CHROM. 4544

A SIMPLE, RAPID, ISOTHERMAL GAS CHROMATOGRAPHIC PROCEDURE FOR THE ANALYSIS OF MONOSACCHARIDE MIXTURES*

P. E. REID**, B. DONALDSON***, D. W. SECRET AND B. BRADFORD§
Department of Pathology, University of British Columbia, Vancouver 8, B.C. (Canada)

(Received December 2nd, 1969)

SUMMARY

A simple, rapid, isothermal gas chromatographic method has been developed for the quantitative analysis of the expected neutral monosaccharide and N-acetyl hexosamine components of glycoproteins and other carbohydrate polymers. Analysis of mixtures of the trimethylsilyl ethers of galactose, glucose, mannose, N-acetyl glucosamine and N-acetyl galactosamine with fucose or with xylose and arabinose gave good agreement between the analytical and theoretical values.

INTRODUCTION

In a recent publication, one of us[1] reported a gas–liquid chromatographic procedure for the analysis of mixtures of arabinose, xylose, galactose, glucose and mannose. In this paper we report an improved, simplified procedure for this analysis and its extension to the common non-acidic constituents of glycoproteins.

The use of gas–liquid chromatography (GLC) in carbohydrate chemistry and biochemistry has been extensively reviewed[2–6]. Methods are available for the analysis of the monosaccharide constituents of compounds such as glycoproteins[7–17], glycolipids[18,19], glycosaminoglycans[20–25], a wide variety of polysaccharides[26–32] and some carbohydrate constituents of serum[33–35] and urine[36–38]. This paper reports a simple, rapid method for the GLC analysis of mixtures of the trimethylsilyl (TMS) ethers of fucose, galactose, glucose, mannose, N-acetyl glucosamine and N-acetyl galactosamine and mixtures of the TMS ethers of arabinose, xylose, galactose, glucose, mannose, N-acetyl glucosamine and N-acetyl galactosamine. This procedure was developed for the analysis of the probable non-acidic carbohydrate components of

* An account of this work was presented at the 12th Annual Meeting of the Canadian Federation of Biological Societies, University of Alberta, Edmonton, Alberta, Canada, June 11th–13th, 1969. Abstract No. 268.

** Scholar of the Medical Research Council of Canada.

*** Present address: Department of Chemistry, University of British Columbia, Vancouver 8, B.C., Canada.

§ Present address: Department of Pharmacology, University of British Columbia, Vancouver 8, B.C., Canada.

glycoproteins but can be applied with equal facility to the hydrolysates of hemicelluloses and other biologically important carbohydrates. Recently the procedure has been applied to the estimation of serum monosaccharides[39], the estimation of serum sorbitol in a patient on peritoneal dialysis[39] and in a model procedure for estimation of the degree of polymerization of carbohydrates[40].

Since free carbohydrates are insufficiently volatile for direct GLC their analysis requires the preparation of volatile derivatives. Although the acetate esters[7–10,28–32,41–45], and the TMS ethers[11–27,33–40,46–53] have proved generally satisfactory for analytical purposes, the existence of the carbohydrates in more than one anomeric form is a complicating factor, since it leads to a multiplicity of peaks which are often difficult to resolve. Conversion of the carbohydrates to the corresponding polyhydric alcohol acetates[6–10,28,30–32,41,43,44] or to the aldono-1,4-lactone TMS ethers[51,52] yields greatly simplified chromatograms with each sugar represented by a single GLC peak. These procedures, however, involve considerable chemical manipulation and the use of the polyhydric alcohol acetates is complicated by the fact that the eight hexoses and four pentoses yield only six and three polyhydric alcohols, respectively. Further, the analysis of ketoses is complicated by the two alcohols produced upon reduction, which would, for example, make difficult the analysis of fructose in serum due to the ubiquitous presence of glucose in the samples. Adequate resolution of some carbohydrate mixtures has also been achieved by the GLC of the acetates and the TMS ethers of the derived methyl glycosides[11–13,18,19,29,49].

Despite the complexity of the chromatograms obtained, GLC of the TMS ethers of monosaccharide mixtures has been successfully used for the quantitative analysis of the monosaccharide components of glycoproteins[14,17] and hemicelluloses[1,26,27]. These procedures use the TMS ethers prepared from the sugar mixtures at mutarotation equilibrium. Silylation of a sugar at mutarotation equilibrium leads to a number of peaks. The ratio of the areas under the peaks can then be precisely determined. Thus, in an incompletely resolved chromatogram, the total peak area of a sugar can be determined if one peak is completely resolved. In some cases[1] it is unnecessary to resolve any of the peaks attributable to a single sugar since the area of one or more peaks can be obtained by difference if the area of the co-chromatographing peaks is known. Therefore, analytical procedures based upon an analysis of the TMS ethers of free monosaccharide mixtures require that the equilibrium composition of the component sugars be known, under the conditions chosen to achieve mutarotation equilibrium. Most published procedures[14,17,27,46,47] achieve mutarotation equilibrium in aqueous solution. This requires the removal of water before silylation. BETHGE, HOLMSTRÖM AND JUHLIN[26] avoided the concentration step by conducting the mutarotation in pyridine containing 0.2% (w/v) lithium perchlorate at 40°. Under these conditions mutarotation equilibrium was achieved in 2 h and the equilibrium solution could be silylated directly. However, it was necessary to extract the sugar TMS ethers with hexane before GLC. We have found that the procedure can be simplified by bringing about mutarotation equilibrium in N,N-dimethylformamide or pyridine at 40° in the presence of the bifunctional catalyst 2-hydroxypyridine[50]. The TMS ether of 2-hydroxypyridine is volatile and does not interfere with the GLC analysis of the sugar TMS ethers. Therefore it is possible to achieve mutarotation equilibrium rapidly, to silylate the solution without concentration and to analyse the sugar TMS ethers without any extraction procedure. In a

previous publication by DUTTON *et al.*[1] it was shown that mixtures of the TMS ethers of arabinose, xylose, galactose, glucose and mannose could be analysed by GLC on columns containing 20% Silicone Fluid SF-96 on 60–80 mesh Diatoport S. This procedure along with some others[17,26,27] requires the use of temperature-programmed GLC. We have found that by the use of columns packed with 10% Silicone Fluid on 80–100 mesh Diatoport S, or 10% SE-52 on 80–100 mesh AW Chromosorb W, and a chromatograph fitted with flame ionisation detection rather than with thermal conductivity detection[1], the TMS ethers of arabinose, xylose, galactose, glucose and mannose can be analysed under isothermal conditions at 190°. Moreover, the same system adequately resolves mixtures of the TMS ethers of fucose, galactose, glucose, mannose, N-acetyl glucosamine and N-acetyl galactosamine and will accommodate the use of sorbitol as an internal standard.

The chromatographic procedure described in this paper is applicable to the constituents of a wide variety of biological materials and is well suited to routine use since it is rapid and combines a minimum of chemical manipulation with simplicity of chromatographic operation.

Prior to the analysis of monosaccharides it is necessary to determine the molar response factors (MRF)[1] of the sugars relative to an internal standard, and the percentage composition of each of the sugars at mutarotation equilibrium under the conditions employed. Initially pyridine was employed as solvent but it was found that the MRF of N-acetyl glucosamine (and that of N-acetyl glucosaminitol) showed large variations which were not apparent when N,N-dimethylformamide (DMF) was used as solvent. Since DMF was found to be a better solvent than pyridine for the

TABLE I

MOLAR RESPONSE FACTORS OF SUGAR TMS ETHERS

Sugar	*Molar response factor*[a]
Fucose	0.73 ± 0.01
Arabinose	0.67 ± 0.01
Xylose	0.66 ± 0.01
Galactose	0.82 ± 0.01
Glucose	0.83 ± 0.01
Mannose	0.83 ± 0.02
N-Acetyl glucosamine	0.63 ± 0.02
N-Acetyl galactosamine	0.60 ± 0.02

[a] Sorbitol = 1.00.

mixtures analysed, it was adopted for routine use except when necessary to isolate the TMS ethers by concentration and by solvent extraction. In a series of preliminary experiments, conditions were determined which brought about mutarotation equilibrium within 3 or 6 h. These times can presumably be decreased by increasing the concentration of 2-hydroxypyridine. In our laboratory mutarotation for 3 h or overnight (*ca.* 16 h) has proved convenient for routine analytical work. Table I shows the MRF values of the sugars determined by this procedure. Table II gives the percentage composition of the sugars determined under a variety of conditions. Ex-

TABLE II

PERCENTAGE COMPOSITION OF SUGARS UNDER DIFFERENT EQUILIBRIUM CONDITIONS

Experimental conditions:
(1) 1.25×10^{-2} *M* sugar, 1.25×10^{-2} *M* sorbitol, 2×10^{-1} *M* 2-hydroxypyridine in DMF 3 h at 40°.
(2) 1.25×10^{-2} *M* sugar, 1.25×10^{-2} *M* sorbitol, 1×10^{-1} *M* 2-hydroxypyridine in DMF 6–24 h at 40°.
(3) 1.25×10^{-2} *M* sugar, 1.25×10^{-2} *M* sorbitol, 1×10^{-1} *M* 2-hydroxypyridine in pyridine 6–24 h at 40°.
(4) 0.2% lithium perchlorate in pyridine at 40° for 2 h. One further galactose peak 2.2 ± 0.75[16].
(5) Refluxing pyridine for 40 min. Glucose one further peak of 3.2%. Xylose only showed three peaks[46].
(6) Water saturated with chloroform, room temperature, 48 h[1].
(7) Equilibration in water[17].
(8) Equilibration in water[27].
(9) Equilibration using 0.3–0.5 *N* hydrochloric acid[17].

Sugar		*No. of anomers*	*% composition*								
			1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
Xylose	1 + 2	4	4.23 ± 0.43	4.37 ± 0.35	2.88 ± 0.54	3.4 ± 0.56	11.3	3.58 ± 0.28	3.4		
	3		44.43 ± 0.72	44.5 ± 0.59	46.19 ± 0.54	46.0 ± 1.05	45.9	96.4 ± 0.28	41.3		
	4		51.34 ± 0.69	51.1 ± 0.75	50.75 ± 0.47	50.6 ± 1.1	42.8		55.2		
Fucose	1	3	16.29 ± 0.38	17.42 ± 0.4	9.7 ± 0.83	11.3					
	2		30.71 ± 0.38	30.34 ± 0.76	35.11 ± 0.84	35.7					
	3		53.0 ± 0.42	52.23 ± 0.62	54.92 ± 0.76	53.0					
Galactose	1	3	21.68 ± 0.50	23.6 ± 1.17	15.36 ± 0.65	13.8 ± 0.48	24.3	8.19 ± 0.80	5.4	10.5	
	2		27.52 ± 0.30	26.91 ± 0.53	31.98 ± 0.45	33.7 ± 0.90	29.9	29.3 ± 0.27	31.9	26.4	
	3		50.80 ± 0.60	49.51 ± 1.06	52.68 ± 0.64	50.4 ± 0.75	45.8	62.6 ± 0.2	62.6	55.9	
Glucose	1	2	40.87 ± 0.87	41.46 ± 0.41	43.75 ± 0.91	45.2 ± 0.39	47.3	38.3 ± 0.33	39.8	40	
	2		59.13 ± 0.87		56.24 ± 1.03	54.8 ± 0.39	49.5	61.7 ± 0.36	60.2	60	
Mannose	1	2	84.54 ± 0.52	85.39 ± 0.56	82.1 ± 0.71	81.0 ± 1.1	78.3	74.5 ± 0.21	72.0	72	68–70
	2		15.46 ± 0.52		17.86 ± 0.71	19.0 ± 1.1	21.7	25.5 ± 0.21	28.0	28	30–32
N-acetyl galactosamine	1 + 2		28 10 ± 2.7		22.33 ± 1.53						
	3		71.90 ± 2.7		77.67 ± 1.53						

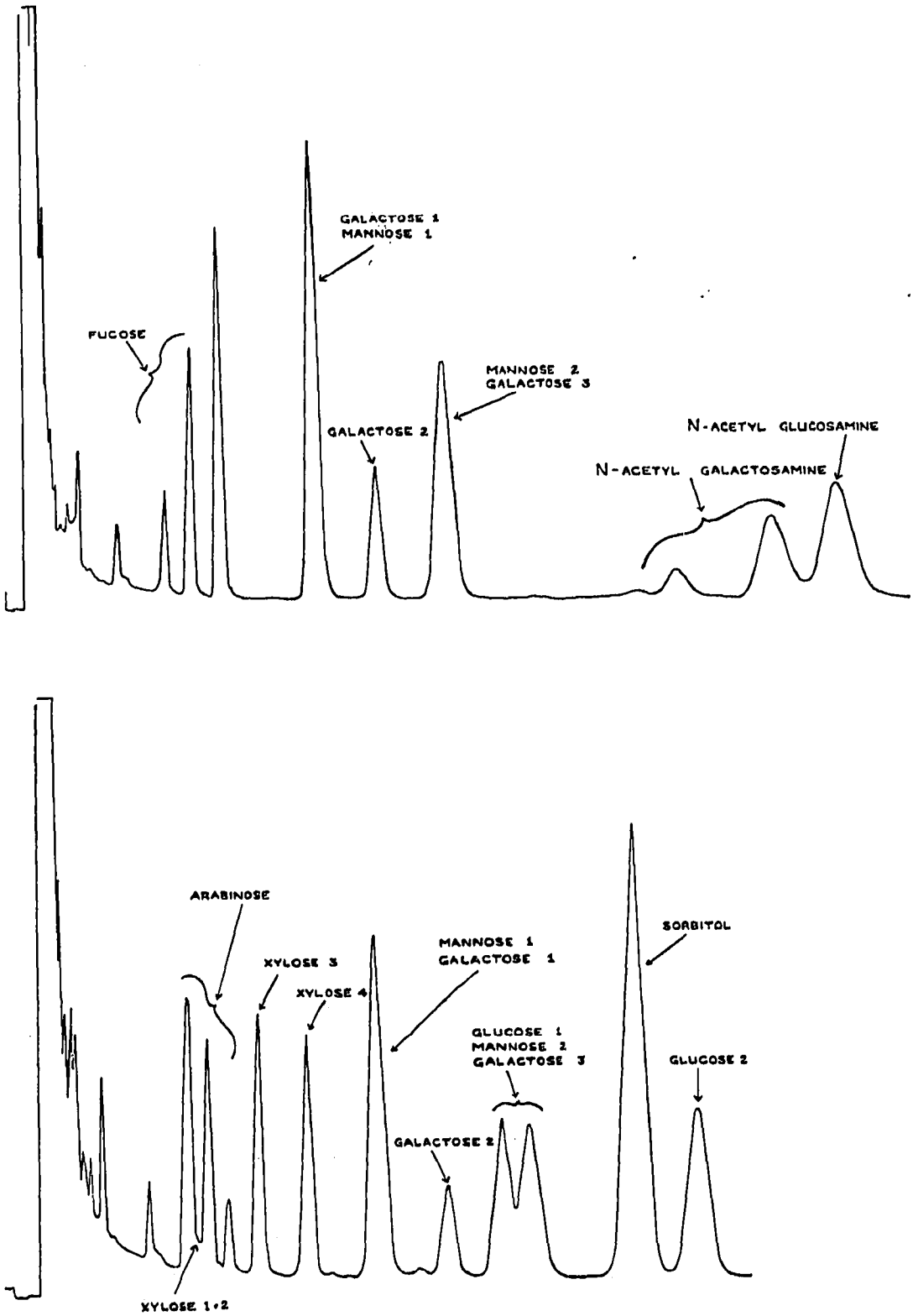

Fig. 1. GLC of the TMS ethers of (a) fucose, galactose, mannose, N-acetyl galactosamine and N-acetyl glucosamine and (b) arabinose, xylose, galactose, mannose and sorbitol on 10% Silicone Fluid SF-96 on 80–100 mesh Diatoport S at 190°.

TABLE III

RELATIVE RETENTION TIMES OF THE ANOMERIC SUGAR TMS ETHERS

Sugars		*Relative retention time*[a]
Arabinose	1	0.247 ± 0.007
	2	0.282 ± 0.007
	3	0.316 ± 0.005
Xylose	1	0.258 ± 0.003
	2	0.275 ± 0.002
	3	0.368 ± 0.005
	4	0.452 ± 0.005
Fucose	1	0.273 ± 0.005
	2	0.323 ± 0.004
	3	0.377 ± 0.01
Mannose	1	0.563 ± 0.01
	2	0.838 ± 0.003
Galactose	1	0.581 ± 0.004
	2	0.685 ± 0.012
	3	0.818 ± 0.004
Glucose	1	0.778 ± 0.004
	2	1.107 ± 0.003
N-Acetyl glucosamine		1.54 ± 0.007
N-Acetyl galactosamine		
	1	1.16 ± 0.005
	2	1.25 ± 0.06
	3	1.43 ± 0.005

[a] Sorbitol = 1.00.

amination of Table II shows clearly that the percentage composition of the sugars at equilibrium is dependent on the composition of the solvent used; and points to the necessity of bringing about mutarotation equilibrium under carefully controlled conditions if accurate analytical results are to be obtained. No percentage composition figures are quoted for the anomers of arabinose because under the conditions employed the GLC peaks were incompletely resolved. Attempts to mutarotate N-acetyl glucosamine in pyridine, DMF and water produced only one GLC peak under the conditions used. Fig. 1a shows a typical chromatogram for the GLC analysis of fucose, galactose, mannose and sorbitol. In Fig. 1a the area of the fucose peaks can be measured directly while the total area of the galactose peaks can be obtained from the galactose-2 peak. The area of the mannose-1 peak (and thus the total peak area of the sugar) is obtained by subtracting the calculated area of the galactose-1 peak from the total area of the combined galactose-1 and mannose-1 peak. The relative areas of the N-acetyl galactosamine-3 and N-acetyl glucosamine peaks are obtained either from the equilibrium data or by the method of BARTLET AND SMITH[55]. The total area of the sugar peaks in chromatograms of mixtures of arabinose, xylose, galactose, glucose, and mannose is

TABLE IV

GLC ANALYSIS OF SUGAR MIXTURES

Analysis No.		*Weight of sugars (mg)*[a]					
		Fucose	*Arabinose*	*Xylose*	*Galactose*	*Glucose*	*Mannose*
1	Actual	20.1	—	—	20.8	—	20.5
	Found	19.9			21.3		20.2
2	Actual	—	23.5	22.9	20.1	20.8	22.8
	Found		24.6	23.9	20.3	20.8	21.8
3	Actual	5.0	—	—	51.9	—	50.0
	Found	4.9			54.4		47.3
4	Actual	46.4	—	—	7.1	—	46.6
	Found	45.2			7.3		44.7
5	Actual	25.1	—	—	27.2	—	5.1
	Found	25.4			27.6		4.7
6	Actual	—	7.3	19.6	19.4	24.6	16.7
	Found		7.5	19.5	18.6	24.1	16.8
7	Actual	—	21.1	6.1	18.2	20.0	22.2
	Found		20.6	6.4	17.6	20.1	22.2
8	Actual	—	21.4	28.2	24.1	7.1	20.7
	Found		22.0	28.6	24.4	7.2	20.6
9	Actual	—	18.2	20.4	19.3	25.7	19.5
	Found		18.3	20.5	18.5	26.4	19.3
10	Actual	—	20.0	22.4	20.1	27.8	4.8
	Found		20.5	22.9	19.9	28.7	4.8
11	Actual	—	20.8	23.1	3.8	20.2	20.6
	Found	—	21.1	23.3	3.3	20.2	20.6

[a] Results are the mean values obtained from four analyses.

obtained in a similar manner. Table III gives the retention times of the sugar anomers relative to sorbitol. Tables IV and V give the results of some analyses of mixtures of known composition. Excellent agreement was obtained between the theoretical and experimental results.

With simpler mixtures of sugars, alternative internal standards such as arabinitol or inositol[30] can be used and the analysis time can be shortened by operating at higher temperatures.

BENTLEY AND BOTLOCK[54] have used GLC to investigate the enzymatic mutarotation of D-glucose. Investigations of the kinetics of mutarotation have generally relied upon polarimetry as a measure of change in anomeric ratio and consequently have used concentrated solutions. GLC should provide an excellent method for an investigation of complicated mutarotation since the anomeric composition can be determined directly, and investigations can be carried out in dilute solutions in a variety of solvents. Such investigations require the determination of the MRF's of the sugar anomers. The MRF's could be obtained directly by the isolation and the re-chromatography of the anomers but might also be obtained indirectly. If it is assumed, as seems probable, that the MRF's of the anomeric components of a sugar are identical, then the MRF of the sugar (determined from the total peak area) should be the same at equilibrium as at some time during the mutarotation when the ratio

TABLE V

GLC ANALYSIS OF SUGAR MIXTURES

Sample		*Sugars molar ratio*[a]							
		Fucose	*Arabinose*	*Xylose*	*Galactose*	*Glucose*	*Mannose*	*N-Acetyl glucosamine*	*N-Acetyl galactosamine*
12	Actual	1.12	—	—	1.00	—	1.13	0.95	—
	Found				1.00		1.09	0.95	
13	Actual	1.00	—	—	1.00	—	1.23	—	0.92
	Found	1.00			1.00		1.16		0.96
14	Actual	—	1.30	1.00	1.14	1.38	1.07	—	—
	Found		1.34	1.00	1.17	1.44	1.07		
15[b]	Actual	0.97	—	—	1.0	—	1.02	0.77	0.71
	Found	0.92			1.0		0.85	0.76	0.72
16[b]	Actual	1.00	—	—	1.57	—	0.79	0.50	1.0
	Found	1.00			1.51		0.76	0.51	1.08
17[c]	Actual					1.0			1.62
	Found					1.0			1.65

[a] Chromatograms run isothermally at 200° in the absence of sorbitol. Results are the mean of four determinations.

[b] The areas of the overlapping peaks of N-acetyl glucosamine and N-acetyl galactosamine were estimated by the method of BARTLETT AND SMITH[55].

[c] Chromatograms run isothermally at 190° on 10% SE 52. Seperation of N-acetyl galactosamine, N:acetyl glucosamine and glucose can be achieved on SF 96 at 190°.

of the peak areas differs from the ratio at equilibrium. In a similar way MRF's determined at equilibrium in different solvents should be identical. Statistical analysis of the results presently at hand has led to no definite conclusion as to whether or not the MRF's of the sugar anomers are identical. Kinetic analysis of mutarotation employing GLC has recently been reported[57].

EXPERIMENTAL

GLC was carried out on an F & M 402 dual column gas chromatograph fitted with flame ionisation detectors and direct on-column injection. The chromatograph was routinely operated isothermally at 190° with a flash heater temperature of 260°, a detector temperature of 270° and helium as carrier gas, using dual copper columns 8 ft. × ¼ in. O.D. containing 10% (w/w) Silicone Fluid SF-96 on 80–100 mesh Diatoport S or 10% (w/w) SE-52 on 80–100 mesh AW Chromosorb W. Columns were packed to contain the same weight of packing (± 0.1 g) and best results were obtained with the SF-96 columns when the packing was sieved after preparation. Columns were conditioned under non-flow conditions for 30 min and then under flow conditions for at least 24 h at temperatures at least 50° above the maximum temperature used for chromatography. Column life was prolonged by regularly (after every fourth injection) alternating the two columns. Performance was improved by regularly removing the deposits which accumulate on the collecting electrodes when silyl ethers are being chromatographed. An F & M gas chromatograph is particularly convenient in this regard since the detector can be rapidly disassembled and easily cleaned. Peak areas were measured with a Disc integrator and when necessary by the method of BARTLET AND SMITH[55].

DETERMINATION OF MOLAR RESPONSE FACTORS AND EQUILIBRIUM PERCENTAGE COMPOSITION

Preliminary experiments to establish conditions for mutarotation equilibrium were carried out on pairs of sugars which were completely separated from each other on GLC. Samples of the two sugars plus sorbitol were dissolved in analytical grade pyridine or N,N-dimethylformamide (8 ml) to give a final concentration of approx. 1.25×10^{-2} *M* in each of the sugars and in sorbitol. These solutions were incubated at 40° in plastic capped glass vials in the presence of varying quantities of 2-hydroxypyridine. Aliquots (1 ml) were withdrawn at intervals and the TMS ethers were prepared by treatment with hexamethyldisilazane (1 ml) and trimethylchlorosilane (0.5 ml)[46]. After 30 min, aliquots (2 μl) were used for GLC analysis and the ratio of the peak areas of the anomers was measured. Mutarotation was allowed to continue until the ratio of the peak areas of the anomers became a constant. On the basis of these experiments the following sets of conditions were selected:

(a) Reagent grade N,N-dimethylformamide was made approximately 1.25×10^{-2} *M* in each of two sugars, 1.25×10^{-2} *M* in sorbitol and 2×10^{-1} *M* in 2-hydroxypyridine. The solutions were incubated at 40° for 3 h.

(b) Conditions as for (a) above using analytical grade pyridine or N,N-dimethyl formamide in the presence of 1×10^{-1} *M* 2-hydroxypyridine at 40° for 6–24 h.

Molar response factors and percentage compositions at equilibrium were determined by quadruplicate analyses on each of six mutarotation mixtures. Statistical analysis indicated that the results obtained from the six separate mutarotation mixtures were in the same population.

MRF's were calculated from the formula:

$$\text{MRF} = \frac{\text{Peak area of sugar} \times \text{moles internal standard}}{\text{Peak area of internal standard} \times \text{moles of sugar}}$$

ANALYSIS OF SUGAR MIXTURES

Mixtures of sugars were brought to mutarotation equilibrium by using procedure (a) above. Samples (1 ml) were silylated with hexamethyldisilazane (1 ml) and trimethylchlorosilane (0.5 ml) and four aliquots (2 μl) were analysed. In these experiments the analyst knew the total weight of the mixture to be analysed but was unaware of its quantitative and qualitative composition.

ACKNOWLEDGEMENTS

The financial assistance of the Medical Research Council of Canada (Grant Nos. MA-2636 and ME-2681) is gratefully acknowledged. One of us (P.E.R.) wishes to thank the Medical Research Council of Canada for the award of an M.R.C. Scholarship.

REFERENCES

1 G. G. S. DUTTON, K. B. GIBNEY, G. D. JENSEN AND P. E. REID, *J. Chromatog.*, 36 (1968) 152.
2 C. T. BISHOP, *Methods Biochem. Anal.*, 10 (1962) 1.
3 J. W. BERRY, *Advan. Chromatog.*, 2 (1962) 271.

4 W. W. WELLS, in H. A. SZYMANSKI (Editor), *Biomedical Applications of Gas Chromatography*, Plenum Press, New York, 1964, p. 169.
5 C. T. BISHOP, *Advan. Carbohyd. Chem.*, 19 (1965) 95.
6 J. H. SLONEKER, in H. A. SZYMANSKI (Editor), *Biomedical Applications of Gas Chromatography*, Vol. 2, Plenum, Press, New York, 1968, p 87.
7 T. TAKAHASHI, P. RAMACHANDRAMURTHY AND E. LIENER, *Biochim. Biophys. Acta*, 133 (1967) 123.
8 J. H. KIM, B. SCHOME, T. A. HSIU LIAO AND J. G. PIERCE, *Anal. Biochem.*, 20 (1967) 258.
9 W. F. LEHNHARDT AND R. J. WINZLER, *J. Chromatog.*, 34 (1968) 471.
10 K. O. LLOYD AND E. A. KABAT, *Carbohydrate Res.*, 4 (1967) 165.
11 C. H. BOLTON, J. R. CLAMP AND L. HOUGH, *Biochem. J.*, 96 (1965) 5c.
12 C. H. BOLTON, J. R. CLAMP, G. DAWSON AND L. HOUGH, *Carbohydrate Res.*, 1 (1965) 333.
13 J. R. CLAMP, G. DAWSON AND L. HOUGH, *Biochim. Biophys. Acta*, 148 (1967) 342.
14 M. D. G. OATES AND J. SCHRAGER, *Biochem. J.*, 97 (1965) 697.
15 D. A. CRAVEN AND G. W. GEHRKE, *J. Chromatog.*, 37 (1968) 414.
16 M. I. HOROWITZ AND M. R. DILMAN, *J. Chromatog.*, 21 (1966) 300.
17 M. D. G. OATES AND J. SCHRAGER, *J. Chromatog.*, 28 (1967) 232.
18 C. C. SWEELEY AND B. WALKER, *Anal. Chem.*, 36 (1964) 1461.
19 R. J. PENICK AND R. H. MCCLUER, *Biochim. Biophys. Acta*, 116 (1966) 288.
20 B. RADHAKRISHNAMURTHY, E. R. DALFERS, JR. AND G. S. BERENSON, *Anal. Biochem.*, 17 (1966) 545.
21 A. A. LEHTONEN, J. K. KARKKAINEN AND E. O. HAAHTI, *Anal. Biochem.*, 16 (1966) 526.
22 A. A. LEHTONEN, J. K. KARKKAINEN AND E. O. HAAHTI, *J. Chromatog.*, 24 (1966) 179.
23 B. RADHAKRISHNAMURTHY, E. R. DALFERS, JR. AND G. S. BERENSON, *Anal., Biochem.*, 24 (1968) 397.
24 J. K. KARKKAINEN, A. A. LEHTONEN AND T. NIKKARI, *J. Chromatog.*, 20 (1965) 457.
25 J. R. CLAMP AND J. E. SCOTT, *Chem. Ind. (London)*, (1969) 652.
26 P. O. BETHGE, C. HOLMSTRÖM AND S. JUHLIN, *Svensk Papperstid.*, 69 (1966) 60.
27 H. E. BROWER, J. E. JEFFREY AND M. W. FOLSOM, *Anal. Chem.*, 38 (1966) 362.
28 E. P. CROWELL AND B. B. BURNETT, *Anal. Chem.*, 39 (1967) 121.
29 H. W. KIRCHER, *Tappi*, 45 (1962) 143.
30 E. SJÖSTRÖM, P. HAGLUND AND J. JANSON, *Svensk Papperstid.*, 69 (1966) 381.
31 P. ALBERSHEIM, D. J. NEVINS, P. D. ENGLISH AND A. KARR, *Carbohydrate Res.*, 5 (1967) 340.
32 J. M. OADES, *J. Chromatog.*, 28 (1967) 246.
33 W. W. WELLS, T. A. PITMAN AND T. J. EGAN, *J. Biol. Chem.*, 240 (1965) 1002.
34 C. C. SWEELEY, W. W. WELLS AND R. BENTLEY, *Methods Enzymol.*, 8 (1966) 95.
35 J. H. COPENHAVER, *Anal. Biochem.*, 17 (1966) 76.
36 T. BHATTI AND J. R. CLAMP, *Clin. Chim. Acta*, 22 (1968) 563.
37 J. K. HUTTUNEN AND T. A. MIETTEN, *Anal. Biochem.*, 29 (1969) 441.
38 M. MUELLER, J. A. WALKER SMITH, D. H. CURTIUS AND A. PRADER, *Clin. Chim. Acta* 24 (1969) 45.
39 P. E. REID AND D. W. SECRET, unpublished data.
40 G. G. S. DUTTON, P. E. REID, J. J. M. ROWE AND K. L. ROWE, *J. Chromatog.*, 47 (1970) 195.
41 S. W. GUNNER, J. K. N. JONES AND M. B. PERRY, *Can. J. Chem.*, 39 (1961) 1892.
42 H. G. JONES AND J. K. N. JONES, *Can. J. Chem.*, 40 (1962) 1559.
43 D. H. SHAW AND G. W. MOSS, *J. Chromatog.*, 41 (1969) 350.
44 J. S. SAWARDEKER, J. H. SLONEKER AND A. JEANES, *Anal. Chem.*, 37 (1965) 1602.
45 M. B. PERRY AND A. C. WEBB, *Can. J. Biochem.*, 46 (1968) 1163.
46 C. C. SWEELEY, R. BENTLEY, M. MAKITA AND W. W. WELLS, *J. Am. Chem. Soc.*, 85 (1963) 2497.
47 J. M. RICHEY, H. G. RICHEY, JR. AND R. SCHRAER, *Anal. Biochem.*, 9 (1964) 272.
48 M. B. PERRY, *Can. J. Biochem.*, 42 (1964) 451.
49 G. WULFF, *J. Chromatog.*, 18 (1965) 285.
50 J. S. SAWARDEKER AND J. H. SLONEKER, *Anal. Chem.*, 37 (1966) 945.
51 M. B. PERRY AND R. K. HULYALKER, *Can. J. Biochem.*, 43 (1965) 573.
52 I. M. MORRISON AND M. B. PERRY, *Can. J. Biochem.*, 44 (1966) 1115.
53 K. M. BROBST AND E. E. LOTT, *Cereal Chem.*, 43 (1966) 35.
54 R. BENTLEY AND N. BOTLOCK, *Anal. Biochem.*, 20 (1967) 312.
55 J. C. BARTLET AND D. M. SMITH, *Can. J. Chem.*, 88 (1960) 2057.
56 C. G. SWAIN AND J. F. BROWN, Jr., *J. Am. Chem. Soc.*, 74 (1952) 2534, 2538.
57 T. E. ACREE, R. S. SHALLENBERGER, C. Y. LEE AND J. W. EINSET, *Carbohyd. Res.*, 10 (1969) 355.