubated at 37 °C for 15 h. The reaction mixture was dried under vacuum for 4 h in a c.v.e. at approx. 45 °C and dissolved in a suitable concentration of electrophoresis sample buffer, so that the concentration of each labelled saccharide was 100 pmol/μl. Labelled saccharides were stored at −70 °C. The standard method was varied to determine the optimal reaction conditions. In these reactions, glucose, lactose and maltopentaose were derivatized together as test saccharides. The quantities of saccharides, the concentration of ANTS and acetic acid and the reaction time were all varied as indicated. The volumes of the ANTS and $NaCNBH_3$ solutions, the concentration of $NaCNBH_3$ and the temperature were kept constant unless otherwise stated.

Enzymic digestions

Heat-hydrolysed wheat starch was suspended, with vigorous mixing, at a concentration of 10 mg/ml in 0.1 M-ammonium acetate buffer, pH 5.5, at 37 °C. To 50 μl of this suspension was added 5 μl of a solution containing 0.75 μg of α-amylase (from *B. subtilis*)/ml. The mixture was incubated at 37 °C for 30 min, after which time the digestion was stopped by the addition of 1 ml of ice-cold ethanol and dried under vacuum using a c.v.e. The digestion products were allowed to react with ANTS using the standard conditions and dissolved subsequently in 50 μl of electrophoresis sample buffer; 2.0 μl was analysed per gel lane.

Oligosaccharides I and II were both treated with β-galactosidase and then allowed to react with ANTS as follows. To approx. 2 nmol of each oligosaccharide in solution in 5 μl of 0.1 M-sodium phosphate buffer, pH 7.4, at 37 °C, was added 1 μl of a solution of β-galactosidase in water [1 unit (supplier's definition)/ml]. The mixture was incubated for 4 h at 37 °C and then freeze-dried in a c.v.e. The dry digest was derivatized with ANTS using the standard conditions. Control assays, in which either no oligosaccharide or no enzyme was used, were carried out simultaneously. Each enzymic digestion and the subsequent derivatization were carried out in the same reaction tube.

Electrophoresis

Saccharides labelled with ANTS were subjected to PAGE using a type SE600 electrophoresis apparatus from Hoefer Scientific Instruments, Newcastle under Lyme, Staffs., U.K. The plates of the gel cassettes were made of either window glass (when the gels were to be photographed) or Pyrex glass (when gels were imaged *in vitro* using the c.c.d. camera).

The electrophoretic buffer used was based on the Tris/HCl/glycine discontinuous system of Laemmli [25], but SDS was omitted throughout. The polyacrylamide gel consisted of a linear gradient from 20 to 40% (w/v) acrylamide containing 0.67–1.06% (w/v) *NN'*-methylenebisacrylamide respectively as cross-linker. The gradient was generated by using a three-channel peristaltic pump. The polymerization of both gels was initiated by the addition of 20 μl of 10% (w/v) ammonium persulphate solution and 10 μl of *NNN'N'*-tetramethylenediamine/12 ml of gel solution. The resolving gel size was 140 mm high × 140 mm wide × approx. 0.5 mm thick. A stacking gel was used. The sample wells were 4 mm wide. Samples were electrophoresed at 100 V for 30 min, 500 V for 30 min and finally at 1000 V for approx. 120 min, until the buffer front reached approx. 5–10 mm from the gel base. All the voltages were held constant. The gels were cooled to 5–7 °C by the surrounding stirred lower electrode buffer.

Photography

Gels were photographed after removal from their cassettes and placing on a u.v. light box (Transilluminator, type TM40; UVP, Cambridge, U.K.) with a maximum emission wavelength of 302 nm and a power of approx. 7000 μW/cm^2. A Polaroid type 55 film (ISO 50), which gave both a negative and a positive photograph, a Wratten 8 gelatin filter (Kodak), an aperture of f4.5 and an exposure time of 50 s were used. The photographic images were approx. 62% of the size of the original gel.

Densitometry

Film negatives were scanned with white light on a Chromoscan 3 densitometer (Joyce–Loebl, Gateshead, Tyne and Wear, U.K.) using a slit size of 0.1 mm × 1.5 mm, except when generating the densitometric profile shown in Fig. 7 (below), in which the slit size was 0.05 mm × 1.5 mm. Densitometric measurements of the bands produced by the fluorescently labelled saccharides were carried out as described previously [26]. All the absorbance measurements were less than 85% of the maximum film density.

Quantification of the fluorescent labelling using [^{14}C-]glucose

ANTS was allowed to react with various quantities of glucose (14–110 nmol/reaction tube), each containing 0.5 μCi of [U^{14}C]glucose. After it had been dried the reaction mixture was dissolved in 100 μl of water and 1.0 μl was applied to a silica-gel t.l.c. plate (Polygram SILG; 20 cm × 20 cm; Macherey–Nagel) and chromatographed in a solution of butan-1-ol/ethanol/water (5:3:2, by vol.). The chromatogram was autoradiographed using Cronex 4 X-ray film (du Pont). Known quantities of unchanged [^{14}C]glucose were chromatographed as standards.

Time-course measurement for the fluorescent labelling

A portion (25 nmol) of each of the saccharides (glucose, lactose and maltopentaose) were allowed to react together at 37 °C with 20 μl of 0.2 M-ANTS in acetic acid/water (3:17, v/v) and 20 μl of 1.0 M-NaCNBH$_3$ in DMSO. Samples (4.0 μl) were removed at intervals and frozen immediately in liquid N_2 until the last sample had been taken. All the samples were dried together for 4 h at approx. 45 °C in a c.v.e., dissolved subsequently in 40 μl of electrophoresis sample buffer and 2.0 μl was electrophoresed. The extent of the reaction was determined by densitometry of the labelled saccharide bands in the film negative of the gel.

Gel imaging using the cooled c.c.d.

Gels were imaged electronically without being removed from their electrophoresis cassettes using the Astromed (Cambridge, U.K.) 2200 Imaging system. Briefly, except for the image in Fig. 4(*a*) (below), the system consisted of a cooled c.c.d. containing an array of 385 × 578 picture elements (pixels) each having dimensions 22 μm × 22 μm, on to which was focused an image of a section of the gel. The gel image was demagnified 5-fold by a lens with an aperture of f1.9. The c.c.d. was cooled to approx. 242 K by a Peltier cooler. Each gel was viewed in six sections and the images joined by the associated computer to give an image of a total area of gel approx. 120 mm × 120 mm. The time for viewing each section of each individual gel was constant for individual gels, but was varied from gel to gel, and was either 10 or 60 s. The c.c.d. response was directly proportional to the imaging time. The illumination was in the plane of the gel from the anodic edge using a fibre-optic light guide with the dimensions 0.5 mm × 200 mm. The light-guide was adjusted so that its aperture was aligned with the gel edge. The gel cassette was placed so that it abutted the light-guide. The light source was a 100 W tungsten–halogen lamp. The excitation and emission interference filters (Omega Optical, Brattleboro, VT, U.S.A.) had transmission maxima of 390 and 492 nm respectively. In the case of images taken for the quantitative data, any unevenness in the gel illumination was removed digitally by using as a reference an image of a gel containing 0.1 mM-ANTS uniformly.

The fluorescence of the saccharide bands in the gels was measured by determining the mean number of photons registered/min per pixel in a defined rectangular area, 40 × 20 pixels, covering each band and subtracting the gel background measured on similar adjacent blank areas in the same gel lane. The image shown in Fig. 4(*a*) (below) was obtained by a slightly more sensitive system than that described above. The essential differences were that the lens aperture was f1.2, the light-guide had exit dimensions 0.5 mm × 90 mm and a 50 W light-source was used. Since the light-guide was less wide than the gel, the latter was positioned before it mechanically on a precision carriage controlled by the computer system.

RESULTS

Derivatization of the saccharides with ANTS

Various reaction conditions were altered to determine the optimal labelling with ANTS of the three chosen test saccharides (glucose, lactose and maltopentaose). The effect of varying the acetic acid concentration between 0 and 20% (v/v) in the standard method was measured. When the concentration of acetic acid was between 5 and 20% (v/v), the degree of

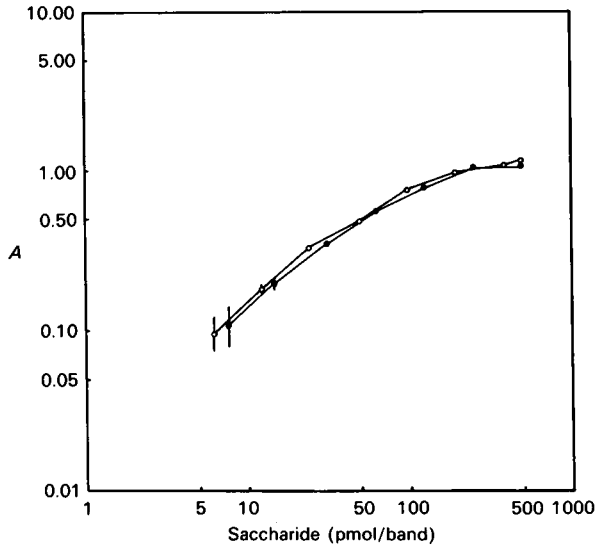

Fig. 2. Variation of the film response with the quantity of ANTS labelled saccharide per gel band

The film absorbances were measured as described in the text. The values shown are obtained for ANTS-labelled maltopentaose. ○, Film absorbances for the samples which were produced by serial dilution in electrophoresis sample buffer of the most concentrated sample shown in which there was a total of 25 nmol of each of the three test saccharides per reaction. In this sample the saccharides were derivatized using the standard conditions, and the products dissolved in 100 μl of electrophoresis sample buffer. For all samples, 2 μl was loaded per gel lane. ●, Film absorbances for samples in which the quantities of the three test saccharides were varied similarly in each reaction and a fixed proportion, $\frac{1}{50}$, of each was electrophoresed. Each point represents the mean of four determinations for the derivatization of maltopentaose. The S.E.M. is shown for the lowest two points on each curve. The rest of the standard errors of the mean were all less than 5%. The data for glucose and lactose were similar.

derivatization was optimal and constant. When the acetic acid concentration was 0%, the degree of derivatization was approx. 84% of the optimum. A solution of acetic acid/water (3:17, v/v) was chosen for the standard conditions. It was found convenient to dissolve the ANTS in this solution, for which gentle warming was required. The time course for the reaction of ANTS with equimolar amounts of the three test saccharides was measured. The time courses for all three saccharides were similar. The reaction was virtually complete by 12 h at 37 °C, and a time of 15 h was used for the standard conditions.

The extent of saccharide derivatization was measured as the concentration of ANTS was varied in the standard method. When 25 nmol of each test saccharide was allowed to react, the maximum derivatization was obtained when the concentration of ANTS was at least 0.1 M, and 0.2 M was used routinely. Similar results were obtained for each saccharide tested and also when the quantity of each was reduced to 2.5 nmol per reaction tube.

Densitometric measurements of the autoradiographs of the t.l.c. analyses of the reaction products of the ANTS labelling of [^{14}C-]glucose showed that, for all the quantities of glucose tested, greater than 99% of the glucose had reacted and that 92% occurred in a new band which, for the higher loadings of glucose, could be seen to be fluorescent when the t.l.c. plate was illuminated by u.v. light. A faint background of radiolabel was found along the length of each sample lane, which accounted for the remaining 8% of the label not in the major band. This may have been caused either by impurities in the ANTS or the glucose or by artefacts of the reaction or the chromatography.

Quantitative analysis and limits of sensitivity

The quantitative characteristics of the photographic-recording method were determined by derivatizing together 25 nmol of each of the three test saccharides using the standard conditions, dissolving the reaction products in 100 μl of electrophoresis sample buffer, serially diluting the solution and analysing 2 μl of each dilution. The film response for maltopentaose is shown in Fig. 2. The results for glucose and lactose were similar. The data

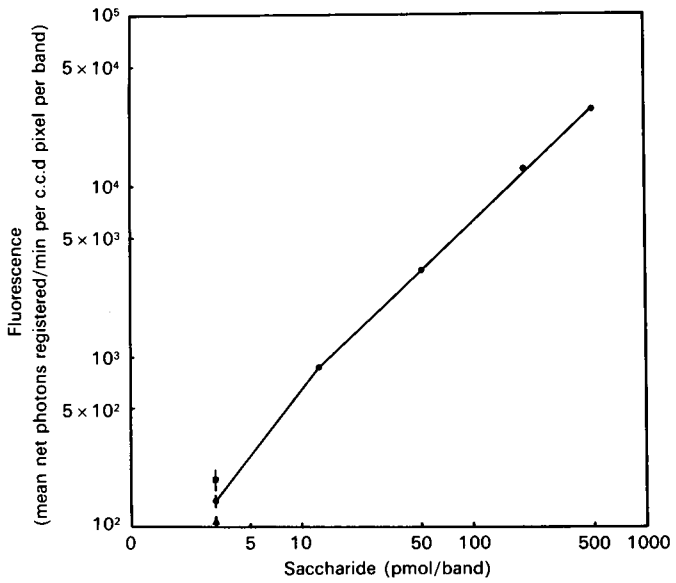

Fig. 3. Variation of the CCD response with the quantity of ANTS-labelled saccharide per gel band

The c.c.d. response was measured as described in the text. ●, Mean values for maltopentaose. The values for glucose and lactose were similar to those for maltopentaose for each of the four highest loadings and for reasons of clarity are not shown. The S.E.M. values were all less than 5.1% unless indicated. For the lowest quantity of saccharide (3.1 pmol) there was considerable variation in the means and S.E.M. values, and these are shown for all three test saccharides; ●, maltopentaose; ■, glucose; ▲, lactose.

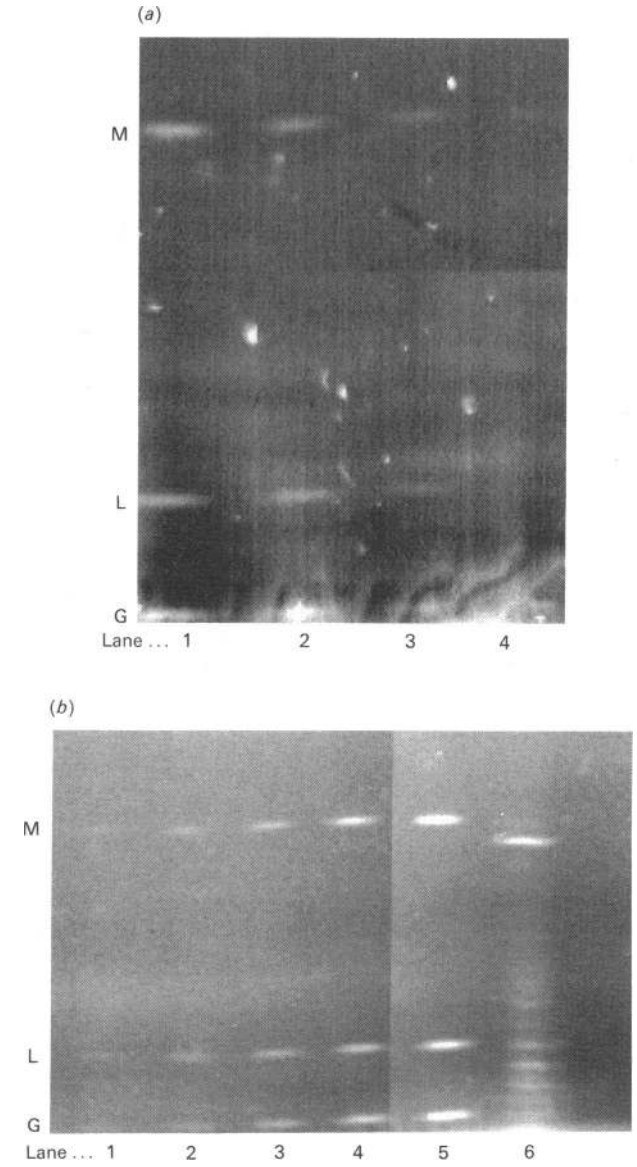

Fig. 4. Photographs of the graphics display of the cooled c.c.d. imaging system, showing the lowest limits of detection

Only the relevant sections of the gels are shown. In both images the contrast has been optimized. Each lane, except (*b*), lane 6, contained equimolar quantities of ANTS-labelled glucose (G), lactose (L) and maltopentaose (M) serially diluted from a standard reaction mixture containing 25 nmol of each saccharide. The samples were loaded in alternate gel lanes. The sample buffer showed no fluorescent bands. (*a*) The gel was imaged using an *f* 1.2 lens, a 50 W lamp and a 0.5 mm × 90 mm light-guide. Lane 1, 0.8 pmol; lane 2, 0.4 pmol; lane 3, 0.2 pmol; lane 4, 0.1 pmol. The sharp horizontal discontinuity in the background is a join between two image sections. This image was processed digitally to smooth the background. (*b*) The gel was imaged using the standard viewing system. Lane 1, 0.4 pmol; lane 2, 0.8 pmol; lane 3, 1.0 pmol; lane 4, 2.0 pmol; lane 5, 4.0 pmol; lane 6, 2% of the standard reaction mixture. Only that section of the lane having significant fluorescent background is shown. The vertical discontinuity in the background between lanes 4 and 5 is the join between two image sections.

are plotted on logarithmic scales to encompass conveniently the wide dilution range. The film response varied non-linearly with the amount of saccharide and decreased markedly when the higher amounts were analysed.

A similar film response (see Fig. 2) was obtained when the quantities of saccharides in each reaction were varied between 0.39 and 25 nmol and a constant proportion (1:50) of each reaction mixture was analysed. Although quantities of saccharides as low as 1 pmol could be detected faintly by eye on the film, it was not found possible to measure accurately the film absorbance at levels below approx. 5 pmol of saccharide per band.

A similar experiment was carried out in which the saccharide content of the reaction mixture was varied, but in this case the reaction mixture was dissolved in various volumes of electrophoresis buffer so that the concentration of each of the three test saccharides was 100 pmol/μl. When 2 μl of each solution was electrophoresed and the fluorescence was measured, it was found that the film response was constant, that is, independent of the quantity of saccharide in the reaction tubes. This result is in agreement with the data from the radiolabelling experiment, which showed that the degree of labelling of glucose was constant irrespective of the quantity in the reaction tube, up to the maximum of 110 nmol.

The quantitative characteristics of the c.c.d. imaging system were determined by electrophoresing samples serially diluted from a reaction mixture containing 25 nmol of each of the test saccharides as described for assessing the photographic method. The gels were imaged for either 10 or 60 s and the results normalized to 60 s. Fig. 3 shows data for maltopentaose for the range 3.1–500 pmol. The c.c.d. responded linearly from 12.5 to 500 pmol, and the S.E.M. for each value was less than 5.1 %. The data for glucose and lactose were similar for the range 12.5–500 pmol. However, considerable variation between the individual saccharides and relatively high S.E.M. values were found for the 3.1 pmol quantity. This reflected the difficulty of obtaining accurate measurements when the signal from the saccharide band was close to that of the gel background. However, it was possible to reveal lower quantities. In Fig. 4 are shown c.c.d. images of gels containing serial dilutions of a sample containing equimolar quantities of the three test saccharides. It was possible to detect as little as 0.2 pmol per band when using a 60 s viewing time (see Fig. 4*a*, lane 3). The level of background obtained when loading 2% of a standard reaction solution containing no saccharides is also shown in Fig. 4(*b*).

Electrophoretic analysis

A diagrammatic representation of an electrophoretogram depicting the separation of 35 different ANTS-labelled saccharides is shown in Fig. 5. It can be seen that, as the size of the saccharides increases, there is a general decrease in electrophoretic mobility. However, numerous saccharides with identical M_r values were well separated. The most notable separations were as follows: 6-deoxyglucose had the highest mobility of all the saccharides tested and was separated clearly from the three other deoxyhexoses. The epimers glucose and galactose were well resolved, but mannose and galactose were not. *N*-Acetylgalactosamine and *N*-acetylglucosamine were resolved, but the latter had the same mobility as galactose.

The disaccharides tested also had a range of mobilities. The isomers galactosylgalactose and galactobiose were resolved, as were maltose, isomaltose and cellobiose. By contrast, the

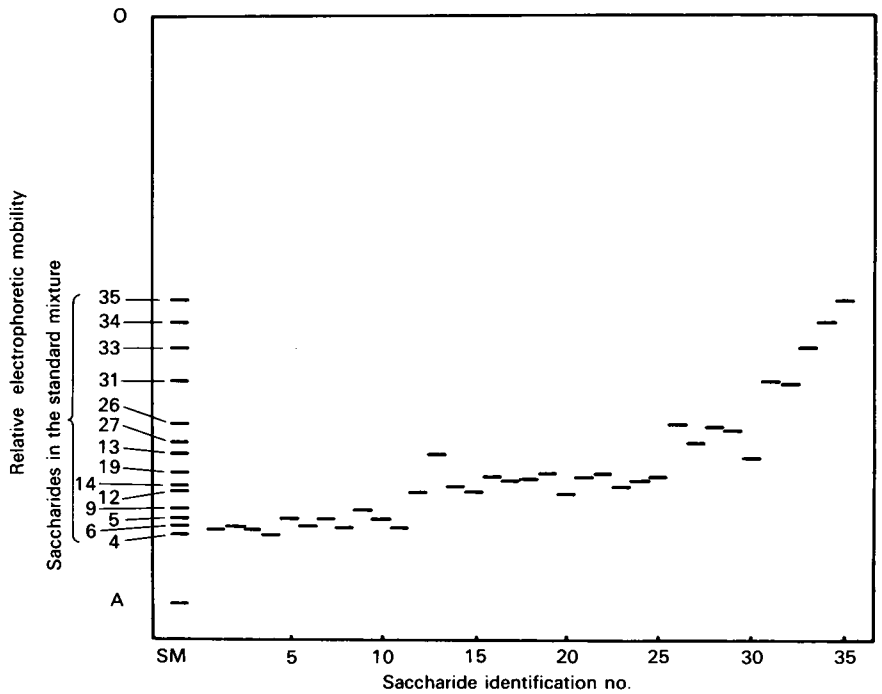

Fig. 5. Diagrammatic representation of an electrophoretogram of ANTS-derivatized saccharides showing their relative electrophoretic mobilities

Each band represents the position of an individual saccharide relative to unchanged ANTS (labelled 'A'), which moved at the buffer front. Each gel track number corresponds to the number of each saccharide shown in Table 1. The track labelled 'SM' shows the separation of the standard mixture of 14 saccharides chosen to give a wide spread of well-resolved bands. In order of decreasing mobilities the identities of the bands are: 4, 6-deoxyglucose; 6, glucose; 5, galactose; 9, *N*-acetylgalactosamine; 12, galactosylgalactose; 14, lactose; 19, maltose; 13, galactobiose; 27, cellotriose; 26, maltotriose; 31, maltotetraose; 33, maltopentaose; 34, maltohexaose; 35, maltoheptaose. The top of the resolving gel is marked by an 'O'.

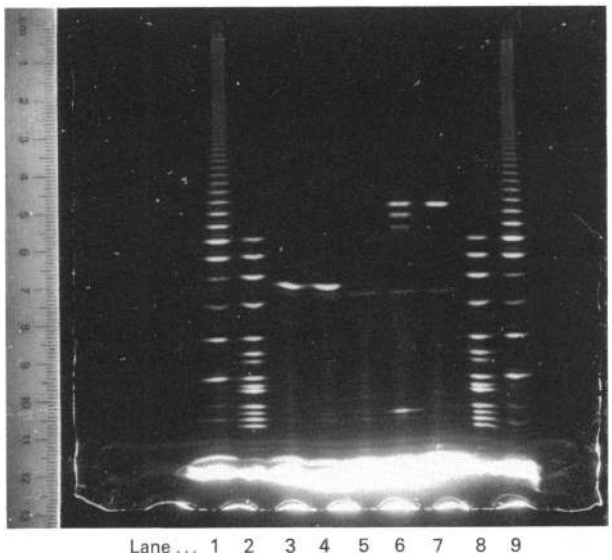

Fig. 6. Photograph of an electrophoretogram of various ANTS-derivatized saccharides

Lanes 1 and 9, partial α-amylase digest of the equivalent of 20 μg of heat-hydrolysed wheat starch; lanes 2 and 8, the standard mixture with each band representing approx. 200 pmol of each derivatized saccharide. In order of decreasing mobilities the identities of the bands are: 6-deoxyglucose, glucose, galactose, *N*-acetylgalactosamine, galactosylgalactose, lactose, maltose, galactobiose, cellotriose, maltotriose, maltotetraose, maltopentaose, maltohexaose, maltoheptaose; lane 3, oligosaccharide I; lane 4, oligosaccharide I treated with β-galactosidase; lane 5, β-galactosidase; lane 6, oligosaccharide II treated with β-galactosidase; lane 7, oligosaccharide II. The strongly fluorescent band at the base of the gel is caused by excess ANTS. The conditions used for the enzyme digestions and the ANTS derivatizations are described in the text. One-tenth of the total reaction mixture was analysed for each sample in lanes 3–7.

corresponding 1→3-linked disaccharides nigerose and laminaribiose, and the 1→6-linked disaccharides isomaltose and gentiobiose had different mobilities, but were not well resolved.

The trisaccharides tested also showed a range of mobilities. Maltotriose had a slightly lower mobility than isomaltotriose and was well resolved from cellotriose. Maltotriose also had a slightly lower mobility than panose, but they were not well resolved. Similarly, maltotetraose had a small mobility difference from α-D-Glc1-6(α-D-Glc1-4)$_2$α-D-Glc.

The 14 saccharides which were in the standard mixture (SM) shown in the left-hand track of Fig. 5 were chosen to give a wide spread of well-resolved bands. It included glucose and all the α1→4-linked straight-chain oligomers of glucose from maltose to maltoheptaose. A photograph of a gel is shown in Fig. 6, which shows the separation of this standard in lanes 2 and 8.

The range and resolving power of the method is also indicated in Fig. 6. Lanes 1 and 9 contained the ANTS-derivatized partial α-amylase digest of heat-hydrolysed wheat starch. Seven bands can be seen to correspond to glucose and its α1→4-linked straight-chain oligomers up to maltoheptaoses. In addition there are 19 other bands having lower mobilities than maltoheptaose, each resolved from the next. Presumably these represent individual polysaccharides differing from one another by a single hexose unit. A number of additional minor bands exist that may represent oligosaccharides containing α1→6-linkages which are not cleaved by the α-amylase. In Fig. 7 is shown a densitometric trace of lane 1 from Fig. 6. The resolution of each band up to 26 hexose units can be seen clearly, and there is also an indication that even-higher-M_r polymers can be resolved.

Lanes 3 and 6 in Fig. 6 contained the complex oligosaccharides I and II respectively (see Fig. 1). They both had higher mobilities than the oligosaccharides containing a similar number of glucose residues. Oligosaccharide I had a mobility between that of maltotetraose and maltopentaose, and oligosaccharide II had a mobility between those of the ninth and tenth major bands in the starch-digest lanes.

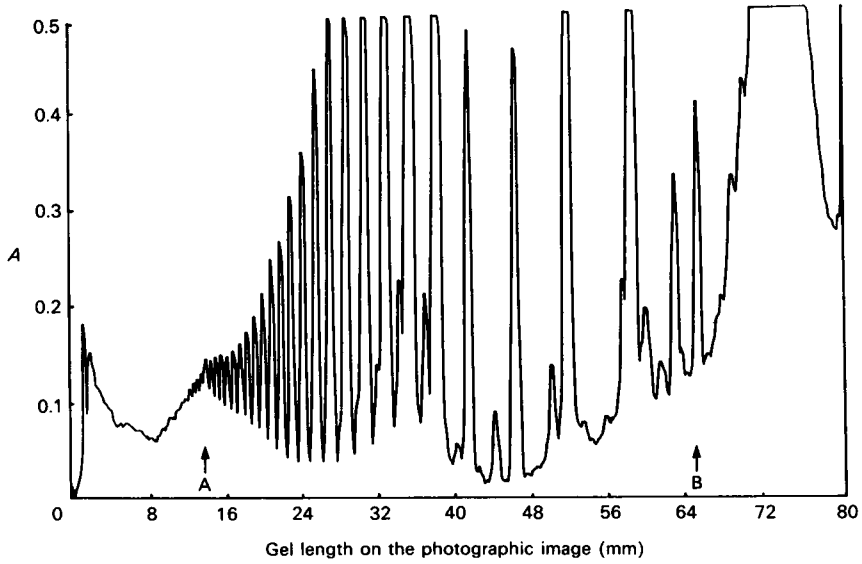

Fig. 7. Densitometric profile of a photographic negative of a PAGE separation of an ANTS-derivatized α-amylase partial digest of heat-hydrolysed wheat starch

The profile was obtained by scanning lane 1 of the negative of the photograph shown in Fig. 8. The densitometer slit size was 0.05 mm × 1.5 mm. The arrow marked 'A' indicates the position of the 26th major band from the glucose band, which is indicated by the arrow marked 'B'.

Lanes 4 and 6 in Fig. 6 show the effect of treating oligosaccharides I and II with β-galactosidase. Oligosaccharide I remained undigested. Oligosaccharide II was partially digested. Three bands with lower mobilities than the original were produced. The two closest to the original band appear to represent oligosaccharide products which have lost one or more galactose residues. The third band has the same mobility as the galactose standard and represents the cleaved galactose. A control reaction mixture containing only β-galactosidase is shown in lane 5. Several faint 'artefactual' bands having mobilities higher than maltotriose can be seen in tracks 3–7. These bands could be seen in all samples, including those which contained no enzymes or saccharides. They only became significant when a relatively high proportion of the reaction mixture was analysed (10% in lanes 3–7 in Fig. 6; see also Fig. 4*b*). In lanes 3–7 in Fig. 6 there was also a faint sharp band which moved slightly faster than oligosaccharide I. This band, of unknown identity, was also present in all samples and could be seen if sufficient was loaded. It can be seen most clearly in Fig. 4(*b*), lane 6. It has a lower emission wavelength than the ANTS. It appears that the β-galactosidase (see lanes 4 and 5, Fig. 6) contained traces of galactose or a contaminant with a similar mobility.

In Fig. 8 is shown a photograph of the graphics display of an image of a gel showing a typical separation of a variety of oligosaccharides and the SM. The area of the original gel represented by the image was approx. 120 mm × 120 mm and was re-proportioned by the imaging system to fit the display screen.

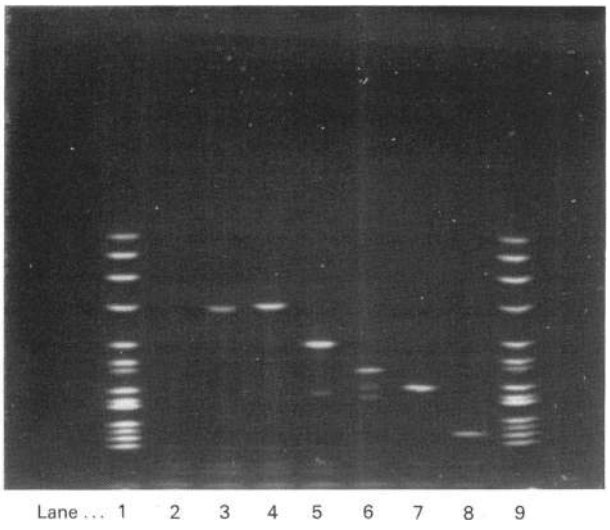

Fig. 8. Photograph of the graphics display of the cooled c.c.d. imaging system, showing an electrophoretogram of various ANTS-derivatized saccharides

Lanes 1 and 9, SM. In order of decreasing mobility the identities of the band are: 6-deoxyglucose, glucose, galactose, *N*-acetylgalactosamine, galactosylgalactose, lactose, maltose, galactobiose, cellotriose maltotriose, maltotetraose, maltopentaose, maltohexaose, maltoheptaose; lane 2, water; lane 3, Glcα1-6(Glcα1-4)$_2$Glc; lane 4, maltotetraose; lane 5, isomaltotriose; lane 6, *N*-acetylneuramin-lactose; lane 7, panose; lane 8, L-fucose; Each band contained approx. 200 pmol of derivatized saccharide. The gel was imaged for 10 s per section. The total image shown consisted of six sections, which were merged by the computing system and then re-proportioned to fill the display. The area of gel shown had the approximate dimensions 120 mm × 120 mm. The whole width of the gel was illuminated along its length from its anodic edge, which was approx. 12 mm from the lower edge of the image. The main band of excess ANTS is beyond the edge of the image, but feint 'artefactual' bands arising from the ANTS can be seen.

The lower edge of the image is approx. 12 mm from the anodic edge of the gel. The broad band of excess ANTS is almost entirely outside the imaged area. Above it are faint additional bands arising from the ANTS. Some saccharide samples appeared to contain impurities. For instance, the single feint band below the main band of isomaltose and the bands below the main band in lane 5 (*N*-acetylneuramin-lactose) were found consistently. In the latter sample the lowest band has the same mobility as lactose. The band above it is of unknown origin, but it is possible that it represents the portion [15% (w/w); manufacturers' data] of the 2→6-linked *N*-acetylneuramin-lactose which was present in the sample.

DISCUSSION

Methods for the covalent labelling of saccharides have been described, but none has used ANTS [11,14,16,17,19,20]. The ANTS derivatization imparts both charge and fluorescence to the saccharides, enabling them to be both electrophoresed and detected. ANTS was found to be the most suitable of the numerous fluorophores tested, since it has a relatively high charge, enabling rapid separations, in particular of the larger oligosaccharides. It also has a large Stokes shift, which facilitates the differential filtration of the excitation and emission wavelengths, thus improving the sensitivity by decreasing the detectable background fluorescence. The excitation wavelength maximum of 370 nm enables inexpensive tungsten–halogen lamp illumination to be used and the yellowish emission, wavelength maximum 515 nm, facilitates direct observation of the band pattern. This wavelength is also well suited for detection by the c.c.d., which is inefficient at detecting blue light.

The labelling procedure is simple to perform and uses inexpensive reagents which are available commercially. The 15 h reaction can be arranged conveniently overnight. It may be possible to accelerate the procedure by raising the reaction temperature. The reaction products are relatively stable and may be stored for several weeks in solution in electrophoresis sample buffer at −70 °C without apparent degradation.

The reaction conditions were optimized so that the derivatization was virtually quantitative for up to at least 110 nmol of saccharide per reaction tube. For optimal labelling DMSO was essential. When it was replaced by water, the degree of labelling was reduced by approx. 20%. The effect of varying the concentration of $NaCNBH_3$ has not yet been investigated rigorously. If it was omitted, derivatives were obtained which had diminished and blue fluorescence. These derivatives had slightly different mobilities from those obtained when $NaCNBH_3$ was included.

The relatively high concentration of ANTS, required for complete derivatization, could be a disadvantage when small quantities of saccharides are being analysed. For instance, when the standard derivatization conditions were used and 10% of the total reaction mixture was applied to the gel, then faint 'artefactual' bands arising from the ANTS were seen (see Fig. 6, lanes 3–7). These bands are also shown in Fig. 4(*b*), lane 6, where 2% of a standard reaction mixture containing no saccharide was imaged at high sensitivity. To ensure that lower-M_r saccharides can be detected against the 'artefactual' bands, it is necessary to allow a minimum of about 100 pmol to react and to load no more than 10%. The practical limits for the proportion of the standard-conditions reaction mixture which could be loaded without causing gross distortions in the electrophoresis band patterns was also about 10%. However, initial experiments show that it is possible to reduce the total reaction volume from 10 to 2 μl. It should be possible to reduce to $\frac{1}{5}$ the total minimum quantity of any saccharide which can be allowed to react and be

analysed as compared with the standard method described. The potential of the technique for the analysis of glycans was demonstrated clearly by the β-galactosidase digestion of oligosaccharide II.

PAGE has been used previously for the separation of oligosaccharides which are charged naturally [27–29] and also for the separation of uncharged oligosaccharides as borate-ion complexes [15]. This latter method appeared to be of relatively low resolution and required radiolabelling of the oligosaccharides for their detection. In contrast, the PAGE method described here is of high resolution and avoids radiolabelling. In addition it has higher sensitivity than paper chromatography, t.l.c. and the non-capillary electrophoretic methods described previously for the analysis of small reducing carbohydrates. The limit of sensitivity of detection by the c.c.d. is similar to that obtained for pre-column-derivatization-h.p.l.c. methods and better than that obtained for pulsed amperometric detection of underivatized saccharides, which has a sensitivity in the range 10–100 pmol [30]. The detector response of the latter method varies for each saccharide and requires calibration, whereas the fluorescent labelling should give the same response per mol of reducing end group labelled, and this was found to be so for the three saccharides when tested rigorously. The resolving power approaches that of h.p.l.c. and capillary-zone electrophoresis and is determined both by the size and structure of the saccharides. The wide range of molecular sizes which can be resolved is at least as good as that obtained by h.p.l.c. This separation range is in part due to the use of a polyacrylamide gradient gel. However, useful separations can also be obtained on uniform-concentration gels (e.g. 30%, w/v). Small-scale gels have also been used successfully to obtain rapid analyses.

The method described has shown an unexpected resolution of some saccharides. The electrophoretic mobility is related mainly to the size of the saccharides, but other factors appear to have an effect. The effective mass of each derivative, and thus its electrophoretic mobility may be determined, in part, by the saccharide conformation, and this may explain, for instance, the separation of maltose and cellobiose. However, it seems unlikely that the effective masses of galactose and glucose differ significantly. The separation of these epimers could depend on a differential interaction of each saccharide with the gel matrix. At present the exact mechanism for the separations is unclear, and may be attributable to a combination of the effects suggested. Two methods of imaging the fluorescent electrophoretograms were used. The photographic method gave good-quality negatives that enabled accurate densitometric measurement of the fluorescent gel bands, but the film response was non-linear. The limit of the film sensitivity was about 1 pmol per band using a 50 s exposure time and an aperture of f4.5. This was also the limit of sensitivity when film exposures of 100 s were used, since the recorded background fluorescence increased to mask the increased band fluorescence. When gels were photographed a second time, significant fading had occurred. Densitometric measurements were made only on the first exposure.

It has been shown previously that polyacrylamide gels can be imaged successfully using the Astromed 2200 cooled c.c.d. system [26], and the present work demonstrates an additional application. The imaging was about 5 times more sensitive than photography, having a limit of detection of about 0.2 pmol. However, visual inspection of the illuminated gels on the viewing platform of the imaging device showed that the c.c.d. was at least 50 times more sensitive than the human eye. This contrasts with the photography, to which the human eye had similar sensitivity. It should be possible to increase significantly the sensitivity of detection by the c.c.d.: (1) the excitation and emission filters could be matched more precisely to the absorbance and fluorescence wavelengths of ANTS; (2) a higher-power lamp could be used, together with a lens with a wider aperture; (3) the c.c.d. could be cooled to a lower temperature (c.c.d. cameras with secondary water cooling are available which operate at about 210 K; this alone should enable the lower detection limit to be about 4-fold less than at present).

The c.c.d. system was more convenient to use than the photographic method, since the gels could be imaged immediately after electrophoresis while they remained clamped in their glass electrophoresis cassettes. It was found convenient to image each gel firstly for 10 s per section and subsequently for 60 s. The c.c.d. response is directly proportional to the exposure time, and the shorter exposure times are required to avoid saturation of the c.c.d. with light from the more intense bands. Fading of the fluorescence was found to be less than 1% when the gels were illuminated for up to 30 min. In contrast with the photographic method, a linear c.c.d. response was obtained for fluorescent bands containing between 12.5 and 500 pmol. However, there was considerable variation when attempting to measure 3.1 pmol of saccharide, since the band fluorescence was close to the background level.

The method described should be particularly useful for the microscale analysis of numerous samples in parallel, for instance, those obtained from the enzymic structural analysis of complex oligosaccharides. Although not all the isomers and epimers tested were separated, the demonstrated resolving power, the low cost and simplicity of the procedure, the widespread availability of the electrophoretic equipment, the sensitivity of the detection and the quantitative characteristics of the derivatization make the method a useful addition to current carbohydrate-analysis methods.

This method not only has considerable potential for further development itself, but it also enables the use of the powerful technique of blotting. Initial experiments have shown that it is possible to transfer, both rapidly and efficiently, the ANTS-derivatized saccharides from gels on to porous membranes for probing with specific carbohydrate-binding proteins such as lectins and specific antibodies.

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