ON THE AUTOMATED ANALYSIS OF NEUTRAL MONOSACCHARIDES IN GLYCOPROTEINS AND POLYSACCHARIDES

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ABSTRACT

An improved method for the automated, quantitative analysis of glycoproteins and polysaccharides for neutral monosaccharide components has been developed, based on the ion-exchange chromatography at pH 7 of sugar–borate complexes. The destruction of sugars during acid hydrolysis has been investigated, and a variety of methods for the neutralization of hydrolysates have been evaluated.

INTRODUCTION

Automated methods of analysis are essential in the investigation of complex macromolecules of biological origin, which are frequently available in only milligram quantities. Gas–liquid chromatography (g.l.c.) methods have been widely applied to carbohydrates in view of the excellent separations obtained for trimethylsilyl ethers of free sugars[1,2] and of methyl glycosides[3,4] and, more recently, for alditol acetates[5–7] formed after reduction of the liberated monosaccharides by sodium borohydride. The inherent disadvantages of these g.l.c. methods are the appearance of several anomeric peaks for any one monosaccharide and/or the need to convert the sugars into volatile derivatives.

Such disadvantages are avoided in the methods based on ion-exchange chromatography, whereby mixtures of monosaccharides can be analysed directly without derivatization. Two basic techniques have been devised. Samuelson and co-workers[8–10] have been developing a system which depends on the differential partitioning of the sugars between the resin and an ethanol–water eluant. The other ion-exchange technique, based on the original experiments of Zill and co-workers[11,12], involves the chromatography of sugar–borate complexes on basic ion-exchange resins in the borate form. Various elaborations[13–17] of this latter technique have been described, and the present paper discusses an improved method which eliminates some of the various disadvantages.

A further, major problem in the analysis of polysaccharides is the quantitative liberation of the constituent monosaccharides on hydrolysis by acid, and a generally applicable procedure is presented which overcomes some of the difficulties involved.

METHODS AND RESULTS

Chromatographic system. — Fig. 1 is a schematic diagram of the system used for the separation and detection of the mixtures of sugars. The borosilicate glass column (6 × 750 mm) containing a resin bed of 700 mm, the Autoanalyzer, the nine-chamber gradient device (Autograd), and associated equipment shown in Fig. 1 were obtained from Technicon Instruments Co. Ltd., Chertsey, Surrey, England.

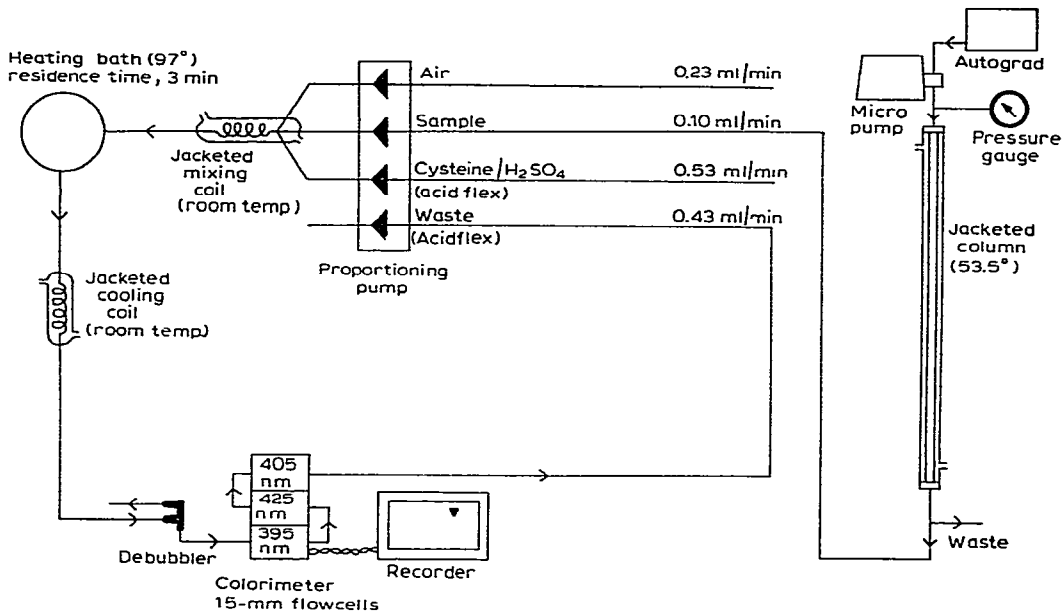

Fig. 1. Schematic diagram of the system.

The Type-S Chromobeads anion-exchange resin, supplied by Technicon, was 8% cross-linked polystyrene of high capacity. The resin was packed into the column in short sections, at a pressure of 200 lb/sq.in., and at the column operating-temperature of 53.5°. This procedure was essential in order to obtain sharp peaks. After 2–3 chromatographic runs, the resin bed had shrunk by ~10 cm, and more resin was then added. After a further 3–4 runs, the resin bed had stabilized. Formation of a dark-brown ring in the top 2 cm of the resin bed, also observed by other workers[14,17], was eliminated by the inclusion of a Whatman filter tube (W. & B. Balston Ltd.) in the feed line of the 10% aqueous potassium tetraborate, which was stored in a polythene bottle to preclude contamination of the resin bed by silicate ions[14].

The effect of various borate–chloride gradients supplied from the Autograd (37 ml/h) on the elution times of various monosaccharides was investigated. After each chromatographic run, the resin was regenerated to the borate form by pumping (20–25 ml/h) aqueous potassium tetraborate (10% w/v) through the column overnight,

and equilibrating with the starting buffer (0.1M boric acid, pH 7.00, 36–38 ml/h) at 53.5° for 1 h the following morning.

The column eluate was analyzed for carbohydrates[18] by reaction with a solution of cysteine hydrochloride (0.07% w/v) in sulphuric acid (86% v/v), and the absorbance was measured at three wavelengths (395, 405, and 425 nm) in 15-mm flow cells. The cysteine–sulphuric acid reagent, which was stable at room temperature indefinitely, was stored in a 5-litre, clear bottle fitted with a dust trap.

Chromatographic separation. — The elution times of the various sugars were critically dependent on the rate of increase of both borate-ion and chloride-ion content of the eluant. An increase in the gradient with respect to both ions shortened the elution times of all sugars, but a considerable degree of selectivity was observed, particularly for borate.

The Autograd compositions for the two elution gradients used routinely in Methods I and II are given in Tables I and II, respectively, and the corresponding separations in Figs. 2 and 3.

TABLE I

AUTOGRAD COMPOSITION FOR METHOD I

Chamber	*0.1M H_3BO_3[a] (ml)*	*0.2M H_3BO_3[a] (ml)*	*0.2M H_3BO_3[a]/0.2M NaCl (ml)*
1	50		
2	35		15
3		25	25
4		25	25
5		25	25
6			50
7			50
8			
9			

[a]Boric acid buffers were titrated to pH 7.00 with 2M sodium hydroxide.

TABLE II

AUTOGRAD COMPOSITION FOR METHOD II

Chamber	*0.1M H_3BO_3[a] (ml)*	*0.4M H_3BO_3[a] (ml)*	*0.4M H_3BO_3[a]/0.2M NaCl (ml)*
1	32		3
2	32		3
3	32		3
4		32	3
5		32	3
6		32	3
7		25	10
8			35
9			35

[a]Boric acid buffers were titrated to pH 7.00 with 2M sodium hydroxide.

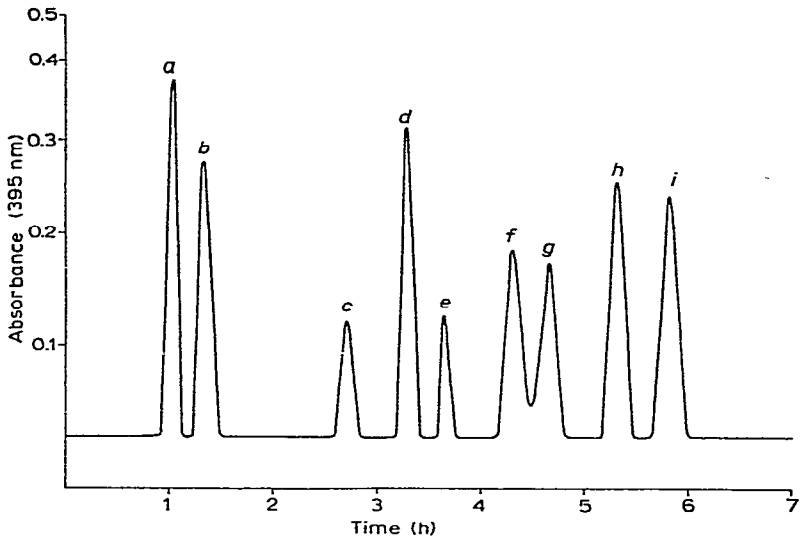

Fig. 2. Separation of various carbohydrates by Method I: (*a*) trehalose, (*b*) cellobiose, (*c*) L-rhamnose, (*d*) D-ribose, (*e*) D-mannose, (*f*) L-arabinose, (*g*) D-galactose, (*h*) D-xylose, (*i*) D-glucose; 560 nmoles of each carbohydrate were used with the exception of trehalose (284 nmoles) and cellobiose (355 nmoles).

Increasing the borate ion concentration to 0.2M in the early stages of elution (chamber 1 of the Autograd) led to marked decreases in the elution times for D-mannose and L-fucose relative to the other sugars, such that D-mannose was eluted

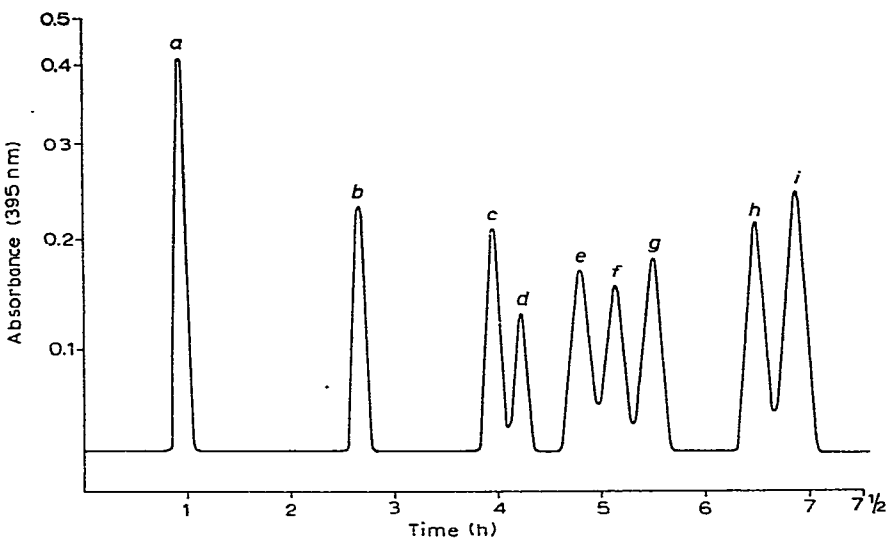

Fig. 3. Separation of various carbohydrates by Method II: (*a*) trehalose, (*b*) L-rhamnose, (*c*) D-ribose, (*d*) D-mannose, (*e*) L-fucose, (*f*) L-arabinose, (*g*) D-galactose, (*h*) D-xylose, (*i*) D-glucose; 580 nmoles of each carbohydrate were used with the exception of trehalose (290 nmoles).

before D-ribose, and L-fucose was eluted immediately after D-ribose. This result was used in an attempt to separate L-fucose and L-arabinose, which had the same elution times in Method I. However, reduction of the borate ion concentration to 0.15M in chamber 1, in order to resolve L-fucose and D-ribose satisfactorily, led to incomplete separation of D-mannose and D-ribose. Increasing the chloride ion concentration to 0.02M in the early stages of elution had little effect on elution times, apart from L-rhamnose, the elution time of which was shortened by 40 min. It was found that if chloride was included to this concentration in the first few chambers, and the borate concentration was raised sharply to 0.4M in chamber 2, the elution order of D-ribose and D-mannose in Method I could be retained, whilst achieving a separation of L-fucose, L-arabinose, and D-galactose, although the resolution obtained was still inadequate. The effect of raising the borate ion concentration less sharply reduced the resolution of L-fucose, L-arabinose, and D-galactose, whereas raising it more sharply reduced the resolution of D-ribose and D-mannose. A compromise solution was found by reducing the chamber volumes from 50 ml to 35 ml, and delaying the sharp increase in borate ion concentration to chamber 4 (Table II). In this way, the detrimental effect of increased borate ion concentration on the D-ribose–D-mannose separation was avoided, whilst the sharp gradient in borate after chamber 3 resolved L-fucose, L-arabinose, and D-galactose (Fig. 3). A later, sharp increase in the chloride ion concentration after chamber 6 (Table II) shortened the elution times of D-xylose and D-glucose, so that the routine analytical run could be carried out in an eight-hour working day.

Since the colour produced in the cysteine–sulphuric acid reaction is to some extent dependent upon the amount of salt present, the reproducibility of peak areas depended on reproducible elution times, so that the Autograd solutions had to be accurately prepared (a fast-running burette was found to be suitable for measuring the solution volumes). Provided this precaution was taken, standard deviations never exceeded $\pm 3\%$ for any sugar, and a linear relationship was observed between the amount of sugar (in the range 100–1000 nmoles) and peak area.

The response at 395 nm was used for quantitation of the chromatograms (Figs. 2 and 3). Only hexoses responded significantly at 425 nm, and the ratio of the heights of the peaks at 405 nm to those at 395 nm was 0.50–0.57 for pentoses and 0.69–0.76 for 6-deoxyhexoses. In this way, the different monosaccharides could be distinguished.

Preparation of polysaccharide hydrolysates for analysis. — Careful studies were made on hydrolysis and subsequent neutralization of simulated polysaccharide preparations. A solution of monosaccharide (500 nmoles of each/ml; 1 ml) in M sulphuric acid was neutralized by five different techniques: (*i*) on a column of Amberlite IRA-400(CO_3^{2-}) resin; (*ii*) on a column (1 × 8 cm) of Amberlite IR-45 resin; (*iii*) on a column (1 × 8 cm) of Amberlite IR-4B resin; (*iv*) with barium carbonate; and (*v*) with *N*,*N*-dioctylmethylamine (20% v/v in chloroform; 5 ml). The results (Table III) show selective losses for all of the techniques except (*v*).

Four conditions of hydrolysis were investigated, using the simulated polysac-

TABLE III

RECOVERIES OF MONOSACCHARIDES FROM DIFFERENT NEUTRALISATION PROCEDURES

Monosaccharide	*Recovery*[a] (%)				
	IRA-400	*IR-45*	*IR-4B*	*$BaCO_3$*	N,N-*Dioctylmethylamine*
L-Rhamnose	110	90	89	95	93
D-Ribose	20	80	77	88	102
D-Mannose	71	99	87	89	101
L-Fucose	93	99	90	83	97
L-Arabinose	57	*c*	*c*	88	101
D-Galactose	65	100	91	89	101
D-Xylose	52	97	85	92	102
D-Glucose	67	102	90	86	96
Efficiency[b] (%)	85	85	80	83	92

[a]Relative to trehalose (= 100). [b] As measured by absolute recovery of trehalose. [c]Not determined.

charide preparation described above, at 100° for 6 h in sealed ampoules: (*a*) M sulphuric acid under air; (*b*) M sulphuric acid under nitrogen; (*c*) 2M hydrochloric acid under nitrogen; (*d*) 2M trifluoroacetic acid. The results are given in Table IV.

The routine procedure adopted for the preparation of polysaccharide samples for analysis was as follows: 2M sulphuric acid (0.5 ml) was added to the polysaccharide solution (0.5 ml, containing 50–1000 nmoles of neutral sugars) in a test tube (1 × 15 cm), which was then flushed with oxygen-free nitrogen for 20 min after drawing out; the sealed ampoule was maintained at 100° for the required time and, after cooling, an aliquot (0.1 ml) of trehalose of cellobiose solution (2500 nmoles/ml) was added as internal standard, rendering quantitative recovery in the neutralization procedure unnecessary. The hydrolysate was then transferred to a stoppered test-tube containing *N,N*-dioctylmethylamine (20% v/v in chloroform; 5 ml), shaken vigorously, and clarified by centrifugation. This was sufficient to neutralize 4 mequiv. of

TABLE IV

RECOVERIES OF MONOSACCHARIDES FROM DIFFERENT HYDROLYSIS CONDITIONS

Monosaccharide	*Recovery* (%)			
	M *H_2SO_4* *in air*	M *H_2SO_4* *in N_2*	2M *HCl* *in N_2*	2M *CF_3CO_2H* *in N_2*
L-Rhamnose	76	93	81	87
D-Ribose	48	52	30	73
D-Mannose	84	86	68	88
L-Fucose	90	93	88	90
D-Galactose	87	90	89	95
D-Xylose	67	69	46	78
D-Glucose	98	97	98	92

sulphuric acid. The chloroform layer was drawn off by pipette, and residual quantities of the amine in the aqueous layer were extracted with chloroform (5 ml). A few drops of 0.4M boric acid (pH 7) were added to the neutralized hydrolysate prior to analysis.

DISCUSSION

Most other analytical systems[13–16] based on the ion-exchange chromatography of sugar–borate complexes utilize buffers exceeding pH 8. In view of the well-documented lability of sugars to alkaline conditions[19,20], especially at elevated temperatures, it seemed desirable to avoid use of buffers exceeding pH 7. Thus, chromatography of D-mannose on the Technicon system[15] yielded a second peak of area greater than 10% of that of the D-mannose peak; this peak decreased to 1% when a similar experiment was conducted on the system reported here (unpublished results). Further evidence for possible alkaline degradation of sugars during chromatography on the Technicon system[15] was a fluctuating baseline. These observations are in agreement with other reports[21–23], although Lee *et al.*[16], who used buffers exceeding pH 9, claim that the short time of exposure of sugars to the alkaline buffers minimizes possible alkaline rearrangements.

The present results show that a good resolution of sugars of common occurrence in biological macromolecules can be obtained by the use of borate buffers at pH 7, and that the inclusion of glycerol[24] or butane-2,3-diol[17,21], which interfere with the convenient colorimetric reactions, is unnecessary. Also, incorporation of an automatic selector valve at the column head[17] is avoided.

Various colorimetric reactions have been used in automated analytical systems, most of which have certain limitations. Several systems[13,15,16] utilize the orcinol–sulphuric acid reagent[25], but our experience has shown that rapid deterioration of the orcinol caused precipitation in the heating coil, resulting in "noisy" base lines. Moreover, the general instability of the reagent necessitated frequent standardization. Systems[14,22] employing the phenol–sulphuric acid reagent[26] require careful design of the apparatus to control the excessive liberation of heat when the 98% sulphuric acid is mixed with the column effluent[22]. The colour reaction with pentane-2,4-dione[27], following periodate oxidation[9] at pH 7.5, possesses complications in reagent preparation, and 6-deoxyhexoses do not respond in the assay. The aniline–acetic and orthophosphoric acid method[28] possesses the severe disadvantage that the analytical system has to be pressurised[17].

The automated cysteine–sulphuric acid assay[18] possesses none of the above disadvantages. The reagent, which appears to be stable indefinitely, can be used to determine accurately less than 100 nmoles of sugars, and, by monitoring the colours at the three wavelengths described, it is possible to distinguish between the different classes of sugar, *e.g.* aldohexose, aldopentose, 6-deoxyaldohexose, etc.

For the type of analysis under consideration, the use of the sugar–borate complex technique as described has several advantages over the partition chromatography system developed by Samuelson and co-workers[8–10]. An equivalent separation

by the latter method required[9] 16 h, although, of course, regeneration of the resin is unnecessary. The fast runs, which utilize high flow-rates, led to a loss in resolution[9]. Moreover, the more-retarded sugars yielded exceptionally broad peaks, reducing the sensitivity; *ca.* 2000 nmoles of glucose are required to produce a peak of reasonable size. The very high pressures employed in the fast runs led to the gradual compaction of the highly porous resin used[29], and high column temperatures are required for good resolutions. A further difficulty encountered was rapid deterioration of the Yellow-Tygon tubing used on the proportioning pump[8].

Whereas important advances have been made in the development of highly sensitive techniques in the automated analysis of saccharides, little progress has been reported on the recurrent problem of the quantitative liberation of constituent monosaccharides from macromolecules, on which the former depends. The hydrolytic conditions required to cleave the glycosidic linkages are such that the liberated sugars are destroyed to a greater or lesser extent, resulting in incomplete recovery. Methanolysis[3,4] overcomes the problem, but introduces the complication of multiple, anomeric products. Moreover, methyl glycosides do not form strong borate complexes, and are therefore unsuitable for analytical methods based on the ion-exchange chromatography of sugar–borate complexes. Other attempts to solve the problem have involved the use of solid, acidic resins such as Dowex-50[30,31] or water-soluble polystyrene sulphonic acid[32]. Although use of such reagents greatly reduces the destruction of the liberated sugars, routine micro-analysis demands simple procedures with a minimum of manipulation.

The results presented in Table IV demonstrate that exclusion of oxygen and choice of hydrolytic agent can be extremely important factors in minimising destruction of sugars during hydrolysis. Although optimal conditions for maximal release of monosaccharides from a particular polysaccharide or glycoprotein require independent evaluation, it is clear that sulphuric acid and trifluoroacetic acid are less destructive than hydrochloric acid, and investigation of other strong acids might well lead to further improvements in recoveries.

An associated problem is the removal of the acid following hydrolysis. The neutralizing agents most widely used are either anion-exchange resins or barium carbonate. A detailed study by Murphy *et al.*[33] revealed a reaction of D-xylose and Amberlite IR-45 resin, which was attributed to the formation of a Schiff's base or glycosylamine. The results given in Table III confirm that a proportion of certain sugars is lost during neutralization on each of the resins. Thus alternative methods of neutralisation were sought. The main disadvantage of the barium carbonate method was the manipulative difficulties associated with its use on hydrolysates of the order of 1 ml. The use of *N*,*N*-dioctylmethylamine in chloroform[34], described above, avoids the various disadvantages encountered with other neutralizing agents.

The analytical system as described is currently being employed in our laboratories for the routine analysis of plant polysaccharides and glycoproteins.

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