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An Assessment of Methanolysis and Other Factors Used in the Analysis of Carbohydrate-Containing Materials

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The stability of monosaccharides in methanolic hydrochloric acid of different strengths and at different temperatures was determined. They are generally stable for 24h in methanolic 1M- and 2M-hydrochloric acid at both 85°C and 100°C, but undergo considerable destruction in methanolic 4M- and 6M-hydrochloric acid at 100°C. Analysis of glycopeptides and oligosaccharides of known composition showed that release of carbohydrate was complete within 3h in methanolic 1M-hydrochloric acid at 85°C. Removal of methanolic hydrochloric acid by rotary evaporation resulted in considerable losses of monosaccharides, which could be prevented by prior neutralization. Methanolysis caused extensive de-*N*-acetylation of acetamidohexoses, so that a re-*N*-acetylation step is necessary in the analytical procedure. The addition of acetic anhydride for this purpose also prevented loss of internal standard by adsorption on the insoluble silver salts used in neutralization. Several trimethylsilylating agents were studied and suitable conditions are recommended. The effects on the analytical system of water and some common organic and inorganic contaminants are assessed.

The first stage in the analysis of covalently linked carbohydrate in biological material is to cleave it to the monosaccharide units. This is usually done by hydrolysis with aqueous acid or with cation-exchange resins in the protonated form, which yields the reducing sugars; numerous studies to determine the optimum conditions for this process have been done (Moggridge & Neuberger, 1938; Sorensen, 1938; Boas, 1953; Strange & Powell, 1954; Gottschalk & Ada, 1956; Foster, Horton & Stacey, 1957; Michon & Bourrillon, 1959; Johansen, Marshall & Neuberger, 1960; Swann & Balazs, 1966; Albersheim, Nevins, English & Karr, 1967; Schrager & Oates, 1968; Niedermeier, 1971).

An alternative method of cleavage is by methanolysis, which yields the methyl glycosides. Unlike aqueous acid hydrolysis, there have been few studies on the process of methanolysis, although this technique may have some advantages (Sweeley & Walker, 1964; Nozawa, Uesaka, Suzuki & Ito, 1969*a*; Bhatti, Chambers & Clamp, 1970). Thus methanolysis appears to be as efficient as hydrolysis at cleaving glycosidic linkages (Reeves, Schwartz & Giddens, 1946; Jeanloz & Jeanloz, 1964; Chan, Hull, Fields & McNall, 1967; Levvy, Hay, Conchie & Strachan, 1970; Entlicher & BeMiller, 1971) and, in addition, causes less destruction of carbohydrate than does aqueous acid, as has been shown with glycosides, oligosaccharides (Wulff, 1965) and uronic acids (Morell, Baur & Link, 1934; Clamp & Scott, 1969). Although the stability of neutral monosaccharides in methanolic hydrochloric acid has been determined (Nozawa, Hiraguri & Ito, 1969*b*), most other studies were done with complex biological materials and did not include a systematic study of the release and stability of the different types of monosaccharide that are likely to be present in such materials. It was decided therefore to establish the most suitable conditions for the release and determination of neutral sugars, acetamidohexoses, *N*-acetylneuraminic acid and hexuronic acids.

MATERIALS

Aristar- or AnalaR-grade chemicals, where available, were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K., unless otherwise stated. *N*-Acetylgalactosamine and *N*-acetylneuraminic acid were obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K., α- and β-methyl D-xylosides from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K., and bis(trimethylsilyl)-acetamide and bis(trimethylsilyl)trifluoroacetamide from Applied Science Laboratories Inc., State College, Pa., U.S.A. β-Methyl D-galactoside, α- and β-methyl D-glucosaminide, β-methyl D-galactosaminide, α-methyl *N*-acetyl-D-glucosaminide, α-methyl *N*-acetyl-D-galactosaminide, the α-methyl glycosides of methyl D-glucuronate and methyl D-galacturonate and the mixed

methyl glycosides of *N*-acetylneuraminate were kindly supplied by Professor L. Hough, University of London; *N*-acetylchitobiose by Dr R. C. W. Berkeley, University of Bristol; lacto-*N*-fucopentaose II by the late Professor R. Kühn, Max Planck Institut, Heidelberg, Germany, and mannose–*N*-acetylglucosamine-containing oligosaccharides by Professor J. Montreuil, Laboratorie de Chimie Biologique, Faculté des Sciences, Lille, France.

METHODS

Preparation of methanolic hydrochloric acid. Methanol was dried by heating it (500ml) together with magnesium turnings (2.5g) and iodine (0.1g) under reflux for 1h. The dry methanol was then distilled into a clean flask and dry HCl gas slowly bubbled in until the desired strength, namely 1M, 2M, 4M or 6M, was reached.

*Methanolysis and re-*N*-acetylation.* The material under investigation, together with the internal standard, mannitol, was added to glass tubes, which were then thoroughly dried in a vacuum desiccator over P_2O_5. Methanolyses were done with methanolic 1M- and 2M-HCl at 85°C and methanolic 1M-, 2M-, 4M- and 6M-HCl at 100°C. Methanolic HCl (0.5ml) was added to the tubes and N_2 was bubbled in for 30s, after which the tubes were sealed and heated for 24h. The acid was then neutralized with Ag_2CO_3 and re-*N*-acetylation performed in the same tube with acetic anhydride (0.05ml) at room temperature for 6h. After thorough trituration, the sample was centrifuged and the supernatant was transferred to a fresh tube. The trituration and centrifugation steps were repeated with three additions of methanol and the pooled supernatants were evaporated under reduced pressure at 35°C. The samples were finally dried for 12h in a vacuum desiccator over P_2O_5.

Gas–liquid chromatography. The methyl glycosides were separated as the trimethylsilyl ethers (Bhatti *et al.* 1970). To the dried material, trimethylsilylating agent (0.05ml) consisting of pyridine, trimethylchlorosilane and hexamethyldisilazane (5:1:1, by vol.) was added and, after thorough trituration, the tube was stoppered and left at room temperature for 30min. After centrifugation, a portion (1–5μl) of the supernatant was injected into the chromatograph. Glass columns (250cm × 0.32cm internal diam.) packed with 3.0% SE-30 on Diatoport S (Hewlett–Packard Ltd., Slough, Bucks., U.K.) were used with O_2-free N_2 (British Oxygen Co. Ltd., Bristol, U.K.) as carrier gas (50ml/min). The chromatograph was equipped with a flame-ionization detection system and was temperature-programmed from 140°C to 200°C at 0.5°C/min, the limit of 200°C being held until the last peak had emerged. Peak areas were determined by disc integration (Disc Instruments Ltd., Hemel Hempstead, Herts., U.K.).

Determination of the acid stability of the internal standard. The peak area of the internal standard (mannitol) was determined after direct trimethylsilylation and also after methanolysis for 24h in methanolic 1M- and 2M-HCl at 85°C and 1M, 2M, 4M and 6M acid at 100°C. The proportion of mannitol destroyed was calculated by reference to an internal standard (arabinitol) added to the sample after methanolysis.

Determination of the acid stability of monosaccharides. Response factors were determined for a range of monosaccharides after methanolysis under the various conditions of temperature and acid strength described above, and compared with those obtained by direct trimethylsilylation of the corresponding reducing sugars and methyl glycosides. The relative stability was measured with respect to internal standard that had been subjected to methanolysis under the same conditions, and absolute stability was determined with respect to internal standard added after methanolysis.

Release of monosaccharides. A series of glycoproteins, glycopeptides and oligosaccharides were methanolysed for 24h in methanolic 1M-HCl at 85°C and methanolic 2M-, 4M- and 6M-HCl at 100°C. Internal standards were added before (mannitol) and after (arabinitol) methanolysis. Analytical results were compared for each substance under the various conditions and, for oligosaccharides, with the theoretical composition. The liberation of monosaccharides by methanolic 1M-HCl was also measured by analysing replicate samples of a glycoprotein and glycopeptides after heating them at 85°C for various lengths of time.

Determination of the effect of water. Methanolysis and trimethylsilylation were done in the presence of various quantities of water. Peak areas were compared with controls that did not contain water.

Determination of the effect of organic and inorganic buffers. Response factors were determined in the presence of different quantities of NaCl. The relative retention times of citrate and tris before and after methanolysis were also determined.

Removal of methanolic hydrochloric acid by rotary evaporation. Monosaccharides were methanolysed and the methanolic HCl was removed by rotary evaporation at room temperature. The samples were finally dried for 12h in a vacuum desiccator over NaOH pellets and P_2O_5. After the addition of methanol 0.5ml), re-*N*-acetylation and evaporation were performed as described above and the response factors were determined.

RESULTS AND DISCUSSION

Choice of internal standard

For the quantitative analysis of monosaccharides by g.l.c. the technique of internal standardization is generally used. In this technique a known amount of the standard is added to the sample at the beginning of the analysis so that any subsequent losses will affect both to an equal extent. The ratio of each monosaccharide to the internal standard at the end of the analysis is then a direct measure of the amount of that sugar in the sample. The choice of internal standard is therefore critical and should satisfy certain requirements. Standards should be readily available in a highly purified form, preferably as solids, be chemically similar to the class of compounds under investigation and give single chromatographic peaks adequately separated from those of the other components. The standards should also be stable under all conditions of the analytical procedure. Glycitols appear to fulfil these requirements for carbohydrate analyses. Thus mannitol is completely stable in

methanolic 1M- and 2M-hydrochloric acid at either 85°C or 100°C and is therefore suitable for analyses under these conditions. More extreme conditions, however, cause considerable destruction, 38% of the mannitol being lost in 4M acid at 100°C and 48% in 6M acid at 100°C.

Stability of monosaccharides

The stabilities under various conditions of methanolysis of those monosaccharides commonly found in biological material are given in Tables 1 and 2. In Table 1, the molar response factors of monosaccharides that have been heated at 85°C for 24h in methanolic 1M-hydrochloric acid are compared with the molar response factors obtained by direct trimethylsilylation of the free reducing sugars and the authentic methyl glycosides. There are no significant differences between the three sets of results, indicating that no losses have occurred. The stability of monosaccharides in stronger acid at higher temperatures is shown in Table 2. The response factors for monosaccharides with respect to mannitol, added before heating, are remarkably constant up to methanolic 4M-hydrochloric acid at 100°C. Mannitol could therefore be used as an internal standard under these conditions. The actual destruction that occurs may be determined from the response factors for arabinitol, which was added after the heating in methanolic hydrochloric acid. All monosaccharides are stable in methanolic 2M-hydrochloric acid at 85°C and methanolic 1M-hydrochloric acid at 100°C, apart from hexuronic acids, of which 3% are lost in 2M acid at 85°C and 9% are lost in 1M acid at 100°C. These results do not confirm those of Nozawa *et al.* (1969*b*) who found some loss of neutral sugars in their system with strengths of methanolic hydrochloric acid as low as 1% (approx. 0.2M). However, in their procedure, ion-exchange chromatography was used for neutralization and some losses might have occurred during this step, which was not separately investigated. In fact, fucose, the hexoses and the acetamidohexoses remain stable in methanolic 2M-hydrochloric acid at 100°C (Table 2), although under these conditions there was some destruction of the hexuronic acids (15%), xylose (11%) and *N*-acetylneuraminic acid (5%). At higher strengths of methanolic hydrochloric acid, all monosaccharides undergo destruction to various extents. Thus, in methanolic 4M-hydrochloric acid at 100°C, 96% of *N*-acetylneuraminic acid, 65% of xylose, 45% of the hexuronic acids, 40% of fucose, 33% of the hexoses and 27% of the acetamidohexoses are

Table 1. *Stability of monosaccharides in methanolic* 1M-*hydrochloric acid at* 85°C *for* 24h

Molar response factors, that is the total peak areas relative to that of the internal standard, were determined with respect to arabinitol (A) and mannitol (M). Results for the reducing sugars and the authentic methyl glycosides were obtained after direct trimethylsilylation. The methyl glycosides of *N*-acetylneuraminic acid (NeuNAc), glucuronic acid and galacturonic acid were chromatographed as methyl esters. Methyl fucoside was not available (n.a.) for study. Arabinitol, which coincides with the first peak of galacturonic acid, was not added to samples containing this monosaccharide.

		Molar response factor		
			Methyl glycoside	
Residue	Internal standard	Reducing monosaccharide	Authentic	After methanolysis
Fuc	A	0.89	n.a.	0.85
	M	0.77	n.a.	0.74
Xyl	A	0.80	0.77	0.78
	M	0.69	0.66	0.67
Man	A	0.99	1.01	1.04
	M	0.83	0.87	0.90
Gal	A	1.05	1.03	1.03
	M	0.90	0.88	0.89
Glc	A	1.04	0.98	1.05
	M	0.90	0.88	0.93
GlcNAc	A	0.85	0.83	0.85
	M	0.76	0.71	0.74
GalNAc	A	0.74	0.79	0.70
	M	0.64	0.68	0.62
NeuNAc	A	0.62	0.67	0.55
	M	0.53	0.58	0.49
GlcA	A	0.75	0.65	0.67
	M	0.65	0.56	0.59
GalA	M	0.63	0.56	0.58

Table 2. *Stability of monosaccharides in methanolic hydrochloric acid of different strengths and at different temperatures*

Molar response factors were determined with respect to internal standards added before (M) and after (A) the methanolysis step. Abbreviations are as in Table 1.

			Molar response factor					
		Concn. of acid ...	1M	2M	1M	2M	4M	6M
Residue	Internal standard	Oven temp. ...	85°C	85°C	100°C	100°C	100°C	100°C
Fuc	A		0.85	0.87	0.85	0.84	0.52	0.35
	M		0.74	0.74	0.74	0.74	0.73	0.63
Xyl	A		0.78	0.77	0.76	0.69	0.27	0.16
	M		0.67	0.66	0.65	0.59	0.37	0.28
Man	A		1.04	1.02	1.04	1.03	0.60	0.43
	M		0.90	0.90	0.93	0.90	0.84	0.74
Gal	A		1.03	1.02	1.07	1.02	0.72	0.51
	M		0.89	0.89	0.93	0.88	1.00	0.92
Glc	A		1.05	1.04	1.03	1.06	0.77	0.62
	M		0.93	0.90	0.88	0.93	1.08	1.16
GlcNAc	A		0.85	0.88	0.87	0.85	0.65	0.60
	M		0.74	0.76	0.74	0.75	0.92	1.01
GalNAc	A		0.70	0.70	0.71	0.69	0.49	0.42
	M		0.62	0.61	0.62	0.62	0.69	0.71
NeuNAc	A		0.55	0.55	0.55	0.52	0.02	0.00
	M		0.49	0.48	0.46	0.47	0.03	0.00
GlcA	A		0.67	0.65	0.61	0.55	0.35	0.23
	M		0.59	0.56	0.53	0.49	0.49	0.39
GalA	M		0.58	0.58	0.56	0.55	0.55	0.41

lost. In methanolic 6M-hydrochloric acid at 100°C, *N*-acetylneuraminic acid is totally destroyed and 80% of xylose, 64% of the hexuronic acids, 59% of fucose, 50% of the hexoses and 35% of the acetamidohexoses are lost. *N*-Acetylneuraminic acid is reasonably stable in methanolic hydrochloric acid, showing little loss even under conditions as rigorous as 2M acid at 100°C and in this respect differs from its behaviour in aqueous acid (Craven & Gehrke, 1968; Yu & Ledeen, 1970; Suttajit & Winzler, 1971).

Release of monosaccharides from biological material

The yields of monosaccharides obtained by heating glycopeptides from immunoglobulins A and M and gastric mucus for 24h in methanolic 1M-hydrochloric acid at 85°C and methanolic 2M- and 4M-hydrochloric acid at 100°C are shown in Table 3. All samples were treated with 1M acid at 85°C to obtain reference values with which the results obtained by heating in methanolic 2M- and 4M-hydrochloric acid at 100°C could be compared. Where sufficient material was available, the same sample was treated with both methanolic 2M- and 4M-hydrochloric acid. The results in Table 3 show that there is no significant increase in the release of monosaccharides, including acetamidohexoses, when strengths greater than 1M acid or temperatures higher than 85°C are used. However, to make certain that complete release of monosaccharides was being achieved, ten *N*-acetylglucosamine-containing oligosaccharides of known composition were subjected to methanolysis for 24h in methanolic 1M-hydrochloric acid at 85°C, and, where sufficient material was available, in methanolic 2M-hydrochloric acid at 100°C. The results, expressed as percentages of the expected monosaccharide content, are given in Table 4. The results obtained for *N*-acetylchitobiose are particularly important, as this compound consists of *N*-acetylglucosamine linked β-(1$\rightarrow$4) to a second *N*-acetylglucosamine. This grouping is likely to be the most resistant to methanolysis that might occur in glycoproteins. If deacetylation of either acetamidohexose occurs the charged species would be highly resistant to cleavage by acid. Table 4 shows that methanolic 1M-hydrochloric acid at 85°C is effective in the quantitative release of acetamidohexose units linked in this way.

It is difficult to assess the efficiency of methanolysis in conditions more extreme than 4M acid at 100°C. Biological samples heated at 100°C for 24h in methanolic 6M-hydrochloric acid give rise to complex chromatograms that are difficult to interpret. A large number of peaks that have not been identified are present, presumably arising from degradation products of carbohydrates and amino acids (Wolfrom & Kashimura, 1969), and these

Table 3. *Release of monosaccharides from glycopeptides under different conditions of methanolysis*

Methanolyses were done for 24h. Monosaccharide compositions are expressed as μmol/mg. Abbreviations are as in Table 1.

Glycopeptide no.	Acid strength	Temp. (°C)	Content (μmol/mg of dried material)					
			Fuc	Man	Gal	GlcNAc	GalNAc	NeuNAc
1	1M	85	0.00	2.67	0.00	0.84	0.00	0.00
	2M	100	0.00	2.64	0.00	0.82	0.00	0.00
	4M	100	0.00	2.65	0.00	0.80	0.00	0.00
2	1M	85	0.28	0.84	0.50	1.12	0.00	0.61
	2M	100	0.28	0.88	0.58	1.22	0.00	0.60
	4M	100	0.26	0.90	0.48	1.11	0.00	0.00
3	1M	85	0.39	1.03	0.94	1.02	0.20	0.00
	4M	100	0.43	0.92	0.88	1.17	0.24	0.00
4	1M	85	0.52	0.75	0.92	1.17	0.22	0.28
	4M	100	0.54	0.68	0.94	1.13	0.21	0.00
5	1M	85	0.64	0.00	1.31	0.89	0.46	0.17
	4M	100	0.69	0.00	1.32	0.85	0.44	0.00
6	1M	85	0.31	0.93	0.58	1.06	0.00	0.19
	2M	100	0.29	0.86	0.54	1.11	0.00	0.16
7	1M	85	0.42	1.01	0.64	1.35	0.00	0.24
	2M	100	0.41	0.98	0.64	1.26	0.00	0.23
8	1M	85	0.00	2.15	0.00	0.65	0.00	0.00
	2M	100	0.00	1.96	0.00	0.59	0.00	0.00
9	1M	85	0.22	0.62	0.40	0.84	0.00	0.49
	2M	100	0.20	0.62	0.44	0.84	0.00	0.41
10	1M	85	0.40	1.02	0.57	1.43	0.00	0.44
	2M	100	0.36	0.95	0.54	1.42	0.00	0.42
11	1M	85	0.07	0.82	0.10	0.39	0.00	0.02
	2M	100	0.07	0.78	0.09	0.39	0.00	0.02
12	1M	85	0.31	0.91	0.56	1.23	0.00	0.41
	2M	100	0.29	0.89	0.55	1.17	0.00	0.38
13	1M	85	0.11	1.30	0.15	0.45	0.00	0.00
	2M	100	0.10	1.15	0.12	0.37	0.00	0.00

Table 4. *Release of monosaccharides from characterized* N*-acetylglucosamine-containing disaccharides, trisaccharides and oligosaccharides*

Methanolyses were done for 24h. Monosaccharide compositions are expressed as a percentage of the actual content of that monosaccharide.

Material	Acid strength	Temp. (°C)	Monosaccharide released (%)				
			Fuc	Man	Gal	Glc	GlcNAc
N-Acetylchitobiose	1M	85	—	—	—	—	91
	2M	100	—	—	—	—	92
Disaccharide	1M	85	—	94	—	—	106
	1M	85	—	102	—	—	98
Trisaccharide	1M	85	—	99	—	—	102
	1M	85	—	101	—	—	99
	1M	85	—	99	—	—	102
Tetrasaccharide	1M	85	—	94	—	—	106
	1M	85	—	102	—	—	98
Lacto-*N*-fucopentaose II	1M	85	100	—	100	95	105
	2M	100	100	—	98	105	100
Pentasaccharide	1M	85	—	100	—	—	99

interfere to a considerable extent with the usual monosaccharide peaks. However, such conditions are not necessary for the methanolysis of glycopeptides and glycoproteins and methanolic 1M-hydrochloric acid at 85°C appears to be entirely adequate. In contrast to Levvy *et al.* (1970) who

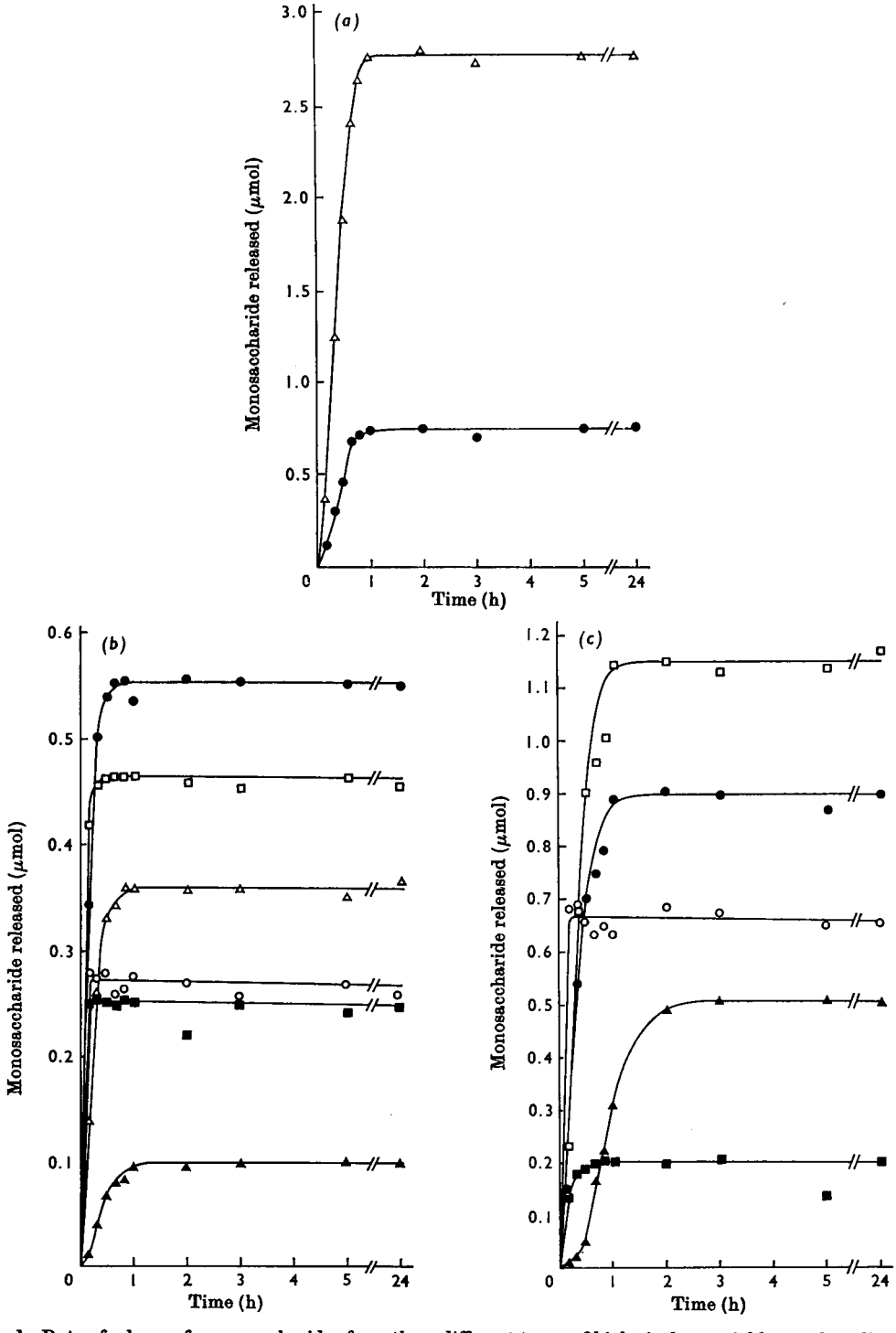

Fig. 1. Rate of release of monosaccharides from three different types of biological material by methanolic 1 M-HCl at 85°C. Details are given in the text. (*a*), Release of mannose (△) and *N*-acetylglucosamine (●) from a glycopeptide derived from immunoglobulin M; (*b*), release of fucose (○), mannose (△), galactose (□), *N*-acetylglucosamine (●), *N*-acetylgalactosamine (▲) and *N*-acetylneuraminic acid (■) from a glycopeptide derived from secretory immunoglobulin A; (*c*), release of carbohydrate from a glycoprotein derived from mucus (symbols as in *b*).

found that, in their system, methanolysis was suitable for neutral sugars but acetamidohexoses were best hydrolysed with aqueous acid, we suggest that methanolysis is a satisfactory procedure for the determination of all monosaccharides, and a system that enables all components to be analysed together in a single procedure offers advantages over other methods (Clamp, Dawson & Hough, 1967).

Rate of release of monosaccharides. The rate at which monosaccharides are liberated by methanolic 1M-hydrochloric acid at 85°C is shown in Fig. 1. Three different samples were chosen as typical examples of the type of material likely to be encountered. The first was a glycopeptide containing mannose and *N*-acetylglucosamine only (Fig. 1*a*); the second was a more complex glycopeptide containing fucose, mannose, galactose, *N*-acetylglucosamine, *N*-acetylgalactosamine and *N*-acetylneuraminic acid (Fig. 1*b*), and the third was a glycoprotein of high molecular weight from mucus (Fig. 1*c*). In every case, total release of all monosaccharides had occurred within 3h. The curves also confirm that monosaccharides are stable in methanolic 1M-hydrochloric acid at 85°C for at least 24h.

Removal of methanolic hydrochloric acid

The use of silver carbonate to neutralize the acid after methanolysis involves subsequent centrifugation of samples and transfer of the supernatants to fresh containers for drying before analysis by g.l.c. Removal of the methanolic hydrochloric acid by rotary evaporation would allow the entire procedure to be done in the same container. However, during this procedure there are extensive losses of monosaccharides, amounting to 47% of *N*-acetylneuraminic acid, 26% of fucose, 20% of galactose and 8% of mannose, although there is no loss of acetamidohexoses. Extraneous peaks occur in the chromatogram which, it has been suggested (Wulff, 1965), arise from partial degradation of methyl glycosides. From these results it would appear that neutralization is best done by the addition of an agent such as silver carbonate before drying.

Re-N-acetylation

A re-*N*-acetylation step is recommended, as methanolysis can cause de-*N*-acetylation of acetamido sugars (Ludowieg & Dorfman, 1960), and the presence of de-*N*-acetylation products in the sample gives rise to complex chromatograms that are difficult to interpret. Thus, acetamidohexoses that had been heated for 24h in methanolic hydrochloric acid, but not re-*N*-acetylated, produced chromatograms that had extra peaks in the region of mannose and galactose, small peaks in the usual acetamidohexose positions and considerable variation in the internal-standard peaks, which in some cases were virtually absent. The peaks in the region of mannose and galactose were identified as methyl hexosaminides by comparison with the authentic compounds. Thus β-methyl D-galactosaminide emerges in the same position as α-methyl D-mannoside, and β-methyl D-glucosaminide coincides with α-methyl D-galactoside. The loss of internal standard cannot be satisfactorily explained by solubility effects. Although glycitols are not particularly soluble in methanol, the volume used (0.5ml) should be well in excess of that required to dissolve 0.2μmol of mannitol, and similar responses were obtained when a series of dried samples containing mannitol, mannose, α-methyl D-mannoside, *N*-acetylglucosamine and α-methyl *N*-acetyl-D-glucosaminide were heated overnight at 85°C in either a mixture of methanol (0.5ml) and acetic anhydride (0.05ml) or in methanol alone. It is more likely that the losses are caused by adsorption on to the silver carbonate–silver chloride precipitate produced during neutralization. The addition of acetic acid in place of acetic anhydride restores the internal standard peaks. This suggests that acetic acid or acetic anhydride in some way prevents the adsorption of glycitols on the insoluble silver salts. The substitution of acetic acid for acetic anhydride for this purpose is useful because it makes possible the estimation of the amount of de-*N*-acetylation taking place during methanolysis. After being treated in methanolic 1M-hydrochloric acid at 85°C for 24h, *N*-acetylglucosamine, *N*-acetylgalactosamine and *N*-acetylneuraminic acid lose 90–100% of their acetyl groups. Re-*N*-acetylation is therefore an essential stage in the procedure.

Trimethylsilylation

Several trimethylsilylating agents are now available and although they may offer advantages for the preparation of derivatives of compounds such as amino acids, nucleosides and steroids, for the analysis of carbohydrates they do not appear to be superior to the original reagent described by Sweeley, Bentley, Makita & Wells (1963). This consists of a mixture of pyridine, trimethylchlorosilane and hexamethyldisilazane. Response factors were determined for a series of monosaccharides by using bis(trimethylsilyl)acetamide and bis(trimethylsilyl)trifluoroacetamide under the same conditions described in the Methods section. Different batches of bis(trimethylsilyl)acetamide varied in their efficiency of trimethylsilylation depending on the source, but with satisfactory batches, response factors were obtained that agreed well with

those shown in Table 1. Bis(trimethylsilyl)trifluoroacetamide, on the other hand, was not satisfactory, producing only small peaks for neutral sugars and none for acetamidohexoses and internal standard. This is probably caused by the insolubility of monosaccharides in the reagent, as the addition of an equal volume of pyridine greatly improved the chromatogram, although several extra peaks were found as shoulders on the main acetamidohexose peaks. However, bis(trimethylsilyl)trifluoroacetamide has been successfully used for carbohydrate analysis, but always in conjunction with another reagent and some degree of heating (Williams & Perry, 1969; Perry, Adams & Shaw, 1969; Sweeley & Dawson, 1969; Stimson, 1971). This requirement makes the procedure more complicated and offers no advantage over procedure with the usual reagent in which trimethylsilylation proceeds rapidly and satisfactorily at room temperature. Although bis(trimethylsilyl)acetamide had given satisfactory results with standard monosaccharides, the analysis of a series of glycopeptides encountered the same problem of insolubility as described above for bis(trimethylsilyl)-trifluoroacetamide. The addition of pyridine again produced multiple peaks for acetamidohexoses, but this problem can similarly be overcome by heating and the addition of other reagents (Kärkkäinen & Vihko, 1969). A third trimethylsilylating agent is trimethylsilylimidazole, but although this reagent has been extensively used with steroids (Chambaz & Horning, 1967, 1969), few reports have so far appeared describing its application to carbohydrates. It has, however, been used for the determination of neutral sugars and hexosamines in glycoproteins (Mullinax, Mullinax, Cohen, Cromwell & Deboe, 1971) although no peak for sialic acid could be obtained. Trimethylsilylimidazole is also useful for conditions in which derivative formation of monosaccharides is normally difficult, for example in aqueous solution (van Ling, 1969) and in syrups (Sennello, 1971).

Gas–liquid chromatography

Temperature programming. The chosen conditions give the most satisfactory separation of all monosaccharides within a reasonable time. Good resolution of mannose and galactose (as the methyl glycosides) is achieved, and the last peak (*N*-acetylneuraminic acid) emerges after approx. 2h. Lower initial temperatures improve the separation, but greatly increase the duration of the chromatogram, whereas a higher initial temperature or faster programme rate (1°C/min) results in considerable loss of resolution.

Column packing. Although SE-30 is recommended as a suitable liquid phase, other similar methyl silicone gum rubbers such as OV-1 are also satisfactory. However, the percentage loading of liquid phase is an important consideration, and the most satisfactory results are obtained with a 3–4% loading. Columns of lower loading (1–2%) were tried, but the separation of neutral sugars was poor and marked tailing of the acetamidohexose peaks occurred, although the response factors remained the same, as shown in Table 1. The use of columns having a liquid-phase loading of greater than 4% resulted in poor resolution and increased retention times.

Effect of common contaminants on monosaccharide analysis

Organic contaminants. Organic contaminants such as those used in buffer systems may not interfere with methanolysis or trimethylsilylation, but may produce peaks in important areas of the chromatogram. The positions of the peaks depend on whether the sample is methanolysed first or chromatographed after direct trimethylsilylation. In this g.l.c. system, after methanolysis tris gives two peaks that are superimposed on the methyl fucoside triplet, whereas citrate gives two peaks that appear in the solvent complex and do not interfere with any monosaccharides, although they might interfere in systems that commence at a lower temperature. On direct trimethylsilylation, however, tris emerges as a single peak in the solvent complex, whereas the single peak of citrate coincides with the first mannose peak. This has also been reported by Langley (1968), who found that in his system succinate was eluted before fucose and was therefore suitable for use in buffer systems. The choice of buffer is therefore an important consideration in experiments involving g.l.c. Carbohydrates themselves can occur as contaminants, often arising from various separation systems. Glucose is often found in samples fractionated on Sephadex or cellulose, including the ion-exchange forms of both, and xylose can be found after separations on paper. Pierce & Liao (1968) also found xylose as a contaminant from cellulose ion-exchange resins. The other major source of organic contamination associated with biological material is lipids, some of which emerge in the region of the hexoses and acetamidohexoses.

Salts. The presence of inorganic salts such as sodium chloride does not appear to affect methanolysis, but does interfere with trimethylsilylation. This may be because sodium chloride, although relatively insoluble in methanolic hydrochloric acid, is appreciably dissolved by the acetic anhydride added for re-*N*-acetylation, and can therefore be carried through to the trimethylsilylation stage. This stage appears to be affected because, on g.l.c.,

samples contaminated with salt give complex chromatograms containing many extra peaks. This problem may be overcome to a large extent by the addition of excess of trimethylsilylating agent, and chromatograms of samples treated in this way revert to the expected pattern. Small amounts of salt can be tolerated and response factors determined in the presence of up to 0.5mg of sodium chloride are the same as those shown in Table 1. In the presence of greater quantities, however, the response factors, especially for acetamidohexoses, are low and it is therefore more satisfactory to remove the salt before analysis.

Water. Methanolic hydrochloric acid that contains appreciable quantities of water can cause the loss of methyl glycoside and internal-standard peaks on g.l.c. Little or no loss occurs in methanolic hydrochloric acid containing up to 2% (v/v) water. Destruction increases thereafter as the proportion of water is raised, so that *N*-acetylneuraminic acid is completely lost at water concentrations of about 40% (v/v), internal standard at about 60% (v/v), acetamidohexoses at about 70% (v/v) and neutral sugars at about 90% (v/v). The loss of methyl glycosides is accompanied by the appearance of new peaks in the chromatogram, which correspond to the reducing sugars. The quantity of reducing sugar detected, however, is small and does not account for the proportion of methyl glycosides lost.

In addition to the methanolysis step, the trimethylsilylation reaction is also extremely sensitive to the presence of water. If the reagent itself becomes contaminated with water, derivative formation of monosaccharides is either incomplete or does not occur at all.

Various proportions of water were added to the reagent and the mixture was then used for trimethylsilylation. Water concentrations as low as 0.2% (v/v) caused considerable losses of all monosaccharides. Concentrations greater than 2% (v/v) resulted in total loss of internal standard, acetamidohexose and *N*-acetylneuraminic acid, and no peaks were found at all when the water concentration exceeded 5% (v/v).

Alternatively, contamination may occur after trimethylsilylation is complete. This causes rapid hydrolysis of the monosaccharide derivatives and loss of peaks on g.l.c The addition of 2% (v/v) water to a sample in which trimethylsilylation had been proceeding for $\frac{1}{2}$h resulted in total loss of *N*-acetylneuraminic acid within $1\frac{1}{2}$h, acetamidohexoses within 4h and neutral sugars within 6h. Similarly, the addition of 3% (v/v) water resulted in total loss of all peaks within 4h. Under anhydrous conditions, these derivatives are stable for at least 24h (Clamp *et al.* 1967). Indication that hydrolysis might have occurred is given by the loss of the multiple small peaks on the tail of the initial solvent peak. The addition of more trimethylsilylating agent to such samples can give satisfactory results although, as was found with samples containing sodium chloride, quantitative recovery of acetamidohexoses is not always obtained.

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REFERENCES

Albersheim, P., Nevins, D. J., English, P. D. & Karr, A. (1967). *Carbohyd. Res.* **5**, 340.
Bhatti, T., Chambers, R. E. & Clamp, J. R. (1970). *Biochim. biophys. Acta*, **222**, 339.
Boas, N. F. (1953). *J. biol. Chem.* **204**, 553.
Chambaz, E. M. & Horning, E. C. (1967). *Analyt. Lett.* **1**, 201.
Chambaz, E. M. & Horning, E. C. (1969). *Analyt. Biochem.* **30**, 7.
Chan, D., Hull, E. W., Fields, M. & McNall, E. (1967). *Clin. Chem.* **13**, 506.
Clamp, J. R., Dawson, G. & Hough, L. (1967). *Biochim. biophys. Acta*, **148**, 342.
Clamp, J. R. & Scott, J. E. (1969). *Chemy Ind.* p. 652.
Craven, D. A. & Gehrke, C. W. (1968). *J. Chromat.* **37**, 414.
Entlicher, G. & BeMiller, J. N. (1971). *Carbohyd. Res.* **16**, 363.
Foster, A. B., Horton, D. & Stacey, M. (1957). *J. chem. Soc.* p. 81.
Gottschalk, A. & Ada, G. L. (1956). *Biochem. J.* **62**, 681.
Jeanloz, R. W. & Jeanloz, D. A. (1964). *Biochemistry, Easton*, **3**, 121.
Johansen, P. G., Marshall, R. D. & Neuberger, A. (1960). *Biochem. J.* **77**, 239.
Kärkkäinen, J. & Vihko, R. (1969). *Carbohyd. Res.* **10**, 113.
Langley, T. J. (1968). *Archs Biochem. Biophys.* **128**, 304.
Levvy, G. A., Hay, A. J., Conchie, J. & Strachan, I. (1970). *Biochim. biophys. Acta*, **222**, 333.
Ludowieg, J. & Dorfman, A. (1960). *Biochim. biophys. Acta*, **38**, 212.
Michon, J. & Bourrillon, R. (1959). *Bull. Soc. Chim. biol.* **41**, 277.
Moggridge, R. C. G. & Neuberger, A. (1938). *J. chem. Soc.* p. 745.
Morell, S., Baur, L. & Link, K. P. (1934). *J. biol. Chem.* **105**, 1.
Mullinax, F., Mullinax, G. L., Cohen, M. R., Cromwell, C. L. & Deboe, J. (1971). *Immunochemistry*, **8**, 551.
Niedermeier, W. (1971). *Analyt. Biochem.* **40**, 465.
Nozawa, Y., Hiraguri, Y. & Ito, Y. (1969*b*). *J. Chromat.* **45**, 244.
Nozawa, Y., Uesaka, H., Suzuki, H. & Ito, Y. (1969*a*). *J. Chromat.* **43**, 528.
Perry, M. B., Adams, G. A. & Shaw, D. H. (1969). *J. Chromat.* **44**, 614.
Pierce, J. G. & Liao, T. H. (1968). *Analyt. Biochem.* **24**, 448.

Reeves, R. E., Schwartz, W. M. & Giddens, J. E. (1946). *J. Am. chem. Soc.* **68**, 1383.
Schrager, J. & Oates, M. D. G. (1968). *Biochem. J.* **106**, 523.
Sennello, L. T. (1971). *J. Chromat.* **56**, 121.
Sorensen, M. (1938). *C. r. Trav. Lab. Carlsberg*, **22**, 487.
Stimson, W. H. (1971). *FEBS Lett.* **13**, 17.
Strange, R. E. & Powell, J. F. (1954). *Biochem. J.* **58**, 80.
Suttajit, M. & Winzler, R. J. (1971). *J. biol. Chem.* **246**, 3398.
Swann, D. A. & Balazs, E. A. (1966). *Biochim. biophys. Acta*, **130**, 112.
Sweeley, C. C., Bentley, R., Makita, M. & Wells, W. W. (1963). *J. Am. chem. Soc.* **85**, 2497.
Sweeley, C. C. & Dawson, G. (1969). *Biochem. biophys. Res. Commun.* **37**, 6.
Sweeley, C. C. & Walker, B. (1964). *Analyt. Chem.* **36**, 1461.
van Ling, G. (1969). *J. Chromat.* **44**, 175.
Williams, D. T. & Perry, M. B. (1969). *Can. J. Biochem.* **47**, 983.
Wolfrom, M. L. & Kashimura, N. (1969). *Carbohyd. Res.* **11**, 151.
Wulff, G. (1965). *J. Chromat.* **18**, 285.
Yu, R. K. & Ledeen, R. W. (1970). *J. Lipid Res.* **11**, 506.