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Incorporation of *N*-acyl-2-amino-2-deoxy-hexoses into glycosphingolipids of the pheochromocytoma cell line PC 12

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The synthesis of *N*-acyl-2-amino-2-deoxy-hexoses, their metabolism and their incorporation into glycosphingolipids of rat pheochromocytoma cell line PC 12 were investigated. The data indicate that in PC 12 cells the *N*-acyl-2-amino-2-deoxy-hexoses, *N*-propanoyl-D-glucosamine and *N*-butanoyl-D-glucosamine are metabolized to the corresponding phosphates, and that *N*-propanoyl-D-glucosamine is also metabolized to *N*-propanoyl neuraminic acid. Using variously radiolabelled *N*-acyl-2-amino-2-deoxy-hexoses, their incorporation into glycosphingolipids was shown.

N-Acyl neuraminic acid biosynthesis; Synthesis of radiolabelled *N*-acyl-2-amino-2-deoxy-hexose; Glycosphingolipid; PC 12 cell

1. INTRODUCTION

Sialic acids are essential constituents of cell surface glycoconjugates. More than 30 different naturally occurring sialic acids have been isolated and characterized from different biological sources [1]. From the data available, some biological functions of sialic acids can be deduced, e.g. masking of antigenic sites [2], physicochemical influence on the structure of glycoconjugates [3], protection against degradation [4]; but on the whole the biological role of sialic acids is poorly understood. Promising approaches to the elucidation of the significance of sialic acids would be to modify the biosynthesis, the transfer or the catabolism of sialic acids.

In an earlier study, we blocked the in vitro biosynthesis of *N*-acetyl neuraminic acid with *N*-acyl- and *N*-alkyl-2-amino-2-deoxyhexoses [5]. The resulting data suggested that in the cell-free rat liver system a small amount of *N*-propanoyl neuraminic acid (Neu5Prop), which is not known to occur naturally, was formed from the corresponding *N*-acyl-2-amino-2-deoxy hexoses.

In this study, we investigated the metabolism and incorporation into glycosphingolipids of different *N*-acyl-2-amino-2-deoxy-hexoses in the pheochromocytoma cell line PC12. The data obtained showed for the first time the in vivo metabolism of *N*-acyl-2-amino-2-deoxy-hexoses and their incorporation into glycosphingolipids.

Abbreviations: HPTLC, high performance thin layer chromatography; PC 12, rat pheochromocytoma cell line; GlcNProp, *N*-propanoyl-D-glucosamine; GlcNBut, *N*-butanoyl-D-glucosamine; Neu5Prop, *N*-propanoyl neuraminic acid.

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2. MATERIALS AND METHODS

2.1. *Materials*

1-[^{14}C]Glucosamine (55 mCi/mM) and 1-[^{14}C]*N*-propanoyl-D-glucosamine (0.3 mCi/ml see Fig. 1 for radiolabel location) were purchased from ARC (St. Louis, USA).

Chemicals and solvents were from Merck (D-6100 Darmstadt, Germany). Neuraminidase from *Clostridium perfringens* (EC 3.2.1.18) was from Boehringer (D-6800 Mannheim, Germany). For quantification of radioactivity a scintillation spectrometer, model 1900 CA (Packard Instruments Inc., CH-8038 Zürich, Switzerland) was used. Radioactive ganglioside standards containing ^{3}H-labelled G_{M1}, G_{M2}, G_{M3}, G_{D1a} and G_{D1b} were a gift from Dr. S. Sonnino.

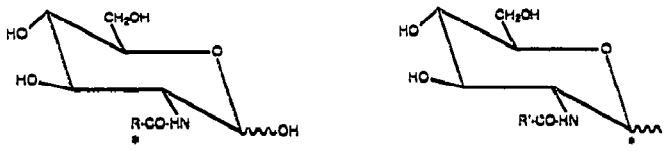
2.2. *Synthesis of N-acyl-2-amino-2-deoxy-hexoses*

N-acyl-2-amino-2-deoxy-hexoses were synthesized according to the method of Roseman and Ludowieg [6]. Structures were confirmed by NMR spectroscopy (data not shown). For the synthesis of ^{14}C-labelled *N*-acyl-2-amino-2-deoxy-hexoses (at the level of the hexose moiety) 50 μCi 1-[^{14}C]GlcN (0.9 μmol) were dissolved in 100 μl water/methanol (1:1 v/v) then shaken with 20 μl of Dowex-1×2 (carbonate form) and 2 μl of propionic acid anhydride or butanoic acid anhydride, respectively, for 90 min at 0–5°C. The mixture was centrifuged at 5,000 × *g* for 10 min to remove Dowex-1×2 and the supernatant was passed through a column containing 1 ml of Amberlite IR-120 (acid form). The effluent was purified by descending paper chromatography (see section 2.3.) and the main product could be obtained by elution with water. Purity was proven by re-chromatography.

2.3. *Chromatographic methods*

Descending paper chromatography was performed on Whatman No. 3MM paper using a solvent system containing *n*-propanol/water/1 mol/l sodium acetate (7:2:1 by vol.), pH 5.0, according to Lewin and Wei [7]. The chromatograms were usually developed for 16 h with UMP as internal standard. To determine radioactivity, paper strips from chromatograms were measured by liquid scintilation counting.

Purified acidic glycosphingolipids were separated by HPTLC on silica gel 60 HPTLC plates using a HPTLC applicator (Linomat III,



R	nomenclature
-C_2H_5	[1'-^{14}C]-N-propanoyl-2-amino-2-deoxy-D-glucose (s)
-C_3H_7	[1'-^{14}C]-N-butanoyl-2-amino-2-deoxy-D-glucose (s)
R'	nomenclature
-C_2H_5	[1-^{14}C]-N-propanoyl-2-amino-2-deoxy-D-glucose (r)
-C_3H_7	[1-^{14}C]-N-butanoyl-2-amino-2-deoxy-D-glucose (r)

Fig. 1. Structure of different radiolabelled *N*-acyl-2-amino-2-deoxy-hexoses. r, ring, radioactive label located in the hexose moiety; s, side chain, radioactive label located in the *N*-acyl moiety; * marks position of label.

Camag, Berlin, Germany) and the solvent system chioroform/methanol/0.2% aqueous calcium chloride (50:45:10 by vol.). For the separation of neutral glycosphingolipids a solvent system containing chloroform/methanol/water (55:20:3 by vol.), was used. After development, the plates were sprayed with an enhancer (Du Pont, Boston, USA) and exposed to fluorography. Radioactive gangliosides were used as standards.

2.4. *Cell culture*

PC 12 cells were cultured as described by Greene and Tischler [8] at 37°C in RPMI medium (Biochrom, Berlin, Germany) containing 10% heat-inactivated horse serum and 5% heat-inactivated calf serum (Gibco, Berlin, Germany) in an atmosphere containing 5% CO_2. Cells were labelled for 24 h in the presence of the appropriate *N*-acyl-2-amino-2-deoxy-hexose (25 μCi/6 × 10^6 cells). The harvested cells were washed 3 times with ice-coid phosphate-buffered saline, stored at −70°C and lyophilized before extraction.

2.5. *Determination of metabolites of* N-*acyl-2-amino-2-deoxy-hexoses*

In order to show in vitro metabolism of *N*-acyl-2-amino-2-deoxy-hexoses, PC 12 cells were harvested in a buffer containing 200 mM Tris–HCl, 2 mM $MgCl_2$ and 75 mM nicotinic acid amide [9]. After incubation of 100 μl PC 12 cell homogenate with 100 nCi of an *N*-acyl-2-amino-2-deoxy-hexose and, in order to uphold the ATP concentration, 20 μl of an ATP regenerating system (100 mM ATP, 100 mM CTP, 100 mM UTP, 200 mM phosphoenolpyruvate and 10 mg/ml pyruvate kinase [9]) for 30 min at 37°C, the reaction was stopped by adding 200 μl ethanol. The solutions were centrifuged at 10,000 × *g* for 15 min and the supernatants were analysed by descending paper chromatography as described above. The *N*-acyl-hexosamine phosphates were identified according to Grünholz et al. [5] by enzymatic digestion with alkaline phosphatase (EC 3.1.3.1).

2.6. *Lipid extraction and purification of glycosphingolipids*

Total lipids from lyophilized cell pellets were extracted and partitioned between solvents as described by Tettamanti et al. [10]. Neutral glycosphingolipids were purified from the dried ether phase of the partition system after removing contaminating phospholipids and neutral lipids by alkaline treatment [11]. The gangliosides were ob-

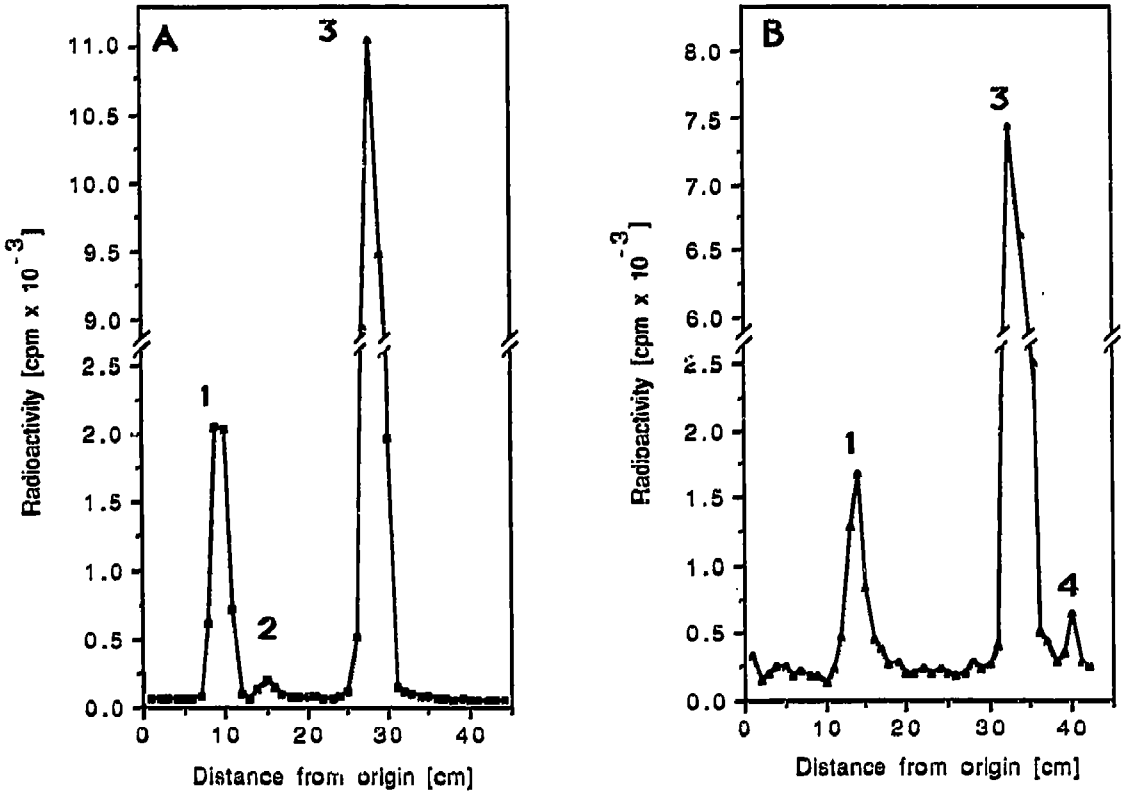


Fig. 2. Metabolites of different *N*-acyl-2-amino-2-deoxy-hexoses. Descending paper chromatographic analysis of the metabolites of different *N*-acyl-2-amino-2-deoxy-hexoses was carried out as described in Materials and Methods. Chromatogram A shows the data for GlcNProp labelling, and chromatogram B for GlcNBut labelling. The peaks represent the following metabolites: (1) *N*-acyl-hexosamine phosphates; (2) *N*-acyl-neuraminic acid; (3) *N*-acyl-hexosamine; (4) unidentified.

tained from the aqueous phase after dialysis against water using the described method.

2.7. *Neuraminidase digestion*

Gangliosides, purified as described above, were dissolved in a buffer containing 50 mM sodium acetate (pH 5.0), and incubated with 175 mU neuraminidase from *Clostridium perfringens* (EC 3.2.1.18, Boehringer-Mannheim, Germany) for 24 h at 37°C. The reaction was stopped by adding 100 μl methanol/chloroform (1:1 v/v) and centrifugation at 5,000 × *g* for 15 min. The organic phases were evaporated to dryness, dissolved in 50 μl water, de-salted by dialysis and applied to HPTLC analysis.

2.8. *Mild acidic hydrolysis of glycosphingolipids*

The glycosphingolipid fraction was purified as described above and dissolved in 2 mol/l acetic acid in order to obtain total removal of sialic acid [12]. The samples were incubated for 2 h at 80°C, lyophilized, de-salted by dialysis, resolved in 50 μl chloroform/methanol (1:1 v/v) and applied to HPTLC analysis.

3. RESULTS AND DISCUSSION

3.1. *Synthesis of* N-*acyl-2-amino-2-deoxy-hexoses*

As precursors for the biosynthesis of gangliosides, various *N*-acyl-2-amino-2-deoxy-hexoses, such as *N*-propanoyl-2-amino-2-deoxy-D-glucose (GlcNProp) and *N*-butanoyl-2-amino-2-deoxy-D-glucose (GlcNBut), were synthesised with a high grade of purity (Fig. 1). The synthesis was carried out to ensure the reaction conditions and the correct structure of the desired precursors with a yield of 76% for GlcNProp (m.p. 180–182°C [17]) and 73% for GlcNBut (m.p. 204–207°C [16]), before synthesizing of the radiolabelled analogues. Yield of radioactive products were 75% for GlcNProp and 83% for GlcNBut. After purification by descending paper chromatography the radioactive *N*-acyl-hexosamines showed a purity of more than 99%.

3.2. *Metabolism of* N-*acyl-2-amino-2-deoxy-hexoses in PC 12 cells*

In an earlier study, we demonstrated that *N*-acyl-2-amino-2-deoxy-hexoses were metabolized in vitro to the corresponding *N*-acyl-neuraminic acids, which indicates that these hexosamine analogues are metabolized like the naturally occurring hexosamines, *N*-acetyl-D-glucosamine and *N*-acetyl-D-mannosamine. In this in vivo study using PC 12 cells, paper chromatographic analysis indicated the formation of *N*-propanoyl neuraminic acid, but no data were obtained in the formation of *N*-butanoyl neuraminic acid (Fig. 2).

3.3. *Incorporation of different radiolabelled* N-*acyl-2-amino-2-deoxy-hexoses into neutral glycosphingolipids and gangliosides*

To investigate the possible incorporation of *N*-acyl-2-amino-2-deoxy-hexoses into glycosphingolipids, analogues were used that were labelled either in the hexose or in the acyl moiety. This approach permits the detection and measurement of possible deacylation. As a control, cells were labelled with radioactive D-glucosamine. After extraction, neutral glycosphingolipids and

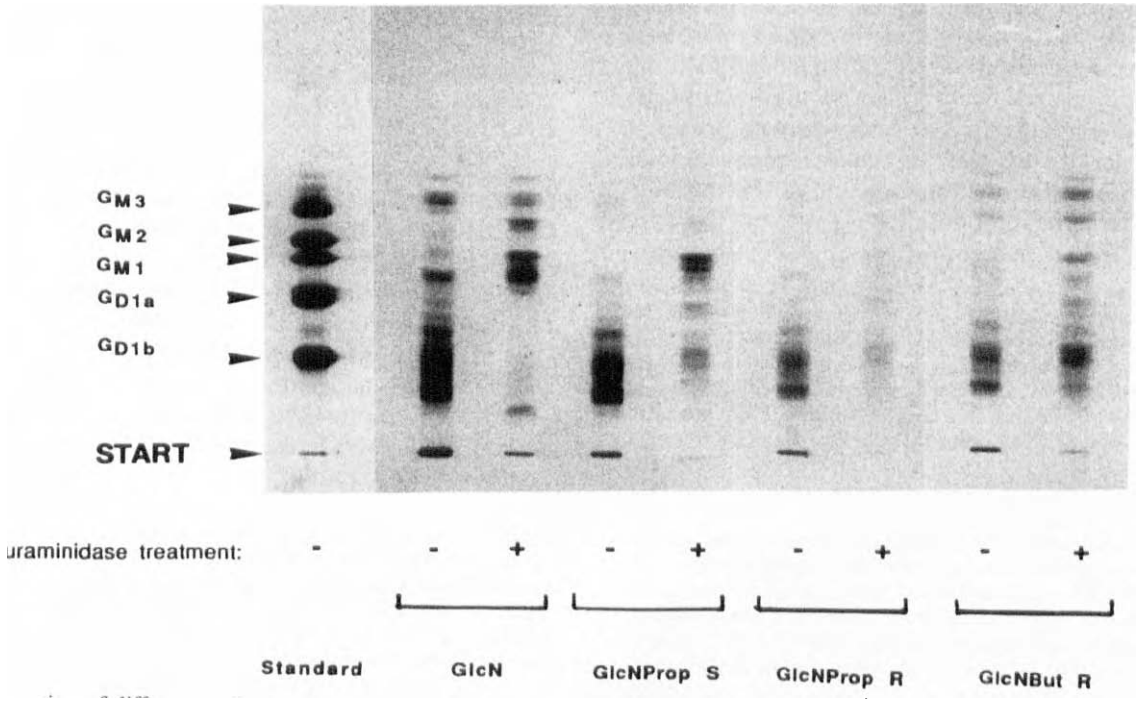


Fig. 3. Incorporation of different radiolabelled *N*-acyl-2-amino-2-deoxy-hexoses into PC 12 gangliosides. PC 12 cells were labelled for 24 h in the presence of the respective *N*-acyl-2-amino-2-deoxy-hexose (25 μCi/6 × 10^6 cells). Analogues were used which were labelled in two different parts of the molecule: in the hexose (R) or the acyl moieties (S), respectively. The harvested cells were washed 3 times with ice-cold phosphate-buffered saline, lyophilized, and lipids extracted and purified as described in Materials and Methods. Neuraminidase sensitivity of the purified lipids was investigated by incubation with (+) and without (−) neuraminidase from *Clostridium perfringens*. D-Glucosamine-labelled lipids were used as control.

gangliosides were separated by solvent partitioning as described. HPTLC analysis of the purified neutral glycosphingolipid fraction showed that *N*-acyl-2-amino-2-deoxy-hexoses were not incorporated, whereas D-glucosamine was well incorporated (data not shown). Therefore we suggest that the formation of the corresponding UDP-derivatives is blocked or that the transfer of the hexoses to the acceptors is not possible by the corresponding glycosyltransferases. In contrast, in *N*-acyl-2-amino-2-deoxy-hexose labelled cells, the label could be visualized in the ganglioside fraction, showing for the first time an incorporation of these non-physiological precursors as *N*-acyl-neuraminic acid into gangliosides. The analysis by HPTLC (Fig. 2) demonstrated that (i) hexose- and *N*-acyl-labelled analogues lead to a similar, (ii) the pattern of labelled lipids is dependent on the acyl moiety of the *N*-acyl-2-amino-2-deoxy-hexose, and (iii) the pattern is similar to the control experiment and to the published ganglioside pattern of PC 12 cells [13]. By digestion of the ganglioside fraction with neuraminidase from *Clostridium perfringens* it was shown that, after labelling with GlcNProp, the bands were neuraminidase-sensitive, whereas after labelling with GlcNBut the bands were neuraminidase-sensitive. These results are in agreement with data published by Meindl and Tuppy [14], who reported that the enzymatic hydrolysis of α-ketosides of *N*-acyl-neuraminic acids depends on the chain length of the acyl moiety. Finally, we suggest that in PC 12 cells, the *N*-acyl-2-amino-2-deoxy-hexoses, GlcNProp and GlcNBut, were metabolized to the corresponding 1- and 6-phosphates, and that in small amounts GlcNProp was also metabolized to the corresponding *N*-acyl-neuraminic acid. Metabolism to D-galactose derivatives and their subsequent incorporation was not unequivocally excluded by this study, because mild acidic hydrolysis could not totally remove the radioactive label of the glycosphingolipids. However, the data obtained indicate an incorporation of *N*-acyl-neuraminic acids that do not occur naturally. Preliminary results by GC/MS studies on serum glycoproteins supported the assumption that *N*-propanoyl-2-amino-2-deoxy-glucose is incorporated as *N*-propanoyl-neuraminic acid. With respect to gangliosides we will attempt to characterize the neuraminic acid analogues by further studies.

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