The occurrence of two novel *N*-acetylglucosaminyltransferase activities in human serum

J. Zieleński and J. Kościelak+

Department of Biochemistry, Institute of Hematology, 00-957 Warsaw, Poland

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1. INTRODUCTION

Many mammalian glycoconjugates and oligosaccharides contain β-D-galactopyranosyl residues substituted at position 3, or at position 3 and 6 with *N*-acetyl-D-glucosamine. Transferases of *N*-acetyl-D-glucosamine responsible for the biosynthesis of these structures have not been described. We now report the presence of two *N*-acetyl-D-glucosamine transferase activities in human serum with lactose as an acceptor. When this work was in its final stage of preparation we obtained information that the paper on the presence of similar enzymic activities in homogenates of Novikoff ascites tumor cells of mice was in press [1]. In this study, asialo-α_1-acid glycoprotein was used as an acceptor.

2. MATERIALS AND METHODS

Samples of human blood were obtained from blood donors. Globotetraosylceramide was isolated from human erythrocytes by procedure A in [2]. Electrophoretically homogeneous preparation of bovine α-lactalbumin was the same as used in [3]. UDP-D-[U-^{14}C]galactose (spec. act. 337 mCi/mmol) and UDP-*N*-acetyl-D-[U-^{14}C]glucosamine (spec. act. 282 mCi/mmol) were purchased from the Radiochemical Centre (Amersham). D-Glucose, *N*-acetyl-D-glucosamine, lactose, UDP-*N*-acetyl-D-glucosamine and UDP-D-galactose were from Serva (Heidelberg). ATP was from Calbiochem.; *N*-tris-[hydroxymethyl]-methyl-2-aminoethane sulfonic acid (TES) from Sigma; sodium cacodylate from IE (England); sodium borohydride from BDH; polygram Sil-G TLC plates from Macherey and Nagel; β-*N*-acetyl-hexosaminidase from Seikagaku Fine Biochemicals; synthetic *N*-acetyllactosamine was a gift from Professor W.M. Watkins.

Lactose labelled in the galactopyranosyl residue was synthesized as in [3] from UDP-D-[^{14}C]galactose and D-glucose using human serum as the source of the component A of lactose synthase (EC 2.4.1.90). The composition of the reaction mixture in 4 ml final vol. was as follows: UDP-D-[^{14}C]galactose, 10 nmol; D-glucose, 20 μmol; lactalbumin, 10 mg; sodium cacodylate (pH 6.0), 180 μmol; $MnCl_2$, 33 μmol; ATP, 8.8 μmol; NaN_3, 8 μmol; human serum, 1 ml. The reaction mixture was incubated at 37°C for 48 h. Lactose from the deproteinized reaction mixture was isolated by fractionation on a 3-layered 18 × 1 cm column packed from top-to-bottom with activated charcoal, Dowex 2 × 8 (acetate form), and Dowex 50W × 8 (H^+ form) as in [3]. The synthesized disaccharide (1.24×10^6 cpm; ~2.8 nmol) exhibited the same chromatographic mobility on

+ To whom correspondence should be addressed

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Whatman 1 paper as a lactose standard in ethyl acetate–pyridine–water (12:5:4, by vol.) solvent mixture (solvent A).

N-Acetyl-D-glucosamine transferase activities were measured by transfer of ^{14}C-labelled aminosugar to lactose, or by transfer of *N*-acetyl-D-glucosamine to [^{14}C]lactose labelled in the galactopyranosyl residue. The reaction mixtures and incubation times are given in the tables and figures. The reactions were terminated by the addition of equal volumes of absolute ethanol. The precipitated proteins were sedimented by centrifugation and washed once with 100 μl aliquots of 50% aqueous ethanol. The pooled supernatants from each reaction mixture were deionized by being passed through 9 × 0.5 cm columns packed with Dowex 50W × 8 (H^+ form) and Dowex 2 × 8 (acetate form). The reaction products were separated by descending chromatography on Whatman 1 paper in solvent A. Chromatographic lanes were then cut into 2 cm long pieces and their radioactivities counted in the Tricarb Packard scintillation counter, model 2450, using a scintillation solution containing 5 g PPO and 100 mg POPOP/toluene.

For methylation analysis the reaction products labelled in the penultimate D-galactopyranosyl residue were reduced with sodium borohydride and mixed with globotetraosylceramide. Methylation, acetolysis, and derivatization of methylated sugars to partially methylated alditol acetates were performed as in [4]. Two external standards (i.e., 2,3,4,6-tetra-*O*-methyl-D-galactitol and 2,3,4,6-tetra-*O*-methyl-D-glucitol) were added. Other standards present were globotetraosylceramide-derived 2,4,6-tri-*O*-methyl-D-galactitol, 2,3,6-tri-*O*-methyl-galactitol and 2,3,6-tri-*O*-methyl-D-glucitol. The mixture was separated by preparative gas chromatography on a 200 × 0.4 cm column packed with 3% ECNSS-M on Gas Chrom Q as in [3]. Vapours of individual methylated sugar derivatives were collected directly to vials containing cold toluene scintillation solutions and counted for radioactivity. Since standards of 2,3,4-tri-*O*-methyl-D-galactitol and 2,4-di-*O*-methyl-D-galactitol were not present in the mixture their positions on the gas chromatogram were calculated from the known retention times relative to 2,3,4,6-tetra-*O*-methyl-D-glucitol.

Enzymic hydrolysis of *N*-acetyl-D-[^{14}C]glucosamine labelled reaction product was carried out for 24 h at 37°C with 0.1 U of β-*N*-acetylhexosaminidase from *Turbo cornutus* in 300 μl total vol. in the presence of 10 μmol sodium citrate (pH 4.0) and 10 μmol NaCl. Deproteinization and deionization of the hydrolysis products were performed as described for isolation of the product of *N*-acetyl-D-glucosamine transfer.

3. RESULTS

3.1. *Incorporation of* N-*acetyl-D-[^{14}C]glucosamine to lactose using human serum as enzyme source*

The incubation of human serum with UDP-*N*-acetyl-D-[^{14}C]glucosamine, lactose and Mn^{2+} resulted in the formation of a new radioactive material which chromatographed on Whatman 1 paper in solvent A with $R_{lactose}$ of 0.65. The formation of this material under different experimental conditions is shown in table 1. A high concentration of lactose was employed to increase the initial reaction velocity and minimize the effect of a rapid

Table 1

Requirements for incorporation of *N*-acetyl-D-[^{14}C]glucosamine to lactose using human serum as enzyme source

Incubation mixture	Incorporation of *N*-acetyl-D-[^{14}C]glucosamine (pmol.mg protein^{-1}.24 h^{-1})
Complete	22.08
− Serum + heated serum (100°C for 5 min)	0.0
− Lactose	0.29
− $MnCl_2$	0.26
− $MnCl_2$ + $MgCl_2$ (2 μmol)	2.69
− ATP	14.42
+ EDTA (4 μmol)	0.21
− Lactose + *N*-acetyl-lactosamine (3 μmol)	27.14

The complete incubation mixture contained in 100 μl total vol.: TES (pH 7.0), 9 μmol; $MnCl_2$, 2 μmol; ATP, 0.56 μmol; NaN_3, 0.5 μmol; UDP-*N*-acetyl-D-[^{14}C]glucosamine, 0.5 nmol, 150000 cpm; lactose, 3.1 μmol; human serum, 40 μl. The incubation was carried out at 37°C for 24 h

decompostion of UDP-*N*-acetyl-[^{14}C]glucosamine, presumably by serum pyrophosphatases. At 3.2 μM UDP-*N*-acetyl-D-[^{14}C]glucosamine, ~47% of the original amount of sugar nucleotide in the reaction mixture was decomposed during 120 min incubation at 37°C in the absence of ATP. The *N*-acetyl-D-glucosamine transfer reaction shows a broad pH optimum between pH 6.8 and pH 7.8 (fig.1).

3.2. *Identification of the reaction product*

N-Acetyl-D-glucosamine was transferred to [^{14}C]lactose labelled on the D-galactopyranosyl residue. The reaction products (17000 cpm) were subjected to methylation analysis employing preparative gas chromatography. The results in table 2 clearly show that the product derived label was recovered only in two partially methylated galactitols, i.e., in 2,3,6-tri-*O*-methyl-D-galactitol and 2,3,4-tri-*O*-methyl-D-galactitol. A rather high radioactivity recovered in 2,3,4,6-tetra *O*-methyl-D-galactitol results from the contamination of the product with [^{14}C]lactose acceptor of 2.5 nmol which was initially present in the reaction mixture (1.2×10^6 cpm).

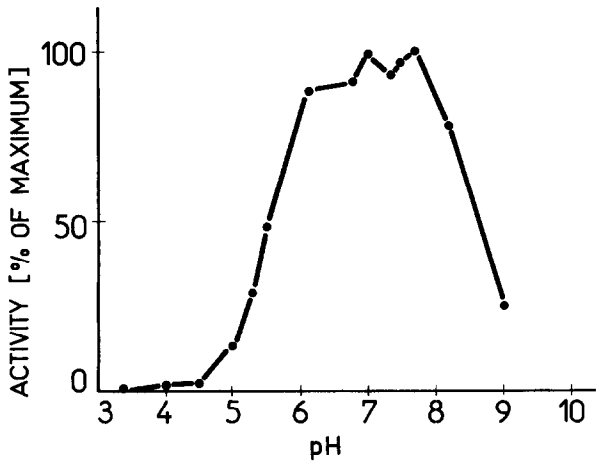

Fig.1. Effect of pH on the transfer of *N*-acetyl-D-[^{14}C]glucosamine to lactose by *N*-acetylglucosaminyl-transferase of human serum. The reaction mixtures were the same as specified in table 1 except that they contained 0.2 M of different buffers: glycyl–glycine (pH 3.4–4.5); 2-(*N*-morpholino)ethanesulfonate (pH 5.0–6.8); TES (pH 6.9–7.3); glycine (pH 7.5–9.0). The pH-values were determined directly in the incubation mixtures.

Table 2

The distribution of radioactivity in a mixture of partially methylated alditol acetates including those derived from methylated product of the transfer of *N*-acetylglucosamine to [^{14}C]lactose

Derivative	cpm
2,3,4,6-Tetra-*O*-methyl-glucitol	0
2,3,4,6-Tetra-*O*-methyl-galactitol	1693
2,4,6-Tri-*O*-methyl-galactitol	1126[a]
2,3,6-Tri-*O*-methyl-galactitol	0[a]
2,3,6-Tri-*O*-methyl-glucitol	13
2,3,4-Tri-*O*-methyl-galactitol	304
2,4-Di-*O*-methyl-galactitol	5
Other regions of the chromatogram	0–32

[a] Corrected for incomplete separation of 2,4,6-tri-*O*-methyl-galactitol and 2,3,6-tri-*O*-methyl-galactitol by the triangle method; the determined count values were 881 and 136 cpm, respectively

The composition of the incubation mixture was the same as specified in table 1 except that it contained non-radioactive UDP-*N*-acetylglucosamine (3 μmol) and lactose labelled in galactopyranosyl residue (2.5 nmol). Values represent ~20% of the total product derived methylated alditol acetates

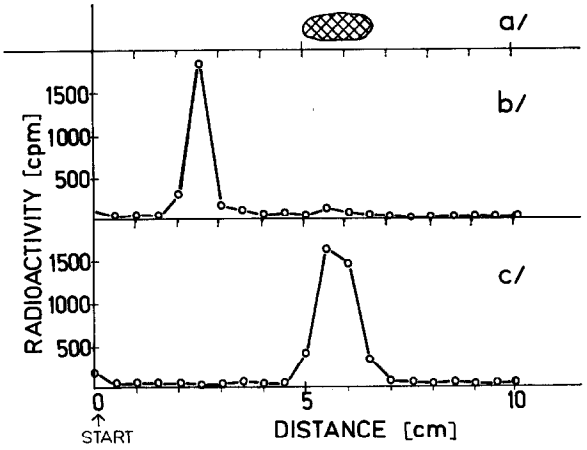

Fig.2. Hydrolysis of *N*-acetyl-D-[^{14}C]glucosamine-labelled product with β-*N*-acetylhexosaminidase. The products of digestion were spotted on Polygram Sil-G plates and developed with *n*-propanol–water (7:3, v/v), together with *N*-acetyl-D-glucosamine standard. The lanes containing hydrolysis products were cut into 0.5 cm long pieces and counted for radioactivity: (a) *N*-acetyl-D-glucosamine standard visualised by charring with H_2SO_4; (b,c) *N*-acetyl-D-[^{14}C]glucosamine-labelled product treated with heat inactivated enzyme (100°C for 5 min) and native enzyme, respectively.

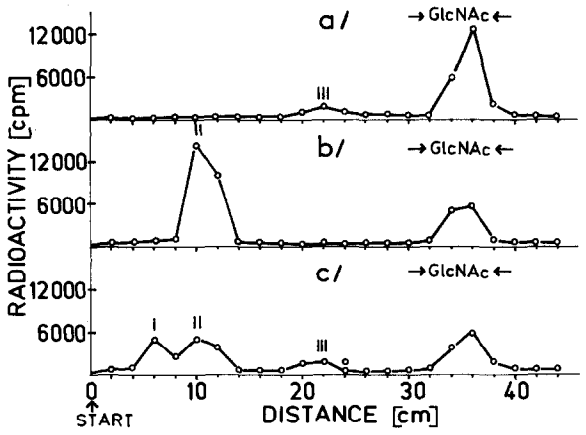

Fig.3. Separation by paper chromatography in solvent A of the products of *N*-acetyl-D-[^{14}C]glucosamine transfer reaction into lactose carried out in the presence of UDP-D-galactose. Compositions of the reaction mixtures were as in table 1 except that some contained 78 nmol of UDP-D-galactose: (a) complete incubation mixture + UDP-D-galactose − lactose; (b) UDP-D-galactose omitted; (c) complete incubation mixture + UDP-D-galactose; peak III represents *N*-acetyllactosamine by-product.

In a separate experiment, the reaction products labelled with *N*-acetyl-D-[^{14}C]glucosamine were treated with *β-N*-acetylhexosaminidase of *Turbo cornutus*. The label was completely released and the ^{14}C-labelled product of digestion migrated on a thin layer of silica gel with the mobility of free *N*-acetyl-D-glucosamine (fig.2c).

3.3. *Transfer of* N-*acetyl-D-[^{14}C]glucosamine to lactose in the presence of UDP-galactose*

When human serum is incubated with UDP-*N*-acetyl-D-[^{14}C]glucosamine, lactose and UDP-D-galactose the formation of a new radioactive product is observed (fig.3c). The mobility of the new product suggests that it is more complex than the product of transfer of *N*-acetylglucosamine to lactose.

4. DISCUSSION

Our data indicate that human serum contains enzyme catalysing transfer of *N*-acetyl-D-glucosamine to lactose and *N*-acetyllactosamine. The reaction requires the presence of Mn^{2+}. The results of methylation analysis strongly suggest that the reaction products with lactose as an acceptor are 2 trisaccharides with *N*-acetyl-D-glucosamine residues linked to position 3 and 6, respectively, of penultimate galactopyranosyl residues. Both *N*-acetyl-D-glucosamine residues are *β*-linked as evidenced by the susceptibility of the reaction products to the action of *β-N*-acetylhexosaminidase of *Turbo cornutus*. Thus the two trisaccharides have the structure: GlcNAc *β*1−3 Gal *β*1−4 Glc, and GlcNAc *β*1−6 Gal *β*1−4 Glc. Hence, the enzymes responsible for their formation should be classified as UDP-GlcNAc:Gal-R, *β*-D-3-*N*-acetylglucosaminyltransferase and UDP-GlcNAc:Gal-R, *β*-D-6-*N*-acetylglucosaminyltransferase, respectively. It is likely that *β*-D-3-*N*-acetylglucosaminyltransferase together with *N*-acetyllactosamine synthase (EC 2.4.1.90) catalyse formation of linear glycans containing alternating *β*-3-*O*-substituted residues of D-galactose and *β*-4-*O*-substituted residues of *N*-acetyl-D-glucosamine. Such structures are present among others in glycosphingolipids of H-II type [1], polyglycosylceramides [5,6] and polyglycosylpeptides (erythroglycan) [7,8] of human erythrocytes. The *β*-D-6-*N*-acetylglucosaminyl transferase should initiate formation of side chains. It was predicted that this enzyme is specified by *I* gene [5,6] and should be a key enzyme in the biosynthesis of I antigen of erythrocytes. Here, we have obtained no evidence for the formation of branched structure; i.e., of an oligosaccharide with both 3 and 6 position of penultimate galactose substituted with *N*-acetylglucosamine residues. We ascribe this either to insufficient *N*-acetylglucosaminyl transferase activity of human serum or unsuitability of the acceptor selected for the study. It is significant that incorporation of *N*-acetyl-D-glucosamine into a more physiological acceptor (i.e., into *N*-acetyllactosamine) was higher than into lactose. It may be so that branching points arise through the concerted action of *N*-acetylglucosaminyltransferase and of *N*-acetyllactosamine synthase. We obtained preliminary evidence for such concerted action of the enzymes in question showing that a more complex oligosaccharide(s) was synthesized if UDP-D-galactose was also present in the reaction mixture. Since human serum contains *N*-acetyllactosamine synthase as the only transferase capable of transferring D-galactose into *N*-acetyl-D-glucosamine residues [3], the effect of UDP-D-galactose must have been mediated through this enzyme.

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