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α-D-GALACTOSYLTRANSFERASE ACTIVITY IN CALF THYMUS
A HIGH-RESOLUTION ^{1}H-NMR STUDY

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Summary. In calf thymus an α-D-galactosyltransferase activity has been detected that transfers galactosyl residues from UDP-galactose to suitable acceptors having galactose at the non-reducing terminus. For example, incubation of UDP--[^{14}C]galactose and Galβ(1→4)GlcNAc* (*N*-acetyllactosamine) in the presence of a calf thymus cell-free suspension containing this galactosyltransferase activity resulted in the enzymic synthesis of a ^{14}C-labelled trisaccharide. 500-MHz ^{1}H-NMR spectroscopic analysis revealed the structure of the trisaccharide to be: Galα(1→3)Galβ(1→4)GlcNAc. This study illustrates the suitability of the ^{1}H-NMR method for the analysis of enzymic conversions of carbohydrate chains.

Introduction. In recent years, there is increasing interest in the biosynthesis and catabolism of the carbohydrate chains of glycoconjugates. In particular, many studies are aimed at the unraveling of metabolic pathways, and at the isolation and characterization of the enzymes involved in these processes. These enzymes appear to be highly specific with regard to the nature of the sugar donor and acceptor, to the type and configuration of the glycosidic linkage to be formed and to the spatial structure of the acceptor carbohydrate chain [1-8].

Among the methods that can be used for studying these metabolic processes *in vitro*, recently ^{1}H-NMR spectroscopy has been shown to be capable of monitoring enzymic conversions of carbohydrate chains, both discontinuously, *e.g.*, by characterization of substrates and products before and after the conversion, respectively, as well as continuously, *i.e.*, by observing the structural alterations during the conversion taking place in the NMR-tube [5,6,8,9]. In this study, the NMR method is applied for the characterization of a galactosyltransferase activity detected in calf thymus, in the discontinuous mode.

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* All sugars mentioned possess the D-configuration.

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Materials and methods. Fresh calf thymus (13 g) was homogenized in 9 parts (v/w) of 0.25 M sucrose at 0°C using a Potter-Elvehjem system. The homogenate was centrifuged for 10 min at 700 x g. The sediment was discarded and the supernatant was centrifuged for 60 min at 120,000 x g. The sediment of this centrifugation was rehomogenized in 26 ml sucrose solution to give a membrane suspension at a concentration of 11.8 mg protein/ml. The suspension was stored at -20°C until use.

The system for the galactosylation of *N*-acetyllactosamine consisted of the following components: 13.1 μmol *N*-acetyllactosamine; 1.95 μmol UDP-[^{14}C]Gal (0.64 Ci/mol); 75 μmol sodium cacodylate buffer pH 6.0; 45 μmol $MnCl_2$; 1.9 μmol β-mercaptoethanol; 6.1 μl Triton X-100; 9.4 μmol D-galactono-1,4-lactone in order to suppress endogenous galactosidase activity; 3.8 μmol sodium azide to prevent bacterial growth and calf thymus cell-free suspension (8.85 mg of protein) in a volume of 750 μl. The system was incubated at 37°C for 7 h; then an additional amount of UDP-[^{14}C]Gal (1.95 μmol) was added to compensate for hydrolysis of the sugar nucleotide, followed by incubation for another 16 h.

After incubation the mixture was applied to a small column (1 ml) of Dowex 1X8 acetate (100-200 mesh) and eluted with 5 ml 0.01 M formic acid. The combined eluate and wash fluid were lyophilized and subsequently applied to a calibrated column of Bio-Gel P-4 (Fig. 1). Fractions containing the [^{14}C]galactosylated *N*-acetyllactosamine were pooled and lyophilized. Remaining traces of detergent were finally removed by passing the product over a small column (1 ml) of Octyl--Sepharose (particle size 40-190 μ) in 4 M ammonium acetate pH 5.2. The radioactive oligosaccharide product was obtained by lyophilization of appropriate fractions, in a yield of 0.64 μmol.

Prior to ^{1}H-NMR spectroscopic analysis, the *N*-acetyllactosamine and the [^{14}C]-galactosylated *N*-acetyllactosamine samples were repeatedly treated with D_2O at room temperature. After each exchange treatment, the materials were lyophilized. Finally, the samples were redissolved in 0.4 ml D_2O (99.96 atom% D, Aldrich, U.S.A.) for examination in a 5 mm-tube (528 PP, Wilmad, U.S.A.). 500-MHz ^{1}H-NMR spectroscopy was performed on a Bruker WM-500 spectrometer (SON-facility, Department of Biophysics, Nijmegen University, The Netherlands), operating in the Fourier transform mode and equipped with a Bruker Aspect 2000 computer. For further experimental details, see [10]. For solvent-peak suppression, a WEFT¶ pulse sequence (180°-τ-90°) was used. Spin decoupling difference spectra were measured as described in [11-13]. NOE difference spectra were obtained according to Wagner and Wüthrich [14] in combination with a DANTE pulse sequence for selective suppression of the HOD-line (DASWEFT-technique; C.A.G. Haasnoot, personal communication). Resolution-enhancement of the spectra was achieved by Lorentzian to Gaussian transformation [10]. The probe temperature was 300 K, and was kept constant within 0.1 K. Chemical shifts (δ) are expressed in ppm downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS), but were actually measured by reference to internal acetone (δ 2.225 in D_2O at 300 K), with an accuracy of 0.002 ppm.

Results. The [^{14}C]galactosylated *N*-acetyllactosamine, being the product of the enzymic *in vitro* incubation, was obtained in a pure state by the combination of ion exchange chromatography, gel filtration and hydrophobic interaction chromatography. In this way, the necessary elimination of contaminating substances, which might diminish the quality of the ^{1}H-NMR spectrum to be recorded, was conveniently accomplished. Furthermore, by Bio-Gel P-4 filtration (Fig. 1) the

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¶ Abbreviations: SDDS, spin decoupling difference spectroscopy; NOE, nuclear Overhauser enhancement (or effect); WEFT, water-elimination Fourier-transform; DANTE, delays alternated with nutation for tailored excitation; DASWEFT, DANTE specific WEFT.

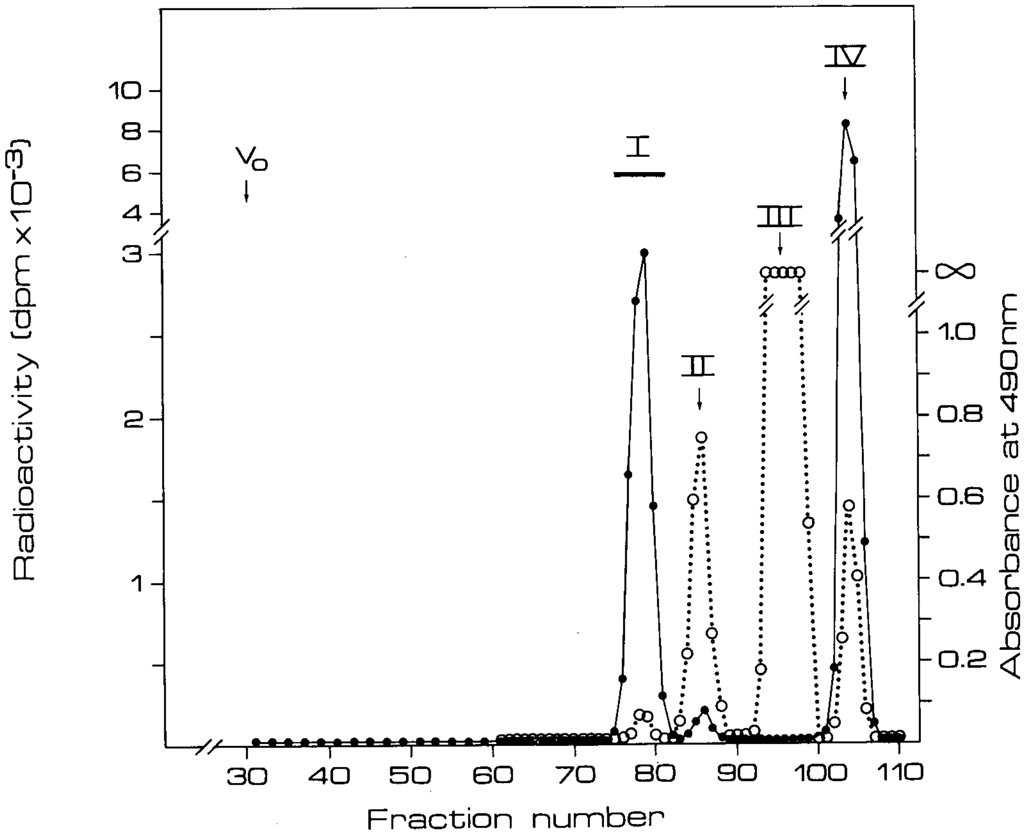

Fig. 1 Isolation of [^{14}C]galactosylated *N*-acetyllactosamine by Bio-Gel P-4 filtration. The neutral oligosaccharide material obtained from the incubation by Dowex 1 chromatography was fractionated on a column (1.6 x 200 cm) of Bio-Gel P-4 (200-400 mesh) maintained at 37°C and equilibrated in 0.05 M ammonium acetate pH 5.2. After addition of 1 mg galactose to the sample, the column was eluted with the same buffer at a flow of 15 ml/h. Fractions of 3.7 ml were collected and aliquots were assayed for ^{14}C-radioactivity (●—●) by liquid scintillation counting, and for neutral sugars by the phenol-sulfuric acid reaction (absorbance at 490 nm, o···o) [17]. Fractions indicated by the bar, containing the [^{14}C]galactosylated oligosaccharide product (I), were pooled and lyophilized.

product (I) could be separated from the substrate *N*-acetyllactosamine (II), sucrose (III) introduced in the incubation system together with the enzyme, and [^{14}C]galactose (IV) originating from UDP-[^{14}C]Gal (and from its degradation product [^{14}C]Gal-1-P) by enzymic hydrolysis. The purity of the isolated oligosaccharide product permitted rapid and reliable determination of the essential structural parameters by ^{1}H-NMR spectroscopy.

The 500-MHz ^{1}H-NMR spectra of *N*-acetyllactosamine and its [^{14}C]galactosylated analogue are presented in Figs. 2 and 3. The spectra of the substrate and of the product of the enzymic incubation are each superpositions of the subspectra of

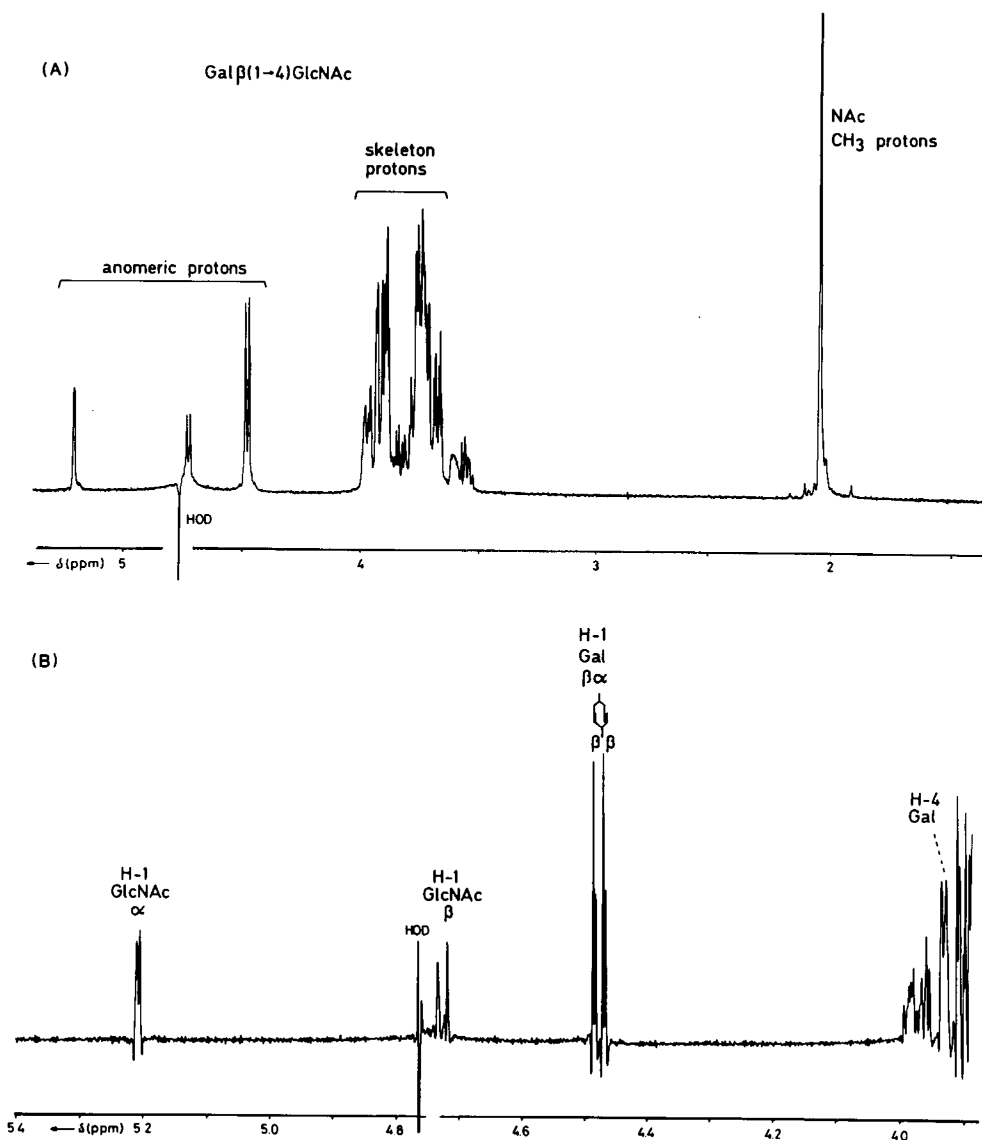

Fig. 2. (A) Overall 500-MHz ^{1}H-NMR spectrum of the substrate disaccharide *N*-acetyllactosamine, in D_2O at 300 K. The HOD-resonance was suppressed by a WEFT-pulse sequence.
(B) Expanded structural-reporter-group regions of the resolution-enhanced 500-MHz ^{1}H-NMR spectrum of *N*-acetyllactosamine.

the two anomers of the reducing oligosaccharides occurring in D_2O solution, having GlcNAc in the α- and β-pyranose form, respectively (compare with [10]).

In the spectrum of *N*-acetyllactosamine (Fig. 2) the following signals are observed clearly separated from the bulk resonance of sugar skeleton protons ($3.4 < \delta < 3.9$). In the anomeric region ($4.3 < \delta < 5.4$), the doublets at δ 5.206

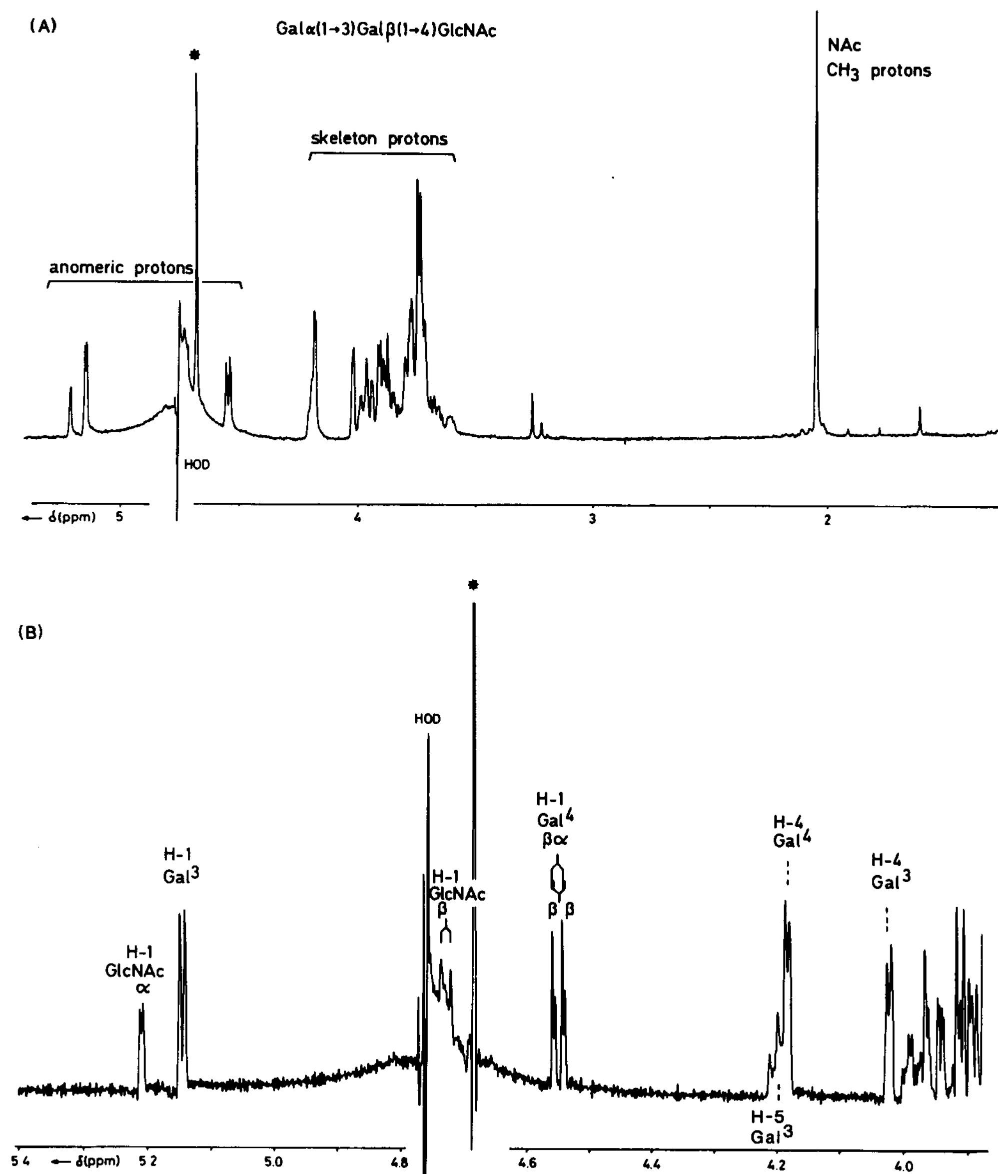

Fig. 3. (A) Overall 500-MHz ^{1}H-NMR spectrum of the product trisaccharide, the galactosylated *N*-acetyllactosamine, in D_2O at 300 K. The HOD-resonance was suppressed by a WEFT-pulse sequence.
(B) Expanded structural-reporter-group regions of the resolution-enhanced 500-MHz ^{1}H-NMR spectrum of galactosylated *N*-acetyllactosamine. A superscript at the name of a sugar residue indicates to which position of the adjacent monosaccharide it is linked. (* = spike)

($J_{1,2}$ = 2.7 Hz) and at δ 4.725 ($J_{1,2}$ = 7.6 Hz), occurring with relative intensities in the ratio of 2:1, are assigned to the α- and β-form of the reducing GlcNAc residue, respectively. The chemical shifts as well as the coupling

constant values are in perfect agreement with those observed for H-1 of the reducing GlcNAc in numerous *N*-acetyllactosamine-type oligosaccharide chains derived from *N*-glycosidic glycan chains of glycoproteins [10] (which bears a Man residue in β(1→4)-linkage). The same holds for the ratio of the α- and β-anomers of the disaccharide, as revealed from spectral integration of the aforementioned doublets [10]. Another pair of doublets occurring in the anomeric ratio is observed at δ 4.478 and 4.474. These signals which have in common a $J_{1,2}$ value of 7.8 Hz, are attributed to H-1 of the non-reducing β(1→4)-linked Gal residue in the α- and β-anomer of the disaccharide, respectively. In the high-field part of the spectrum, two singlets occur, at δ 2.044 and 2.042, which belong to the *N*-acetyl methyl protons of GlcNAc in the α- and β-form, respectively.

The 500-MHz ^{1}H-NMR spectrum of the galactosylated *N*-acetyllactosamine (Fig. 3) shows again a set of doublets at δ 5.209 ($J_{1,2}$ = 2.4 Hz; α-anomer) and 4.727 ($J_{1,2}$ = 7.6 Hz; β-anomer) for H-1 of the reducing GlcNAc residue. The *N*-acetyl signal coincides for both anomers at δ 2.043. A pair of anomeric doublets separated by a relatively small difference in chemical shift due to anomerization ($\Delta\delta_{\alpha-\beta}$ = 0.004 ppm; intensity ratio 2:1), is found at δ 4.550 and 4.546 ($J_{1,2}$ = 7.8 Hz for both), indicating the presence of a β-linked Gal residue in the trisaccharide, that possesses its anomeric proton in the sphere of influence of the anomeric centre of GlcNAc. Finally, a single doublet occurs at δ 5.145 ($J_{1,2}$ = 3.75 Hz). The combination of the H-1 chemical shift and the $J_{1,2}$ value points to an α-glycosidic linkage for this third constituent monosaccharide, being the transferred Gal. The H-1 chemical shift value is unaffected by anomerization of the trisaccharide which indicates that this Gal residue is more remote from the GlcNAc anomeric centre than the former one.

The type of linkage between the two Gal residues has been established to be (1→3), by combination of SDDS and NOE experiments, essentially analogous to those applied for solving a similar structural problem in ceramide oligosaccharides [11-13]. These double-resonance techniques enable to detect neighbouring, scalar--coupled (HCC'H') and dipolar-coupled (HCC'H', but also, *e.g.*, HCOC'H') protons, respectively. Preirradiation of the H-1 doublet of the α-Gal residue at δ 5.145 gives rise to an NOE difference signal at δ 3.863, representing the H-2 resonance of the same sugar ring ($J_{1,2}$ = 3.8 Hz; $J_{2,3}$ = 10.5 Hz), and two more, at δ 3.79 and 4.182, corresponding to the H-3 ($J_{2,3}$ = 10.4 Hz; $J_{3,4}$ = 3.4 Hz) and H-4 ($J_{3,4}$ = 3.2 Hz; $J_{4,5}$ ≃ 1.0 Hz) signals of the β-Gal residue, respectively. Therefore, the theoretical possibilities of a Galα(1→2)Galβ or a Galα(1→6)Galβ type of linkage can already be ruled out. The sequence Galα(1→•)Galβ is unambiguously determined to be (1→3) rather than (1→4), on the basis of the chemical shifts of H-2 of β-Gal (δ 3.663, α-anomer, and δ 3.669, β-anomer of trisaccharide) found by SDDS upon irradiation of the H-1 doublets at δ ≃ 4.55. Owing to α-galactosylation, H-2 of β-Gal is shifted downfield by Δδ ≃ 0.11 ppm as compared to a

terminal β-linked Gal residue (see also [15]). Such a shift may be expected for (1→3), but not for (1→4) substitution [11]. An α(1→4)-linkage would require δ ≈ 3.58 for H-2 of the β-linked Gal [H. van Halbeek, unpublished results] (*cf.* [11-13]). On the other hand, the discovery of the H-2 resonances of β-Gal enabled to verify the identity of the aforementioned H-3 and H-4 signals, with the aid of SDDS of H-2 and H-3, respectively.

Once the occurrence of the Galα(1→3)Galβ(1→•) structural element in the product trisaccharide has been established, its recognition by means of chemical shifts of structural-reporter groups only is greatly facilitated. In addition to the chemical shifts of the anomeric protons of both Gal residues, it should be emphasized that the attachment of the second Gal in α(1→3)-linkage leads to a shift increment for H-1 of the substituted, β-Gal ($\Delta\delta$ = 0.07 ppm). This effect is very similar to that of the attachment of sialic acid in α(2→3)-linkage to such a Gal residue [10]. Moreover, H-4 of the penultimate Gal undergoes a shift increment from δ 3.929 for the disaccharide to δ 4.182 in the trisaccharide spectrum, while the H-4 signal of the terminal, α-linked Gal is found at δ 4.021. The H-5 signal of α-Gal is found at δ 4.194; this relatively downfield position makes H-5 a very suitable reporter for recognition of the α-Gal residue (*cf.* [16]). Finally, it is noteworthy that the structural elements of Galα(1→3)-Galβ(1→•) and Galα(1→4)Galβ(1→•) can be clearly distinguished by the chemical shift of H-1 of the α-linked Gal residue (δ 4.946 in the latter case) [H. van Halbeek *et al.*, unpublished results]. The trend observed for ^{1}H chemical shifts of the Galα(1→3)Gal unit as compared to those of the Galα(1→4)Gal structural element, when investigating oligosaccharides in aqueous solution, is similar to that described for ceramide oligosaccharides in dimethylsulfoxide-d_6 [11-13].

Discussion. The structure of the [^{14}C]galactosylated *N*-acetyllactosamine isolated from the *in vitro* incubation mixture clearly indicates that calf thymus contains a UDP-Gal : *N*-acetyllactosamine galactosyltransferase activity capable of attaching galactosyl residues in α(1→3)-linkage to galactose of Galβ(1→4)-GlcNAc. The enzyme appears to be quite distinct from the blood-group B associated α(1→3) galactosyltransferase since the latter enzyme requires a fucosyl residue α(1→2)-linked to the galactose of the acceptor substrate [18]. A galactosyltransferase which seems to be closely related to the calf thymus enzyme has been described to occur in Ehrlich ascites tumour cells [19]. This enzyme acts on Galβ(1→4)GlcNAc producing a trisaccharide structure which is identical to that of the galactosylated *N*-acetyllactosamine described in this study. Further details regarding the properties and specificity of the α-galactosyltransferase in calf thymus will be published elsewhere.

This report is an example of the applicability of high-resolution ^{1}H-NMR spectroscopy in the study of the specificity of enzymes involved in the metabo-

lism of the carbohydrate chains of glycoconjugates. The method is capable of assigning rapidly and non-destructively, alterations in oligosaccharide structures in relation to enzymic conversions. Even, the determination of the sugar sequence and of the sites of glycosidic attachments is feasible on the basis of ^{1}H-NMR spectroscopy, only. The present study deals with relatively small oligosaccharides, but it can be anticipated that, on the basis of the parameters which are defined above for the Galα(1→3)Galβ(1→4)GlcNAc sequence, also in small amounts of larger substrate molecules, such structural elements and alterations in it can be analyzed adequately.

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