Journal of Chromatography, 180 (1979) 69–82

CHROM. 12,172

METHYLATION ANALYSIS IN GLYCOPROTEIN CHEMISTRY

GENERAL PROCEDURE FOR QUANTIFICATION OF THE PRODUCTS OF SOLVOLYSIS OF PERMETHYLATED GLYCOPEPTIDES AND GLYCOPROTEINS

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(Received June 25th, 1979)

SUMMARY

A technique for the gas chromatographic analysis of the products of solvolysis of permethylated glycopeptides and glycoproteins has been developed. It involves methanolysis of a permethylated compound, quantitative transformation of methyl ethers of methyl glycosides into the corresponding O-trimethylsilyl(TMS)-O-methylalditols [2-deoxy-2-(N-methyl)acetamido-O-TMS-O-methylalditols in the case of hexosamine derivatives] and gas chromatographic quantification using a single column packed with 0.4% OV-225 on surface-modified Chromosorb.

INTRODUCTION

In a previous paper[1] we reported the gas chromatographic (GC) conditions that allow the separation and identification of the methylated derivatives of mannose, galactose and N-acetylglucosamine as the corresponding O-trimethylsilyl(TMS)-O-methyl(N-acetyl-N-methyl)alditols. In this paper, quantitative GC parameters (relative responses) of these compounds are presented. Using proton-catalysed methanolysis to cleave glycosidic linkages and GC analysis to monitor the completeness of the stages of transformation of the methyl ethers of methyl glycosides into the corresponding O-TMS-O-methylalditols, we have succeeded in elaborating a general procedure for the quantification of the methylated sugar derivatives that are obtained in the solvolysis of permethylated glycoproteins and glycopeptides.

EXPERIMENTAL

Materials

Methyl ethers of methyl-α-D-mannopyranoside, methyl-α-D-galactopyranoside and methyl-2-deoxy-2-(N-methyl)acetamido-α-D-glucopyranoside were prepared as described previously[1]. Lactose, arabitol and mannitol used were of "reinst"

grade (Serva, Heidelberg, G.F.R.). Five-times recrystallized ovalbumin was purchased from Reakhim (Olaine, U.S.S.R.). A total glycopeptide fraction of the pronase digest (with Pronase P; Serva) of ovalbumin was isolated by gel chromatography on a column packed with Bio-Gel P-6 (Bio-Rad Labs, Richmond, Calif., U.S.A.) and equilibrated with 0.1 *M* ammonium hydrocarbonate solution. The monosaccharide composition of the glycopeptide fraction was shown to be the same as that of the parent glycoprotein, and the major amino acid found was asparagine.

A 2 *N* solution of trifluoroacetic acid was prepared by dilution of the sequenal-grade reagent (Beckman Instruments, Palo Alto, Calif., U.S.A.) with water doubly distilled in an all-glass apparatus. A 4 *N* solution of hydrochloric acid was prepared from constant-boiling hydrochloric acid redistilled under freshly recrystallized tin (II) chloride. Other reagents were purified as described previously[2].

General methods

All evaporations were carried out using a rotary evaporator equipped with a water aspirator, the bath temperature being 30–35°. Methanolysis and hydrolysis steps were performed in sealed, thoroughly evacuated glass ampoules at 100° (if not stated otherwise).

Chromatographic conditions

Gas chromatography was performed with a Chrom 41 instrument (Laboratorní Přistroje, Prague, Czecoslovakia) equipped with flame-ionization detectors, a linear temperature programmer and an electromechanical integrator. A coiled glass column (300 cm × 2.5 mm I.D.) was silanized and packed with 0.4% OV-225 on surface-modified[3] Chromosorb W AW, 80–100 mesh (Johns-Manville, Denver, Colo., U.S.A.). The packing was prepared as described previously[1]. The carrier gas was helium at a flow-rate of 40 ml/min. The temperatures of the injection port and the detector were 180° and 250°, respectively. After the injection of a sample the column was kept at 130° for 2 min and then heated to 200° at a rate of 1°/min.

Permethylation

The samples (0.1–2 mg of carbohydrates) dried over phosphorus pentoxide *in vacuo* for 3–5 days were dissolved in dimethylsulphoxide and permethylated according to Hakomori[4] as described by Lindberg[5]. Ovalbumin and the glycopeptides derived from it were subjected to prior N-acetylation[6]. With lactose and the glycopeptides the permethylated products were extracted from the reaction mixture with chloroform[5], and with ovalbumin continuous dialysis, first against running tap water and then against distilled water, was employed to isolate the methylated glycoprotein. With ovalbumin the methylation procedure was repeated twice.

Methanolysis and hydrolyses

A permethylated sample in water was placed in an ampoule, evaporated to dryness and then dried for 24–48 h over phosphorus pentoxide *in vacuo*. Methanolysis with 1–2 ml of 0.5 *N* hydrochloric acid in anhydrous methanol was carried out for 24 h[2]. Then the ampoule seal was broken, insoluble material (if present) was separated by centrifugation at 1500–2000 *g* for 5–10 min, washed with methanol (three 0.5-ml volumes) and the supernatant combined with the methanol washings

was transferred to another ampoule. Pyridine (50–100 μl) was added and the solution was evaporated almost to dryness (this step is critical; the use of an oil vacuum pump or a bath with a temperature above 35° as well as complete evaporation of the solvent may result in significant losses of volatile methyl ethers of methyl glycosides).

Hydrolysis with 1–2 ml of 2*N* trifluoroacetic acid was performed for 6 h. Then the acid was evaporated, the sample was dissolved in 2 ml of 50% aqueous methanol and applied on a column (20 × 0.5 cm) packed with freshly regenerated Dowex 50W-X2 (H^+) resin, (100–200 mesh; Serva) and equilibrated with 50% methanol. Derivatives of neutral sugars were eluted with 50% methanol and methyl ethers of methylglucosaminide with 2 *N* ammonia solution in 50% methanol[7]. Then methylglucosaminide derivatives were placed in an ampoule, evaporated to dryness and hydrolysed with 1–2 ml of 4 *N* hydrochloric acid for 3 h.

Reduction

The methylated monosaccharides (derivatives of neutral sugars and those of glucosamine separately or together) were placed in a small flask, evaporated to dryness and the residue was dissolved in 0.5–1 ml of water, the pH was adjusted to 9–10 with a few drops of dilute sodium hydroxide solution and then 10–20 mg of sodium borohydride were added. After 3–16 h at 0–4° the excess of borohydride was destroyed with a few drops of 50% acetic acid (to pH *ca.* 5). Methanol was added to a 50% concentration and the sample was applied to a Dowex column (the same as above). Methylated derivatives of neutral sugars were eluted with 50% methanol; boric acid was removed by repeated co-evaporation with acidified methanol[8].

Methylated derivatives of glucosamine were eluted with 2 *N* ammonia solution in 50% methanol and, after evaporation, were combined with derivatives of neutral sugars. A suitable amount of an internal standard solution (mannitol and/or arabitol) was added and the mixture was evaporated to dryness and kept in a vacuum desiccator over phosphorus pentoxide at least overnight.

Trimethylsilylation and re-N-acetylation

A 100–500-μl volume of silylating mixture[9] was added to the sample. The mixture was stirred and left for 30 min at room temperature, then acetic anhydride was added (about one tenth of the volume of the silylating mixture) and the mixture was left for a further 30 min. A 1–5-μl volume of the mixture was injected directly on to the chromatographic column.

RESULTS

Methanolysis

Quantitative degradation of carbohydrate moieties of glycoproteins to monosaccharide units is known to be a complicated task. With permethylated glycopeptides and glycoproteins extra difficulties arise owing to the possible side reactions resulting in partial demethylation of the products of solvolysis and the very high resistance of the glycosidic linkages formed by N-methylhexosamine residues to acid solvolysis[5,10]. To overcome these difficulties various solvolytic conditions have been proposed[5,6,10–12].

Acetolysis with 95% acetic acid containing 0.5 *N* sulphuric acid[10] has been most frequently employed.

Proton-catalysed methanolysis, first applied to glycoproteins by Clamp *et al.*[13], is now being increasingly used for the quantitative cleavage of glycosidic linkages in glycoproteins. The use of methanolysis to degrade the methylated oligosaccharides[14,15] demonstrated that the side processes of destruction and, in particular, demethylation of monosaccharide units are less pronounced in this instance when compared with acid hydrolysis. However, in structural investigations of carbohydrate moieties of glycoproteins methanolysis of permethylated glycopeptides has not yet become a conventional procedure. Stellner *et al.*[10] claimed that in methanolysis complete cleavage of linkages formed by hexosamines residues had not occurred. Other investigators used methanolysis to cleave only labile residues of N-acetylneuraminic acid[16]. However, Montreuil and co-workers developed a GC technique for the analysis of methylated methyl glycosides[17–20], which, in combination with methanolysis, has been used successfully in investigations of complex oligosaccharides derived from glycoproteins[21–23].

In our preliminary experiments on the methylation analysis of the glycopeptide fraction of pronase digest of ovalbumin, it was found that methanolysis gives complete cleavage of all glycosidic linkages, with no demethylation accompanying the solvolysis. The N-acetyl groups of the N-acetylglucosamine residues remained intact, which was apparently the result of the N-methylation.

The problem was investigated in more detail using the standard methyl ethers of methyl N-acetyl-N-methylglucosaminide (3,4,6-tri-O-methyl, 3,6- and 3,4-di-O-methyl and 3-mono-O-methyl derivatives). As can be seen from Fig. 1a and b, in methanolysis with 0.5 *N* hydrochloric acid in anhydrous methanol no de-N-acetylation occurs. Quantitative de-N-acetylation of the methylated methylglucosaminide derivatives took place when heating with 2 *N* trifluoroacetic acid for 6 h at 100°, *i.e.*, under conditions providing quantitative hydrolysis of the methylated methyl glycosides of mannose and galactose (see below). This is illustrated in Fig. 1c and d.

To examine the stability of methylated sugar derivatives towards methanolysis, series of samples of standard methyl ethers of methyl glycosides were heated with methanolic hydrochloric acid in the presence of arabitol as an internal standard and then analysed using gas chromatography of the O-TMS (N-acetyl) derivatives. The chromatograms of these samples showed no peaks due to derivatives whose degree of methylation (number of methoxy groups in a molecule) was lower than that of an authentic compound. This confirmed the absence of the demethylation under the conditions used. Destruction of the methylated derivatives in the methanolysis was insignificant (Table I).

Hydrolysis of methyl glycosides

Methylated methyl glycosides may be analysed directly after acetylation or trimethylsilylation of free hydroxyl groups and N-acetylation of derivatives of hexosamines[19,20]. However, as was mentioned previously[1], complete separation of a large number of such closely related compounds is difficult to achieve even when employing high-efficiency packed columns. The conversion of methylated methyl glycosides into the corresponding O-TMS-O-methylalditols seems to be a rational

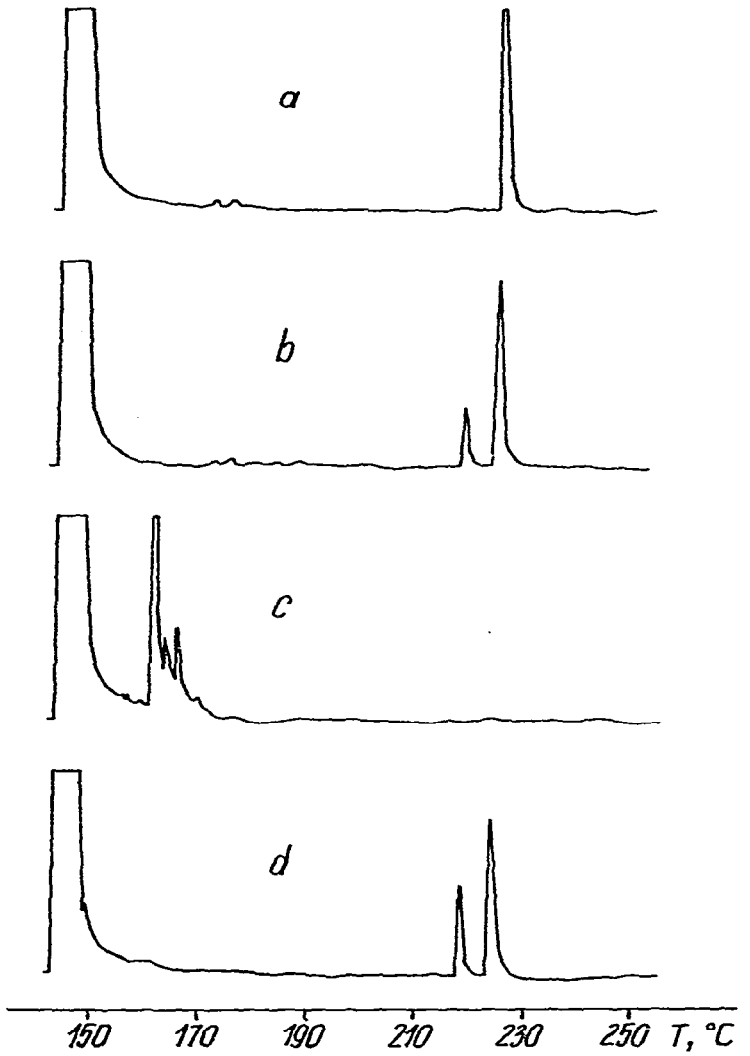


Fig. 1. Stability of N-acetyl group of methyl 3-mono-O-methyl-N-acetyl-N-methyl-α-D-glucosaminide towards solvolysis. (a) Chromatogram of the authentic compound; (b) chromatogram after methanolysis with 0.5 *N* methanolic HCl for 24 h at 100°; (c) chromatogram after hydrolysis with 2 *N* CF_3COOH for 6 h at 100°; (d) same chromatogram as in (c), but with re-N-acetylation using co-injection of acetic anhydride. The column (see Experimental) was heated at a rate of 4°/min.

approach. Hydrolysis of methyl glycosides evidently ought to be the first stage of a modification procedure.

In a search for the optimal conditions for performing this stage, we studied the hydrolysis of a series of the standard methyl ethers of methyl glycosides of mannose, galactose and N-acetylglucosamine with sulphuric, hydrochloric and trifluoroacetic acids of various concentration with different temperatures and durations of the hydrolysis. With mineral acids of concentration 1–4 *N* and in the temperature range from 80 to 110°, we failed to find suitable conditions for the hydrolysis of methylated methyl glycosides of the neutral sugars. The rate of hydrolysis depends

TABLE I

STABILITY OF METHYL O-METHYLGLYCOSIDES TOWARDS METHANOLYSIS WITH 0.5 *N* HYDROCHLORIC ACID IN ANHYDROUS METHANOL FOR 24 h AT 100°

Parent monosaccharide	*Location of methoxy groups*	*Yield (%)* *
Mannose	2, 3, 4, 6	96
	2, 4, 6	101
	2, 3, 4	98
	3, 6	98
	2, 4	97
	6	100
	2	95
Galactose	2, 3, 4, 6	95
	2, 3, 4	94
	3, 4, 6	98
N-Acetylglucosamine	3, 4, 6	102
	3, 6	100
	3, 4	100
	3	96

* The yield was determined as the ratio of the sum of the relative areas of the peaks of anomers obtained by methanolysis to the relative area of the peak of the parent methyl-α-D-glycoside.

on the nature of a parent sugar and decreases with increase in the degree of methylation. For example, heating with 2 *N* sulphuric acid for 3 h at 100° afforded complete hydrolysis of the methylated galactosides, but the extent of hydrolysis of permethylated methyl mannoside was only 30%, of trimethyl derivatives of mannoside 40–60%, of dimethyl derivatives 50–70% and of monomethyl derivatives 70–90%. Further increases in the acid concentration, temperature or duration of the hydrolysis led to considerable destruction of methyl glycosides. With hydrochloric acid the processes of destruction and demethylation were more pronounced.

Hydrolysis with trifluoroacetic acid[24] is widely used for the cleavage of glycosidic linkages in oligosaccharides and glycoproteins. Recently, we have also reported[25] that heating with 2 *N* trifluoroacetic acid for 6 h at 100° results in the quantitative cleavage of glycosidic linkages formed by neutral sugar residues in the oligosaccharide chains of some glycopeptides and glycoproteins. These conditions happened to be optimal for the hydrolysis of methylated methyl glycosides of mannose and galactose also. The hydrolysis with trifluoroacetic acid is not accompanied by demethylation or destruction of methylated methyl glycosides. On varying duration of the hydrolysis, we found that the rate of hydrolysis depended on the degree of methylation in the opposite manner to hydrolysis with mineral acids, *i.e.*, the maximal rate occurred with permethylated compounds and the minimal rate with monomethyl derivatives (Fig. 2). Nevertheless, quantitative hydrolysis of all methylated methyl mannosides and methyl galactosides is achieved in 6 h, the destruction of the monosaccharides formed being negligible.

As has already been mentioned, on heating with trifluoroacetic acid under the conditions indicated, complete de-N-acetylation of methylated methyl N-acetyl-N-methylglucosaminides occurs. In this instance, glycosidic linkages are not hydrolysed, evidently due to the formation of a strongly charged secondary amino group

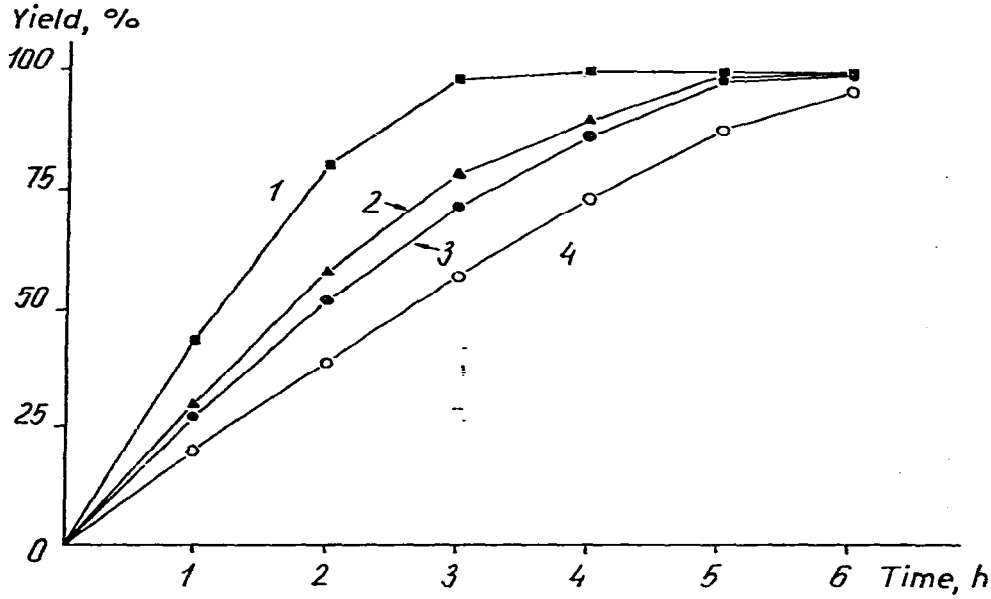


Fig. 2. Hydrolysis of methylated methyl-α-D-mannopyranosides with 2 *N* CF_3COOH at 100°. (1) 2,3,4,6-Tetra-O-methylmannoside; (2) 2,4,6-tri-O-methylmannoside; (3) 3,6-di-O-methylmannoside; (4) 2-mono-O-methylmannoside.

at the neighbouring position. This requires severer conditions for the hydrolysis of the methylated methylglucosaminides. We found heating with 4 *N* hydrochloric acid for 3 h at 100° to be optimal. Under these conditions, the methylated glucosaminides are hydrolysed quantitatively (Table II), with demethylation virtually absent.

TABLE II

HYDROLYSIS OF METHYLATED N-ACETYLGLUCOSAMINIDES WITH 4 *N* HYDROCHLORIC ACID FOR 3 h AT 100°

Location of methoxy groups	*Completeness of hydrolysis (%)* *
3, 4, 6	98
3, 6	100
3, 4	97
3	96

* Determined as the ratio of the peak area of the methylated monosaccharide to the sum of the areas of the peaks of the methylated monosaccharide and the methylated methyl glycoside.

Thus, for the hydrolysis of methylated methyl glycosides of neutral sugars and those of hexosamines different condititons are required, which necessitates the preliminary separation of these compounds. De-N-acetylation of methylglucosaminides on heating with trifluoroacetic acid favours the use of cation-exchange resins for this purpose.

Reduction

Reduction with sodium borohydride is a common procedure for the conversion of methylated sugars into the corresponding O-methylalditols. Nevertheless,

there is no agreement in the literature about definite conditions for this stage with respect to pH of the medium, reaction time, ratio of reagents, etc.[5,7,10]. Our experience is that the optimal conditions providing quantitative reduction of methylated derivatives of mannose, galactose and glucosamine are the following: 100–500-fold molar excess of sodium borohydride, pH 9–10, 3 h at 0–4°.

Trimethylsilylation and re-N-acetylation

In a previous paper[1], we showed that complete separation of the mannose and galactose derivatives is achieved when they are analysed as O-TMS-O-methylalditols, whereas methylated derivatives of glucosamine can be analysed both as 2-deoxy-2-(N-methyl)acetamido-O-acetyl-O-methylalditols and as the corresponding N-acetyl-O-TMS derivatives. The use of the trimethylsilyl protection of free hydroxyl groups is preferable, as it allows the simultaneous analysis of methylated derivatives of neutral sugars in a single GC run. Without the re-N-acetylation, the glucosamine derivatives tend to decompose on the GC column[1] and their peaks interfere with those of the neutral sugar derivatives. Treatment with acetic anhydride in the silylating mixture is a convenient one-step procedure for the protection of hydroxy and amino groups.

The presence of even moderate amounts of inorganic salts in a sample hinders the modification, distorting the quantitative data and often leading to non-interpretable chromatograms. Hence the stage of reduction of monosaccharides must be followed by ion-exchange clean-up and removal of boric acid from the neutral sugar fraction.

It has been reported[26] that alditols have limited solubility in pyridine, which may result in incomplete trimethylsilylation and the appearance of several peaks for one compound in a chromatogram. We have not attempted a special investigation of the solubility in pyridine of methylated alditols or of the arabitol and mannitol used as internal standards. Our experience is that with *ca.* 100 μl of the silylating mixture per 1–2 μmol of alditols no difficulties associated with limited solubility arise.

Quantification

Fig. 3 is a chromatogram of a mixture of O-TMS-O-methylamnnitols, O-TMS-O-methylgalactitols and 2-deoxy-2-(N-methyl)acetamido-O-TMS-O-methylglucitols, illustrating the possibility of using GC for the analysis of the products of the solvolysis of permethylated oligosaccharide components of glycoproteins. Good separation of the peaks allows the unambiguous identification and reliable quantification of methylated sugars using GC only.

An accurate GC quantification must be based on the determination of reliable detector responses for individual methylated sugars. There is no agreement in the literature on this question. Some authors[5,27] claimed that relative molar responses (RMRs) can be considered to be identical for all of the methylated derivatives of hexoses regardless of the degree of methylation. An alternative opinion[20], which possibly reflects some shortcomings of the techniques used, was that even for isomers with an identical degree of methylation, the RMR values were different, with no correlations between the number of methoxy groups in a molecule and an RMR value. However, from the recent work by Conchie and Strachan[28], in which special

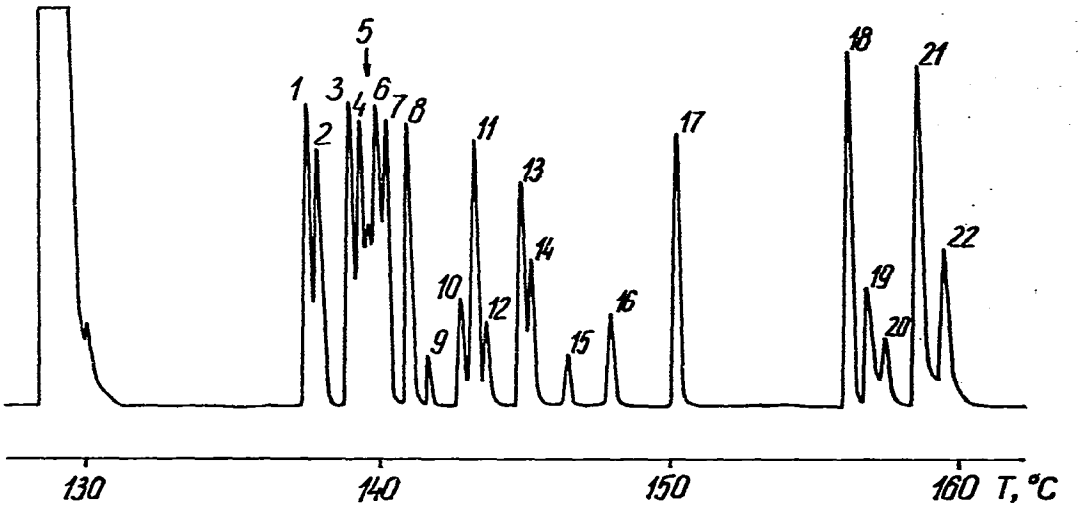


Fig. 3. GC separation of a mixture of methylated derivatives of mannose, galactose and N-acetylglucosamine as the corresponding O-TMS-O-methylalditols and 2-deoxy-2-(N-methyl)acetamido-O-TMS-O-methylalditols. 1 = 2,3,4,6-Tetra-O-methyl-; 3 = 2,4,6-tri-O-methyl-; 4 = 2,3,6-tri-O-methyl-; 8 = 3,4,6-tri-O-methyl-; 10 = 2,3,4-tri-O-methyl-; 11 = 3,6-di-O-methyl-; 12 = 4,6-di-O-methyl-; 13 = 3,4-di-O-methyl-; 14 = 2,4-di-O-methyl-; 15 = 2,6-di-O-methyl-; 16 = 6-mono-O-methyl-; 17 = 2-mono-O-methylmannitols. 2 = 2,3,4,6-Tetra-O-methyl-; 5 = 2,4,6-tri-O-methyl-; 6 = 2,3,6-tri-O-methyl-; 7 = 3,4,6-tri-O-methyl-; 9 = 2,3,4-tri-O-methylgalactitols. 19 = 3,4,6-Tri-O-methyl-; 20 = 3,6-di-O-methyl-; 21 = 3,4-di-O-methyl-; 22 = 3-mono-O-methyl-2-deoxy-2-(N-methyl)acetamidoglucitols. 18 = Mannitol (internal standard).

attention was paid to the quantitative aspects of the analytical procedure used, it follows that (i) RMR values can be regarded as identical for isomers with the same degree of methylation, and (ii) RMR values vary with the number of methoxy groups in the molecules of methylated sugars.

Having developed a reliable procedure for the quantitative conversion of methyl O-methylglycosides into the corresponding O-TMS-O-methylalditols, we carried out a detailed investigation. For this purpose, a series of samples of the standard methyl ethers of methyl glycosides that underwent all stages of the proposed modification procedure, including methanolysis, were analysed and RMRs were determined for individual methylated derivatives. The results of this investigation with tetra- and trimethyl derivatives of mannose and galactose as examples are presented in Table III. From these data it follows that, within experimental error, the RMRs for the isomeric compounds with equal degrees of methylation can be considered identical. The RMRs of dimethyl and monomethyl derivatives, determined in the same manner, are 0.81 ± 0.09 and 0.89 ± 0.09, respectively. Hence the dependence of the RMRs of O-TMS-O-methylalditols on the number of methoxy groups in the molecules is clear, *i.e.*, the RMR values decrease as the degree of methylation increases.

With methylated derivatives of N-acetylglucosamine with different degrees of methylation, the RMRs differ only slightly and, within experimental error (as great as *ca.* 15%), are equal to 0.65. This may be associated with partial destruction of the derivatives of glucosamine under severe hydrolysis conditions.

TABLE III

RELATIVE MOLAR RESPONSES WITH RESPECT TO MANNITOL FOR TETRA- AND TRI-O-METHYL-O-TMS-ALDITOLS

Based on ten determinations.

Parent monosaccharide	*Location of methoxy groups*	*RMR**	*Average RMR**
Mannose	2, 3, 4, 6	0.60 ± 0.07	0.62 ± 0.07
Galactose	2, 3, 4, 6	0.63 ± 0.07	
Mannose	2, 3, 6	0.70 ± 0.05	0.71 ± 0.09
	2, 4, 6	0.74 ± 0.08	
	3, 4, 6	0.72 ± 0.06	
	2, 3, 4	0.68 ± 0.06	
Galactose	2, 4, 6	0.72 ± 0.06	
	2, 3, 6	0.71 ± 0.07	
	3, 4, 6	0.71 ± 0.09	
	2, 3, 4	0.67 ± 0.09	

* RMR ± mean relative error.

It should be emphasized that the RMR values obtained in the described manner together with the mean errors reflect the accuracy and reproducibility of the procedure developed as a whole.

Methylation of lactose

The analysis of the methanolysis products of permethylated lactose (1 μmol) as O-TMS-O-methylalditols (Fig. 4) confirmed that the RMRs for derivatives of methylated hexoses with equal degrees of methylation were independent of the nature of the parent monosaccharide residue and the location of methoxy groups. The use of the mean RMR values given in Table III for the quantification of the 2,3,4,6-tetra-O-methyl derivative of galactose and the 2,3,6-tri-O-methyl derivative

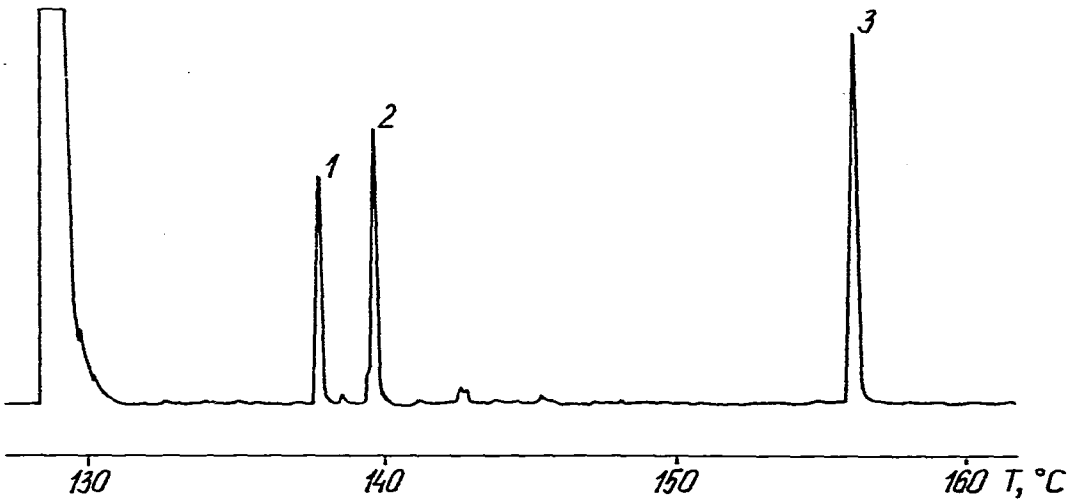


Fig. 4. Analysis of the products of the methanolysis of permethylated lactose as O-TMS-O-methylalditols. 1 = 2,3,4,6-Tetra-O-methylgalactitol; 2 = 2,3,6-tri-O-methylglucitol; 3 = mannitol.

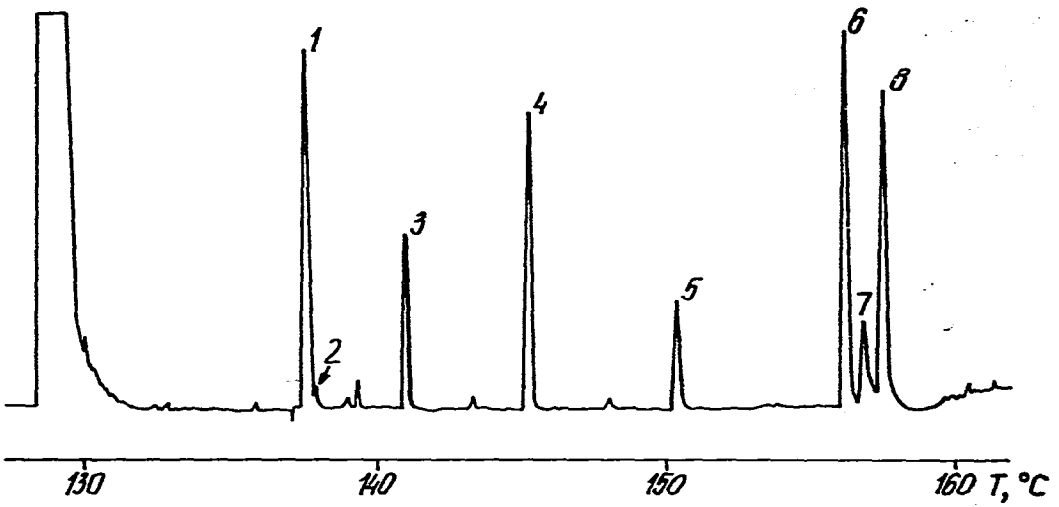


Fig. 5. Analysis of the products of the methanolysis of permethylated ovalbumin. 1 = 2,3,4,6-Tetra-O-methyl-; 3 = 3,4,6-tri-O-methyl-; 4 = 2,4-di-O-methyl-; 5 = 2-mono-O-methylmannitols. 2 = 2,3,4,6-Tetra-O-methylgalactitol; 7 = 3,4,6-tri-O-methyl-; 8 = 3,6-di-O-methyl-2-deoxy-2-(N-methyl)acetamidoglucitols. 6 = Mannitol.

of glucose gives identical values of 0.52 μmol for both substances. The ratio of galactose to glucose (1.0) exactly reflects the monosaccharide composition of lactose.

The introduction of an internal standard allows one to evaluate the efficiency of isolation of a permethylated sample from the reaction mixture and makes the choice of the method of isolation easier.

Methylation of ovalbumin

Fig. 5 shows a chromatogram of the methanolysis products of permethylated ovalbumin. Although this glycoprotein contains only *ca.* 3% (w/w) of sugars[29], there are no false peaks in the chromatogram. This fact is additional evidence for the preference of methanolysis over other methods of solvolysis. Ovalbumin is known[28,30] to be a typical representative of glycoproteins with pronounced microheterogeneity of the carbohydrate moiety. The yields of methylated derivatives of mannose

TABLE IV

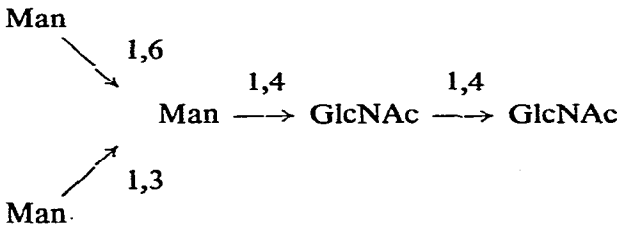
PRODUCTS OF THE METHANOLYSIS OF PERMETHYLATED OVALBUMIN

From 1 μmol of the glycoprotein.

Parent monosaccharide	*Location of methoxy groups*	*Yield (μmol)*
Mannose	2, 3, 4, 6	1.44
	3, 4, 6	0.65
	2, 4	0.95
	2	0.45
		Total: 3.49
N-Acetylglucosamine	3, 4, 6	0.55
	3, 6	1.50
		Total: 2.05

and N-acetylglucosamine expressed in moles per mole of ovalbumin (Table IV) reveals this fact.

The profile of methylated derivatives of mannose and N-acetylglucosamine obtained agrees well with recently established[28] structures of the major glycopeptides of ovalbumin. Thus, the presence of 2,4-di-O-methyl and 3,4,6-tri-O-methyl derivatives of mannose and the predominance of the 3,6-di-O-methyl derivative among the derivatives of N-acetylglucosamine reveals the presence of a typical structural element[31]:



The presence of the 2-mono-O-methyl derivative of mannose indicates that in some molecules there are two branchings at the "core" mannose residue. The terminal (non-reducing) positions of oligosaccharide chains are occupied by mannose and, to a lesser extent, by N-acetylglucosamine residues, affording 2,3,4,6-tetra-O-methyl and 3,4,6-tri-O-methyl derivatives, respectively. In accordance with recently reported data[28,32], all of the galactose residues, present in ovalbumin in minor amounts, occupy terminal positions (2,3,4,6-tetra-O-methyl derivatives only).

The ovalbumin preparation used in this work contained 5.1 mannose residues and 3.0 N-acetylglucosamine residues per molecule of the glycoprotein[2]. As follows from Table IV, the total yields of the methylated derivatives comprise *ca.* 70% of the above values, reflecting a partial loss of the permethylated glycoprotein when treating the mixture after the permethylation. It is important that the ratio of the total amount of the methylated mannoses to that of the methylated N-acetylglucosamines, 1.70, is in good agreement with the monosaccharide composition of ovalbumin [Man/GlcNAc = 1.70 (ref. 2)]. Hence one can conclude that (i) methanolysis of the permethylated glycoprotein led to quantitatvie cleavage of the linkages formed both by neutral sugars residues and by those of glucosamine, and (ii) the technique developed provides for the quantitative transformation of methyl O-methylglycosides into corresponding O-TMS-O-methylalditols and an accurate quantification of methylated sugar derivatives. The data obtained from methylation analysis of the glycopeptide fraction derived from ovalbumin were similar to those obtained with the glycoprotein.

DISCUSSION

A reliable and reproducible technique for the analysis of the products of the solvolysis of permethylated glycopeptides and glycoproteins has been developed. The main stages of the analytical procedure are presented in Fig. 6. The quantitative yields of the stages, the reliability of identification of methylated sugars and the accuracy and sensitivity of their quantitative determination allow one to obtain

detailed information on oligosaccharide structures using very small samples ($\leqslant$ 1 mg of sugars). This leads to the possibility that the technique may become a reasonable alternative to conventional gas chromatography–mass spectrometry techniques[5,10] and may be useful in structural investigations of glycoproteins and other carbohydrate-containing natural compounds.

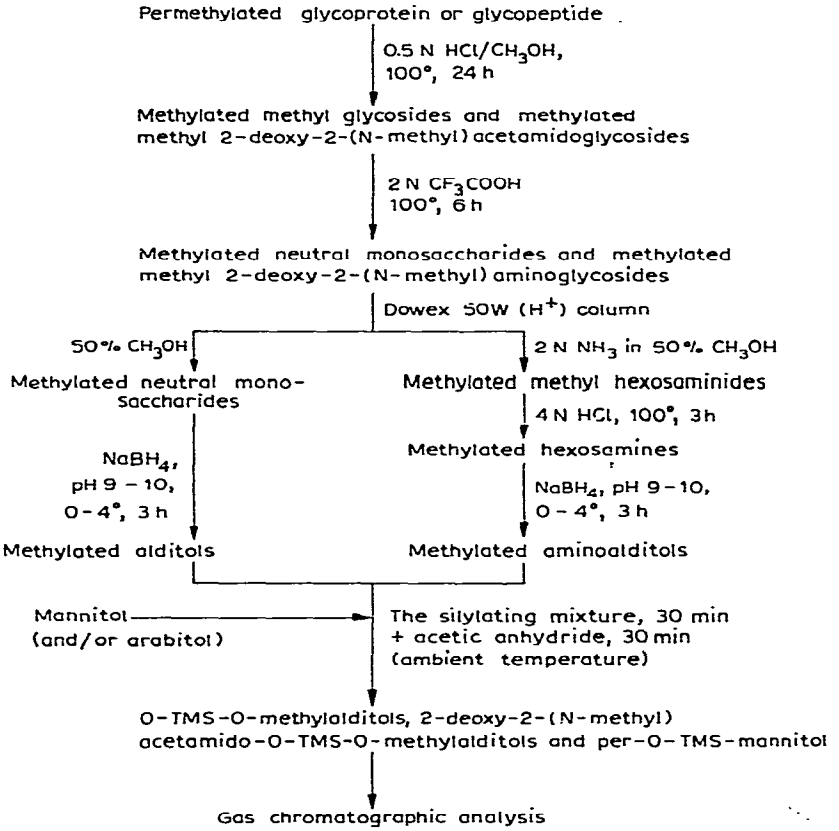


Fig. 6. The main stages of the procedure for the modification of the products of methanolysis of permethylated glycopeptides and glycoproteins for GC analysis.

The general principles of the structural organization of carbohydrate moieties of a number of glycoproteins have already been established[20,31,33,34]. However, investigations revealed a certain individuality in the structures of oligosaccharide chains occurring in a particular glycoprotein molecule.

Structural investigations of glycoproteins are usually associated with laborious stages of separation and isolation of individual glycopeptides or oligosaccharides. The technique developed here permits methylation analysis to be used for obtaining detailed structural information with the glycoprotein directly. The excellent GC separation of the methylated derivatives of the major monosaccharides encountered in glycoproteins together with their quantification allows one to obtain a kind of "fingerprints" of carbohydrate moieties of glycoproteins or glycopeptides and to speculate about their special organization, *i.e.*, (i) the type of structure and the number

of oligosaccharide chains, (ii) the presence and nature of branchings and (iii) the nature of terminal monosaccharide residues. For some purposes (*e.g.*, medical) such "fingerprints" may prove to be sufficient for characterizing a carbohydrate component of a glycoprotein. Moreover, the opportunity of obtaining detailed preliminary information on the nature of oligosaccharide chains facilitates the choise of techniques for the separation of glycopeptides and a strategy for determining the structure of the carbohydrate moiety.

The "fingerprints" of glycopeptides may be useful for estimating localization of individual oligosaccharide chains on a polypeptide chain when establishing the complete covalent structure of a glycoprotein. In combination with methods of selective degradation of native glycoproteins, "fingerprints" may be used in the study of the three-dimensional structures of glycoprotein molecules.

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