Glycoconjugate Journal (1992) **9**:251–264

Reassessment of the acceptor specificity and general properties of the Lewis blood-group gene associated α-3/4-fucosyltransferase purified from human milk

PHILIP H. JOHNSON[1,2], ALASTAIR S. R. DONALD[1]†, JAMES FEENEY[3] and WINIFRED M. WATKINS[1,4]*

[1] *Division of Immunochemical Genetics, MRC Clinical Research Centre, Watford Road, Harrow, Middlesex, HA1 3UJ, UK*

[2] *MRC Human Biochemical Genetics Unit, University College London, Wolfson House, 4 Stephenson Way, London, NW1 2HE, UK*

[3] *Biomedical Nuclear Magnetic Resonance Centre, National Institute for Medical Research, Mill Hill, London, NW7 1AA, UK*

[4] *Department of Haematology, Royal Postgraduate Medical School, Hammersmith Hospital, Du Cane Road, London, W12 0NN, UK*

Received 23 June 1992

The acceptor specificity and general properties of a Lewis blood-group gene associated α-3/4-L-fucosyltransferase isolated from human milk have been examined at the penultimate purification stage involving affinity chromatography on GDP-hexanolamine Sepharose, and after a subsequent gel filtration step on Sephacryl S-200. Both preparations transferred fucose to the *O*-4 position of *N*-acetylglucosamine in Type 1 (Galβ1-3GlcNAc-R) acceptors and the *O*-3 position of glucose in lactose-based (Galβ1-4Glc) oligosaccharides, and both used Type 1 sialylated compounds when the terminal *N*-acetylneuraminic acid was present in α-2,3 linkage. The striking difference between the two preparations was in their reactivity with Type 2 (Galβ1-4GlcNAc-R) chains; after Sephacryl S-200 chromatography the apparent K_M values for the α-3/4- preparation with unsubstituted low-molecular-weight Type 2 oligosaccharides were considerably increased. Substitution of the terminal galactose with sialic acid in α-2,3 linkage decreased the K_M values for low-molecular-weight oligosaccharides but no detectable incorporation of fucose was observed into *N*-acetyllactosamine end-groups of glycoproteins with *N*-linked oligosaccharide chains, irrespective of the presence of sialic acid in the terminal sequences.

Keywords: α-3/4-L-fucosyltransferase, Lewis blood-group gene encoded enzyme, Lewis antigens, sialyl-Le[a]; X-antigen, sialyl-X antigen, human milk

Purification of the Lewis blood-group gene associated α-3/4-fucosyltransferase from human milk up to a stage when it was eluted from the affinity absorbent, GDP-hexanolamine Sepharose 4B, yielded a 500 000-fold purified enzyme preparation (GDP-HS Eluate) that transferred fucose to *N*-acetylglucosamine in both Type 1 (Galβ1-3GlcNAc-R) and Type 2 (Galβ1-4GlcNAc-R) acceptors at approximately the same rate [1]. In this respect the preparation resembled the highly purified α-3/4-L-fucosyltransferase described earlier by Prieels *et al.* [2] which had the capacity to function as both an α-3- and α-4-fucosyltransferase with respect to *N*-acetylglucosamine and as an α-3-fucosyltransferase with respect to D-glucose in lactose-based structures. The co-purification of these activities through a series of purification steps led the authors to suggest that the three activities were combined in a single enzyme encoded by the Lewis blood-group gene. However, gel filtration of the GDP-HS Eluate on Sephacryl S-200 or Sephadex G-150, was found to remove a contaminant of α-3-fucosyltransferase catalysing the addition of fucose exclusively to *N*-acetylglucosamine in Type 2 chains that had remained associated with the α-3/4-fucosyltransferase up to this final purification stage [1]. The residual α-3/4-enzyme (S-200 Eluate 3) retained the capacity to transfer fucose to the *O*-4 position in Type 1 chains and to the *O*-3 position of glucose in lactose-based structures, but had very restricted activity with *N*-acetylglucosamine in Type 2 acceptors.

† Deceased 25 June 1991.

* To whom correspondence should be addressed.

The X [Galβ1-4(Fucα1-3)GlcNAc-R], Y [Fucα1-2Galβ1-4(Fucα1-3)GlcNAc-R] and sialyl-X [NeuAcα2-3Galβ1-4(Fucα1-3)GlcNAc-R] cell surface structures have for some time commanded attention because of their rôle as tumour-associated [3, 4] and differentiation antigens [5] and, more recently, much interest in these structures has been generated by the discovery of their function as possible ligands for adhesion molecules involved in inflammatory reactions [6, 7]. In view of the importance of deciding whether the Lewis-gene-encoded enzyme is responsible for the biosynthesis of X, Y and sialyl-X structures in addition to Le^a [Galβ1-3(Fucα1-4)GlcNAc-R], Le^b [Fucα1-2Galβ-1-3(Fucα1-4)GlcNAC-R] and sialyl-Le^a [NeuAcα2-3Galβ1-4(Fucα-1-3)GlcNAc-R] structures, the acceptor specificities and general properties of the α-3/4-fucosyltransferase purified 500 000-fold up to the affinity chromatography stage (GDP-HS Eluate) have been compared with those of the preparation obtained by gel filtration of this fraction on Sephacryl S-200 (S-200 Eluate 3) [1].

Materials and methods

Substrates

GDP-L-[U-^{14}C]Fucose (254 Ci mol^{-1}) was purchased from Amersham International. Unlabelled GDP-L-fucose was synthesized by the method of Nunez *et al.* [8], and the structure was confirmed by ^{1}H NMR analysis.

Low-molecular-weight acceptors

N-Acetyllactosamine (Galβ1-4GlcNAc) and lacto-*N*-biose 1 (Galβ1-3GlcNAc) were synthesized by the methods of Alais and Veyrières [9] and Flowers [10], respectively. Galβ1-4ManAc was isolated as a by-product of the synthesis of *N*-acetyllactosamine and Galβ1-3Glc was synthesized by the method of Kuhn and Baer [11]. 2′-Fucosyllactose (Fucα1-2Galβ1-4Glc), lacto-*N*-tetraose (Galβ1-3GlcNAcβ1-3Galβ1-4Glc), lacto-*N*-neotetraose (Galβ1-4GlcNAc-β1-3Galβ1-4Glc), lacto-*N*-fucopentaose I (Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc), lacto-*N*-fucopentaose II [Galβ1-3-(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc] and lacto-*N*-fucopentaose III [Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc] were recovered from human milk as described by Donald and Feeney [12] and the purity and structures of all these oligosaccharides were confirmed by ^{1}H NMR analysis. Lactose was purchased from BDH Biochemicals (UK) and recrystallized before use. Lactitol (Galβ1-4Glc-ol) was prepared by sodium borohydride reduction of lactose. Synthetic 2′-fucosyl-*N*-acetyllactosamine (Fucα1-2Galβ1-4GlcNAc) [13] was a gift of Professor P. Sinaÿ, Université d'Orléans, France, and the Type 1 and Type 2 disaccharides with spacer arms, Galβ1-3GlcNAc-*O*-$(CH_2)_8$COOMe and Galβ1-4GlcNAc-*O*-$(CH_2)_8$COOMe were kindly provided by Professor R. U. Lemieux, University of Alberta, Canada. The blood-group A-active trisaccharide GalNAcα1-3Galβ1-4GlcNAc was isolated from an A-active ovarian cyst glycoprotein as described [14], and the tetrasaccharide GalNAcα1-3(Fucα1-2)Galβ-1-4Glc was purchased from BioCarb Chemicals AB, Sweden. Galβ1-3Ara, Galβ-1-4Man and α- and β-phenyl *N*-acetylglucosaminides were purchased from Sigma Chemical Co. (UK).

The sialic acid-containing oligosacharides 3′-sialyllactose (NeuAcα2-3Galβ1-4Glc), 6′-sialyllactose (NeuAcα2-6Galβ1-4Glc), 3′-sialyl-*N*-acetyllactosamine (NeuAcα2-3Galβ1-4GlcNAc) and 6′-sialyl-*N*-acetyllactosamine (NeuAcα2-6Galβ1-4GlcNAc) were isolated by Dr Cecilia Soh, MRC Clinical Research Centre, from human urine by the method of Parkkinen and Finne [15]. Di-sialyllactose (NeuAcα2-8NeuAcα2-3Galβ1-4Glc) was a gift of Dr A. Corfield, University of Bristol, UK, and sialyllacto-*N*-tetraose (NeuAcα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc) was kindly provided by Dr D. Smith, University of Virginia, USA.

The glycoproteins fetuin, human transferrin and α_1-acid glycoprotein were purchased from Sigma Chemical Co. (UK). A blood-group precursor glycoprotein (No 484) was isolated from an ovarian cyst fluid as described [16]. The patient was a nonsecretor of Lewis or ABH blood group activity and the glycoprotein therefore contained either unsubstituted Type 1 and Type 2 chain endings or these structures substituted with sialic acid or α-3 linked fucose on Type 2 chains [17]. Type XIV pneumococcal polysaccharide was a gift from the late Dr John Humphrey, National Institute for Medical Research (UK). Asialoglycoproteins were prepared by treatment of a 1% solution of the glycoproteins with 0.05 M H_2SO_4 for 1 h at 80 °C. The products were thoroughly dialysed against distilled water and freeze-dried.

The glycolipids lactosylceramide, trihexosylceramide, globoside and lacto-*N*-tetraosylceramide were gifts of Professor J. Koscielak, Institute of Haematology, Warsaw, Poland.

Glycosidases

Neuraminidase from *Vibrio cholerae* was purchased from Koch-Light (UK). α-2- and α-3/4-Fucosidases from *Trichomonas foetus* were separated as described [18] and tested for specificity with lacto-*N*-fucopentaose I and lacto-*N*-fucopentaose II [1]. α-*N*-Acetylgalactosaminidase was isolated from *T. foetus* [19] and tested for specificity with α- and β-nitrophenyl-*N*-acetylgalactosaminides.

Paper and thin layer chromatography

Chromatography papers Nos 40, DE 81 and 3MM were purchased from Whatman Ltd. (UK) and HPTLC silica gel 60 plates were supplied by BDH Chemicals Ltd. (UK). Unlabelled standard sugars were located on paper chromatograms with alkaline silver nitrate reagent [20] and on TLC plates with 0.5% (w/v) orcinol in ethanol containing 0.5% H_2SO_4 (v/v).

The following solvent systems were used: solvent 1, ethyl acetate–pyridine–water (10:4:3 by vol); solvent 2, ethyl acetate–pyridine–water (2:1:1 by vol); solvent 3, ethyl acetate–pyridine–water (12:5:4 by vol); solvent 4, propan-1-ol–ethyl acetate–pyridine–water (5:1:1:3 by vol); solvent 5, phenol–propan-2-ol–formic acid (85:5:10:100 by vol); solvent 6, ethanol–butanol–pyridine–water–acetic acid (500:50:50:150:15 by vol); solvent 7, butanol–ethanol–water (5:3:2 by vol); solvent 8, 1% sodium tetraborate pH 9.1.

Column chromatography media

The ion exchange resin AG1-X4 (200–400 mesh) was obtained from Bio-Rad Laboratories (UK) and Fractogel TSK HW-40(S) from BDH Chemicals (UK). Concanavalin A, purchased from Sigma Chemical Co. (UK), was coupled with cyanogen bromide activated Sepharose 4B (Pharmacia Fine Chemicals Ltd, UK) by the method of Cuatrecasas [21].

Enzyme inhibitors

N-Ethylmaleimide and *p*-hydroxymercuribenzoate were purchased from Sigma Chemical Co. (UK).

Binding of fucosyltransferases to Concanavalin A–Sepharose 4B

Fucosyltransferase preparations in 25 mM cacodylate-HCl buffer, pH 7.3, containing 0.5 M NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5% glycerol and 0.1% bovine serum albumin (0.8 ml) were loaded on 0.4 ml columns of Concanavalin A–Sepharose 4B (substitution 4 mg ml^{-1}). The columns were washed with 0.8 ml of the same buffer and then bound glycoproteins were released by inclusion in the eluting buffer of 0.25 M benzyl α-*N*-acetylglucosaminide. The capacity of the 0.4 ml columns was calculated as 3 mg ml^{-1} for the glycoprotein transferrin and no binding was observed with unglycosylated bovine serum albumin.

Fucosyltransferase assays

Oligosaccharide acceptors. Standard reaction mixtures for α-3- and α-4-fucosyltransferases contained in a total volume of 70 µl; 0.5 µmol acceptor, 0.2 nmol (70 000 counts min^{-1}) GDP-[^{14}C]fucose, 1 µmol $MnCl_2$, 5 µmol sodium cacodylate/HCl buffer, pH 7.3, 5 µg NaN_3 and 250 µg bovine serum albumin. *N*-Acetyllactosamine and 2′-fucosyllactose were the substrates used for routine assays of α-3-fucosyltransferase activity, and lacto-*N*-biose 1 the substrate for routine assays of α-4-fucosyltransferase activity. The reaction mixtures were incubated at 37 °C for between 30 min and 16 h according to the activity of the transferases. After incubation, the mixtures containing neutral oligosaccharides as acceptor sugars were developed by paper chromatography in a solvent system appropriate for the size of the fucosylated oligosaccharides required to be separated and the papers were scanned in a Packard radiochromatogram scanner. The radioactive areas were cut out and counted in a Nuclear Chicago scintillation counter. Products were provisionally characterized by their mobility relative to known reference standards and where the possibility existed that more than one compound could have been synthesized with a given acceptor the product was re-run in a different solvent system under more stringent separation conditions.

When sialylated oligosaccharides were used as acceptor sugars the reaction mixtures were first subjected to paper electrophoresis on Whatman 3MM paper in 40 mM pyridine–acetate buffer, pH 5.4, on a Southern-Shandon electrophoresis apparatus at 4 kV and 80 mA for 2 h. Mobilities ($R_{picrate}$) were calculated relative to a picric acid marker. The papers were scanned in a radiochromatogram scanner and the oligosaccharide product areas were cut out, eluted, and the products were further examined by paper chromatography in solvent 2, and scanned and counted as above.

Glycoprotein acceptors. The reaction mixtures were the same as for the oligosaccharide acceptors except that 100 µg glycoprotein was used as acceptor substrate. The reaction products were separated by chromatography in solvent 4 on Whatman No 40 paper for 20 h. The papers were scanned and counted as above. Radiolabelled glycoproteins remained at the origin of the chromatogram and were thereby separated from the unused GDP-fucose and breakdown products which moved away from the origin. Assay mixtures without acceptor substrate were included to correct for endogenous incorporation of [^{14}C]fucose.

Glycolipid acceptors. The reaction mixtures were the same as for the oligosaccharide acceptors except that 0.025 µmol glycolipid was used in place of the oligosaccharide acceptor and 5 µl 1% Triton X-100 was included in the reaction mixture. At the end of the reaction the glycolipids were extracted from the reaction mixture with chloroform–methanol (2:1 by vol, 0.6 ml) as described by Pacuszka and Koscielak [22] and subjected to chromatography for 16 h on Whatman 3MM paper in solvent 8. Radioactive areas were detected and counted as above.

Large scale biosynthesis of fucosylated products

Oligosaccharide acceptors (3–6 mg) and unlabelled GDP-fucose (5–8 mg) plus 120 000 counts min^{-1} GDP-[^{14}C]fucose were incubated at 37 °C for 48 h with 50 µmol sodium cacodylate/HCl buffer, pH 7.3, 5 µmol $MnCl_2$, 1 mg bovine serum albumin and purified fucosyltransferase preparation in a total volume of 250 µl. Mixtures containing neutral products were spotted on Whatman No 40 paper and run in solvent 1 and detected as above. The appropriate area of the chromatogram was eluted with water, dried by rotary evaporation under reduced pressure at 30 °C, made up to 1 ml with water and loaded on a 190 cm × 1 cm column of Fractogel TSK

40(S). The column was eluted with water at 15 ml h^{-1}. The purity of the fucosylated derivative was assessed by TLC in Solvent 7.

The products containing sialic acid were spotted on pre-moistened Whatman 3MM paper and electrophoresed in 40 mM pyridine–acetate buffer, pH 5.4, on a Shandon-Southern model L24 high voltage electrophoresis apparatus at 4 kV and 80 mA for 1 h. Mobilities were measured relative to a picrate marker. The radioactive products were detected as above and eluted from the paper with 100 mM pyridine–acetate buffer, pH 5.4. The eluate was concentrated by rotary evaporation under reduced pressure at 25 °C, and pyridine–acetate was removed by repeatedly redissolving the product in water and re-drying. The product was reconstituted in 1 ml of water and loaded on a column (160 cm × 0.9 cm) of AG 1-X4 (Ac^-), 200–400 mesh, ion exchange resin equilibrated with 5 mM pyridine–acetate buffer, pH 5.4. The column was eluted with this same buffer at a rate of 15 ml h^{-1}. The fractions were tested for purity by TLC in solvent 6. Final purification was achieved by HPLC chromatography on a Spherisorb-NH2 (pore size 5 μm) column eluted for 5 min with 80% acetonitrile–20% 15 mM KH_2PO_4, pH 5.2, and then with a linear gradient changing from 80% acetonitrile–50% 15 mM KH_2PO_4, pH 5.2, to 50% acetonitrile–50% 15 mM KH_2PO_4, pH 5.2, over 60 min: the fucosylated products formed with 3′-sialyllactose and 3′-sialyl-*N*-acetyllactosamine emerged after 25 min.

Methylation analysis

Methylation with methyl iodide in dimethylformamide with BaO–$Ba(OH)_2$ as catalyst was carried out as described [23]. After hydrolysis with triethylacetic acid for 2 h at 100 °C the monosaccharide methyl ethers were separated into neutral and basic fractions by passage through ion exchange columns and the amino sugar methyl ether fraction was examined on an amino acid analyser [23]. The neutral sugar methyl ethers were reduced with $NaBH_4$ and examined by GLC.

^{1}H-NMR analysis

Samples for ^{1}H-NMR spectroscopy were repeatedly evaporated from ^2H_2O to replace all exchangeable hydrogens. The 500 MHz ^{1}H-NMR spectroscopy was carried out on a Bruker AM500 spectrometer with a probe temperature of 295 K as described previously [12]. Chemical shifts were measured from an acetone internal standard and then expressed as ppm from sodium 4,4-dimethyl-4-silapentane-1-sulfonate (taken as 2.225 ppm from the acetone signal).

Purification of α-3/4-fucosyltransferase

The Lewis blood-group gene associated α-3/4-fucosyltransferase in human milk was purified by chromatography on Phenyl Sepharose 4B, CM-Sephadex C 50 and affinity chromatography on GDP-hexanolamine Sepharose 4B as described previously [1]. The active preparation eluted from GDP-hexanolamine Sepharose 4B was purified 500 000-fold and designated GDP-HS Eluate. Gel filtration of this fraction on Sephacryl S-200 or Sephadex G150 separated a GDP-fucose: galactosyl β1-4-*N*-acetylglucosaminide α-3-fucosyltransferase from the α-3/4-fucosyltransferase [1]. The residual α-3/4-fucosyltransferase preparation eluted from Sephacryl S-200 with sodium cacodylate buffer, pH 5.5, containing 5% glycerol, was designated S-200 Eluate 3 and in preliminary experiments this preparation reacted primarily with Type 1 and lactose-based oligosaccharide chains [1]. These two eluates, GDP-HS Eluate and S-200 Eluate 3, were used in the following experiments.

Results

Effect of pH and divalent cations on the activities of GDP-HS Eluate and S-200 Eluate 3

With the Type 2 acceptor *N*-acetyllactosamine as substrate, GDP-HS Eluate transferred fucose at pHs ranging from 5 to 10 with a broad optimum between pH 7 and pH 9 whereas, with the Type 1 acceptor lacto-*N*-biose 1 as substrate, there was a much sharper optimum between pH 8.7 and 9.0 (Fig. 1a). The further purified S-200 Eluate 3 gave a pH profile with lacto-*N*-biose 1 as acceptor that was similar to that given by GDP-HS Eluate with an optimum at pH 9.2 (Fig. 1b).

The GDP-HS Eluate was active with both lacto-*N*-biose 1 and *N*-acetyllactosamine in the absence of added cations but activity was stimulated with both acceptors by the addition of 20 mM Mn^{2+}, Ca^{2+}, Mg^{2+}, and Co^{2+} ions (Table 1). Mn^{2+} ions were the most effective for both these acceptors, but the activation curves differed in that maximum enhancement with *N*-acetyllactosamine as substrate occurred at 20 mM metal ion concentration and decreased only marginally up to a concentration of 100 mM,

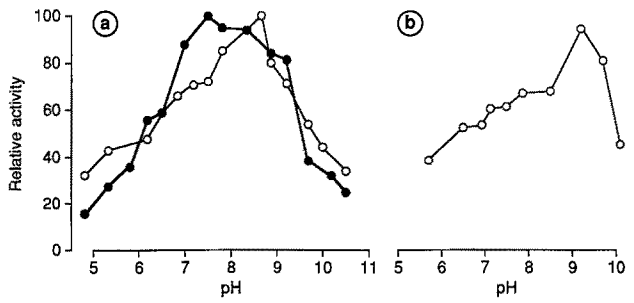

Figure 1. Effect of pH on fucosyltransferase activity: (a) GDP-HS Eluate with Galβ1-4GlcNAc. (●) and Galβ1-3GlcNAc (○) as acceptors (b) S-200 Eluate 3 with Galβ1-3GlcNAc as acceptor. Fucosyltransferase activity was assayed with standard incubation mixtures except that the pH of the buffer was varied as shown.

Table 1. Influence of divalent cations on the fucosyl-transferase activity of GDP-HS Eluate and S-200 Eluate 3.

Divalent cation	*Relative activity* [a]		
	GDP-HS Eluate		*S-200 Eluate 3*
	3-Fucosyl-transferase	*4-Fucosyl-transferase*	*4-Fucosyl-transferase*
No added ion	100	100	100
Mn^{2+}	395	241	300
Ca^{2+}	205	205	320
Mg^{2+}	217	226	200
Ni^{2+}	46	82	80
Co^{2+}	148	158	133
Cu^{2+}	4	4	9
Zn^{2+}	18	79	51

[a] Activity relative to that found in the absence of added divalent cation. Fucosyltransferase activity assayed under standard conditions except that 20 mM $MnCl_2$ was replaced by chlorides of other divalent cations. Galβ1-4GlcNAc and Galβ1-3GlcNAc were the substrates, respectively, for the α-3- and α-4-fucosyltransferase activities.

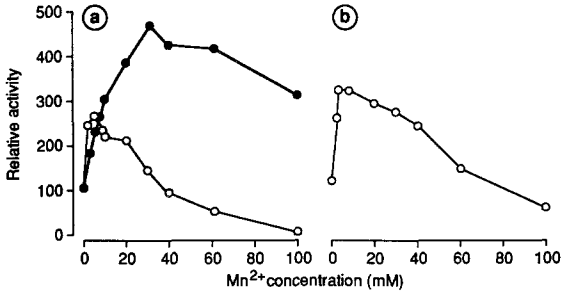

Figure 2. Effect of Mn^{2+} ion concentration on fucosyltransferase activity: (a) GDP-HS Eluate with Galβ1-4GlcNAc (●) and Galβ1-3GlcNAc (○) as acceptors and (b) S-200 Eluate 3 with Galβ1-3GlcNAc as acceptor. Fucosyltransferase activity was assayed with the standard incubation mixture except that the Mn^{2+} concentration was varied as shown.

whereas with lacto-*N*-biose maximum activation occurred at 2.5–5 mM concentration and decreased relatively sharply at higher concentrations (Fig. 2a). The S-200 Eluate 3 gave a similar activation profile with lacto-*N*-biose 1 as substrate (Fig. 2b).

Sensitivity of GDP-HS Eluate to N-*ethylmaleimide and p-hydroxymercuribenzoate*

The activity of GDP-HS Eluate with both *N*-acetyllactosamine and lacto-*N*-biose 1 as acceptors was relatively resistant to *N*-ethylmaleimide (Fig. 3a). At 10 mM concentration 88% of the activity remained with *N*-acetyllactosamine and 70% of the activity with lacto-*N*-biose 1. The activity with both substrates was inhibited to a greater extent by *p*-hydroxymercuribenzoate although with this reagent the α-4-fucosyltransferase activity appeared slightly more resistant than the α-3-fucosyltransferase activity (Fig. 3b). The susceptibility of the

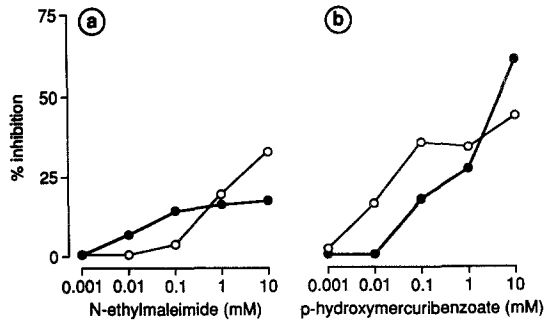

Figure 3. Effect of (a) *N*-ethylmaleimide and (b) *p*-hydroxymercuribenzoate on the fucosyltransferase activity of GDP-HS Eluate with Galβ1-4GlcNAc (●) and Galβ1-3GlcNAc (○) as acceptors. Varying concentrations of the inhibitors were added to reaction mixtures that contained the enzyme preparation and all the standard ingredients except BSA. The tubes were left at 4 °C for 45 min and then BSA was added and the mixtures were incubated for 3 h at 37 °C.

further purified S-200 Eluate 3 to sulfhydryl reagents was not determined.

Binding of GDP-HS Eluate to Concanavalin A–Sepharose 4B

Application of the GDP-HS Eluate to a column of Concanavalin A–Sepharose 4B reduced the activity in the eluent with both lacto-*N*-biose 1 (88%) and *N*-acetyllactosamine (92%). Subsequent elution of the column with 0.25 M benzyl α-*N*-acetylglucosaminide released 48% of the α-3-fucosyltransferase activity with *N*-acetyllactosamine and 30% of the α-4-fucosyltransferase activity with lacto-*N*-biose 1. The results are therefore consistent with the interpretation that both the α-3- and α-4- activities in the preparation are associated with glycoprotein molecules with *N*-linked oligosaccharide chains [24].

Substrate specificity with oligosaccharide acceptors

With GDP-HS Eluate as the enzyme source very similar values were obtained for the relative rates, K_M values and maximum velocities with the Type 1 and Type 2 disaccharides, lacto-*N*-biose 1 (1, Table 2) and *N*-acetyllactosamine (2, Table 2). The apparent K_M values were considerably decreased when the disaccharides were linked to the hydrophobic spacer arm, $(CH_2)_8COOMe$, (3 and 4, Table 2) but the ratio of activities was unchanged. With the S-200 Eluate 3, however, the apparent K_M value for the Type 2 disaccharide was markedly increased, and the relative rate decreased, in comparison with the Type 1 structure. *N*-Acetyllactosamine was only about 10% as effective an acceptor as lacto-*N*-biose 1 at 10 mM concentration and, although the K_M values for both disaccharides were again lowered when they were attached to the hydrophobic spacer arm, $(CH_2)_8COOMe$, the ratio of the activities was not altered.

Substitution of the terminal nonreducing galactose residue

Table 2. Acceptor specificity of fucosyltransferases in GDP-HS Eluate and S-200 Eluate with low-molecular-weight oligosaccharide substrates.

Substrate	*GDP-HS Eluate*			*S-200 Eluate*		
	Relative activity[a]	*App.* K_M (mM)	V_{max} ($\mu mol\ mg^{-1}\ min^{-1}$)	*Relative activity*[a]	*App.* K_M (mM)	V_{max} ($\mu mol\ mg^{-1}\ min^{-1}$)
1 Galβ1-3GlcNAc	100	2.4	2.9	100	4	0.8
2 Galβ1-4GlcNAc	106	2.0	2.8	11	17	0.2
3 Galβ1-3GlcNAc-*O*-$(CH_2)_8$COOMe	131	0.2	3.4	235	0.5	1.6
4 Galβ1-4GlcNAc-*O*-$(CH_2)_8$COOMe	135	0.3	3.9	21	3	0.2
5 Fucα1-2Galβ1-4GlcNAc	94	0.5	2.6	nd[b]	–	–
6 NeuAcα2-3Galβ1-4GlcNAc	88	0.3	2.4	38	2	0.2
7 NeuAcα2-6Galβ1-4GlcNAc	0	–	–	0	–	–
8 GalNAcα1-3Galβ1-4GlcNAc	105	1.0	2.7	nd	–	–
9 Galβ1-3GlcNAcβ1-3Galβ1-4Glc	123	2.4	3.6	113	3	0.9
10 Galβ1-4GlcNAcβ1-3Galβ1-4Glc	141	1.5	4.1	131	4	1.1
11 Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc	126	0.5	3.5	123	4	1.0
12 NeuAcα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc	88	0.5	2.3	56	1.2	0.4
13 Galβ1-4Glc	25	37	2.7	37	75	2.2
14 Fucα1-2Galβ1-4Glc	69	6	3.4	91	5	0.9
15 NeuAcα2-3Galβ1-4Glc	40	6	2.4	30	12	0.4
16 NeuAcα2-6Galβ1-4Glc	0	–	–	0	–	–
17 GalNAcα1-3(Fucα1-2)Galβ1-4Glc	107	2.2	3.2	nd	–	–
18 Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc	78	14	4.6	24	12	0.4
19 Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc	65	18	4.2	nd	–	–
20 Galβ1-3Glc	12	34	2.7	nd	–	–

[a] Activity relative to Galβ1-3GlcNAc at 10 mM concentration except for the two disaccharides, 3 and 4, with attached hydrophobic spacer arms [$(CH_2)_8$COOMe] which were tested at a concentration of 0.8 mM.
[b] nd, not determined.

in the Type 2 disaccharide at the *O*-2 or *O*-3 hydroxyl group gave compounds that had an increased affinity for the GDP-HS Eluate; the K_M values for Fucα1-2Galβ1-4GlcNAc (5, Table 2), NeuAcα-2-3Galβ1-4GlcNAc (6, Table 2) and GalNAcα1-3Galβ1-4GlcNAc (8, Table 2) were each lower than the K_M for the unsubstituted disaccharide. Substitution of the galactose residue on the *O*-6 position by sialic acid in NeuAcα2-6Galβ1-4GlcNAc (7, Table 2), on the other hand, completely abolished the capacity of the compound to function as an acceptor. With S-200 Eluate 3 as the enzyme source, substitution of the terminal galactosyl residue with 2–3 linked sialic acid (6, Table 2) considerably decreased the apparent K_M value, and increased the relative rate of incorporation of fucose into the Type 2 substrate in comparison with the parent disaccharide.

Lactose (13, Table 2) was a relatively poor substrate for both the GDP-HS Eluate and S-200 Eluate 3. Substitution of the terminal galactose with α-2-linked fucose or α-3-linked sialic acid (14 and 15, Table 2) again yielded trisaccharides that were better acceptors than lactose itself. Indeed at 10 mM concentration the trisaccharide 2′-fucosyllactose (14, Table 2) was almost as good an acceptor for S-200 Eluate 3 as the Type 1 acceptor, lacto-*N*-biose 1. Substitution of the galactosyl residue with both fucose and *N*-acetylgalactosamine, as in the blood-group A-active tetrasaccharide GalNAcα1-3(Fucα1-2)Gal-β1-4Glc (17, Table 2), rendered the compound a good acceptor for GDP-HS Eluate.

The synthetic disaccharide Galβ1-3Glc (20, Table 2) was a poor, but definite acceptor for GDP-HS Eluate; a result indicating that the α-3/4-fucosyltransferase has a limited capacity to transfer fucose to the *O*-4 position of glucose as well as to the *O*-4 position of *N*-acetylglucosamine. The larger oligosaccharides (9, 10, 11 and 12, Table 2) each contained both *N*-acetylglucosamine and glucose residues and hence each had two potential acceptor sites for fucosyl transfer. Lacto-*N*-tetraose (9), lacto-*N*-neotetraose (10) and lacto-*N*-fucopentaose 1 (11) were good acceptors for both enzyme preparations, but at this level of testing it was not possible to know to which of the possible acceptor sugars the fucose had been transferred. 3′-Sialyllacto-*N*-tetraose (12) was also a relatively good acceptor for both enzyme preparations but, in this instance, substitution of sialic acid on lacto-*N*-tetraose reduced rather than enhanced the capacity of the oligosaccharide to function as an acceptor.

Various other compounds were tested as possible acceptors with GDP-HS Eluate; Galβ1-3Ara, Galβ1-4Man and Galβ1-4-ManNAc were relatively weak acceptors and

Table 3. ^{1}H Chemical shifts of the constituent monosaccharides of the alditols of the products synthesized by enzymatic transfer of fucose to *N*-acetyllactosamine (NAL), lacto-*N*-biose 1 (LNB-1) and 2′-fucosyllactose (2′-FL).

Residue	*Reporter group*	*Chemical shifts (ppm)*						
		Alditols of enzymatic products formed with			*Alditols of reference compounds*[a]			
		NAL	*LNB-1*	*2′-FL*	*3-FNAL*	*NAL*	*LNB-1*	*LDFT*
Fuc(1-2)	H-1	–	–	5.417	–	–	–	5.417
	H-5	–	–	4.212	–	–	–	4.212
	H-6	–	–	1.223	–	–	–	1.223
Fuc(1-3)	H-1	5.065	–	5.069	5.065	–	–	5.069
	H-5	4.302	–	4.186	4.301	–	–	4.185
	H-6	1.210	–	1.221	1.210	–	–	1.221
Fuc(1-4)	H-1	–	5.076	–	–	–	–	–
	H-5	–	4.223	–	–	–	–	–
	H-6	–	1.203	–	–	–	–	–
Gal	H-1	4.518	4.503	4.556	4.517	4.499	4.499	4.556
	H-2	3.544	3.575	3.680	3.544	3.564	3.552	3.680
	H-3	3.647	3.668	–	3.647	3.656	3.674	3.864
	H-4	3.918	3.924	3.889	3.919	3.917	3.900	3.889
GlcNAc-ol	H-2	4.426	4.307	–	4.424	4.311	4.251	–
	H-3	4.252	4.167	–	4.252	3.780	4.166	–
	H-4	3.988	4.015	–	3.988	3.862	3.551	–
	NAc	2.044	2.044	–	2.044	2.052	2.033	–
Glc-ol	H-2	–	–	4.140	–	–	–	4.140
	H-3	–	–	4.061	–	–	–	4.061

[a] Reference compounds: 3-FNAL, Galβ1-4(Fucα1-3)GlcNAc; NAL, Galβ1-4GlcNAc; LNB-1, Galβ1-3GlcNAc; LDFT, Fucα1-2Galβ1-4(Fucα1-3)Glc.

at 10 mM concentration incorporated, respectively, 25, 4 and 11% of the radioactivity incorporated by lacto-*N*-biose 1. Disialyllactose was not an acceptor at 10 mM concentration. Lactitol (Galβ1-4Glc-ol), cellobiose (Glcβ1-4Glc), phenyl α-*N*-acetylglucosaminide, and *N*-acetylglucosamine were also not acceptors under the standard incubation conditions used for testing the oligosaccharides listed in Table 2, but the undiluted enzyme preparation incorporated very small amounts of fucose into these compounds on prolonged incubation. Under the same stringent incubation conditions the enzyme failed to incorporate [^{14}C]fucose into galactose, glucose, *N*-acetylgalactosamine, fucose or phenyl β-*N*-acetylglucosaminide.

Characterization of oligosaccharide products

N-*acetyllactosamine product.* With GDP-HS Eluate as the enzyme source the product formed with GDP-fucose and *N*-acetyllactosamine was synthesized on a mg scale (yield, 2.3 mg) as described in the Materials and methods section. Methylation of the product gave 2,3,4-*O*-methylfucose, 2,3,4,5-*O*-methylgalactose and 6-*O*-methylglucosamine; demonstrating that both the *O*-3 and *O*-4 positions of *N*-acetylglucosamine were occupied and hence that, since the *O*-4 position in the parent compound was substituted with the galactosyl residue, the fucose had been transferred to the *O*-3 position. ^{1}H-NMR analysis of the reduced trisaccharide gave a spectrum virtually identical with that of an authentic sample of Galβ1-4(Fucα1-3)GlcNAc-ol. In comparison with the parent substrate, the major change observed in the product was in the chemical shift for the H-3 of GlcNAc-ol (Table 3). The small amount of radioactive product synthesized by S-200 Eluate 3 with GDP-[^{14}C]fucose and *N*-acetyllactosamine as acceptor substrate had a chromatographic mobility (R_{Lac} = 0.78 in solvent 1) identical with that of the product formed with GDP-HS Eluate, and radioactive fucose was released by α-3/4-fucosidase and not by α-2-fucosidase.

Lacto-N-*biose 1 product.* The large scale product (yield 0.7 mg) formed from GDP-fucose and lacto-*N*-biose 1 with GDP HS Eluate as the enzyme source again gave 2,3,4-tri-*O*-methylfucose, 2,3,4,6-tetra-*O*-methyl galactose and 6-*O*-methylglucosamine on methylation analysis, showing that in this compound the fucose had been transferred to the *O*-4 position of the *N*-acetylglucosamine residue. ^{1}H-NMR data were not available for the reduced trisaccharide Galβ1-3(Fucα1-4)GlcNAc-ol, but comparison of the chemical shifts for the reduced parent compound and fucosylated derivative revealed that the values for galactose were little changed whereas large changes were observed for the H-4 of GlcNAc-ol (Table 3). The similarity to the changes found for the values of H-3 of the GlcNAc-ol in the *N*-acetylglucosamine product supported the conclusion that the *N*-acetyllactosamine residue of the lacto-*N*-biose 1 product was 3,4-disubstituted (Table 3). The radioactive product synthesized with GDP-[^{14}C]fucose and

lacto-*N*-biose 1 with S-200 Eluate 3 as the enzyme source co-chromatographed with the authenticated product [Galβ1-3(Fucα-1-4)GlcNAc, $R_{Lac} = 0.68$ in solvent 1] formed with GDP-HS Eluate. [^{14}C]Fucose was released from this trisaccharide by the α-3/4-fucosidase.

2′-Fucosyllactose product. The reduced form of the product synthesized on a mg scale from GDP-fucose and 2′-fucosyllactose with GDP-HS Eluate (yield 1.4 mg) gave a ^{1}H-NMR spectrum that was identical with that of an authentic specimen of reduced lactodifucotetraose [Fucα1-2Galβ1-4(Fucα1-3)Glc-ol] [12] and thus confirmed that the fucose had been transferred to the *O*-3 position of the glucose residue (Table 3). The radioactive product formed with GDP-[^{14}C]fucose and 2′-fucosyllactose with S-200 Eluate 3 as the enzyme source co-chromatographed with the authentic sample of lactodifucotetraose, and fucose was released from the prouct by α-3/4-fucosidase.

3′-Sialyllactose product. The radioactive products formed with GDP-[^{14}C]fucose and 3′-sialyllactose by both GDP-HS Eluate and S-200 Eluate 3 were separated from the reaction mixtures by paper electrophoresis in pyridine–acetate buffer, pH 5.4 ($R_{picrate} = 0.55$), eluted and chromatographed in solvent 2 ($R_{Lac} = 0.28$). The fucosylated compounds were initially not susceptible to hydrolysis by either the α-2- or α-3/4-fucosidases but, after removal of sialic acid with neuraminidase, the radioactive products co-chromatographed with 3′-fucosyllactose ($R_{Lac} = 0.70$ in solvent 2) and became susceptible to hydrolysis by α-3/4-fucosidase and not by α-2-fucosidase.

Confirmation that the fucose had been transferred to the *O*-3 position of glucose was obtained by reduction and methylation of the product formed on a larger scale with 3′-sialyllactose (yield 1.5 mg). The identified products were 2,3,4-tri-*O*-methylfucose, 2,4,6-tri-*O*-methylgalactose and 1,2,5,6-tetra-*O*-methylglucitol demonstrating that the fucose had been transferred to the *O*-3 position of the glucose residue. The ^{1}H-NMR spectrum for the reduced product showed significant changes in the chemical shifts for the glucitol residue that were consistent with the transfer of fucose to this sugar (Table 4). The product formed with both enzyme preparations therefore had the structure NeuAcα2-3Galβ1-4(Fucα1-3)Glc.

*3′-Sialyl-*N*-acetyllactosamine.* The radioactive products formed with GDP-fucose and 3′-sialyl-*N*-acetyllactosamine as substrate with either GDP-HS Eluate or S-200 Eluate 3 as the enzyme source were separated from the reaction mixtures by paper electrophoresis in pyridine–acetate buffer, pH 5.4 ($R_{picrate} = 0.57$) and then eluted and chromatographed in solvent 2 ($R_{Lac} = 0.30$). These products resembled the products formed with 3′-sialyllactose in that they were resistant to hydrolysis by the α-2- or α-3/4-fucosidases until after removal of the terminal sialic acid residue. The products formed by neuraminidase treatment co-chromatographed with authentic 3′-fucosyl-*N*-acetyllactosamine in solvent 1 ($R_{Lac} = 0.70$) and became susceptible to hydrolysis by the α-3/4-fucosidase.

Table 4. ^{1}H Chemical shifts of the constituent monosaccharides of the alditols of the trisaccharides 3′-sialyllactose (3′SL) and 3′-sialyl-*N*-acetyllactosamine (3′SNAL) and of the tetrasaccharides formed by enzymatic transfer of L-fucose to these compounds.

Residue	*Reporter group*	*Chemical shifts (ppm)*			
		Alditols of the enzymatic products formed with		*Alditols of the parent trisaccharides*	
		3′-SL	*3′-SNAL*	*3′-SL*	*3′-SNAL*
Fuc(1-3)	H-1	5.072	5.069	–	–
	H-5	4.282	4.314	–	–
	H-6	1.213	1.303	–	–
Gal	H-1	4.559	4.594	4.581	4.576
	H-2	3.568	3.581	3.580	3.600
	H-3	4.094	4.110	4.125	4.135
	H-4	3.948	3.951	3.958	3.953
NeuAc(2-3)	H-3 eq.	2.765	2.760	2.753	2.762
	H-3 ax.	1.803	1.829	1.832	1.846
	NAc	2.030	2.031	2.032	2.031
Glc-ol	H-2	4.164	–	–[a]	–
	H-3	4.051	–	–[a]	–
GlcNAc-ol	H-2	–	4.410	–	4.284
	H-3	–	4.234	–	3.926
	NAc	–	2.040	–	2.050

[a] Signals for these protons are located amongst the bulk of the skeletal protons (3.5–3.7 ppm).

Confirmation of the structure of the product formed on a larger scale with 3′-sialyl-*N*-acetyllactosamine (yield 2.9 mg) was obtained by comparison of the ^{1}H-NMR spectrum of the reduced form with the spectra of the reduced forms of the parent compound and 3-fucosyl-*N*-acetyllactosamine. The NeuAc H-3 equatorial and axial shifts and the Gal H-1, H-2, H-3 and H-4 shifts, which are highly characteristic of the NeuAcα2-3Gal- grouping [25], were scarcely altered by the addition of a fucose residue, whereas those of the H-2 and H-3 of GlcNAc-ol were markedly changed (Table 4). Comparison of the changes in the shifts of the GlcNAc-ol residue when fucose is transferred to *N*-acetyllactosamine to form 3-fucosyl-*N*-acetyllactosamine (Table 3) support the conclusion that the fucose had been added to the *O*-3 position of the *N*-acetylglucosamine residue in sialyl-*N*-acetyllactosamine.

The radioactive product formed with GDP-[^{14}C]fucose and 3′-sialyl-*N*-acetyllactosamine when S-200 Eluate 3 was the enzyme source had the same electrophoretic mobility in pyridine–acetate buffer, pH 5.4 ($R_{picrate} = 0.57$), and chromatographic mobility in solvent 2 ($R_{Lac} = 0.30$), as the product formed with GDP-HS Eluate enzyme preparation; demonstrating that both products had the structure NeuAcα2-3Galβ1-4(Fucα1-3)GlcNAc.

Lacto-N-*tetraose.* The products formed with lacto-*N*-tetraose and either GDP-HS Eluate or S-200 Eluate 3 as enzyme source were synthesized only with high specific activity GDP-[^{14}C]fucose as the sugar donor. The product formed with the GDP-HS Eluate ran as a single spot in solvent 1 under standard conditions but elution of the radioactive area and re-chromatography in solvent 3 for 10 days revealed two radioactive peaks; the mobilities relative to a lacto-*N*-tetraose standard (R_{LNT}) corresponded to lacto-*N*-fucopentaose II ($R_{LNT} = 0.52$; 87% of the radioactivity) and lacto-*N*-fucopentaose V [26] ($R_{LNT} = 0.57$; 13% of the radioactivity). The labelled fucose in both products was released by α-3/4-fucosidase and not by the α-2-fucosidase. Since, in the parent compound, lacto-*N*-tetraose, the *O*-3 position of the *N*-acetylglucosamine and the *O*-4 position of the glucose residue are already substituted, it may be deduced that in the major product the fucose had been transferred to the *O*-4 position of *N*-acetylglucosamine to give Galβ1-3(Fucα1-4)-GlcNAcβ1-3Galβ1-4Glc and in the minor product to the *O*-3 position of D-glucose to give Galβ1-3GlcNAcβ1-3Galβ1-4(Fucα-1-3)Glc.

With S-200 Eluate 3 as the enzyme source only one radioactive spot was observed in the pentasaccharide area when the reaction products were chromatographed first in solvent 1 and then eluted and re-run for a prolonged period in solvent 3; the product co-chromatographed with authentic lacto-*N*-fucopentaose II ($R_{LNT} = 0.52$), showing that the fucose had been transferred to the *O*-4 position of *N*-acetylglucosamine.

Lacto-N-*neotetraose.* The products formed with GDP-fucose and lacto-*N*-tetraose with the GDP-HS Eluate preparation were first chromatographed in solvent 1 and then the product area was eluted and rechromatographed in solvent 3. Two radioactive peaks were detected in the pentasaccharide area of the chromatogram: the major peak ($R_{LNT} = 0.50$) contained 85% of the radioactivity and co-chromatographed with authentic lacto-*N*-fucopentaose III. The minor peak ($R_{LNT} = 0.55$) was tentatively identified as lacto-*N*-fucopentaose VI [Galβ1-4GlcNAcβ1-3Galβ1-4(Fucα1-3)Glc]. Both products were degraded by α-3/4-fucosidase. In these two products, therefore, it could be inferred that the fucose had been transferred to the *O*-3 position of *N*-acetylglucosamine in the slower running compound, to give Galβ1-4(Fucα1-3)GlcNAcβ1-3-Galβ1-4Glc and to the *O*-3 position of glucose in the faster running product, to give Galβ1-4GlcNAcβ1-3Galβ1-4(Fucα1-3)Glc.

In contrast, with S-200 Eluate 3 as the enzyme source, the product with lacto-*N*-neotetraose ($R_{Lac} = 0.32$ in solvent 2) gave only one product when rechromatographed in solvent 3 ($R_{LNT} = 0.55$) which corresponded to the minor product formed with GDP-HS Eluate. Elution of the product, followed by treatment with Jack bean β-galactosidase and rechromatography in solvent 2 gave a faster running radioactive product ($R_{Lac} = 0.62$). Since preliminary experiments with unlabelled standards had confirmed that substitution of the subterminal *N*-acetylglucosamine residue prevents hydrolysis of the terminal galactosyl residue by the Jack bean β-galactosidase, this result strongly supports the inference that the fucose label was attached to the terminal reducing glucose residue of lacto-*N*-tetraose and not to the subterminal nonreducing *N*-acetylglucosamine unit.

3′-Sialyllacto-N-*tetraose.* The fucosylated products synthesized from GDP-[^{14}C]fucose and 3′-sialyllacto-*N*-tetraose by GDP-HS Eluate were separated first by paper electrophoresis in pyridine–acetate buffer, pH 5.4, and then by paper chromatography in solvent 2; the mobility of the major product (85% of the radioactivity), measured relative to lacto-*N*-fucopentaose II ($R_{LNP\,II}$) was 0.46. The eluted product was not susceptible, initially, to digestion by either α-2- or α-3/4-fucosidases. However, removal of sialic acid with neuraminidase gave a radioactive compound that co-chromatographed with lacto-*N*-fucopentaose II in solvent 2 and was susceptible to hydrolysis by the α-3/4-fucosidase and not by the α-2-fucosidase. The fucose had therefore been transferred to the *O*-4 position of *N*-acetylglucosamine to give NeuAcα2-3Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc. Minor products were not further identified. With S-200 Eluate 3 the product synthesized with 2,3-sialyllacto-*N*-tetraose co-chromatographed in solvent 2 ($R_{LNP\,II} = 0.46$) with the major product formed with GDP-HS Eluate as the enzyme source. Therefore, both these enzyme preparations catalysed the formation of hexasaccharides containing terminal sialyl-Le^a sequences.

Lacto-N-*fucopentaose I.* The radioactive product synthesized by GDP-HS Eluate with GDP-[^{14}]fucose and lacto-*N*-fucopentaose I ($R_{Lac} = 0.12$ in solvent 1) was resolved into two fractions on rechromatography for 10 days in solvent 3; the major spot ($R_{LNT} = 0.26$), which accounted for 92% of the radioactivity, was susceptible to hydrolysis by α-3/4-fucosidase and co-chromatographed with authentic lacto-*N*-difucohexaose I, Fucα1-2Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc. The minor product also had the chromatographic mobility of a difucohexaose ($R_{LNT} = 0.33$) and most probably was Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4(Fucα1-3)Glc. Only one product was detected after incubation of GDP-[^{14}C]fucose with lacto-*N*-fucopentaose 1 when the enzyme source was S-200 Eluate 3; this product co-chromatographed with authentic lacto-*N*-difucohexaose 1 ($R_{LNT} = 0.26$ in solvent 3).

Lacto-N-*fucopentaose II.* The radioactive product formed with GDP-[^{14}C]fucose and lacto-*N*-fucopentaose II with either GDP-HS Eluate or S-200 Eluate 3 as an enzyme source had $R_{Lac} = 0.12$ in solvent 1. On re-chromatography in solvent 3 for 10 days only one radioactive spot was

detected which co-chromatographed ($R_{LNT} = 0.33$) with an authentic sample of lacto-*N*-difucohexaose II [Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4(Fucα1-3)Glc] and was not further resolved. The fucose had therefore been transferred to the *O*-3 position of the terminal reducing glucose residue.

GalNAcα1-3Galβ1-4GlcNAc. The product ($R_{Lac} = 0.35$ in solvent 4) synthesized by GDP-HS Eluate with GDP-[^{14}C]fucose and the blood group A-active trisaccharide was initially resistant to hydrolysis by the α-2- or α-3/4-fucosidases. Treatment with α-*N*-acetylgalactosaminidase gave a compound ($R_{Lac} = 0.70$ in solvent 4) which co-chromatographed with authentic 3-fucosyl-*N*-acetyllactosamine and was then susceptible to hydrolysis by α-3/4-fucosidase and not by α-2-fucosidase; supporting the conclusion that the fucose had been transferred to the *O*-3 position of the *N*-acetylglucosamine residue to give the novel compound GalNAcα1-3Galβ1-4(Fucα1-3)-GlcNAc.

Galβ1-3GlcNAc-O-$(CH_2)_8$COOMe and Galβ1-4GlcNAc-O-$(CH_2)_8$COOMe. When either GDP-HS Eluate or S-200 Eluate 3 were used as the enzyme source the Type 1 and Type 2 disaccharides with hydrophobic spacer arms gave radioactive products on incubation with GDP-[^{14}C]fucose which had $R_{Lac} = 1.3$ in solvent 2. The radioactive fucose was completely released from both products by treatment with α-3/4-fucosidase.

Lactose. The radioactive products synthesized by both GDP-HS Eluate and S-200 Eluate 3 with GDP[^{14}C]-fucose and lactose had mobilities $R_{Lac} = 0.70$ in solvent 1. Elution of the radioactive areas and rechromatography in solvent 5, which separates 2′-fucosyllactose ($R_{Lac} = 1.0$) from 3-fucosyllactose ($R_{Lac} = 0.8$) [27], showed that the product co-chromatographed with an authentic sample of 3-fucosyllactose.

Other low-molecular weight acceptors. The A-active tetrasaccharide GalNAcα1-3(Fucα1-2)Galβ1-4Glc gave a product ($R_{Lac} = 0.21$ in solvent 1) which was inferred to be the difucosyl A-structure GalNAcα1-3(Fucα1-2)Galβ1-4(Fucα1-3)Glc. Other acceptors tested with GDP-HS Eluate and characterized only by their chromatographic mobilities in solvent 1 were Galβ1-3Glc product ($R_{Lac} = 0.57$); Galβ1-4Glc-ol product ($R_{Lac} = 0.58$); Galβ-1-4Man product ($R_{Lac} = 0.80$); Galβ1-4ManNAc product ($R_{Lac} = 0.85$); Galβ1-3Ara product ($R_{Lac} = 0.55$).

Acceptor specificity with glycoprotein and glycolipid acceptors. Striking differences were observed when GDP-HS Eluate and S-200 Eluate 3 were assayed with glycoprotein acceptors. Transferrin, fetuin and α_1-acid glycoprotein were each acceptors for the GDP-HS Eluate. In agreement with the specificity established with the low-molecular-weight oligosaccharide substrates containing terminal sialic acid residues, transferrin, which has α-2,6 linked sialic acid on Type 2 chain endings [28], was a more effective acceptor after removal of this sugar (Table 5); in contrast, incorporation of fucose into fetuin and α_1-acid glycoprotein, which have predominantly α-2,3 linked sialic acid on Type 2 chains [29, 30] was not markedly changed by loss of this sugar (Table 5). The Type XIV pneumococcal polysaccharide which has repeating Galβ1-4GlcNAc side chains [31] was an excellent acceptor for the fucosyltransferase in the GDP-HS Eluate. The ovarian cyst precursor blood-group glycoprotein, the only macromolecular substrate tested that has large numbers of oligosaccharide chains terminating in Type 1 sequences in addition to those terminating in Type 2 sequences [32], was also a good acceptor for the enzyme(s) in the GDP-HS Eluate; the positional linkages of sialic acid in this ovarian cyst glycoprotein have not been established, but removal of this sugar gave increased incorporation of fucose, indicating the presence of α-2,6 linkages.

In sharp contrast to the results obtained with GDP-HS Eluate, the further purified S-200 Eluate 3 failed to transfer detectable amounts of fucose to any of the glycoproteins with predominately Type 2 chain endings. The precursor blood-group substance was the only glycoprotein that retained activity with this enzyme preparation (Table 5), indicating that only GDP-fucose: Galβ1-3GlcNAc α-4-fucosyltransferase activity directed towards Type 1

Table 5. Acceptor specificity of fucosyltransferase preparations GDP-HS Eluate and S-200 Eluate 3 with glycoprotein and glycolipid acceptors.

Substrate	*Major sialic acid linkage*	*Major chain type*	*Relative activity*[a]	
			GDP-HS Eluate	*S-200 Eluate*
Glycoproteins				
Transferrin	2–6	2	3.4	0.5
Asialo-transferrin			15.6	0.2
α_1-Acid glycoprotein	2–3	2	29.0	0.4
Asialo-α_1-acid glycoprotein			30.1	0.3
Fetuin	2–3	2	21.1	0.7
Asialo-fetuin			20.9	1.6
Blood-group precursor 484	?	1 and 2	28.6	29.1
Asialo-blood-group precursor 484			113	35.9
Type XIV pneumococcal polysaccharide		2	108	0.3
Glycolipids				
Lacto-*N*-neotetraosyl ceramide		2	5.6	nd
Globoside			0.9	nd
Ceramide trihexoside			0.7	nd
Lactosylceramide			0.6	nd

[a] Incorporation of [^{14}C]fucose into 100 μg glycoprotein or 0.025 μmol glycolipid compared with the incorporation into 0.5 μmol Galβ1-3GlcNAc under the same assay conditions.

sequences was demonstrable with these glycoprotein acceptors.

Glycolipid acceptors were examined as substrates only with the GDP-HS Eluate; of those tested, lacto-*N*-neotetraosyl ceramide, which has a terminal Type 2 disaccharide structure, was the only one that accepted fucose (Table 5). The other three glycolipids each contained a glucose unit adjacent to the ceramide moiety but this residue, although part of a lactose structure, appears not to function as an acceptor for the α-3-fucosyltransferases in the milk enzyme preparation. The further purified S-200 Eluate 3 was not tested with the glycolipid acceptors.

Apparent K_M value for GDP-fucose

With GDP-HS Eluate as the enzyme source, the apparent K_M values for GDP-fucose at saturating concentrations (10 mM) of *N*-acetyllactosamine and lacto-*N*-biose 1 were 5 μM and 4 μM, respectively. The apparent K_M for the donor substrate was not determined for the further purified S-200 Eluate 3.

Competition experiments with S-200 Eluate 3

Insufficient material was available to determine whether the α-3/4-fucosyltransferase obtained after the final gel filtration step had been purified to homogeneity. It was therefore not possible to rule out completely the possibility that a small amount of *N*-acetylglucosaminide: α-3-fucosyltransferase remained associated with the α-3/4- enzyme and was responsible for the residual activity detected with low-molecular-weight Type 2 acceptors. The large difference in the apparent K_Ms for the Type 2 disaccharides measured with the two enzyme preparations (2 and 4, Table 2) indicated that different enzymes were involved in these reactions and competition experiments were carried out to examine this further.

Previously, competition experiments with GDP-HS Eluate as the fucosyltransferase source showed that the Type 1 disaccharide, lacto-*N*-biose 1 plus the spacer arm [Galβ1-3GlcNAc-*O*-$(CH_2)_8$COOMe], was not a competitive inhibitor of the transfer of fucose to the Type 2 disaccharide *N*-acetyllactosamine [1]. However, in contrast, despite the low incorporation of fucose into *N*-acetyllactosamine, competition experiments with S-200 Eluate 3 and the same acceptor and inhibitor indicated competitive inhibition (Fig. 4a).

Experiments with the Type 1 disaccharide lacto-*N*-biose 1 as the acceptor and 2′-fucosyllactose as inhibitor showed strong competitive inhibition (Fig. 4b). These results therefore suggested that a single enzyme species was present that was able to utilize the low-molecular-weight acceptors lacto-*N*-biose, 1, 2′-fucosyllactose and, to a much more limited extent, *N*-acetyllactosamine.

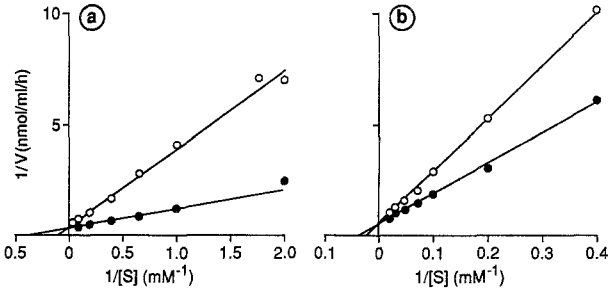

Figure 4. Inhibition of the transfer of fucose to acceptors by S-200 Eluate 3: (a) Galβ1-4GlcNAc substrate (●, no inhibitor; ○, plus 0.9 mM Galβ1-3GlcNAc-*O*-$(CH_2)_8$COOMe); (b) Galβ1-3GlcNAc substrate (●, no inhibitor; ○, plus 15 mM Fucα1-2Galβ1-4Glc). Fucosyltransferase assays were carried out under standard conditions except that the substrate concentration was varied as shown, the GDP-fucose concentration was adjusted to 0.8 nmol (140 000 counts min^{-1}) and inhibitors were added at the concentrations given above. Separation of the products from (a) was carried out by chromatography in solvent 2 for 12 h and from (b) by chromatography in solvent 4 for 20 h.

Discussion

Much biochemical and molecular genetic evidence is accumulating that points to the existence of a family of human α-3-L-fucosyltransferases that differ in their chromosomal location [33, 34], polymorphic expression [35–37], tissue distribution [38, 39] ability to transfer fucose to sialylated acceptors [39, 40] and DNA structure [41–43]. The product of the Lewis blood-group gene was proposed to be an α-4-fucosyltransferase on the basis of structural studies on the Lewis blood-group antigenic determinants but investigations on the distribution of α-3- and α-4-fucosyltransferase activities in salivas from individuals of different Lewis groups established an association between the inheritance of a Lewis blood-group gene and the ability to transfer fucose to the *O*-3 position of glucose in lactose-based oligosaccharides [44]. The co-presence of another α-3-fucosyltransferase in saliva, and most other tissues and body fluids, that is expressed independently of the inheritance of a Lewis gene and transfers fucose solely onto the *O*-3 position of *N*-acetylglucosamine in Type 2 structures, meant that it was not possible to decide from enzyme assays on crude fluids and tissue extracts whether the Lewis gene-encoded enzyme also acts on Type 2 structures. In the purification scheme described previously [1], the enzyme activity in milk from Le(a − b+) donors that catalysed the transfer of fucose to the *O*-3 position of glucose in lactose-based oligosaccharides co-purified through all the stages with the enzyme transferring fucose to the *O*-4 position of *N*-acetylglucosamine, whereas the final gel filtration step removed most of the residual capacity to transfer fucose to the *O*-3 position of *N*-acetylglucosamine in Type 2 chains.

Specificity studies with a range of low-molecular-weight

oligosaccharide acceptors revealed that, of those with only one potential acceptor site for the α-3/4-fucosyltransferase, the synthetic Type 1 and Type 2 disaccharides with the attached hydrophobic arms [$(CH_2)_8COOMe$] were the most effective substrates for the enzyme preparation eluted from the GDP-hexanolamine Sepharose column (GDP-HS Eluate). The Type 1 disaccharide with the hydrophobic spacer arm was also the most effective acceptor with the further purified S-200 Eluate 3, but the activity was very much reduced with the synthetic Type 2 disaccharide plus spacer arm. Of the Type 1 compounds tested that have potential acceptor sites for the addition of fucose both to the *O*-4 position of *N*-acetylglucosamine and the *O*-3 position of glucose, namely lacto-*N*-tetraose, lacto-*N*-fucopentaose I and 3′-sialyllacto-*N*-tetraose, the major product formed with both GDP-HS Eluate and S-200 Eluate was in each case the one containing the fucose added to the *O*-4 position; this demonstrates that the enzyme had a preference for this site (Table 2). The Type 2 tetrasaccharide, lacto-*N*-neotetraose, was a good acceptor for both the eluates but, whereas the major product formed with GDP-HS Eluate was lacto-*N*-fucopentaose III, showing that the fucose had been transferred to the *O*-3 position of *N*-acetylglucosamine, the only product formed with S-200 Eluate had fucose transferred to the *O*-3 position of glucose.

Experiments with the further purified enzyme to determine the influence on the acceptor specificity of substituents on the terminal β-galactosyl residue of the Type 1 and 2 structures revealed a complex pattern of interactions. *N*-Acetyllactosamine itself was a poor acceptor for the enzyme, but substitution of the disaccharide with α-3 linked sialic acid increased its affinity for this substrate (Table 2). Similarly lactose had a low affinity for the enzyme, but substitution of the galactosyl residue with α-2 linked fucose lowered the K_M considerably, rendering 2′-fucosyllactose almost as good a substrate at 10 mM concentration as lacto-*N*-biose 1. Substitution of lactose with α-2,3 linked sialic acid on the β-galactosyl residue also lowered the K_M for the substrate, although to a less marked extent than substitution with fucose. However, despite the fact that these changes in affinity were observed with the low-molecular-weight substrates, with the glycoprotein acceptors with Type 2 chain endings, fetuin, transferrin and α_1-acid glycoprotein, no detectable transfer of fucose occurred irrespective of whether sialic acid was present or absent from the terminal carbohydrate structures. In contrast, the blood-group precursor glycoprotein with *O*-linked oligosaccharide chains containing both Type 1 and Type 2 terminal structures remained a good acceptor for the further purified enzyme, and the activity with this substrate was enhanced by the removal of sialic acid. These results suggest that *in vivo* the enzyme might be expected to synthesize Le^a, Le^b and sialyl-Le^a structures and not X, Y or sialyl-X structures. However, the great variety of glycoprotein and glycolipid molecules carrying potential acceptor sites, which may be differentially influenced by the nature of substituents or of the structures internal to the terminal *N*-acetyllactosamine groupings, make it difficult to predict with any degree of certainty whether any of the Type 2 fucosylated structures on cell surfaces result from the small residual affinity of the α-3/4-fucosyltransferase for Type 2 acceptors that was demonstrable with low-molecular-weight substrates.

Earlier, Prieels *et al.* [2] had co-purified the Lewis blood-group *N*-acetylglucosaminide α-4-fucosyltransferase and an *N*-acetylglucosaminide α-3-fucosyltransferase from human milk and proposed that both activities reside in a single molecular species. Part of the evidence for this proposal came from the demonstration that the activities had identical pH and divalent metal ion dependencies, and identical rates of inactivation upon heating. However, the α-3- and α-4- activities were measured with the substrates 2′-fucosyllactose and lacto-*N*-fucopentaose I, respectively, and from our findings it would appear that even if more than one enzyme had been present in the preparation obtained by these authors both of the substrates would have been utilized by the α-3/4-enzyme and the properties of any contaminating α-3-fucosyl transferase acting solely on *N*-acetylglucosamine in Type 2 chains would not have been measured. In the present investigation, the α-3/4-fucosyltransferase preparation isolated by affinity purification of the milk enzyme on GDP-hexanolamine Sepharose (GDP-HS Eluate) gave different pH profiles when *N*-acetyllactosamine and lacto-*N*-biose 1 were used as substrates (Fig. 1a). The sharp pH optimum at approximately 9.0 for the further purified S-200 Eluate 3 with lacto-*N*-biose 1 was in agreement with the optimum obtained for this substrate with the GDP-HS Eluate (Fig. 1b). Similarly, the curves for manganese activation differed when the two disaccharide substrates were used in experiments with GDP-HS Eluate 1 (Fig. 2); the α-4-fucosyltransferase activity was maximally stimulated by far lower concentrations of the cation than the α-3-fucosyltransferase activity, and more readily inhibited at higher concentrations. More difficult to reconcile with the results presented in this paper are the competition experiments recorded by Prieels *et al.* [2]. These authors found that 2′-fucosyllactose and lacto-*N*-fucopentaitol I were competitive inhibitors of the transfer of fucose to asialotransferrin, which has oligosaccharide chains terminating in Type 2 structures, indicating that the activities were catalysed by a single enzyme. Inhibition of the transfer of fucose by GDP-HS Eluate to glycoproteins by the low-molecular-weight inhibitors was not studied in the present investigation, but the further purified α-3/4-fucosyltransferase in S-200 Eluate failed to transfer detectable amounts of fucose to glycoproteins with Type 2 chain endings; this evidence therefore supports the view that reactivity of the GDP-HS Eluate with these glycoprotein acceptors was dependent on the presence of the contaminating *N*-acetylglucosaminide: α-3-fucosyltransferase.

Another purification of the α-3/4-fucosyltransferase from

human milk was reported by Eppenberger-Castori *et al.* [45]. These authors also included as a last stage a gel filtration step on an HPLC column that separated some *N*-acetylglucosaminide α-3-fucosyltransferase activity from the final α-3/4 preparation. They nevertheless described their purified preparation as an α-3/4-fucosyltransferase acting on both Type 1 and Type 2 chains, and it is not clear how much of the capacity to transfer fucose to Type 2 chains had been removed. In our laboratory, purification of the Lewis-gene-associated glycosyltransferase secreted into the medium from the human A431 cell line as the only major fucosyltransferase species has yielded a preparation that in its specificity properties closely resembles the milk α-3/4-fucosyltransferase described in the present paper [46]; the enzyme transfers fucose readily to lacto-*N*-biose 1 and 2′-fucosyllactose but has low activity with *N*-acetyllactosamine and has virtually no detectable activity with asialoglycoproteins, transferrin, α_1-acid glycoprotein and fetuin. No other mammalian fucosyltransferases with α-4-activity have been described in detail, but a plant enzyme extracted from Mung bean seedlings has been found to catalyse the transfer of fucose to Type 1 chain acceptors to form Le^a structures [47]. This enzyme has no capacity to utilize Type 2 oligosaccharides and appears to have more limited activity towards the trisaccharide 2′-fucosyllactose than does the purified human milk α-3/4-fucosyltransferase.

A definitive answer to the specificity of the Lewis blood-group gene encoded enzyme appeared to have been given when Kukowska-Latallo *et al.* [41] cloned a fucosyltransferase gene believed to be the product of the Lewis genetic locus on human chromosome 19. This DNA when expressed in COS-1 cells as a portion of a secreted protein-A fusion peptide gave rise to fucosyltransferase activity with both Type 1 and Type 2 acceptors and to cell surface expression of both X (SSEA 1 or CD15) and Le^a antigens. However, although products of activity of the enzyme from the transfected cells with the substrates lactose, lacto-*N*-biose 1, *N*-acetyllactosamine and 2′-fucosyllactose were identified by their chromatographic mobilities, initially no quantitative or kinetic data were recorded for the enzyme. More recently, a brief comparison of the relative activity of the recombinant enzyme with these substrates indicated a marked preference of the fucosyltransferase for the Type 1 disaccharide [43]; clearly a more detailed investigation of the kinetics of the recombinant enzyme is needed to establish precisely the relative activities with Type 1 and Type 2 acceptors. Nevertheless, the strong expression of X antigenic determinants on the transfected COS-1 cell surface would not be expected if the enzyme had the same specificity as the purified milk α-3/4-fucosyltransferase described in this paper. The enzymes from milk and A 431 cells that we have examined are soluble species and are therefore presumably lacking in the transmembrane anchoring segment of the fucosyltransferase present in the Golgi membrane-bound form. It could be argued that the solubilization process causes conformational changes in the enzyme that alter its specificity. However, the recombinant enzyme expressed in COS-1 cells represented only the carboxy-terminal domain of the enzyme protein [41], and therefore also lacked the transmembrane segment. The reason for the apparent discrepancy between the specificity of the milk α-3/4-fucosyltransferase described in the present paper and that of the recombinant enzyme remains to be clarified.

Acknowledgements

We are most grateful to Professors R. U. Lemieux, P. Sinaÿ and J. Koscielak and Drs A. Corfield, D. Smith and C. P. C. Soh for gifts of oligosaccharides used as substrates in this investigation.

References

1. Johnson PH, Watkins WM (1992) *Glycoconjugate J* **9**:241–9.
2. Prieels JP, Monnom D, Dolmans M, Beyer TA, Hill RL (1981) *J Biol Chem* **256**:10456–63.
3. Fukushi Y, Nudelman E, Levery S, Hakomori S (1984) *J Biol Chem* **259**:10511–7.
4. Hakomori S (1985) *Cancer Res* **45**:2405–14.
5. Feizi T (1988) *J Biol Chem* **263**:10186–91.
6. Brandley BK, Swiedler SJ, Robbins PW (1990) *Cell* **63**:861–3.
7. Springer TA, Laskey LA (1990) *Nature* **349**:196–7.
8. Nunez HA, O'Connor JV, Rosevear PR, Barker R (1981) *Can J Chem* **29**:2086–95.
9. Alais J, Veyrières A (1981) *Carbohydr Res* **93**:164–5.
10. Flowers HM (1972) *Methods Carbohydr Chem* **6**:474–80.
11. Kuhn R, Baer HH (1954) *Chem Ber* **10**:1560–7.
12. Donald ASR, Feeney J (1988) *Carbohydr Res* **178**:79–91.
13. Jacquinet J-C, Sinaÿ P (1976) *Tetrahedron* **32**:1693–7.
14. Cheese IAF, Morgan WTJ (1961) *Nature* **191**:149–50.
15. Parkkinen J, Finne J (1983) *Eur J Biochem* **140**:427–31.
16. Morgan WTJ (1967) *Methods Immunol Immunochem* **1**:75–81.
17. Watkins WM (1972) In *Glycoproteins: Their Composition, Structure and Function*, Vol 5 (Gottschalk A, ed.) pp. 830–91. Amsterdam: Elsevier.
18. Stealey JR, Watkins WM (1971) *Biochem J* **116**:16–7P.
19. Harrap GJ, Watkins WM (1964) *Biochem J* **93**:9–10.
20. Trevelyan NE, Proctor P, Harrison JS (1950) *Nature* **166**:444–5.
21. Cuatrecasas P (1970) *J Biol Chem* **245**:3059–65.
22. Pacuszka T, Koscielak J (1974) *FEBS Lett* **41**:348–51.
23. Donald ASR (1981) *Eur J Biochem* **120**:234–9.
24. Sharon N, Lis H (1982) In *The Proteins*, Vol 5 (Neurath H, Hill RL eds) pp. 1–145. New York: Academic Press.
25. Vliegenthart JFG, Van Halbeek H, Dorland L (1981) *Pure Appl Chem* **53**:45–77.
26. Kobata A (1972) *Methods Enzymol* **28**:262–71.
27. Montreuil J (1960) *Bull Soc Chim Biol* **42**:1398–427.
28. Spik G, Bayard B, Fournet B, Strecker G, Bouquelet S, Montreuil J (1975) *FEBS Lett* **50**:296–9.
29. Nilsson B, Norden NE, Svensson S (1979) *J Biol Chem* **255**:4545–52.

30. Paulson JC, Weinstein J, Dorland L, Van Halbeek H, Vliegenthart JFG (1982) *J Biol Chem* **257**:12734–8.
31. Lindberg B, Lönngren J, Powell DA (1977) *Carbohydr Res* **58**:177–85.
32. Rege VP, Painter TJ, Watkins WM, Morgan WTJ (1963) *Nature* **200**:532–4.
33. Tetteroo PAT, de Heij HT, Van den Eynden DH, Visser FJ, Schoenmaker E, Guerts van Kessel AHM (1987) *J Biol Chem* **263**:15984–9.
34. Coulin P, Mollicone R, Grisard MC, Gibaud A, Ravise N, Feingold J, Oriol R (1991) *Cytogenet Cell Genet* **56**:108–11.
35. Greenwell P, Johnson PH, Edwards JM, Reed RM, Moores PP, Bird A, Graham HA, Watkins WM (1986) *Blood Trans Immunohaematol* **29**:233–49.
36. Caillard T, Le Pendu J, Ventura M, Mada M, Rault G, Mannoni P, Oriol R (1988) *Expl Clin Immunogenet* **5**: 15–23.
37. Johnson PH, Skacel PO, Greenwell P, Watkins WM (1989) *Biochem Soc Trans* **17**:133–4.
38. Mollicone R, Gibaud A, Francis A, Ratcliffe M, Oriol R (1990) *Eur J Biochem* **191**:169–76.
39. Johnson PH, Watkins WM (1987) *Biochem Soc Trans* **15**:396.
40. Skacel PO, Edwards AJ, Harrison CT, Watkins WM (1991) *Blood* **78**:1452–60.
41. Kukowska-Latallo JF, Larsen RD, Nair RP, Lowe JB (1990) *Genes & Dev* **4**:1288–1303.
42. Lowe JB, Stoolman LM, Nair RP, Larsen RD, Berhend T, Marks RM (1990) *Cell* **63**:475–84.
43. Weston BW, Nair RP, Larsen RD, Lowe JB (1992) *J Biol Chem* **267**:4152–60.
44. Johnson PH, Yates AD, Watkins WM (1981) *Biochem Biophys Res Commun* (1981) **100**:1611–8.
45. Eppenberger-Castori S, Lotscher H, Finne J (1989) *Glycoconjugate J* **6**:101–14.
46. Johnson PH (1988) PhD Thesis, Council for National Academic Awards, UK.
47. Crawley SC, Hindsgaul O, Ratcliffe RM, Lamontagne LR, Palcic MM (1989) *Carbohydr Res* **193**:249–56.