Carbohydrate Research, 72 (1979) 257–260

Note

Use of triphenylmethane as an indicator of complete methylation of glycolipids and glycopeptides

HEIKKI RAUVALA

Department of Medical Chemistry, University of Helsinki, Siltavuorenpenger 10A, SF-00170 Helsinki 17 (Finland)

(Received March 29th, 1978; accepted for publication, August 3rd, 1978)

Methylsulphinyl carbanion is used to generate the reactive sugar alkoxides in the Hakomori[1] method, and methylation is then easily accomplished by adding methyl iodide. The extent of methylation can be assessed by the i.r. absorption for hydroxyl[1]. However, for amounts of carbohydrates in the nanomolar or micromolar range, such assessment is not possible. Undermethylation may be caused by an insufficiency of the carbanion, which can be consumed by proton donors other than carbohydrates. The generation of alkoxides may therefore be incomplete, and undermethylated products may result when methyl iodide is added. Glycolipid and glycopeptide saccharides will be completely methylated if there is an excess of the carbanion, and this can be demonstrated after the time needed for alkoxide formation by using triphenylmethane, which gives a red colour in the presence of carbanion. It is not necessary then to exclude water rigorously.

Figs. 1A and 1B show the methylated sugars formed when rat-brain gangliosides were methylated after treatment with an excess (positive triphenylmethane test) and an insufficiency (negative test) of carbanion. The undermethylation shown in Fig. 1B was observed when the amount of the carbanion solution was $\leqq 25\%$ of that needed for a positive triphenylmethane test. Undermethylation was slight or undetectable with larger amounts of the anion. Above the detection limit with triphenylmethane, the ratio of the sugar peaks remained constant when increasing amounts of carbanion were used. Analysis of methylated sugars from simple, neutral glycolipids (a mixture of lactosylceramide and glucosylceramide) and from a glycopeptide mixture of rat brain suggested that the amount of the carbanion needed for complete methylation was somewhat smaller than that required for the ganglioside mixture.

Methylation experiments were performed by using carbanion solution to which $\sim 3\%$ of water had been added, and sufficient of this solution was used to give a positive triphenylmethane test. Large g.l.c. peaks due to non-sugar material were detected in these experiments, but the ratios of the peaks for methylated sugars suggested that no undermethylation had occurred. Addition of 1% of water to the

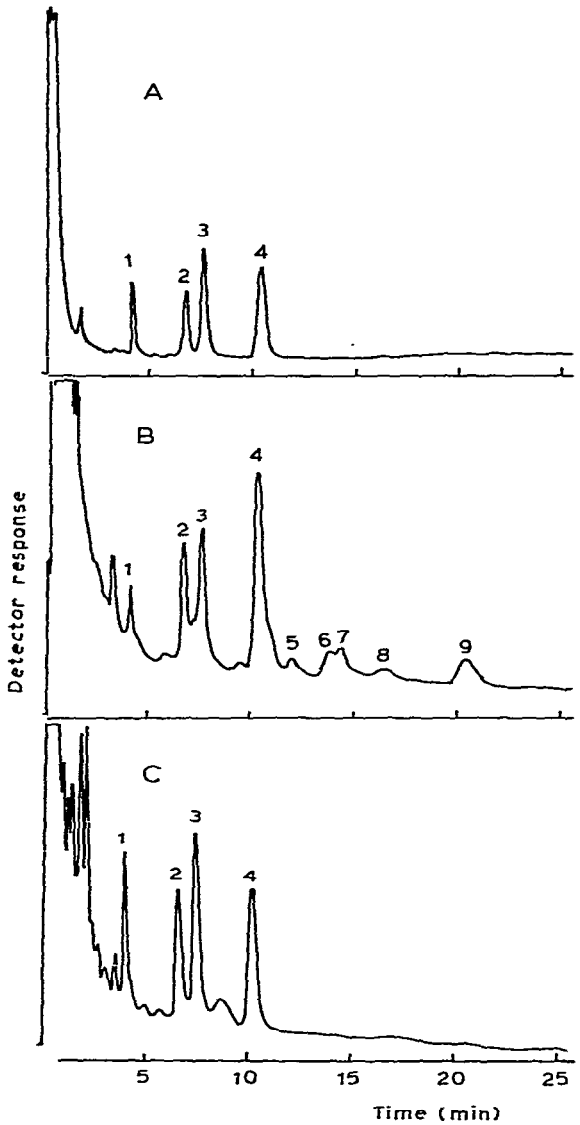

Fig. 1. G.l.c. of methylated hexitol acetates from a mixture of rat-brain gangliosides: *A*, methylation of a reaction mixture showing a positive triphenylmethane test; *B*, methylation of a reaction mixture that was negative in the triphenylmethane test; *C*, methylation in the presence of water (triphenylmethane test positive). Key: 1, 2,3,4,6-Me_4-Gal; 2, 2,4,6-Me_3-Gal; 3, 2,3,6-Me_3-Glc; 4, 2,6-Me_2-Gal; 5, 3,6-Me_2-Hex (the galactose and glucose derivatives are not separated in the conditions used); 6, 6-Me-Gal; 7, 2,3-Me_2-Gal; 8, 2,4-Me_2-Gal +6-Me-Glc; 9, 2-Me-Hex (the galactose and glucose derivatives are not separated). Conditions: 1% OV-225, 175°.

carbanion solution caused a moderate increase in the intensity of the non-sugar peaks (Fig. 1C). Thus, the use of the triphenylmethane test abolishes the possible errors of analysis that are due to wetting and inactivation of the carbanion solution during storage and handling of the reagent. The test can also ensure that only a small excess

of carbanion solution is used, so that the intensity of the g.l.c. peaks that originate from the reagent can be minimised.

Although triphenylmethane has been used as an indicator of methylsulphinyl carbanion[2,3], its utilisation in the methylation procedure has not been shown hitherto. This extremely simple test should be of value in the methylation of compounds that are freely soluble in sodium methylsulphinylmethanide–methyl sulphoxide.

EXPERIMENTAL

A mixture of rat-brain gangliosides was prepared[4] from a chloroform–methanol extract of brain tissue by using chloroform–methanol–water partition. A mixture of lactosyl- and glucosyl-ceramides was prepared from a hematoside isolated from human kidney[5] by mild hydrolysis with acid (0.1M HCl, 100°, 1 h). The acid hydrolysate was passed through a Dowex-1 X8 column, and the glycolipids were eluted with methanol–water (1:1). A pronase digest of delipidated brain-tissue, purified with a Sephadex G-25 column, was used as the mixture of rat-brain glycopeptides[6].

Methyl sulphoxide (containing $<0.03\%$ of water) was used in the methylation procedure and in the preparation[3] of sodium methylsulphinylmethanide. Sodium hydride (55–60% suspension in oil) was extracted (6×) with hexane before mixing with methyl sulphoxide. The carbanion solution was centrifuged, and the clear supernatant was used for methylations. On the basis of titration to the phenolphthalein end-point[3], the base was 2M. The reagent was stored in screw-capped tubes, filled with nitrogen, in a desiccator at 4°.

Methylation was carried out by the Hakomori method[1]. The derivatisation was performed in screw-capped tubes filled with nitrogen, and 100 µl of the carbanion solution in 100 µl of methyl sulphoxide was normally used. In experiments with low amounts of methylsulphinyl carbanion, 5 µl of the carbanion solution was mixed with 500 µl of methyl sulphoxide (0.2–0.5 µmol of glycolipid or glycopeptide). Samples (100 µl) were taken for methylation after 1-h intervals with further additions of 20, 30, and 50 µl of the anion solution to the remaining reactive mixture. In separate experiments, derivatisation was started with 5, 20, 40, 60, 80, and 100 µl of the carbanion solution/500 µl of methyl sulphoxide, and reaction was continued for 2 h in an ultrasonic bath at room temperature. In all experiments, 150 µl of methyl iodide was used for the methylation.

The methylation reaction mixture was tested for carbanion before addition of methyl iodide by applying a drop of the solution onto dry, powdered triphenylmethane. The presence of the carbanion was clearly indicated by the red colour due to triphenyl carbanion[3]. The sensitivity of the test was shown by using a solution of sodium methylsulphinylmethanide into which increasing amounts of water had been added. The detection became negative when the carbanion solution was made 2M with respect to water. The result was the same when tested in normal laboratory conditions (22°; relative humidity, 45%) or in a moist atmosphere (36°; relative humidity, 70%). The indication was instantaneous, but the colour faded in 1–3 min,

due to the moisture present. Use of triphenylmethane indicator in the reaction mixture did not increase the sensitivity, but it caused high disturbing peaks to appear in the subsequent g.l.c. of the partially methylated alditols.

Methylated sugars from glycolipids and glycopeptides were analysed after acetolysis–acid hydrolysis[7]. A reference mixture of partially methylated galactose derivatives was prepared by Kuhn methylation[8] from phenyl β-D-galactoside, which was dissolved in methanol and treated with methyl iodide and silver oxide for ~40 h at 100°. Mass spectra of *m/e* 40–600 were recorded at 70 eV by using a Varian MAT CH-7 instrument equipped with a SpectoSystem 100 MS data-processing system and an ionization current of 300 μA.

ACKNOWLEDGMENTS

Mrs. Liisa Kuivalainen and Mrs. Hilkka Rönkkö are thanked for technical assistance, and the Sigrid Jusélius Foundation for support.

REFERENCES

1 S. Hakomori, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.
2 S. K. Kundu, R. W. Ledeen, and P. A. J. Gorin, *Carbohydr. Res.*, 39 (1975) 179–191.
3 E. J. Corey and M. Chaykovsky, *J. Am. Chem. Soc.*, 87 (1965) 1345–1353.
4 J. Folch, M. Lees, and G. H. Sloane Stanley, *J. Biol. Chem.*, 226 (1957) 497–509.
5 H. Rauvala, *Biochim. Biophys. Acta*, 424 (1976) 284–295.
6 T. Krusius, J. Finne, J. Kärkkäinen, and J. Järnefelt, *Biochem. Biophys. Acta*, 365 (1974) 80–92.
7 K. Stellner, H. Saito, and S. Hakomori, *Arch. Biochim. Biophys.*, 155 (1973) 467–472.
8 R. Kuhn, H. Trischmann, and I. Löw, *Angew. Chem.*, 67 (1955) 32.