USE OF TWO CHEMICALLY SYNTHESISED H ACCEPTORS AS SUBSTRATES FOR A AND B BLOOD GROUP GENE-SPECIFIED GLYCOSYLTRANSFERASES

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1. Introduction

Modern advances in the chemistry of the glycosidic linkage make the total synthesis of blood-group antigenic determinants a realistic challenge. It was very recently taken up by Lemieux and Driguez [1,2] and by Jacquinet and Sinaÿ [3] who described for the first time the step by step synthesis of reducing trisaccharides carrying B, H and Lewis a specificities. In addition, a 'synthetic' antigen related to the human blood-group Lewis a was obtained by Lemieux et al. [4], after attachment of the oligosaccharide to high molecular weight substances.

As demonstrated with oligosaccharides found in minute amounts in natural sources or obtained by various degradations of blood-group substances, these products are useful for biochemical purposes such as hapten inhibition [5–7], affinity chromatography [8–10], raising of antibodies in animals [4,11] or substrate specificity studies of glycosidases [5,6,12] and blood-group gene glycosyltransferases [13–16].

Although ABH oligosaccharides conjugated to solid carriers will be of prime importance in the future for the large scale production of specific antisera, the reducing compounds are useful for inhibition and enzymatic studies.

In this work synthetic H substances [3], namely the 2′-fucopyranosyl-*N*-acetyllactosamine (2′FLNAc) and the 2′-fucosylgalactose (2′FG), were submitted to agglutination inhibition and enzymatic studies.

2. Materials and methods

UDP-*N*-acetyl-D-[1-C^{14}]galactosamine (49.5 mCi/mmole) and UDP-D-[1-^{14}]galactose (281.5 mCi/mmole) were purchased from Nen Chemicals (Frankfurt) and respectively used in 50% and 2% ethanolic solutions.

2′Fucosyllactose (2′FL): *O*-α-L-fucopyranosyl (1→2)-*O*-β-D-galactopyranosyl-(1→4)-D-glucopyranose, was extracted from selected human milk.

2′Fucosylgalactose (2′FG): *O*-α-L-fucopyranosyl-(1→2)-D-galactopyranose and 2′fucopyranosyl-*N*-acetyllactosamine (2′FLNAc): *O*-α-L-fucopyranosyl-(1→2)-*O*-β-D-galactopyranosyl(1→4)-*N*-acetyl-D-glucosamine were chemically synthesised [3].

Serum samples from A_1, A_2, B and O blood-groups donors were collected and stored at −80°C until used for glycosyltransferase assays.

The serological reactivities of the low molecular weight acceptors: 2′FG, 2′FL and 2′FLNAc have been tested by haemagglutination inhibition of appropriate blood-group systems (A, B, H and Lewis) in microtiter plates [17]: ten microliters of a specific antibody or lectin dilution (human anti-A: 1/64;

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human anti-B: 1/512; anti-H from *Ulex europaeus* 1/2; anti-H from Bombay individual: 1/32; human anti-Le^x: 1/4; eel serum: 1/256; and *Lotus tetragonolobus* extract: 1/2), were carefully mixed 10 μl or serial dilutions of inhibitors and incubated 1 h at room temperature. Twenty microliters of 0.5% RBC suspensions (A_2, B, O, Le(a+) and Le(b+) RBC's) were then added and the agglutination reaction read microscopically after standing 16 h at 4°C. The degree of inhibition was expressed as the minimum amount of substance (μg in 40 μl) giving complete inhibition of the systems.

2.2. *Enzyme assays*

α-*N*-acetylgalactosaminyltransferase activities were directly measured on whole sera, in a mixture containing the following components to a total volume of 45 μl: Mn^{2+} 2.0 μmol; sodium cacodylate buffer pH 6 or pH 7, 2.0 μmol; 2'FL, 0.28 μmol or 2'FG, 0.37 μmol, or 2'FLNAc, 0.23 μmol; ATP, 0.2 μmol; [1-^{14}C]UDPGalNAc, 1.0 nmol (≃ 75 000 cpm); serum, 10 μl.

α-*D*-galactosyltransferase activities were performed in a mixture containing under 50 μl total volume: Mn^{2+}, 1.0 μmol; sodium cacodylate buffer pH 6.5, 2.0 μmol; 2'FL, 0.41 μmol or 2'FG, 0.74 μmol, or 2'FLNAc, 0.46 μmol; ATP, 0.5 μmol; serum, 10 μl; [^{14}C]UDPGal, 0.23 nmol (≃ 90 000 cpm). After standing 18 h at 37°C, the synthesised neutral saccharides were separated from unreacted UDP-sugars by high voltage electrophoresis in 0.1 M ammonium formiate buffer pH 3.5 (Whatman paper n° 40; 3000 V/200 mA).

Free [1-^{14}C]GalNAc or [^{14}C]Gal released during incubation process were eliminated by descending chromatography in solvent *b* and the radioactive saccharides were then detected on a radiochromatogram scanner (Packard, 7201) and estimated by liquid scintillation counting (Nuclear Chicago, Mark II).

2.3. *Characterisation of products by paper chromatography*

The A and B active saccharides synthesised after enzyme assays were eluted from Whatman 40 paper strips in lactose $8 \cdot 10^{-6}$ M, desalted on Biodeminrolit, (14–52 mesh, Hopkin and Willians) previously equilibrated with lactose 10 mM, concentrated under vacuum and submitted to descending paper chromatography (Whatman 40) in the following solvents: solvent a: ethyl acetate–pyridine–water (2 : 1 : 2), upper phase, solvent b: ethyl acetate–pyridine–acetic acid–water (5 : 5 : 1 : 3).

The mobility of each compound has been expressed as RLac, in terms of the mobility compared to a reference solution of lactose.

3. Results

3.1. *Synthesis and properties of 2'FLNAc and 2'FG*

The synthesis of 2'FLNAc was performed by using benzyl ethers as temporary blocking groups of hydroxylic functions. Benzyl 2-acetamido-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranoside [18] was first glycosylated by 3, 4, 6-tri-*O*-benzyl-1,2-*O*-(tert-butoxyethylidene)-α-D-galactopyranose. After *O*-deacetylation, the obtained compound was glycosylated by 2, 3, 4-tri-*O*-benzyl-α-L-fucopyranosyl bromide [19] using the bromide ion catalysed reaction [20]. 2'FLNAc was obtained after hydrogenolysis as a pure amorphous powder, $[\alpha]_D^{20} = -46.5°C$ (c 0.5, H_2O), RLac = 1.00 (ethyl acetate–pyridine–water, 10 : 4 : 3).

2'FG was synthesised by glycosylation of benzyl 3, 4, 6-tri-*O*-benzyl-β-D-galactopyranoside with 2, 3, 4-tri-*O*-benzyl-α-L-fucopyranosyl bromide, using the bromide ion catalysed reaction. $[\alpha]_D^{20} = -56°C$ (c 1.2, H_2O), RLac = 1.58 (ethyl acetate–pyridine–water, 10 : 4 : 3). These properties are in close agreement with those previously reported [21].

3.2. *Serological activity of the synthetic sugars*

2'FG and 2'FLNAc were tested in the haemagglutination inhibition of A, B, H and Lewis human RBC by antisera or lectins with appropriate specificity.

As shown in table 1, minimum amount of sugars completely inhibiting red cell agglutinability are reported for 2'FG and 2'FLNAc but also for 2'FL (from human milk) and L-fucose.

As it could be expected, no inhibition is observed in the A, B or Lewis systems. On the contrary, all these compounds inhibit the H/anti-H agglutinating systems but to different extent. The anti-H from *Lotus tetragonolobus* and eel serum are highly inhibited by the substances tested with the exception

Table 1
Inhibition of haemagglutination reactions by oligosaccharides

Inhibitors		Minimum amount (μg/40 μl) of substance giving complete inhibition of agglutination in the following systems							
		A	B	Le^a	Le^b	Anti-H reagents			
Structure	Short name					human (Bh)	*Ulex europaeus*	*Lotus tetragonolobus*	eel serum
α-L-Fuc-(1→2)-Gal	2'FG	>120	>120	>120	>120	>120	1.9	3.75	3.75
α-L-Fuc (1→2) Gal β (1→4) GlNAc	2'FLNAc	>120	>120	>120	>120	30	1.9	3.75	7.5
α-L-Fuc (1→2) Gal β (1→4) Gl	2'FL (from human milk)	>120	>120	>120	>120	35	2.2	3.87	61
L-Fuc (commercial)		nt	nt	nt	nt	>100	50	0.78	3.12

Abbreviations: Gal = D-galactopyranosyl; Gl = D-glucopyranose; GlNAc = N-acetyl-D-glucosamine; L-Fuc = L-fucose.

of 2'FL from human milk which apparently is a rather poor inhibitor of the eel serum. It has been reported that both 2'FL from human milk and a trisaccharide similar to 2'FLNAc isolated after alkaline hydrolysis of blood group substances, are relatively poor inhibitors of eel serum as compared to 2'FG or L-fusoce [6]. It is assumed that the strong inhibition of eel serum by synthetic 2'FLNAc, come both from its high degree of purity and from the primary role of the *N*-acetyl residue in such activity. The presence of some deacetylated molecules in the 2'FLNAc obtained by chemical degradation together with the known heterogeneity of anti-H reagents [22] cannot be excluded and may provide a possible explanation to the poor inhibitory activity of such oligosaccharide toward eel serum [6].

Using an anti-H seed extract from *Ulex europaeus*, 2'FG, 2'FLNAc and 2'FL are equally good inhibitors, while L-fucose itself is much less active. Using a human anti-H from a 'Bombay' (B_h) individual, only 2'FLNAc and 2'FL are found to be highly active. 2'FG and L-fucose are not inhibitors, probably because the human antibody combining site has to be complementary of an antigenic structure involving at least three saccharide units. Furthermore these inhibition studies support the proposed differences of specificities between the various anti-H reagents [22].

3.3. *Glycosyltransferase activities on low molecular weight acceptors*

The enzyme activities in human sera of A_1 (BAD. and ROP.), A_2 (SAL. and RAH.), B (BI : RUF. ; B II : DOI. ; B III : KEI.) and 0 individuals were simultaneously tested on 2'FG, 2'FL and 2'FLNAc, each measurement being done in duplicate (table 2). Among A sera, whatever the enzyme substrate, the A_1 and A_2 transferases were distinguished according to their expected optimum pH: 6.0 for A_1 samples, and 7.0 for A_2 samples [23,24]. In all cases, enzyme activity measurements show that among the three oligosaccharides used, 2'FLNAc appears to be the best acceptor. However it can also be demonstrated that 2'FG is a much poorer substrate for A_2 enzymes.

Among B sera, where one sample of each α-D-galactosyltransferase group B I, B II and B III [25] have been tested at pH 6.5, the 2'FLNAc was also the most efficient acceptor, In variance with the result obtains with A_2 enzymes, 2'FG seems an equally good substrate for B galactosyltransferases.

The structures of A and B reaction products obtained with the various acceptors have not been elucidated by chemical means, but were only identified by paper chromatography in solvents a and b (table 2). It then appears that R_{Lac} values reported for tri- and tetrasaccharides (A and B)

Table 2
Glycosyltransferase activities in A_1, A_2, B and O human sera using low molecular weight acceptors

Serum	Phenotype	Optimum pH	Characteristics of reaction products using[a] 2′fucosylgalactose activity	Rlac (solvent *b*)	Rlac (solvent *a*)	2′fucosyllactose activity	Rlac (solvent *b*)	Rlac (solvent *a*)	2′fucosyl-N-acetyl-lactosamine activity	Rlac (solvent *b*)	Rlac (solvent *a*)
BAD.	A_1	6.0	0.287	1.12	0.81	0.354	0.51	0.39	0.378	0.80	0.60
ROP.	A_1	6.0	0.507	1.12	0.81	0.584	0.51	0.39	0.628	0.80	0.60
SAL.	A_2	7.0	0.086	1.12	0.81	0.193	0.51	0.39	0.228	0.80	0.60
RAH.	A_2	7.0	0.088	1.12	0.81	0.189	0.51	0.39	0.242		
RUF.	B (B_I)[b]	6.5	0.029	0.87	0.70	0.030	0.47	0.36	0.044	0.66	0.51
DOI.	B (B_{II})	6.5	0.073	0.87	0.70	0.070	0.47	0.36	0.083	0.66	0.51
KEI.	B (B_{III})	6.5	0.165	0.87	0.70	0.156	0.47	0.36	0.20	0.66	0.51
Controls: 5 Group O sera		-	< 0.002	-	-	< 0.002	-	-	< 0.002	-	-

[a] Enzyme activities are expressed as the ratio of total radioactivity added in the incubation mixture (mean of two determinations). The reaction products are characterized by paper chromatography in solvent *a* and *b*. The mobilities of non substituted acceptors were as follows: 2′FG (Rlac = 1.45 solvent *a*); 2′FL (Rlac = 0.69 in solvent *a*; Rlac = 0.79 in solvent *b*); 2′FINAc (Rlac = 1.0 in solvent *a*; Rlac = 1.05 in solvent *b*).

[b] Groups of α-D-galactosyltransferase activities in human sera [25].

formed after enzyme assays using 2'FG and 2'FL are in good agreement with previously reported values on well characterised substances (14,26,27). On the other hand, A and B tetrasaccharides derived from 2'FLNAc are similar to the structures isolated by alkaline degradation of human A and B ovarian cyst glycoproteins [28].

4. Conclusions

Chemically synthesised 2'fucosylgalactose (2'FG) and 2'fucosyl-*N*-acetyllactosamine (2'FLNAc) can be obtained in pure form on a gram scale.

These compounds retain their biochemical properties when evaluated by haemagglutination inhibitions and substrate specificity for glycosyltransferases, studies which support their high degree of purity established by chemical analysis.

Considering that substrates are not the limiting factors, 2'FLNAc appears to be an even better acceptor for A and B blood-group enzymes than 2'FL, usually extracted from human milk and in which glucose is the terminal reducing sugar.

However, the differences between acceptors are very small especially with α-D-galactosyltransferase, possibly because the catalytic site of this enzyme is of more restricted area. On the contrary, 2'FG is approximatively three times less efficient than 2'FLNAc in assays of A_2 serum enzymes, such a difference being not observed with A_1 enzymes.

However, the actual difference between A_1 and A_2 blood types is still not clear, even if, as far as serum enzymes are concerned, A_1 and A_2 transferases can be distinguished according to their kinetic properties on low molecular weight acceptors or in enzymic conversion of 0 RBC into A RBC [23,24,29].

Furthermore, the A_1 and A_2 enzymes act on type 1 (LNF-I) and type 2 (2'FG, 2'FL, 2'FLNAc) low molecular weight acceptors, but A_2 serum transferases exhibit higher K_M values than A_1 serum enzymes do.

Keeping in mind first that A_1 and A_2 specificities are easily recognized on red cells but not clearly in secreted glycoproteins [30–32] and secondly that only ABO fucolipids containing type 2 chains have been isolated from human red cell stroma [33], it became likely that reasons to explain A_1 and A_2 specificities should not be based on the existence of type 1 chains in blood-group substances [34]. At the biochemical level, Hakomori et al. [35] have also shown that A glycolipids are highly polymorphic, four antigenic structures A^a, A^b, A^c and A^d being isolated from A_1 RBC. If only trace amounts of the branched glycolipids A^c and A^d are found in A_2 RBC membranes [36], it can be postulated that A_1 enzymes should be able to convey *N*-acetylgalactosamine on linear and branched precursors with H specificity, whereas A_2 enzymes should only act on simplest non-ramified H precursors.

The demonstration of such hypothesis needs further investigations using macromolecular H acceptors, such as described by Stellner et al. [37] and Watanabe et al. [38]. Development of synthetic determinants would therefore be also of a considerable value in this field.

Finally it becomes obvious that A_1 and A_2 gene products identified in human sera have distinct properties, a fact which is further supported by recent estimations of their isoelectric points: pH_i = 10 (A_1 sera) or pH_i = 6.2 (A_2 sera) [39]. However, properties of A_1 and A_2 transferases partially purified from plasma are claimed to be similar [40], an observation to be joined to the identical isoelectric points of these gene products in ovarian cyst fluids [38] (A_1 and A_2 enzymes, pH_i = 9.5 to 10).

It is therefore to be investigated whether or not properties of transferases evaluated on whole sera do represent true characteristics of A_1 and A_2 gene-products.

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References

[1] Lemieux, R. U. and Driguez, H. (1975) J. Am. Chem. Soc. 97, 4063.

[2] Lemieux, R. U. and Driguez, H. (1975) J. Am. Chem. Soc. 97, 4069.
[3] Jacquinet, J. C. and Sinaÿ, P. Tetrahedron, in the press.
[4] Lemieux, R. U., Bundle, D. R. and Baker, D. A. (1975) J. Am. Chem. Soc. 97, 4076.
[5] Watkins, W. M. (1962) Immunology 5, 245.
[6] Watkins, W. M. (1966) in: Glycoproteins, p. 462 (A. Gottschalk, ed.) Elsevier, Amsterdam.
[7] Kabat, E. A. (1956) Blood group substances, p. 184 Academic Press, New York.
[8] Bahl, O. P. (1970) J. Biol. Chem. 245, 299.
[9] Jeffrey, A., Zopf, D. A. and Ginsburg, V. (1975) Biochem. Biophys. Res. Comm. 62, 608.
[10] Kristiansen, T. (1974) Biochim. Biophys. Acta 362, 567.
[11] Zopf, D. A. and Ginsburg, V. (1975) Arch. Biochem. Biophys. 167, 345.
[12] Li, S. C., Mazzotta, M. Y., Chien, S. F. and Li, Y. T. (1975) J. Biol. Chem. 250, 6786.
[13] Kobata, A., Grollman, E. F. and Ginsburg, V. (1968) Biochem. Biophys. Res. Comm. 32, 272.
[14] Kobata, A., Grollman, E. F. and Ginsburg, V. (1968) Arch. Biochem. Biophys. 124, 609.
[15] Hearn, V. M., Smith, Z. G. and Watkins, W. M. (1968) Biochem. J. 109, 315.
[16] Grollman, E. F., Kobata, A. and Ginsburg, V. (1969) J. Clin. Invest. 48, 1489.
[17] Wegmann, T. G. and Smithies, O. (1966) Transfusion (Phil.) 6, 67.
[18] Jacquinet, J. C., Petit, J. M. and Sinaÿ, P. (1974) Carbohydr. Res. 38, 305.
[19] Dejter-Juszynski, M. and Flowers, H. M. (1971) Carbohydr. Res. 18, 219.
[20] Lemieux, R. U., Hendriks, K. B., Stick, R. V. and James, K. (1975) J. Am. Chem. Soc. 97, 4056.
[21] Levy, A., Flowers, H. M. and Sharon, N. (1967) Carbohydr. Res. 4, 305.
[22] Matsumoto, I. and Osawa, T. (1971) Vox Sang. 21, 548.
[23] Schachter, J., Michaels, W. A., Tilley, C. A., Crookston, M. C. and Crookston, J. H. (1973) Proc. Nat. Acad. Sci., Wash. 70, 220.
[24] Cartron, J. P., Gerbal, A., Badet, J., Ropars, C. and Salmon, Ch. (1975) Vox Sang. 28, 347.
[25] Badet, J., Ropars, C., Cartron, J. P. and Salmon, Ch. (1974) Biomedicine 21, 230.
[26] Hearn, V. M., Race, C. and Watkins, W. M. (1972) Biochem. Biophys. Res. Commun. 46, 948.
[27] Race, C. and Watkins, W. M. (1970) FEBS Lett. 10, 279.
[28] Painter, T. J., Watkins, W. M. and Morgan, W. T. J. (1965) Nature (London) 206, 594.
[29] Schenkel-Brunner, H. and Tuppy, H. (1969) Nature (London) 223, 1272.
[30] Boettcher, B. (1967a) Aust. J. Exp. Biol. Med. Sci. 45, 485.
[31] Boettcher, B. (1967b) Aust. J. Exp. Biol. Med. Sci. 45, 495.
[32] Bar-Shany, S., Ben-David, A., Nusbacher, J., Szymanski, T., Kochwa, S. and Rosenfield, R. E. (1970) Ann. N.Y. Acad. Sci. 169, 217.
[33] Hakomori, S. I. and Kobata, A. (1974) in: The Antigens, p. 79 (Sela ed.) Academic Press, New York.
[34] Moreno, C., Lundblad, A. and Kabat, E. A. (1971) J. Exp. Med. 134, 439.
[35] Hakomori, S. I., Stellner, K. and Watanabe, K. (1972) Biochem. Biophys. Res. Commun. 49, 1061.
[36] Hakomori, S. I. (1973) 2ème Symposium International sur les Glycoconjugués, Villeneuve d'Ascq (France) 20 au 27 juin Tome 2, p. 954.
[37] Stellner, K. and Hakomori, S. I. (1973) Biochem. Biophys. Res. Commun. 55, 439.
[38] Watanabe, K., Laine, R. A. and Hakomori, S. I. (1975) Biochemistry 14, 2725.
[39] Topping, M. D. and Watkins, W. M. (1975) Biochem. Biophys. Res. Commun. 64, 89.
[40] Whitehead, J. S., Bella Jr, A. and Kim, Y. S. (1974) J. Biol. Chem. 249, 3448.