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CHARACTERIZATION OF *N*-ACETYL-β-D-GALACTOSAMINYL-TRANSFERASE FROM GUINEA-PIG KIDNEY INVOLVED IN THE BIOSYNTHESIS OF Sd^a ANTIGEN ASSOCIATED WITH TAMM–HORSFALL GLYCOPROTEIN*

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ABSTRACT

This study reports the catalytic activity of *N*-acetyl-β-D-galactosaminyltransferase from guinea-pig kidney towards such non-glycoprotein acceptors as small oligosaccharides and glycolipids, having a carbohydrate structure similar to that of the Sd^a antigen associated with human Tamm–Horsfall glycoprotein. 3′-*O*-Sialyllactose, but not 6′-*O*-sialyllactose or lactose, was an effective acceptor of the glycosyltransferase. On the basis of enzymic and chemical treatment of the tetrasaccharide obtained by the transfer of [^{14}C]GalNAc to 3′-*O*-sialyllactose, we propose that the glycosyltransferase attaches β-D-GalNAc to O-4 of the galactose residue that is substituted at O-3 by sialic acid. The G_{M3} ganglioside, in which the identical carbohydrate moiety of 3′-*O*-sialyllactose is bound to a ceramide residue, did not serve as an acceptor of the kidney-*N*-acetyl-β-D-galactosaminyltransferase and did not behave as a competitive inhibitor of the Tamm–Horsfall glycoprotein in the transferase assay. These results indicate that the hydrophobic moiety in the ganglioside hinders the action of *N*-acetylgalactosaminyltransferase. Study of the transferase activity towards a heterogeneous glycopeptide species prepared from a $Sd^{(a-)}$ Tamm–Horsfall glycoprotein indicated that guinea-pig kidney enzyme preferentially transferred [^{14}C]GalNAc to the oligosaccharides having a tetra-antennary branching-structure.

INTRODUCTION

N-Acetyl-D-galactosamine (2-acetamido-2-deoxy-D-galactose) in β-glycosidic linkage is the immunodominant sugar of the blood group Sd^a antigen, which is also carried by Tamm–Horsfall (T-H) glycoprotein, the major glycoprotein of human urine[1,2]. In a previous study, we demonstrated that guinea-pig kidney microsomal preparations contain an D-*N*-acetyl-β-galactosaminyltransferase that preferentially transfers the sugar to $Sd^{(a-)}$ T-H glycoproteins practically devoid of *N*-acetyl-D-

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galactosamine[3]. The activity of this enzyme requires the presence of sialic acid in the acceptor, and we proposed that GalNAc was transferred to O-4 of a galactose residue that is substituted by sialic acid[3]. These results were consistent with the characterization[4] of a Sd^a-active pentasaccharide, isolated from a preparation of $Sd^{(a+)}$ T-H glycoprotein, that has the following structure: β-D-GalNAc-(1→4)-[α-D-NeuAc-(2→3)]-β-D-Gal-(1→4)-β-D-GlcNAc-(1→3)-D-Gal.

Several lines of evidence[3,5–9] indicate that the carbohydrate moieties of T-H glycoprotein are heterogeneous, being comprised of oligosaccharides *N*-linked to asparagine, that diverge both in the number of *N*-acetyllactosamine groups bound to the trimannosyl core, and in the type and number of substituent sugars inserted at each *N*-acetyllactosamine chain. Some of the T-H glycoprotein oligosaccharides have the branching pattern referred to as the tetraantennary type[9] and there are convincing indications that GalNAc is preferentially located, as a non-reducing terminal sugar, in chains of this types[8]. We[8] suggested that this GalNAc location could reflect an *N*-acetyl-D-galactosaminyltransferase activity that would preferentially transfer GalNAc to the *N*-acetyllactosamine branch attached to O-6 of an α-D-mannose residue of the trimannosyl group. This type of branching pattern usually occurs in tetraantennary oligosaccharides.

The present study on characterization of the substrate specificity of the guinea-pig kidney *N*-acetyl-β-D-galactosaminyltransferase focused on the following points. (*a*) In order to ascertain the substrate specificity of the enzyme with regard to the type of isomeric orientation of sialic acid in the acceptor, the ability of 3′-*O*-sialyllactose and 6′-*O*-sialyllactose to serve as acceptors was investigated. (*b*) As the Sd^a-active portion of T-H glycoprotein is very similar to the carbohydrate chain of G_{M2} ganglioside, the activity of the kidney enzyme in transferring GalNAc to an appropriate ganglioside, such as G_{M3}, and to produce G_{M2} ganglioside was assayed. (*c*) Because tetraantennary oligosaccharides may be fractionated, as retained components, by leucoagglutinin–agarose[10], multibranched T-H glycopeptides to which [^{14}C]GalNAc had been transferred by *N*-acetyl-D-galactosaminyltransferase were chromatographed on leucoagglutinin–agarose in order to evaluate the distribution of transferred GalNAc in the oligosaccharides differing in the branching pattern.

EXPERIMENTAL

Materials. — UDP-*N*-acetyl-D-[^{14}C]galactosamine (61.5 Ci/mol) was from Radiochemical Centre Amersham U.K. and unlabeled UDP-GalNAc from Sigma. Leucoagglutinin (Pharmacia, Uppsala, Sweden) was immobilized to Affi-Gel 15 (Bio-Rad, Richmond, CA) by a procedure detailed elsewhere[11]. Antiserum to T-H glycoprotein was raised in rabbits as described[12]. Silica gel 60 and plates of h.p.l.c. silica gel 60 were from Merck Mannheim, Germany. Neuraminidase from *Vibrio cholerae* was from Boehringwerke, Marburg, Germany; *N*-acetyl-α-D-galactosaminidase *(Charonia lampus)* was from Seigakaku Kogyo, Tokyo, Japan; and β-

hexosaminidase *(jack bean)* was from Sigma. All chemicals were of reagent grade.

Preparation of acceptors. — T-H glycoprotein devoid of GalNAc was prepared from urine of a $Sd^{(a-)}$ individual (B.N.) by a procedure that involved three salt-precipitation steps as described by Tamm and Horsfall[13]. A portion of this glycoprotein preparation was subjected to pronase digestion and the multibranched, sialylated glycopeptides (T-H glycopeptide) was fractionated by chromatography on Bio-Gel P-10 and DEAE-Sephacel as described[3]. Both 3′- and 6′-*O*-sialyllactose were purified from a commercial mixture of the two isomers (Sigma) by ascending chromatography on Whatman No. 3 paper in 5:5:1:3 (v/v) ethyl acetate–pyridine–acetic acid–water (solvent *A*), developed twice as suggested by Bartholomew *et al.*[14]. Under these chromatographic conditions, 6′-*O*-sialyllactose has a mobility markedly lower than that of the 3′-isomer. For preparative separation, 25 mg of the commercial mixture was placed on the paper in 20 spots. Marginal strips were cut from the dried chromatogram and the positions of the two isomers were detected by silver nitrate reagent[15]. On the basis of the chromatographic migration of the two compounds, selected zones were cut from the paper and eluted with water. The eluted samples were lyophilized and passed through a column of Bio-Gel P-2 equilibrated and eluted with water. The trisaccharide recovered in the void volume from the column was analyzed for carbohydrate composition. The molar ratio between sialic acid and neutral sugars was found to be 0.9:2. Sialic acid was determined by the Warren method[16] and neutral sugars by phenol–sulfuric acid assay[17]. Both 3′-*O*-sialyllactose and 6′-*O*-sialyllactose migrated as single compounds both in paper chromatography (solvent *A*) and in t.l.c. (developed with 100:10:10:3:30 ethanol–pyridine–1-butanol–acetic acid–water, solvent *B*). The G_{M2} and G_{M3} gangliosides were kindly provided by G. A. Schwarting (Eunice Kennedy-Shriver Institute for Mental Retardation, Waltham, MA, U.S.A.).

Preparation of microsomes as a source of the glycosyltransferase. — Guinea-pigs were killed by decapitation and the kidneys and brain removed and placed on ice. Microsomes were prepared from both tissues as previously described[3]. The microsomal pellets from both tissues were suspended in 0.2M Tris-HCl buffer, pH 7.5, (20 mg of protein/mL); stored at −80°, they did not lose transferase activity within one month.

Incubation mixture for the glycosyltransferase assay. — The standard incubation-mixture had the following final concentration in a total volume of 50 μL: 0.1M Tris-HCl, pH 7.5, 20mM $MnCl_2$, 0.5% Triton X-100, and 0.4mM UDP-[^{14}C]GalNAc (13,000 d.p.m./nmol). The concentrations of the various acceptors are given in the Figures or table legends. The microsomes from kidney or brain were added to a final concentration of 1 mg/mL of protein, and the incubation was performed for 1 h at 37°. The reaction was stopped by the addition of 10 volumes of cold water. The incorporation of [^{14}C]GalNAc into T-H glycoprotein or to T-H glycopeptide was determined as previously described[3].

Separation and analysis of tetrasaccharide obtained from the transfer of

[^{14}C]GalNAc. — The incubation mixture containing 3′- or 6′-*O*-sialyllactose was centrifuged at 100,000*g* for 1 h at 4° and the supernatant solution was loaded onto a column (1 × 10 cm) of DEAE-Sephacel equilibrated with 0.04M Tris-HCl, pH 8. The column was eluted with a linear gradient of NaCl (50 mL of 0.04M Tris-HCl, pH 8, and 50 mL of the same buffer containing 0.5M NaCl). Fractions of 1 mL were collected at a rate of 10 mL/h and the radioactivity was monitored in each fraction. The fractions containing *O*-sialyllactoses were pooled, lyophilized, and desalted on a column of Bio-Gel P-2 equilibrated with water. The products were then analyzed by t.l.c. on plates of silica gel, twice developed with solvent *B*. Chromatographic mobilities were compared with those of oligosaccharide standards. Labeled products were made visible by autoradiography and quantitated by liquid-scintillation counting after scraping off the gel corresponding to each radioactive spots and suspending it in 0.5 mL of water. Unlabeled oligosaccharides were detected with anisaldehyde reagent[18].

Anomeric configuration of GalNAc transferred to O-sialyllactose. — The configuration was ascertained after treatment of the tetrasaccharide with 0.05M H_2SO_4 for 1 h at 80°, in order to remove sialic acid. Digestions with *N*-acetyl-α-D-galactosaminidase and with β-hexosaminidase were performed as previously described[3]. At the end of the enzyme treatments, the products were *N*-reacetylated in saturated $NaHCO_3$ with two additions of acetic anhydride (10 μL) and kept overnight at room temperature. The products were then desalted by coupled columns of Dowex 1 and Dowex 50 resins, and applied to silica gel plates. T.l.c. plates were developed with solvent *B* and the radioactive compounds quantitated as already described.

N-*Acetylgalactosaminyltransferase activity towards gangliosides.* — Standard assay-conditions used a final volume of 25 μL and 25 μg of G_{M3} ganglioside as an acceptor. At the end of the incubation time (1 h), the entire mixture was applied to plates (10 × 10 cm) of h.p.l.c. silica gel having wicks of Whatman No. 3 paper clamped to the tops of the plates. Chromatograms were developed with 11:9 (v/v) methanol–water for 7 h to remove all hydrophilic compounds from the origin. The plates were then dried and redeveloped with 60:40:9, methanol–chloroform–water containing 0.02% $CaCl_2$, in order to separate the gangliosides. Unlabeled G_{M2} and G_{M3} were detected by resorcinol[19], and radioactive compounds were located by autoradiography. Quantitation of radioactive spots was determined as already described.

Affinity chromatography on leucoagglutinin–agarose. — [^{14}C]GalNAc-labeled T-H glycopeptide was separated from the glycosyltransferase assay-mixture by a column (1 × 80 cm) of Bio-Gel P-10 equilibrated and eluted with 0.1M pyridine acetate buffer, pH 5. The labeled T-H glycopeptide recovered in the elution position of multi-branched glycopeptides was lyophilized and subjected to affinity chromatography on leucoagglutinin–agarose. The column (0.5 × 15 cm) was eluted first with 0.05M sodium phosphate buffer, pH 6.5, and then with sodium acetate buffer, pH 4, containing M NaCl.

Carbohydrate analysis. — This was performed by g.l.c. after methanolysis as described by Dunstan *et al.*[20].

RESULTS

N-Acetyl-D-galactosaminyltransferase activity towards 3'- and 6'-O-sialyllactose. — The substrate specificity of the enzyme with respect to the substitution position of sialic acid in the acceptor was determined by using 3'- and 6'-*O*-sialyllactose purified by paper chromatography. When microsomes from guinea-pig kidney, solubilized by Triton X-100, and UDP-[^{14}C]GalNAc were incubated with 3'-*O*-sialyllactose, a radiolabeled product was recovered by chromatography on DEAE-Sephacel which, from its elution position, was neither UDP-GalNAc nor GalNAc (Fig. 1A). GalNAc was produced, under the incubation conditions, by the degradation of nucleotide sugars by kidney microsomal pyrophosphatases (Fig. 1A). As the labeled product coeluted with *O*-sialyllactoses and its formation was proportional to the amount of 3'-*O*-sialyllactose present in the incubation mixture (Fig. 2), it was assumed to be the product of enzymic transfer of GalNAc to the 3'-*O*-sialyllactose, namely [^{14}C]-*N*-acetyl-D-galactosaminyl-[sialyl-(2→3)]-lactose. No analogous product was fractionated from an identical incubation-mixture containing 6'-*O*-sialyllactose as the acceptor (Fig. 1B). This result indicates that the activity of the kidney transferase was inhibited if the acceptor contained sialic acid bound to O-6 of the galactose. [^{14}C]-*N*-Acetyl-D-galactosaminyl-[sialyl-(2→3)]-lactose fractionated by DEAE-Sephacel chromatography migrated in paper chromatography (solvent *A*) and in t.l.c. (solvent *B*) as an homogeneous compound with a mobility, in both systems, lower than that of the 3'-*O*-sialyllactose. Lactose

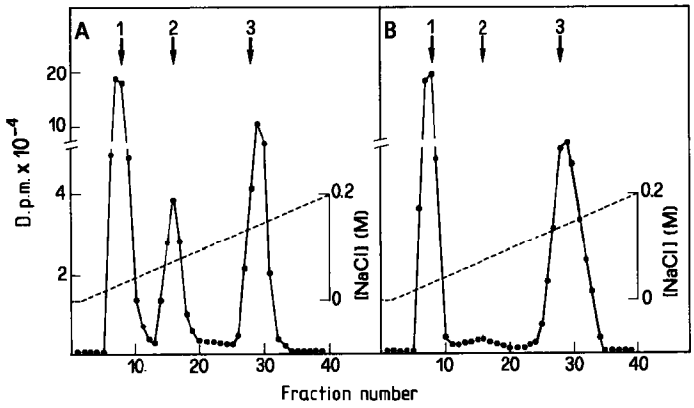

Fig. 1. *N*-Acetyl-D-galactosaminyltransferase activity towards 3'- and 6'-*O*-sialyllactose: Standard assay-conditions employed 50 μg of acceptor in a total volume of 50 μL. The supernatant solution from the incubation reaction was chromatographed on a column of DEAE-Sephacel as described in the Experimental section. Arrows indicated the elution position of GalNAc (1), 3'- or 6'-sialyllactose (2), and of UDP-GalNAc (3). *A*, assay with 3'-*O*-sialyllactose; *B*, with 6'-*O*-sialyllactose.

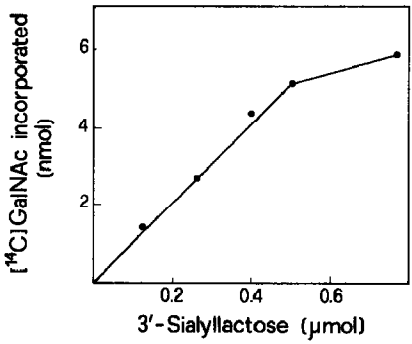

Fig. 2. *N*-Acetyl-D-galactosaminyltransferase activity as a function of concentration of 3'-sialyllactose. The incubation conditions and the separation of 3'-sialyllactose that had incorporated [^{14}C]GalNAc were as described in Fig. 1.

did not serve as an acceptor of the kidney enzyme, in that no product derived from the addition of [^{14}C]GalNAc to the disaccharide could be detected by paper chromatography or t.l.c. when it was added to the glycosyltransferase reaction-mixture. This result indicated that, even with a small oligosaccharide acceptor, sialic acid in the substrate is required for *N*-acetyl-β-galactosaminyltransferase activity.

Anomeric configuration of [^{14}C]GalNAc transferred to 3'-O-sialyllactose. — As a previous study[3] had shown that GalNAc added to T-H glycopeptide by the kidney transferase was released by β-hexosaminidase only after the desialylation of the glycopeptide, [^{14}C]-*N*-acetyl-D-galactosaminyl-[sialyl-(2→3)]-lactose was subjected to procedures that usually remove sialic acid and subsequently digested with β-hexosaminidase. T.l.c. of the tetrasaccharide exhaustively treated with *Vibrio cholerae* neuraminidase revealed that it was resistant to the enzyme, in that no modification of the chromatographic mobility was observed after this treatment (see lanes a and b of Fig. 3). To remove sialic acid, mild acid hydrolysis was performed with 0.05M H_2SO_4 for 1 h at 80°. The effect of this treatment was detected by subjecting the mild acid-treated tetrasaccharide to t.l.c. and by quantitative evaluation of the labeled products; 38% of the tetrasaccharide remained unmodified, whereas 39% was transformed into a faster-migrating product that was assumed to be the expected asialo-trisaccharide (*N*-acetyl-D-galactosaminyl-lactose). Of the remaining radioactivity, 9% was recovered at the position for free GalNAc and 14% was detected as a product having a chromatographic mobility between that of GalNAc and the asialo-trisaccharide (Fig. 3, lane c). Very probably the former product arose by hydrolysis of the terminal *N*-acetyl-D-galactosaminyl linkage and the latter through internal splitting of the β-galactosidic bond of the lactose residue. The lower extent of scission of the terminal GalNAc linkage in comparison with that of the internal galactosidic linkage may be attributable to the fact that, although terminal GalNAc is fairly readily split by acid, if deacetylation would occur prior to this scission, the resultant charged amino group would make

Fig. 3. T.l.c. autoradiography of β-D-GalNAc-(1→4)-[α-D-NeuAc-(2→3)]-β-D-Gal-(1→4)-D-Glc subjected to mild acid hydrolysis and glycosidase digestions: chromatography was performed on silica gel plates in solvent *B*, and the labeled products made visible by autoradiography; (*a*) tetrasaccharide obtained by enzymic addition of [^{14}C]GalNAc to 3′-*O*-sialyllactose, and separated as in Fig. 1; (*b*) the same compound treated with neuraminidase, or (*c*) treated with 0.05M H_2SO_4 for 1 h at 80° and subsequently *N*-reacetylated; (*d*) the compound treated as in (*c*) and subjected to digestion by *N*-acetyl-α-D-galactosaminidase; (*e*) the compound treated as in (*c*) and subjected to digestion by β-hexosaminidase; (*f*) [^{14}C]GalNAc.

the glycosidic linkage more resistant towards acid[21]. In fact a significant amount of GalNAc underwent deacetylation under the conditions of mild-acid treatment, and *N*-reacetylation was necessary before the t.l.c. analysis.

Digestion of mild-acid treated tetrasaccharide with *N*-acetyl-α-D-galactosaminidase did not change the chromatographic mobilities of any compounds (Fig. 3, lane d). In contrast, digestion by β-hexosaminidase cleaved GalNAc almost entirely from the asialo products, leaving the tetrasaccharide unmodified (Fig. 3, lane e). Extension of the mild-acid treatment to 2 h converted the tetrasaccharide almost entirely into the asialotrisaccharide, which was 80% susceptible to digestion by β-hexosaminidase, indicating that GalNAc had been transferred in the β-anomeric configuration by the kidney enzyme.

The resistance of the tetrasaccharide to neuraminidase and to β-hexosaminidase, prior to desialylation, as well as the increased stability of the sialyl bond to the mild acid hydrolysis, were consistent with previous results indicating that GalNAc is transferred by the kidney enzyme to O-4 of a galactose residue substituted at O-3 by sialic acid. In fact, in either the Sd^a-active oligosaccharide isolated from T-H glycoprotein[4,22] or in G_{M2} ganglioside[23], the contiguity of sialic

acid and GalNAc as substituents at O-3 and O-4, respectively, of the same galactose residue was found to hinder the action of both glycosidases.

N-*Acetyl-D-galactosaminyltransferase activity towards gangliosides.* — The occurrence of *N*-acetyl-D-galactosaminyltransferase that attach the sugar in β-anomeric configuration to O-4 of galactose has been reported in some mammalian tissues, in particular in nerve tissue from embryo or adult animals[24-29]. Such enzymes are involved in the biosynthesis of gangliosides, specifically in the conversion of G_{M3} into G_{M2} ganglioside[24]. The similarity between the carbohydrate moiety of G_{M2} ganglioside and the Sd^a-active terminal, nonreducing portion of T-H glycoprotein was pointed out in previous studies[3,4]. It was thus of interest to determine if the *N*-acetyl-D-galactosaminyltransferase from guinea-pig kidney was able to act towards G_{M3} ganglioside and to convert it into G_{M2} ganglioside. As biosynthesis of the gangliosides is very active in nerve tissue, microsomes prepared from guinea-pig brain were incubated with UDP-[^{14}C]GalNAc and G_{M3} ganglioside. The results reported in Table I show that brain microsomes exhibit *N*-acetyl-D-galactosaminyltransferase activity towards the ganglioside, in that a significant amount of GalNAc-labeled G_{M2} ganglioside was recovered from the incubation mixture containing G_{M3} ganglioside as an acceptor. The same brain microsomes did not transfer [^{14}C]GalNAc to $Sd^{(a-)}$ T-H glycoprotein. When kidney microsomes were assayed for transferase activity towards G_{M3} ganglioside or $Sd^{(a-)}$ T-H glycoprotein, the results were opposite to those obtained with brain microsomes; G_{M3} ganglioside was a very poor acceptor of [^{14}C]GalNAc in comparison with the glycoprotein, indicating that the substrate acceptor-specificity of the two enzyme was different. To confirm that kidney enzyme was active only towards the glycoprotein, the effect of an excess of G_{M3} ganglioside on the glycosyltransferase activity towards T-H glycoprotein was determined. In this experiment, the amount of [^{14}C]GalNAc transferred to T-H glycoprotein was quantitated by precipitating the glycoprotein with anti-(T-H glycoprotein) antiserum. Fig. 4 shows that even an excess of G_{M3} ganglioside did not affect the extent of [^{14}C]GalNAc incorporation in the T-H glycoprotein. Altogether these results indicated that the kidney *N*-acetyl-D-galactosaminyltransferase has no catalytic activity towards the ganglioside.

TABLE I

DIFFERENCE IN THE ACTIVITY OF *N*-ACETYL-D-GALACTOSAMINYLTRANSFERASE FROM KIDNEY AND BRAIN

Enzyme source	[^{14}C]*GalNAc transferred*[a] *(d.p.m.)*	
	G_{M3} *ganglioside*	$Sd^{(a-)}$ *T-H glycoprotein*
Kidney microsomes	152	2130
Brain microsomes	1243	0

[a]Standard assays were performed by using 100 and 25 μg of T-H glycoprotein and ganglioside, respectively, as acceptors. The final volume was 25 μL in both cases. The values of GalNAc transferred to T-H glycoprotein were corrected for endogenous incorporation, and are averages of two separate experiments with each acceptor.

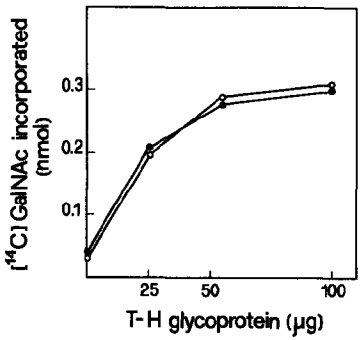

Fig. 4. Effect of adding G_{M3} ganglioside on kidney *N*-acetyl-D-galactosaminyltransferase activity towards $Sd^{(a-)}$ T-H glycoprotein: Standard assay-conditions were used with 100 μg of T-H glycoprotein in a final volume 50 μL. After 1 h of incubation, 100 μL of anti-(T-H glycoprotein) antiserum was added to each mixture and these were kept overnight at 4°. Under these conditions, the antiserum precipitated 95% of the T-H glycoprotein in the concentration range used. This result was confirmed by precipitating an identical solution of [^{3}H]-T-H glycoprotein that had been labeled in the sialic acid by the procedure described in ref. 8; ○, with 25 μg of G_{M3} ganglioside; ●, without G_{M3} ganglioside.

Fractionation of the T-H glycopeptide to which [^{14}C]GalNAc had been transferred by N-acetyl-β-D-galactosaminyltransferase. — We previously reported that, by affinity chromatography on leucoagglutinin–agarose, a tetraantennary glycopeptide could be separated, as a retained component, from the sialylated fraction of T-H glycopeptides[8]. In order to evaluate if the guinea-pig kidney glycosyltransferase differentiates oligosaccharides having higher branching-patterns, T-H glycopeptides, to which [^{14}C]GalNAc had been transferred by *N*-acetyl-β-D-galactosaminyltransferase, were fractionated by leucoagglutinin–agarose. The T-H glycopeptide species used for this experiment was purified by chromatography on DEAE-Sephacel from pronase-digested T-H glycoprotein of a $Sd^{(a-)}$ individual (B.N.). As expected, no GalNAc was detectable in this T-H glycopeptide species and the carbohydrate composition indicated a structure of *N*-linked, *N*-acetyl-

TABLE II

CARBOHYDRATE COMPOSITION OF A T-H GLYCOPEPTIDE FRACTION SEPARATED BY CHROMATOGRAPHY ON DEAE-SEPHACEL FROM PRONASE-DIGESTED T-H GLYCOPROTEIN OF A $Sd^{(a-)}$ INDIVIDUAL (B.N.)

Monosaccharide	*Residue × mol^{-1}* [a]
Fucose	1.3
Mannose	3.0
Galactose	4.8
N-Acetyl-D-glucosamine	5.5
N-Acetyl-D-galactosamine	0.1
N-Acetylneuraminic acid	3.8
Total carbohydrate[b]	91.5

[a]Calculated as residues/3.0 of mannose. [b]The value for total carbohydrate is given as g/100 g of glycopeptide and was obtained by summing the values for the individual carbohydrate.

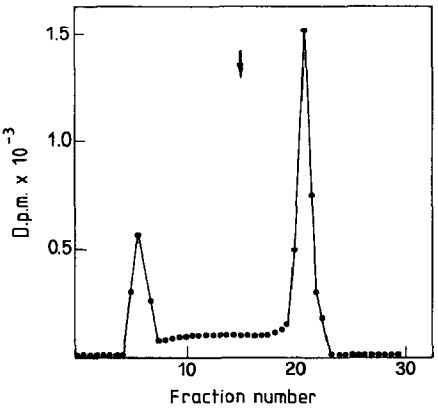

Fig. 5. Fractionation of [^{14}C]GalNAc-labeled T-H glycopeptide by affinity chromatography on leucoagglutinin–agarose: [^{14}C]GalNAc-labeled T-H glycopeptide was isolated from the *N*-acetylgalactosaminyltransferase assay-mixture on a column of Bio-Gel P-10 equilibrated and eluted with 0.1M pyridine acetate buffer, pH 5. The labeled glycopeptide emerged in the elution position of multibranched *N*-linked glycopeptides, and was lyophilized and chromatographed on a leucoagglutinin–agarose column equilibrated with 0.05M sodium phosphate buffer, pH 6.5. This buffer was also used as the first eluent. The arrow indicates the change of the eluent (0.1M acetate buffer, pH 4, containing M NaCl). Fractions of 1 mL were collected and monitored for radioactivity. The recovery of radioactivity was >90%.

lactosamine-type oligosaccharides (Table II). Under our affinity-chromatographic conditions, only 27% of the glycopeptide (calculated on the basis of neutral-sugar analysis) was bound to the lectin-gel and was eluted by an acidic buffer of high-ionic strength. When [^{14}C]GalNAc-labeled T-H glycopeptide from the glycosyltransferase assay-mixture was subjected to leucoagglutinin–agarose chromatography, >50% of glycopeptide was retained by the lectin-gel (Fig. 5), indicating preferential transfer of GalNAc to the tetraantennary glycopeptide.

DISCUSSION

Current data indicate that the *N*-acetyl-β-D-galactosaminyltransferase of guinea-pig kidney is able to act towards small-molecular-weight sialylated oligosaccharides, such as sialyllactose, but it requires sialic acid to be (2→3)-linked to the acceptor for activity. This type of sialidic linkage was found in the Sd^a-active oligosaccharide isolated from T-H glycoprotein[4]. This result therefore strengthens the idea that a similar enzyme in human kidney is involved in the biosynthesis of Sd$^{(a+)}$ T-H glycoprotein. Our results also show that, although G_{M3} ganglioside carries an oligosaccharide indistinguishable from 3′-*O*-sialyllactose, it does not behave as an effective acceptor of the kidney enzyme, suggesting that the hydrophobic moiety of the ganglioside hinders the action of *N*-acetyl-β-D-galactosaminyltransferase. Despite the similarity between the Sd^a antigen and the oligosaccharide portion of G_{M2} ganglioside, liposomes formed with this ganglioside do not inhibit

the interaction between Sd^a antigen and anti-Sd^a antibodies[4]. The inability of the ganglioside to interact with the binding site of anti-Sd^a antibodies, or to serve as an acceptor of *N*-acetyl-β-D-galactosaminyltransferase, suggests that when 3′-*O*-sialyl lactose is bound to ceramide, its three-dimensional conformation is different from that of the free oligosaccharide. Even the preferential action of the glycosyltransferase towards the tetraantennary glycopeptide of T-H glycoprotein suggests that the conformation of the molecule in the vicinity of the acceptor site affects the activity of the enzyme. The tetraantennary branching-pattern might facilitate the accessibility of the terminal portion of antennae to the *N*-acetyl-β-D-galactosaminyltransferase or, alternatively, the enzyme might work preferentially in adding GalNAc to the branch linked to O-6 of an α-mannose residue of the trimannosyl core. This type of branch linkage is typical of the tetraantennary oligosaccharides. A sialyltransferase and a galactosyltransferase that preferentially transfer the sugar to an individual branch of the polyantennary, *N*-linked oligosaccharides have already described[30,31]. On the other hand, the *N*-linked poly-branched structure does not seem to be required by this enzyme, as Conzelmann and Kornfeld[32] found, in a murine, cytotoxic T-lymphocyte cell-line, a similar, if not identical, glycosyltransferase that is able to add β-D-GalNAc both to *O*-linked chains of glycophorin and to *N*-linked chains of T-H glycoprotein. Glycophorin in human erythrocytes was found as a major carrier of the Cad specificity[33]; this specificity is very similar to that of Sd^a, in that both determinants share the same terminal trisaccharide unit, β-D-GalNAc-(1→4)-[α-D-NeuAc-(2→3)]-D-Gal[4,33]. However, the nature of the Sd^a-determinant carrier on the erythrocyte surface is still unknown, and thus further investigation is required to ascertain if the enzyme characterized here is also responsible for the addition of the Sd^a-immunodominant sugar to the oligosaccharide located on the erythrocyte surface.

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