Vol. 94, No. 2, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 30, 1980 Pages 567-572

IDENTIFICATION OF 9-O-ACETYL-N-ACETYLNEURAMINIC ACID ON THE SURFACE OF BALB/c MOUSE ERYTHROCYTES

Gerd Reuter[a,b], Johannes F.G. Vliegenthart[a], Margret Wember[b], Roland Schauer[b], and Russell J. Howard[c*]

[a]Department Bio-Organic Chemistry, State University of Utrecht, Croesestraat 79, 3522 AD Utrecht (The Netherlands)
[b]Biochemisches Institut der Christian-Albrechts-Universität, Olshausenstr. 40-60, D-2300 Kiel 1 (Federal Republic of Germany) and
[c]The Walter and Eliza Hall Institute of Medical Research, Post Office, Royal Melbourne Hospital, Victoria, 3050 (Australia)

Received April 11,1980

SUMMARY

For the first time 9-O-acetyl-N-acetylneuraminic acid has been unequivocally identified as the almost exclusive sialic acid of BALB/c mouse erythrocytes by gas-liquid chromatography/mass spectrometry. In human erythrocytes which were processed simultaneously N-acetylneuraminic acid could be identified as the only sialic acid. In 10^{10} human erythrocytes 350 nmoles of sialic acid were found and in the same number of mouse erythrocytes 440 nmoles.

INTRODUCTION

The presence of sialic acids as a major negatively charged constituent of glycoconjugates of the cell surface membrane (1) has led to the suggestion that they play an important role in cell-cell recognition phenomena such as sequestration of aged erythrocytes in liver and spleen (2) and viral entry into cells (3). Furthermore, the lifetime of serum sialoglycoproteins has been shown to be determined by their sialic acid content (4). This finding has stimulated the search for similar control processes mediated by sialic acids in cellular interactions.

Recently some evidence was obtained that O-acetylation of sialic acids might influence the lifetime of red blood cells

*Present address: National Institutes of Health, Laboratory of Parasitic Diseases, Malaria Section, Bldg. 8, Rm 316, Bethesda, Maryland 20014 (USA)

0006-291X/80/100567-06$01.00/0

(5,6). Therefore it seems necessary to identify the nature of these membrane-bound sialic acids. We describe here the unambiguous identification and quantitative analysis of the sialic acids of mouse erythrocytes. Human erythrocytes were analysed in parallel as a control.

MATERIALS AND METHODS

Sialic acid isolation and analysis were performed by standard techniques (7). Unless otherwise stated all experiments were carried out at 2 to 4°C. Erythrocytes of man and mouse (BALB/c) were prepared in parallel. Human blood was obtained by venipuncture and mice were bled from the retro-orbital plexus. The red cells were immediately washed six times in human or mouse tonicity phosphate buffered saline (respectively: 20 mM sodium phosphate, 0.120 M NaCl, pH 7.3; 20 mM sodium phosphate, 0.149 M NaCl, pH 7.3). Leucocyte contamination was reduced to <0.1 % of the total white cells by buffy coat removal. The erythrocytes were lyophilised and the sialic acids cleaved from the erythrocytes (1-1.5 x 10^{10} human erythrocytes and 5.8-9 x 10^{10} mouse erythrocytes) by hydrolysis in 2-10 ml formic acid (pH 2) for 60 min at 70°C. Each sample was dialysed against 50-100 ml of distilled water for 12 h. Hydrolysis was repeated in 0.1 N HCl for 60 min at 80°C and dialysis was carried out accordingly. This procedure has been shown to result in nearly complete hydrolysis of the sialic acids with relatively low (50 %) removal of O-acetyl groups (8). The dialysates were combined and eluted from a column (3 ml) of Dowex 50W X 8, H^+ form (20-50 mesh) with 15 ml of distilled water. The eluate was concentrated to 2 ml by rotary evaporation at 30°C and loaded onto a column (3 ml) of Dowex 2 X 8, $HCOO^-$ form (200-400 mesh). Elution was carried out with 15 ml of 1 N formic acid and the eluate lyophilised.

The sialic acid content of the fractions from column chromatography was determined by the orcinol/Fe^{3+}/HCl and the periodic acid/thiobarbituric acid reagents (8). The latter analysis was also carried out after saponification of the O-acetyl groups with dilute NaOH. The amount of O-acyl groups of the sialic acids was determined by the Hestrin method (8).

For TLC analysis 0.2 mm cellulose on plastic sheets or 0.2 mm silica gel 60 on plastic sheets (both from E. Merck, Darmstadt) were used. The cellulose plates were prerun in 0.1 N HCl and dried. The solvents were butan-1-ol/propan-1-ol/0.1 N HCl (1/2/1; by vol) for cellulose and propan-1-ol/water (7/3; by vol) for silica gel (8). Crystalline N-acetylneuraminic acid and 9-O-acetyl-N-acetylneuraminic acid isolated from bovine submandibular glands (7) were used as reference compounds. The sialic acid spots were visualised using the orcinol/Fe^{3+}/HCl spray reagent (8).

For GLC and GLC-MS 80-100 µg of the lyophilised sialic acids were treated with diazomethane to give the methyl ester and then trimethylsilylated with pyridine/hexamethyldisilazane/trimethylchlorosilane as described earlier (9). GLC was carried out on a Varian aerograph 2740-30-01 gas chromatograph with a dual flame

ionisation detector and glass columns (2.00 x 4.0 mm i.d.) packed with 3.8 % SE-30 on Chromosorb W/AW-DMCS, HP, 80-100 mesh. The column oven temperature was 215°C and the gas flow rate for N_2 40 ml/min. The 75eV mass spectra were recorded on a Jeol JGC 1100/JMS-07 combination with SE-30 as column material and an oven temperature of 200°C. The ion source temperature was 250°C, the accelerating voltage 1.5 kV and the ionising current 300 µA.

RESULTS AND DISCUSSION

From 10^{10} human erythrocytes 350 nmoles (mean value of 2 experiments) of sialic acids could be obtained in relatively pure form. Correspondingly, 440 nmoles of sialic acids were isolated from 10^{10} mouse erythrocytes (mean value of 4 experiments). On TLC the sample of human erythrocytes showed one single spot which comigrated with a reference of N-acetylneuraminic acid in both systems. The mouse erythrocytes gave two spots the intensities of which were in a ratio of about 1:1 (Fig. 1). The slower migrating component was identical with N-acetylneuraminic acid, the other one had the same chromatographic behaviour as 9-O-acetyl-N-acetylneuraminic acid. The corresponding R_f values of the two compounds were 0.46 and 0.64 on cellulose and 0.40 and 0.50 on silica gel.

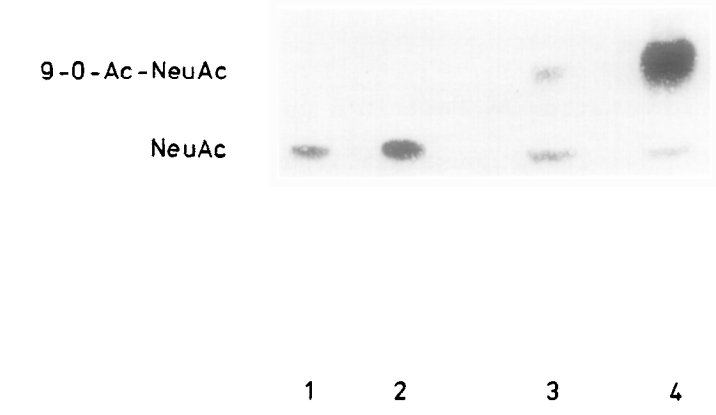

Fig. 1. Thin-layer chromatography on cellulose in butan-1-ol/propan-1-ol/0.1 N HCl (1/2/1; by vol) of sialic acids from human erythrocytes (1) and mouse erythrocytes (3). As references N-acetylneuraminic acid (NeuAc, 2) and a mixture (4) of NeuAc and 9-O-acetyl-N-acetylneuraminic acid (9-O-Ac-NeuAc) were used.

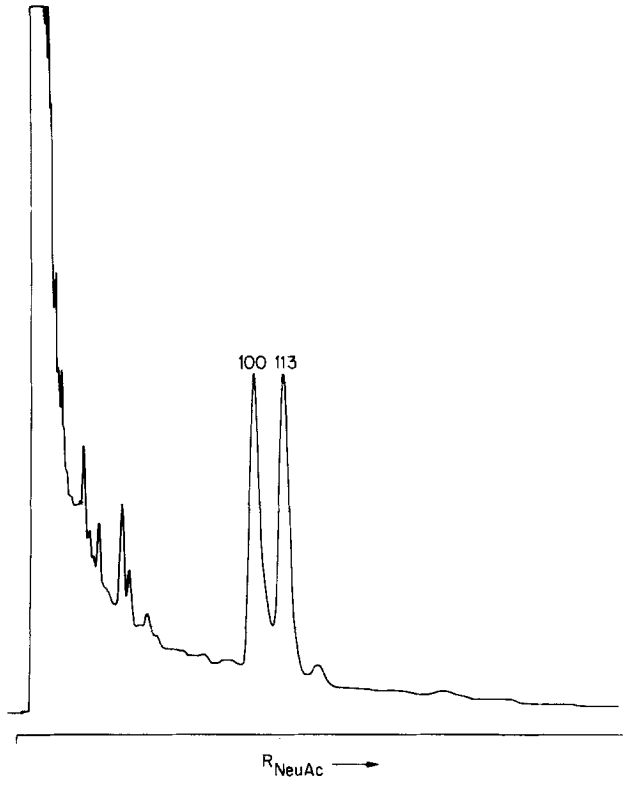

Fig. 2. Gas-liquid chromatography on 3.8 % SE-30 at 215°C of sialic acids from mouse erythrocytes as their methyl esters pertrimethylsilyl ethers. Peaks, identified by combined gas-liquid chromatography mass spectrometry: R_{NeuAc} 1.00, N-acetylneuraminic acid; R_{NeuAc} 1.13, 9-O-acetyl-N-acetylneuraminic acid.

Ester determination by Hestrin's method showed an O-acetyl content of the isolated mouse sialic acids of 60 %. From this, and the relationship of N-acetylneuraminic acid and its 9-O-acetylated derivative on TLC, it can be concluded that about 50 % of the isolated sialic acids represent 9-O-acetyl-N-acetylneuraminic acid. Accordingly, the value for the sialic acid content was reduced by about 50 % in the periodic acid/thiobarbituric acid assay as can be expected for 9-O-acetylated sialic acids (8). After saponification this value became about the same as with the orcinol/Fe^{3+}/HCl method.

About 50 % of the ester groups are usually lost during the hydrolytic release of the sialic acids (7). Therefore, 9-O-acetyl-N-acetylneuraminic acid appears to be the almost exclusive sialic acid in mouse erythrocytes.

One major peak with a retention time of 1.00 relative to per-O-trimethylsilyl-N-acetylneuraminic acid methyl ester could be detected from human erythrocytes by GLC. The sialic acid sample of mouse erythrocytes showed two main peaks (Fig. 2) with relative retention times (R_{NeuAc}) of 1.00 and 1.13. This is in agreement with N-acetylneuraminic acid and 9-O-acetyl-N-acetylneuraminic acid (10). By combined GLC-MS these identifications were confirmed (10) and no other peaks representing sialic acids could be detected.

Thus, as shown by TLC, GLC and GLC-MS, human erythrocytes contain N-acetylneuraminic acid as the only sialic acid while mouse erythrocytes mainly contain 9-O-acetyl-N-acetylneuraminic acid. The absence of detectable amounts of O-acetylated sialic acids in human erythrocytes confirms earlier observations (e.g. (11,12)). This is the first report of an unambiguous identification of an O-acetylated sialic acid from mouse erythrocytes by GLC-MS. In other laboratories (13,14) also indications for the occurrence of O-acylated sialic acids are found, based on GLC alone, but in none of these cases structural analysis has been carried out.

It should be noted that 9-O-acetyl-N-acetylneuraminic acid has recently been identified as a constituent of the trisialoganglioside O-Ac-GT1b from mouse brain (15).

ACKNOWLEDGEMENTS

The financial support of the Deutsche Forschungsgemeinschaft (grants Re 445/2 and Scha 202/9) is greatly acknowledged. We thank P.M. Smith for technical assistance and Prof. G.J.V. Nossal and Dr. G.F. Mitchell for their encouragement.

REFERENCES

1. Hughes, R.C. (1976) in: Membrane Glycoproteins, pp. 1-27, Butterworths, London-Boston.
2. Jancik, J.M., Schauer, R., Andres, K.H. and von Düring, M. (1978) Cell Tiss. Res. 186, 209-226.
3. Gottschalk, A., Belyavin, G. and Biddle, F. (1972) in: Glycoproteins (Gottschalk, A., ed.), part B, 2nd ed., pp. 1082-1096, Elsevier, New York.
4. Ashwell, G. and Morell, A.G. (1974) Adv. Enzymol. 41, 99-128.
5. Howard, R.J., Smith, P.M. and Mitchell, G.F. (1980) Parasitology (in press).
6. Roth, R.L. and Herman, R. (1979) Exp. Parasitology 47, 169-179.
7. Buscher, H.P., Casals-Stenzel, J. and Schauer, R. (1974) Eur. J. Biochem. 50, 71-82.
8. Schauer, R. (1978) Meth. Enzymol. 50, 64-89.
9. Kamerling, J.P., Vliegenthart, J.F.G., Versluis, C. and Schauer, R. (1975) Carbohyd. Res. 41, 7-17.
10. Kamerling, J.P., Haverkamp, J., Vliegenthart, J.F.G., Versluis, C. and Schauer, R. (1978) in: Recent Developments in Mass Spectrometry in Biochemistry and Medicine (Frigerio, A., ed.) vol.1, pp. 503-520, Plenum Publishing Corporation, New York.
11. Schauer, R., Corfield, A.P., Wember, M. and Danon, D. (1975) Hoppe-Seyler's Z. Physiol. Chem. 356, 1727-1732.
12. Baxter, A. and Beeley, J.G. (1975) Biochem. Soc. Trans. 3,134-136.
13. Sarris, A.H. and Palade, G.E. (1979) J. Biol. Chem. 254,6724-6731.
14. Varki, A. and Kornfeld, S., personal communication.
15. Ghidoni, R., Sonnino, S., Tettamanti, G., Baumann, N., Reuter, G. and Schauer, R. (1980) J. Biol. Chem., in press.