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UDP-*N*-ACETYL-D-GALACTOSAMINE AS A DONOR SUBSTRATE FOR THE GLYCOSYLTRANSFERASE ENCODED BY THE *B* GENE AT THE HUMAN BLOOD GROUP *ABO* LOCUS

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ABSTRACT

The properties of the enzyme in the serum of blood group B individuals that catalyses the transfer of small amounts of *N*-acetyl-D-galactosamine to H-active precursor structures were compared with those of the blood group *B* gene-associated α-(1→3)-D-galactosyltransferase and with the blood group *A* gene-associated α-(1→3)-*N*-acetyl-D-galactosaminyltransferases in the serum of blood group A_1 and A_2 individuals. The biosynthetic products formed by the enzyme in B serum were identical with the A-active structures synthesised by the A^1 and A^2 gene-associated α-(1→3)-*N*-acetyl-D-galactosaminyltransferases but the enzyme differed from the A^1 and A^2 transferases in its apparent K_m for UDP-*N*-acetyl-D-galactosamine, its heat susceptibility, its failure to bind to Sepharose 4B, and its adsorption to H-active sites on group O red cell ghosts under conditions which bind the *B* transferase but fail to adsorb the A^1 and A^2 transferases. The correlation between the levels of α-(1→3)-D-galactosyltransferase and α-(1→3)-*N*-acetyl-D-galactosaminyltransferase activities in all the group B serum samples tested, the maintenance of the same ratio of activities after successive cycles of binding to group O red cell ghosts, the retention of the ability to convert blood group O to A-active cells after treatment of the serum with Sepharose 4B, and the failure to detect any comparable activity in group O serum samples tested under the same conditions indicated that the enzyme in group B serum that utilises UDP-*N*-acetyl-D-galactosamine to make blood group A-active structures is the *B* gene-associated α-(1→3)-D-galactosyltransferase.

INTRODUCTION

The mechanism of inheritance of the ABO blood groups that is generally accepted today is the model of multiple alleles at one locus proposed by Bernstein[1]. However, the *ABO* genetic locus is unique amongst the loci so far investigated in mammalian genetic systems in that the structural alleles *A* and *B* code for enzymes with qualitatively different specificities. The *A* gene encodes an α-(1→3)-*N*-acetyl-

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D-galactosaminyltransferase that uses UDP-*N*-acetyl-D-galactosamine as the donor substrate, whereas the α-(1→3)-D-galactosyltransferase encoded by the *B* gene uses UDP-D-galactose. Both these enzymes catalyse the transfer of their respective donor sugars to acceptor substrates containing terminal, nonreducing H-specific structures [α-L-Fuc*p*-(1→2)-β-D-Gal*p*-R] to synthesise the blood group A {α-D-Gal*p*NAc-(1→3)-[α-L-Fuc*p*-(1→2)]-β-D-Gal*p*-R} and B {α-D-Gal*p*-(1→3)-[α-L-Fuc*p*-(1→2)]-β-D-Gal*p*-R} antigenic determinants[2]. Despite the fact that the Bernstein model fits well with the family and population data on the inheritance of the A and B antigens[3], the difference in sugar donor substrate specificity of the enzymes required to synthesise these antigens has led some geneticists to doubt whether the two transferases can be the products of allelic genes[4].

Antibodies raised in rabbits in response to the human *A* gene-associated α-(1→3)-*N*-acetyl-D-galactosaminyltransferase cross react with the *B* gene-associated α-(1→3)-D-galactosyltransferase[5–7], and this immunological homology between the two transferases provides support for the allelic status of the genes. Further biochemical evidence which favours the view that these two enzymes are very closely related has come from more searching experiments on the donor substrate specificities of the transferases. Serum samples derived from group B individuals who, according to the classical mechanism of inheritance of *ABO,* cannot be carrying an *A* gene[3], were found, under certain *in vitro* conditions, to transfer small amounts of *N*-acetyl-D-galactosamine to the H-active acceptor 2′-fucosyllactose to give a product that was indistinguishable structurally and serologically from the tetrasaccharide, α-D-Gal*p*NAc-(1→3)-[α-L-Fuc*p*-(1→2)]-β-D-Gal*p*-(1→4)-D-Glc, synthesised from the same acceptor substrate by the *A* gene-specified transferase derived from the serum of group A individuals[8,9]. The group B serum also converted blood group O red cells into blood group A-active cells in the presence of the appropriate additives[10]. Subsequently, serum from group A individuals was found to have a weak capacity to utilise UDP-D-galactose to form a product with 2′-fucosyllactose that was chromatographically identical with the B-active tetrasaccharide[11], α-D-Gal*p*-(1→3)-[α-L-Fuc*p*-(1→2)]-β-D-Gal*p*-(1→4)-D-Glc. Serum from group O individuals, tested under the same conditions as the A and B serum samples, had no capacity to transfer either *N*-acetyl-D-galactosamine or D-galactose to H-active structures[8,11]. Although manipulation of the reaction conditions is necessary in order to demonstrate the atypical functions of the transferases, the findings suggest that the *A* and *B* genes encode enzymes that have the potential to utilise both UDP-*N*-acetyl-D-galactosamine and UDP-D-galactose as donor substrates. Nevertheless, before firm conclusions can be reached that these apparently overlapping functions are inherent properties of the *A* and *B* gene-encoded enzymes, it is imperative to eliminate the possibility that the low yields of "alternative" products arise from the presence of minor contaminants of an α-(1→3)-*N*-acetyl-D-galactosaminyltransferase in the group B serum and an α-(1→3)-D-galactosyltransferase in the group A serum.

The present paper describes further studies on the properties of the enzyme

in group B serum that utilises UDP-*N*-acetyl-D-galactosamine as a donor substrate and our reasons for concluding that the transferase involved is the enzyme encoded by the blood group *B* gene.

EXPERIMENTAL

Materials. — UDP-D-[U-^{14}C]galactose (11.4 GBq/mmol), UDP-*N*-acetyl-D-[1-^{14}C]galactosamine (2.3 GBq/mmol), UDP-D-[U-^{14}C]glucose (10.8 GBq/mmol), and UDP-*N*-acetyl-D-[U-^{14}C]glucosamine (11.1 GBq/mmol) were obtained from Amersham International, U.K. Unlabelled UDP-*N*-acetyl-D-galactosamine was prepared according to the method of Carlson *et al.*[12] and unlabelled UDP-D-galactose, UDP-D-glucose, UDP-*N*-acetyl-D-glucosamine, and UDP were purchased from Sigma Chemical Co. Ltd., U.K. Sepharose 4B was supplied by Pharmacia, U.K.

Synthetic *N*-acetyllactosamine [β-D-Gal*p*-(1→4)-D-GlcNAc] prepared by a modification of the method of Kuhn and Kirschenlohr[13], lacto-*N*-biose I [β-D-Gal*p*-(1→3)-D-GlcNAc] synthesised by a modification of the method of Flowers[14], and 2′-fucosyllactose [α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→4)-D-Glc], 3-fucosyllactose {α-L-Fuc*p*-(1→3)-[β-D-Gal*p*-(1→4)]-D-Glc}, lacto-*N*-tetraose [β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-D-Glc], and lacto-*N*-neotetraose [β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-D-Glc] isolated from human milk by the method of Anderson and Donald[15], were kindly supplied by Dr. A. S. R. Donald, MRC Clinical Research Centre, Harrow, U.K. 2-Fucosylgalactose [α-L-Fuc*p*-(1→2)-D-Gal], lacto-*N*-fucopentaose I [α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-D-Glc], lacto-*N*-fucopentaose II {β-D-Gal*p*-(1→3)-[α-L-Fuc*p*-(1→4)]-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-D-Glc}, lactodifucotetraose {α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→4)-[α-L-Fuc*p*-(1→3)]-D-Glc}, lacto-*N*-difucohexaose I {α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→3)-[α-L-Fuc*p*-(1→4)]-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-D-Glc} and lacto-*N*-difucohexaose II {β-D-Gal*p*-(1→3)-[α-L-Fuc*p*-(1→4)]-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-[α-L-Fuc*p*-(1→3)]-D-Glc} were gifts of the late Professor R. Kuhn, Max-Planck-Institut für Medizinische Forschung, Heidelberg, F.R.G. Synthetic 2′-fucosyl-*N*-acetyllactosamine [α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→4)-D-GlcNAc] and difuco-*N*-acetyllactosamine {α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→4)-[α-L-Fuc*p*-(1→3)]-D-GlcNAc} were kindly supplied by Professor P. Sinaÿ, Université d'Orléans, France. The disaccharide α-L-Fuc*p*-(1→4)-D-GlcNAc was a gift from Professor R. W. Jeanloz, Harvard Medical School, Boston, Massachusetts, U.S.A.

The phenol-extraction procedure[16] was used to isolate blood group H, Le^a, and precursor glycoproteins from ovarian cyst fluids and blood group H-active glycoproteins from homogenates of human blood group O submaxillary glands and stomachs that had been digested with pepsin. An H-active poly(glycosyl)ceramide preparation[17] and H glycosphingolipid S22 (ref. 18) isolated from human red cell membranes were kindly supplied by Professor J. Koscielak, Institute of Haematology, Warsaw, Poland and Dr. A. Gardas, Medical Centre of Postgraduate Education, Warsaw, Poland, respectively.

Red cell ghosts were prepared by the method of Dodge *et al.*[19] from outdated group O cells supplied by the North London Blood Transfusion Centre, London, U.K. The ghosts were washed ten times with large volumes of distilled water and freeze dried. When required for use, the dried ghosts were suspended in water, washed a further three times, and then resuspended in the required volume of water in a calibrated tube.

Concentration and purification of the B *gene-specified transferase.* — The *B* gene-specified transferase binds to blood group H receptors on group O red cell ghosts and the affinity-bound enzyme can be specifically eluted with 2′-fucosyllactose to yield a highly purified preparation[20]. The enzyme is more stable in the

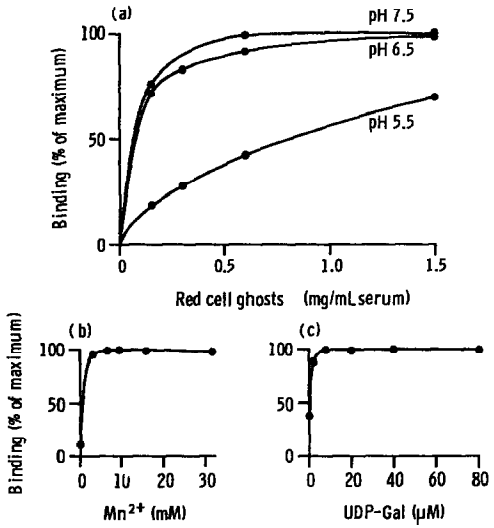

Fig. 1. Optimum conditions for binding α-(1→3)-D-galactosyltransferase to blood group O red cell ghosts: (a) The effect of pH and red cell ghost concentration. Group B serum (1 mL) was treated with various concentrations of group O red cell ghosts in the presence of 16mM $MnCl_2$, 30μM UDP-D-galactose, and 0.2M sodium cacodylate buffer at pH 5.5, 6.5, or 7.5 in a total volume of 1225 μL. (b) The effect of Mn^{2+} concentration. Group B serum (500 μL) was treated with 50 μL of a 1.5% (w/v) suspension of group O red cell ghost in the presence of 20μM UDP-D-galactose, 0.2M sodium cacodylate buffer (pH 7.5), and various concentrations of $MnCl_2$ in a total volume of 625 μL. (c) The effect of UDP-D-galactose concentration. Group B serum (500 μL) was treated with 1.5% (w/v) suspension of group O red cell ghosts (50 μL), 0.2M sodium cacodylate buffer (pH 7.5), 16mM $MnCl_2$, and various concentrations of UDP-D-galactose in a total volume of 625 μL. The mixtures in (a), (b), and (c) were kept for 15 min at 4° and the red cell ghosts were then removed by centrifugation at 16 000 r.p.m. in a Sorvall SS 34 rotor for 30 min. A control was set up for each mixture containing B serum and all the additives except the red cell ghosts. The control serum and the supernatant serum remaining after removal of the ghosts were assayed for α-(1→3)-D-galactosyltransferase activity with the following reaction mixture: UDP-D-[^{14}C]galactose (0.25 nmol; 125 000 c.p.m.), $MnCl_2$ (2 μmol), ATP (0.5 μmol), 2′-fucosyllactose (0.25 μmol), NaN_3 (0.8 μmol), sodium cacodylate buffer (pH 6.5; 5 μmol), and control serum or supernatant after removal of ghosts (20 μL). The total volume of the incubation mixture was 100 μL. The mixtures were incubated for 16 h at 37° and the reaction products separated by chromatography on Whatman DE-81 paper developed with solvent (*b*). The percentage binding of α-(1→3)-D-galactosyltransferase was estimated from the difference in the activity measured in the control serum from that found in the supernatant after removal of the red cell ghosts.

bound form than it is in free solution and, since long incubation times are required for the detection of the products of *N*-acetyl-D-galactosaminyl transfer, the *B*-transferase bound to group O red cell ghosts was used as a concentrated source of the enzyme for many of the experiments to be described. Preliminary investigations to determine the optimum conditions for binding the *B* transferase (Fig. 1a, b, and c) established that at 4° binding was maximal at pH 7.5 in the presence of at least 7.5mM $MnCl_2$ and 8μM UDP-D-galactose. On the basis of these results, the following procedure was adopted. 2M Sodium cacodylate buffer (3 mL; pH 7.5), M $MnCl_2$ (0.6 mL), 10mM UDP-D-galactose (30 μL), and a 0.75% suspension of group O red cell ghosts (3 mL) were added to 30 mL of group B serum. The final concentrations in the mixture were UDP-D-galactose 8μM, $MnCl_2$ 16mM, and red cell ghosts 0.6 mg/mL. The suspension was kept for 10 min and then centrifuged in a Sorvall SS 34 rotor at 16 000 r.p.m. for 10 min. The supernatant was discarded and the deposited ghosts were washed twice with 0.15M NaCl (25 mL) containing 8μM UDP-D-Gal and 16mM $MnCl_2$, and then resuspended in the required volume of 0.15M NaCl. All the binding and washing procedures were carried out at 4°. Details of the amounts of ghost-bound enzyme used for the various experiments are given in the Tables and Figures.

In experiments in which it was impractical to use the enzyme in bound form, the transferase was eluted from the ghosts as follows. Red cell ghosts (45 mg) to which the *B* transferase from 60 mL of group B serum had been bound were washed twice with 0.15M NaCl containing 8μM UDP-galactose and 16mM $MnCl_2$, and then resuspended in a mixture of 0.15M NaCl (0.5 mL), 1% bovine serum albumin (50 μL), and 50mM 2′-fucosyllactose (50 μL). The mixture was kept at 4° for 90 min and then centrifuged for 10 min at 16 000 r.p.m. to sediment the red cell ghosts. The supernatant was removed and used without further treatment for those experiments in which the presence of a small amount of 2′-fucosyllactose was not disadvantageous. When an enzyme preparation free from 2′-fucosyllactose was required, the volume of 0.15M NaCl in the elution mixture was reduced to 0.4 mL and, after centrifugation, the supernatant was dialysed against four changes of 250 mL of 0.15M NaCl at 4°. At the end of the dialysis period, the volume of the eluted enzyme was adjusted to 0.6 mL with 0.15M NaCl. The *B* transferase obtained in this way was purified about 10 000-fold. A much higher degree of purification can be achieved by carrying out the binding step in a column[20] in place of the batch procedure described here, but the enzyme obtained is less stable and hence less suitable for the prolonged incubations required to detect the α-(1→3)-*N*-acetylgalactosaminyltransferase activity.

Transferase assays. — α-(1→3)-*N*-Acetyl-D-galactosaminyltransferase and α-(1→3)-D-galactosyltransferase activities were measured by the transfer of *N*-acetyl-D-[^{14}C]galactosamine or D-[^{14}C]galactose, respectively, to oligosaccharide or macromolecular acceptors. The reaction mixtures and incubation times are given in the Tables and Figures. At the end of the incubation period, the reactions were stopped by immediately freezing the samples at −40°. For the experiments on the

acceptor specificity of the transferases, the incubation mixtures containing the oligosaccharide acceptors were treated as described previously[21] to separate the neutral sugars from the charged compounds. The neutral products were then chromatographed on Whatman No. 40 paper developed with 2:1:2 (v/v, upper phase) ethyl acetate–pyridine–water (solvent *a*). The incubation mixtures containing glycolipid and glycoprotein acceptors were subjected directly to chromatography on Whatman 3MM paper developed with 5:1:1:3 (v/v) propan-1-ol–pyridine–ethyl acetate–water (solvent *b*). In this solvent, the nucleotide sugars and breakdown products migrated away from the origin of the chromatogram, leaving behind the macromolecular materials. Controls from which the acceptor had been omitted were included as a measure of endogenous incorporation. For all experiments other than the acceptor-specificity studies, the reaction mixtures containing 2′-fucosyllactose as acceptor substrate were subjected to chromatography on Whatman DE-81 paper developed with solvent *b*. Radioactive peaks were detected on a Packard Radiochromatogram scanner, the radioactive areas were cut out, and the radioactivity was counted in a Nuclear Chicago Scintillation Counter.

A second method used for the detection of α-(1→3)-*N*-acetyl-D-galactosaminyltransferase activity was conversion of group O red cells to A-active cells[22]. The *B* transferase was bound to red cell ghosts as described. The reaction mixtures for cell conversion contained the enzyme source (100 μL), 0.4M $MnCl_2$ (5 μL), 10μM UDP-*N*-acetyl-D-galactosamine (15 μL), and a 50% suspension of group O red cells in 0.15M NaCl (10 μL). The mixtures were incubated for 16 h at 37°, centrifuged at low speed, and the red cells then washed twice with 0.15M NaCl (500 μL) and finally resuspended in 0.15M NaCl (250 μL). The suspension was titrated against serial dilutions of antiserum and the haemagglutination titre was taken as the reciprocal of the last dilution of the serum at which clumps of two or three cells were visible under the low-power of the microscope. Rabbit immune anti-A was prepared as described[23] and the mouse hybridoma-derived monoclonal anti-A 6D4 (ref. 24) was a gift from Dr. D. Voak, Regional Transfusion Centre, Cambridge, U.K.

Anomeric configuration. — The anomeric configuration of the *N*-acetyl-D-galactosaminyl group transferred to soluble acceptor substrates was ascertained by treatment with an *N*-acetyl-α-D-galactosaminidase from *Trichomonas foetus*[25]. This preparation released nitrophenol from *p*-nitrophenyl *N*-acetyl-α-D-galactosaminide and had no detectable activity with *p*-nitrophenyl *N*-acetyl-β-D-galactosaminide. The products of *N*-acetyl-D-[^{14}C]galactosamine transfer were eluted from the chromatograms with water, taken to dryness, and then dissolved in water (50 μL). *T. foetus* enzyme (5 mU, where 1 unit hydrolyses 1.0 μmol of *p*-nitrophenyl *N*-acetyl-α-D-galactosaminide per min at pH 7.0 and 37°) was added and the mixture incubated for 18 h at 37°. The reaction products were separated by chromatography on Whatman No. 40 paper developed with 10:4:3 (v/v) ethyl acetate–pyridine–water (solvent *c*).

RESULTS

Properties of the enzyme in blood group B serum transferring N-*acetyl*-D-*galactosamine.* — (*a*) *Optimum pH.* Serum from a group B donor was tested at a range of pH values for the capacity to transfer *N*-acetyl-D-[^{14}C]galactosamine from UDP-*N*-acetyl-D-[^{14}C]galactosamine to 2′-fucosyllactose. A similar experiment was carried out with the serum from the same donor but with UDP-D-[^{14}C]galactose as the donor substrate. At the optimum pH for the transfer of D-galactose (pH 6.5), incorporation of *N*-acetyl-D-[^{14}C]galactosamine was <50% of the maximum, and the optimum pH for the transfer of this sugar was 8.0 (Fig. 2). The amount of D-[^{14}C]galactose transferred to 2′-fucosyllactose at pH 6.5 was 368 pmol.h^{-1}.mL^{-1} serum whereas the amount of *N*-acetyl-D-[^{14}C]galactosamine transferred at pH 8.0 was 4.7 pmol.h^{-1}.mL^{-1} serum. Thus, at the respective pH optima for the two donor substrates, ~80 times as much D-galactose as *N*-acetyl-D-galactosamine was incorporated into the H-active acceptor substrate.

No change was observed in the optima for the transfer of D-galactose or *N*-acetyl-D-galactosamine when the *B* transferase was concentrated by adsorption onto red cell ghosts or eluted from the ghosts with 2′-fucosyllactose. All subsequent experiments involving the transfer of *N*-acetyl-D-galactosamine to soluble substrates by enzymes present in, or isolated from, group B serum were performed at pH 8.0. Experiments involving the conversion of group O cells to A-active cells were carried out at pH 7.0.

(*b*) *Metal ion requirement.* — The transfer of *N*-acetyl-D-[^{14}C]galactosamine from UDP-*N*-acetyl-D-[^{14}C]galactosamine by the enzyme in group B serum

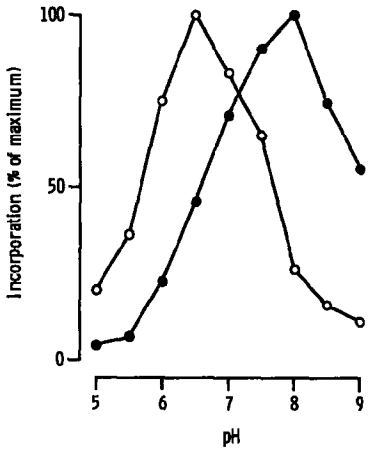

Fig. 2. The effect of pH on the transfer of D-galactose (—○—○—) or *N*-acetyl-D-galactosamine (—●—●—) by enzyme(s) in group B serum. The reaction mixtures and incubation times were the same as described in the legend to Fig. 1, except that for *N*-acetyl-D-galactosaminyl transfer UDP-*N*-acetyl-D-[^{14}C]galactosamine (1.25 nmol; 125 000 c.p.m.) replaced UDP-D-[^{14}C]galactose. Buffers (5 μmol) were included at a range of pH values (sodium cacodylate buffer, pH 5.0–7.0, Tris · HCl buffer pH 7.5–9.0). Activity is expressed as a percentage of the maximum incorporation obtained.

TABLE I

TRANSFER OF D-[^{14}C]GALACTOSE AND *N*-ACETYL-D-[^{14}C]GALACTOSAMINE TO LOW-MOLECULAR-WEIGHT ACCEPTOR SUBSTRATES BY A CONCENTRATED PREPARATION OF α-D-(1→3)-GALACTOSYLTRANSFERASE[a]

Acceptor substrate	*Donor substrate*			
	UDP-[^{14}C]Gal		*UDP-[^{14}C]GalNAc*	
	Product (R_{Lac})[b]	*[^{14}C]Gal incorporated (c.p.m.)*	*Product (R_{Lac})*[b]	*[^{14}C]GalNAc incorporated (c.p.m.)*
β-D-Gal*p*-(1→4)-D-GlcNAc (*N*-Acetyllactosamine)		0		0
α-L-Fuc*p*-(1→4)-D-GlcNAc (4-Fucosyl-*N*-acetyl-D-glucosamine)		0		0
α-L-Fuc*p*-(1→2)-D-Gal (2-Fucosylgalactose)	0.95	45 382	1.01	3713
α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→4)-D-Glc (2′-Fucosyllactose)	0.43	59 818	0.50	6500
α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→4)-D-GlcNAc (2′-Fucosyl-*N*-acetyllactosamine)	0.57	68 431	0.68	8025
α-L-Fuc*p*-(1→3)-[β-D-Gal*p*-(1→4)]-D-Glc (3-Fucosyllactose)	0.44	1984		36

α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→4)-[α-L-Fuc*p*-(1→3)]-D-GlcNAc (Difucosyl-*N*-acetyllactosamine)		226		0
α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→4)-[α-L-Fuc*p*-(1→3)]-D-Glc (Lacto-*N*-difucotetraose)	0.25	2542		0
β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-D-Glc (Lacto-*N*-tetraose)		0		0
β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-D-Glc (Lacto-*N*-neotetraose)		55		0
α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-D-Glc (Lacto-*N*-fucopentaose I)	0.19	40 584	0.19	2585
α-L-Fuc*p*-(1→4)-[β-D-Gal*p*-(1→3)]-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-D-Glc (Lacto-*N*-fucopentaose II)		27		0
α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→3)-[α-L-Fuc*p*-(1→4)]-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-D-Glc (Lactodifucohexaose I)		48		0
α-L-Fuc*p*-(1→4)-[β-D-Gal*p*-(1→3)]-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-[α-L-Fuc*p*-(1→3)]-D-Glc (Lactodifucohexaose II)		340		0

[a]The reaction mixtures for the transfer of D-[^{14}C]galactose contained, in a total volume of 135 μL, UDP-[^{14}C]Gal (0.25 nmol, 125 000 c.p.m.), $MnCl_2$ (2 μmol), ATP (0.5 μmol), acceptor substrate (0.25 μmol), NaN_3 (0.8 μmol), sodium cacodylate buffer (pH 6.5, 5 μmol), and a group O red cell ghost suspension (20 μL) containing enzyme adsorbed from 0.8 mL of group B serum. The mixtures were incubated for 20 h at 37°. The reaction mixture for the transfer of *N*-acetyl-D-[^{14}C]galactosamine contained, in a total volume of 135 μL, UDP-[^{14}C]GalNAc (1.25 nmol, 125 000 c.p.m.), $MnCl_2$ (2 μmol), ATP (0.5 μmol), acceptor substrate (0.25 μmol), NaN_3 (0.8 μmol), Tris · HCl buffer (pH 8.0, 5 μmol), and a group O red cell ghost suspension (50 μL) containing enzyme adsorbed from 2 mL of group B serum. The mixtures were incubated for 64 h at 37°. The reaction products were separated and the radioactivity counted as described in the Experimental section. [b]Mobility relative to lactose on Whatman No. 40 paper developed with solvent (*a*).

required divalent cations. When $MnCl_2$ was added at a series of concentrations (from 2mM to 80mM), the activation curve for the transfer of either *N*-acetyl-D-[^{14}C]galactosamine or D-[^{14}C]galactose showed optimal activation at a concentration of ~15mM. At this concentration, Mg^{2+} ions did not give detectable activation.

(*c*) *Effect of incubation time.* In order to examine the linearity of the reactions with respect to time, a preparation of α-(1→3)-D-galactosyltransferase, purified by binding to group O red cell ghosts and elution with 2′-fucosyllactose, was tested for its capacity to transfer D-[^{14}C]galactose or *N*-acetyl-D-[^{14}C]galactosamine to 2′-fucosyllactose at a series of time intervals up to 64 h. Incorporation of *N*-acetyl-D-[^{14}C]galactosamine was approximately linear for 12 h and continued for up to 64 h. Incorporation of D-[^{14}C]galactose reached a maximum after 15 h and the reaction was approximately linear for 4 h. As the enzyme is less stable in the eluted form than when bound to the red cell ghosts, it has been assumed that the reactions were linear for at least the same length of time when the *B*-transferase bound to red cell ghosts was used as the enzyme source.

(*d*) *Acceptor substrate specificity.* For the experiments with low-molecular-weight acceptor substrates, the enzyme source was transferase from group B serum adsorbed onto red cell ghosts. Details of the reaction mixtures are given in Table I. Incorporation of either D-[^{14}C]galactose or *N*-acetyl-D-[^{14}C]galactosamine was obtained with the acceptors containing an α-L-fucosyl-(1→2)-β-D-galactosyl group at the nonreducing terminal, except when the oligosaccharides contained a second L-fucosyl group attached to the sugar subterminal to the β-D-galactosyl residue. Structurally similar compounds lacking the L-fucosyl group at O-2 of the β-D-galactosyl residue did not function as acceptors.

A preparation of *B* transferase which had been partially purified by adsorption onto group O red cell ghosts, followed by elution with 2′-fucosyllactose, was used as the enzyme source for the experiments with glycoprotein and glycolipid acceptors. The purified enzyme preparation was dialysed to remove 2′-fucosyllactose. Details of the incubation mixtures are given in Table II. The H-active glycolipids isolated from red cells and the H-active glycoproteins isolated from ovarian cyst fluids, submaxillary gland, and stomach mucosal tissue extracts were acceptors for both D-[^{14}C]galactose and *N*-acetyl-D-[^{14}C]galactosamine, whereas the blood group precursor glycoprotein and Le^a-active glycoproteins, which lack H acceptor sites, failed to function as substrates.

(*e*) *Characterisation of products of* N-*acetyl*-D-*galactosaminyl transfer.* Characterisation by methylation analysis, high resolution ^{1}H-n.m.r. spectroscopy, exoglycosidase digestion, and haemagglutination inhibition of the tetrasaccharide formed by the transfer of *N*-acetyl-D-galactosamine to 2′-fucosyllactose catalysed by a partially purified α-(1→3)-D-galactosyltransferase preparation has been described elsewhere[9]. The product was structurally and serologically identical with that made from the same donor and acceptor substrates when the blood group *A* gene-associated α-(1→3)-*N*-acetyl-D-galactosaminyltransferase was used as the enzyme source. The chromatographic mobilities in solvent (*a*) of the products

TABLE II

TRANSFER OF D-[^{14}C]GALACTOSE AND *N*-ACETYL-D-[^{14}C]GALACTOSAMINE TO GLYCOPROTEINS AND GLYCOLIPIDS BY A PARTIALLY PURIFIED PREPARATION OF α-(1→3)-D-GALACTOSYLTRANSFERASE[a]

Acceptor substrate	*Blood group specificity*	*Donor substrate (c.p.m. transferred)*	
		UDP-[^{14}C]Gal	*UDP-[^{14}C]GalNAc*
Ovarian cyst glycoproteins			
484	Precursor	456	0
277	H	75 009	5458
445	Le^a	144	0
579	Le^a	0	0
Stomach mucosal glycoprotein			
34	H	60 549	2849
Glycolipids from red cells			
S 22	H	83 659	10 036
Poly(glycosyl)ceramide	H	66 118	7264

[a]The reaction mixtures and incubation times were the same as in Table I, except that the enzyme source (20 μL in the mixtures containing UDP-D-[^{14}C]galactose and 50 μL in those containing UDP-*N*-acetyl-D-[^{14}C]galactosamine) was transferase eluted from red cell ghosts and 200 μg of each acceptor was used. In addition, bovine serum albumin (5 μL of a 1% solution) was included in all the reaction mixtures and Triton X-100 (5 μL of a 10% solution) was added to those mixtures containing glycolipid acceptors. The total volume of the mixtures was 150 μL. At the end of the incubation period, the reaction products were separated by chromatography on Whatman 3MM paper developed with solvent (*b*). The figures for c.p.m. ^{14}C-sugar incorporated were corrected for endogenous incorporation found in the absence of added substrate.

synthesised with the other low-molecular-weight oligosaccharides (Table I) were also identical with those formed by *A* transferase-catalysed addition of *N*-acetyl-D-galactosamine to the same substrates (data not shown).

The anomeric configuration of the sugar transferred from UDP-*N*-acetyl-D-[^{14}C]galactosamine to both the oligosaccharides and high-molecular-weight acceptors was determined by treatment with an *N*-acetyl-α-D-galactosaminidase from *T. foetus*. All the radioactive products were completely hydrolysed by this exoglycosidase preparation and the labelled sugar released cochromatographed with *N*-acetyl-D-galactosamine.

(*f*) *Donor substrate specificity*. In order to test whether the donor sugar specificity of the α-(1→3)-D-galactosyltransferase becomes altogether less rigid at pH 8, UDP-D-[^{14}C]glucose and UDP-*N*-acetyl-D-[^{14}C]glucosamine were also examined for their capacity to act as donor substrates under the conditions given in Table I for the transfer of *N*-acetyl-D-[^{14}C]galactosamine to 2′-fucosyllactose. With untreated group B or O serum as the enzyme source, low levels of radioactivity were found in the tri- and tetra-saccharide areas of the chromatograms when the reaction mixtures contained UDP-*N*-acetyl-D-[^{14}C]glucosamine. However, since

serum contains a β-(1→3)-*N*-acetyl-D-glucosaminyltransferase known to utilise lactose as a substrate[26], it was suspected that the product arose from the transfer of *N*-acetylglucosamine to endogenous lactose present in the samples. With preparations of α-(1→3)-D-galactosyltransferase from group B serum bound to red cell ghosts as the enzyme source, neither UDP-D-[^{14}C]glucose nor UDP-*N*-acetyl-D-[^{14}C]glucosamine functioned as donor substrates.

(*g*) *Apparent* K_m *and* V_{max} *values for donor substrates.* Substrate concentration curves were set up with varying concentrations of either UDP-D-galactose or UDP-*N*-acetyl-D-galactosamine as the donor substrates, 2′-fucosyllactose as the acceptor substrate, and untreated group B serum as the enzyme source (Fig. 3). For the purposes of comparison, all these experiments were carried out at pH 8.0. At this pH, the V_{max} value for the transfer of D-galactose by the *B* transferase was approximately the same as the V_{max} value for the transfer of *N*-acetyl-D-galactosamine, but the apparent K_m value for UDP-D-galactose was 11μM, whereas the value for UDP-*N*-acetyl-D-galactosamine was 285μM. Under the same conditions, the A^1 gene-associated transferase had an apparent K_m of 14μM and the A^2 gene-associated transferase had an apparent K_m of 35μM (data not shown).

Relationship between the N-*acetyl-*D-*galactosaminyl-transferring activity in group B serum and the* B *gene-associated* α-*(1→3)-*D-*galactosyltransferase.* — The

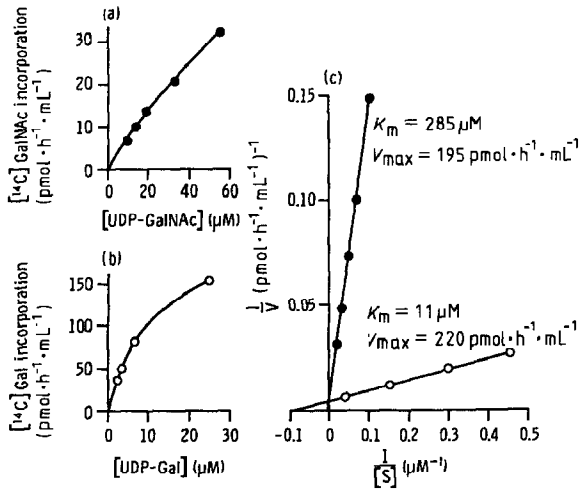

Fig. 3. The apparent K_m and V_{max} values for the transfer of D-galactose from UDP-D-galactose, and *N*-acetyl-D-galactosamine from UDP-*N*-acetyl-D-galactosamine, by enzyme(s) in group B serum. (a) Substrate concentration curve for the transfer of *N*-acetyl-D-[^{14}C]galactosamine; (b) substrate concentration curve for the transfer of D-[^{14}C]galactose; and (c) Lineweaver–Burk plots derived from (a) and (b). The reaction mixtures contained: UDP-*N*-acetyl-D-[^{14}C]galactosamine at a range of concentrations from 9.7 to 55.2μM (each containing 125 000 c.p.m.) or UDP-D-[^{14}C]galactose at a range of concentrations from 2.2 to 24.9μM (each containing 125 000 c.p.m.), $MnCl_2$ (2 μmol); ATP (0.5 μmol), 2′-fucosyllactose (0.25 μmol), NaN_3 0.8 μmol, Tris · HCl buffer (pH 8.0, 5 μmol), and group B serum (20 μL). The total volume of the reaction mixtures was 110 μL. The mixtures containing UDP-*N*-acetyl-D-[^{14}C]galactosamine were incubated for 64 h at 37° and those containing UDP-D-[^{14}C]galactose were incubated for 16 h at 37°.

preceding experiments have confirmed the presence, in unfractionated blood group B serum and in partially purified preparations of the α-(1→3)-D-galactosyltransferase, of an enzyme activity that mimics the blood group *A* gene-associated transferase in its capacity to catalyse the addition of *N*-acetyl-D-galactosamine to acceptor substrates containing terminal H-active structures. The following experiments were designed to test whether this activity is indeed a function of the α-(1→3)-D-galactosyltransferase or whether it arises from the ubiquitous presence in the serum of group B individuals of a small amount of an independent α-(1→3)-*N*-acetyl-D-galactosaminyltransferase.

(*a*) *Correlation between the capacity to transfer* D-*galactose and* N-*acetyl*-D-*galactosamine.* The α-(1→3)-D-galactosyltransferase in 48 different samples of group B serum were concentrated by adsorption onto red cell ghosts and tested for their capacity to transfer D-[^{14}C]galactose or *N*-acetyl-D-[^{14}C]galactosamine to the acceptor substrate 2′-fucosyllactose. All the enzyme preparations catalysed the addition of small amounts of *N*-acetyl-D-[^{14}C]galactosamine and there was an obvious correlation between the activities measured with the two donor substrates (Fig. 4). Under the conditions of pH and incubation time used for these experiments, the mean value for the ratio of D-galactose to *N*-acetyl-D-galactosamine transferred to 2′-fucosyllactose was 70:1.

(*b*) *Group O red cell ghosts treated with group O serum in the presence of UDP-*D-*galactose and tested for the transfer of* N-*acetyl*-D-*galactosamine.* In order to

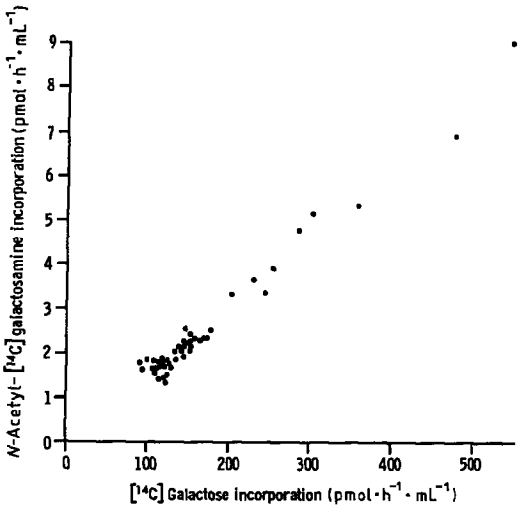

Fig. 4. Relationship between the transfer of D-galactose and *N*-acetyl-D-galactosamine by the α-(1→3)-D-galactosyltransferase preparation from group B serum. Each reaction mixture contained group O red cell ghosts to which the enzyme from 0.5 mL of group B serum had been bound as described. The remainder of the reaction mixtures were as described in the legend to Fig. 3, except the same amount of nucleotide sugar (1.25 nmol; 125 000 c.p.m.) was used for all experiments and the buffer in the mixtures containing UDP-D-[^{14}C]galactose was sodium cacodylate (pH 6.5). The total volume of the reaction mixtures was 100 μL. Mixtures containing UDP-*N*-acetyl-D-[^{14}C]galactosamine were incubated for 64 h at 37° and those containing UDP-D-[^{14}C]galactose were incubated for 3 h at 37°.

confirm that the capacity to transfer small amounts of *N*-acetyl-D-galactosamine to H acceptor molecules is not a property of all human serum samples irrespective of ABO blood group, a further 48 serum samples from blood group O donors were treated with group O red cell ghosts by the method used for the adsorption of the α-(1→3)-D-galactosyltransferase from the group B serum samples. No incorporation of either D-[^{14}C]galactose or *N*-acetyl-D-[^{14}C]galactosamine into 2′-fucosyllactose was detectable when these red cell ghost preparations were tested with UDP-D-[^{14}C]galactose or UDP-*N*-acetyl-D-[^{14}C]galactosamine, and the appropriate additives, under conditions identical with those employed to measure the corresponding activities of the ghost-bound α-(1→3)-D-galactosyltransferase preparations.

(*c*) *Binding of α-(1→3)-N-acetyl-D-galactosaminyltransferases in the serum of blood group A_1 and A_2 individuals to group O red cell ghosts.* Concentrated preparations of the α-(1→3)-*N*-acetyl-D-galactosaminyltransferases from the serum of group A_1 individuals can be made by binding the enzyme to group O red cell ghosts in the presence of UDP-*N*-acetyl-D-galactosamine and Mn^{2+} ions[11]. However, when group A_1 and A_2 serum samples were treated with group O red cell ghosts in the presence of $MnCl_2$ and UDP-D-galactose, under the conditions described in the Experimental section for binding the α-(1→3)-D-galactosyltransferase in group B serum, there was virtually no adsorption of the α-(1→3)-*N*-acetylgalactosaminyltransferase from either sample (Table III). The same treatment applied to group B serum consistently removed 75–80% of both the α-

TABLE III

BINDING OF BLOOD GROUP *A* GENE-ASSOCIATED α-(1→3)-*N*-ACETYL-D-GALACTOSAMINYLTRANSFERASE TO GROUP O RED CELL GHOSTS IN THE PRESENCE OF UDP-D-GALACTOSE[a]

Enzyme source	*Incorporation of ^{14}C-sugar*					
	α-(1→3)-GalNAc transferase			*α-(1→3)-Gal transferase*		
	Before absorption (c.p.m.)	*After absorption (c.p.m.)*	*Percentage bound*	*Before absorption (c.p.m.)*	*After absorption (c.p.m.)*	*Percentage bound*
Group A_1 serum	22 905	22 138	3			
Group A_2 serum	16 801	16 850	0			
Group B serum	624	150	76	15 422	3478	77

[a]Group A_1, A_2, or B serum samples were treated with group O red cell ghosts under the conditions described in the Experimental section for the binding of the *B* gene-associated α-(1→3)-D-galactosyltransferase. Assays for α-(1→3)-*N*-acetyl-D-galactosaminyltransferase and α-(1→3)-D-galactosyltransferase with 2′-fucosyllactose as the acceptor molecule were carried out on the serum before addition of the red cell ghosts and after their removal. The percentage of binding of the enzyme activities to the red cell membranes was calculated from the difference in the two results. Assays for α-(1→3)-*N*-acetyl-D-galactosaminyltransferase were performed at pH 6.0 for the A_1 enzyme, and at pH 8.0 for the A_2 and B enzymes. Assays for α-(1→3)-D-galactosyltransferase were performed at pH 6.5. Otherwise the reaction mixtures were as in Table I.

(1→3)-D-galactosyltransferase activity and the α-(1→3)-*N*-acetyl-D-galactosaminyltransferase activity.

(*d*) *α-(1→3)-D-Galactosyltransferase subjected to two cycles of binding to group O red cell ghosts.* The preceding experiment demonstrated that the α-*N*-acetyl-D-galactosaminyltransferases in normal group A serum samples fail to bind to group O red cell ghosts under the conditions used to bind the α-(1→3)-D-galactosyltransferase from B serum. Hence, if the capacity to transfer *N*-acetyl-D-galactosamine is attributable to a minor α-(1→3)-*N*-acetyl-D-galactosaminyltransferase component in the B serum having the properties of a normal *A* gene-associated transferase, the ratio of activities with UDP-D-galactose and UDP-*N*-acetyl-D-galactosamine would be expected to change in successive cycles of binding to the membranes. Group B serum (30 mL) was treated with group O red cell ghosts (22.5 mg) as described and the washed recovered ghosts were resuspended in 0.15M NaCl (1.2 mL). An aliquot of the suspension (0.4 mL) was removed for subsequent testing and the α-(1→3)-D-galactosyltransferase was eluted from the ghosts in the remaining 0.8 mL of suspension by treatment with 50mM 2′-fucosyllactose (0.1 mL) and 1% bovine serum albumin (0.1 mL) as described. The eluate was dialysed against 0.15M NaCl to remove 2′-fucosyllactose, the volume was then adjusted to 10 mL with 0.15M NaCl, and the enzyme was rebound to fresh group O red cell ghosts with the quantities of all the additives reduced by two-thirds. The washed ghosts were resuspended in a final volume of 0.5 mL of 0.15M NaCl, and the suspensions from the first and second cycles of binding were tested for their capacity to transfer D-[^{14}C]galactose and *N*-acetyl-D-[^{14}C]galactosamine to 2′-fucosyllactose. Although some enzyme activity was lost in this procedure, the ratio of the activities with the two donor substrates was the same for the preparation bound twice to the red cell membranes as it was in the preparation bound once to the membranes (Table IV). The *N*-acetyl-D-galactosamine-transferring ability is thus bound and rebound to the ghosts in parallel with the D-galactose transferring ability.

TABLE IV

RATIO OF D-[^{14}C]GALACTOSE TO *N*-ACETYL-D-[^{14}C]GALACTOSAMINE INCORPORATED INTO 2′-FUCOSYLLACTOSE BY α-(1→3)-D-GALACTOSYLTRANSFERASE SUBJECTED TO TWO CYCLES OF BINDING TO GROUP O RED CELL GHOSTS[a]

Enzyme source	*Cycles of binding*	[^{14}C]*Gal incorporation*		[^{14}C]*GalNAc incorporation*		*Ratio* [^{14}C]*Gal:* [^{14}C]*GalNAc*
		c.p.m.	*pMol · h^{-1} · mL^{-1}*	*c.p.m.*	*pMol · h^{-1} · mL^{-1}*	
B Transferase bound to red cell ghosts	First	16 395	109.3	5288	1.65	66
	Second	8357	27.9	2617	0.41	68

[a]The reaction mixtures were the same as in Fig. 4. Those containing UDP-D-[^{14}C]-galactose were incubated for 3 h at 37° and those containing UDP-*N*-acetyl-D-[^{14}C]-galactosamine for 64 h at 37°.

(*e*) *Binding experiments with Sepharose 4B.* The *A* gene-associated α-(1→3)-*N*-acetyl-D-galactosaminyltransferase adsorbs to agarose gel beads from which it can be eluted[27,28] with UDP. The basis of this affinity binding has yet to be established but other glycosyltransferases, including the *B* gene-associated α-(1→3)-D-galactosyltransferase, fail to bind to this adsorbant[27]. In order to test whether an independent α-(1→3)-*N*-acetyl-D-galactosaminyltransferase coexists with the α-(1→3)-D-galactosyltransferase in group B serum, attempts were made to remove the *A* transferase activity by adsorption onto the agarose preparation Sepharose 4B. The supernatant serum remaining after treatment with Sepharose 4B retained the capacity to transfer both D-[^{14}C]galactose and *N*-acetyl-D-[^{14}C]galactosamine to 2′-fucosyllactose and the ratio of the two activities was the same as in the untreated serum (Table V). A slight drop in both D-[^{14}C]galactose- and *N*-acetyl-D-[^{14}C]-galactosamine-transferring activity was observed in the supernatant serum, but no activity was detectable in the eluate obtained by treating the Sepharose 4B with 20μM UDP. A blood group A_1 serum, diluted 1:50 and treated in the same way, lost 80% of the *A* transferase activity from the supernatant serum, and elution of the Sepharose 4B with 20μM UDP gave a highly active eluate (Table V).

The same serum samples were also tested for their capacity to convert group O into A-active cells before and after treatment with Sepharose 4B. The enzyme in 1 mL of the group B serum, and 1 mL of the supernatant remaining after removal of the Sepharose 4B, were concentrated by binding to red cell ghosts as described. The concentrated enzyme was incubated with group O red cells and UDP-*N*-acetyl-D-galactosamine under the conditions given in the Experimental section. After incubation, the washed red cells were tested for agglutination with rabbit immune anti-A serum. Irrespective of whether the enzyme source was untreated group B serum or the supernatant serum remaining after treatment with Sepharose 4B, haemagglutination titres of 512 and 8, respectively, were obtained with rabbit and monoclonal anti-A reagents. Group O red cells similarly incubated with 100 μL of the A_1 serum (diluted 1:50) and UDP-*N*-acetyl-D-galactosamine became agglutinable to a titre of 256 with rabbit anti-A serum, but the supernatant remaining after adsorption of the diluted A_1 serum with Sepharose 4B lost the capacity to give detectable conversion of O to A-active cells. Therefore, adsorption with an amount of Sepharose 4B that had depleted the α-(1→3)-*N*-acetyl-D-galactosaminyltransferase activity of the group A_1 serum had not appreciably altered the capacity of the enzyme in group B serum to convert H into A-active sites on red cells.

(*f*) *Effect of heating on the* N-*acetyl*-D-*galactosamine-transferring activity of group B serum.* The α-(1→3)-D-galactosyltransferase activity in unfractionated group B serum is more resistant to heat than the α-(1→3)-*N*-acetyl-D-galactosaminyltransferase in group A_1 and A_2 serum samples[29]. In a further attempt to ascertain whether the *N*-acetyl-D-galactosaminyltransferase activity of group B serum is attributable to the presence of an independent *A* transferase, serum samples from group A_1, A_2, and B individuals were heated at 60° for 1 and 2 h. The A_1 and A_2 serum samples were diluted with group O serum to give a level of

TABLE V

ATTEMPTED BINDING OF TRANSFERASES IN GROUP A_1 AND B SERUM SAMPLES TO SEPHAROSE 4B[a]

Enzyme source	*[^{14}C]Gal incorporation*		*[^{14}C]GalNAc incorporation*		*Ratio [^{14}C]Gal:[^{14}C]GalNAc*
	c.p.m.	*pMol · h^{-1} · mL^{-1}*	*c.p.m.*	*pMol · h^{-1} · mL^{-1}*	
Group B serum					
(1) Untreated serum	23 000	755	1013	8	85
(2) Serum after treatment with Sepharose 4B	21 000	655	828	7	86
(3) Eluate from Sepharose 4B	0	0	0	0	
Group A_1 serum diluted 1:50					
(1) Untreated serum			2849	22	
(2) Serum after treatment with Sepharose 4B			553	4	
(3) Eluate from Sepharose 4B			3305	165	

[a]One volume of group A_1 serum was diluted with 49 volumes of group O serum. The diluted A_1 serum (200 mL) and untreated group B serum (200 mL) were each mixed with Sepharose 4B (2 mL) and kept for 1 h at 4°. The mixtures were then poured into columns (0.7-cm diameter), the residual serum was collected, and the Sepharose 4B was washed with 0.1M sodium cacodylate buffer (pH 6.5) containing mM EDTA and 2mM $MnCl_2$ (3 mL). The enzyme was eluted with 10 mL of the same buffer containing 20μM UDP. Aliquots of the starting serum (20 μL), the supernatant after removal of the Sepharose 4B (20 μL), and the eluate (100 μL) were assayed at pH 6.0 for the α-(1→3)-*N*-acetyl-D-galactosaminyltransferase activity of the A_1 serum, at pH 8.0 for the α-(1→3)-*N*-acetyl-D-galactosaminyltransferase activity of the B serum, and at pH 6.5 for the α-(1→3)-D-galactosyltransferase activity of the B serum. Otherwise, the reaction mixtures were as in Fig. 4. The mixtures containing UDP-*N*-acetyl-D-[^{14}C]galactosamine were incubated for 64 h at 37°, except those containing the eluted A_1 transferase which were incubated for 2 h at 37°. The mixtures containing UDP-D-[^{14}C]galactose were incubated for 16 h at 37°.

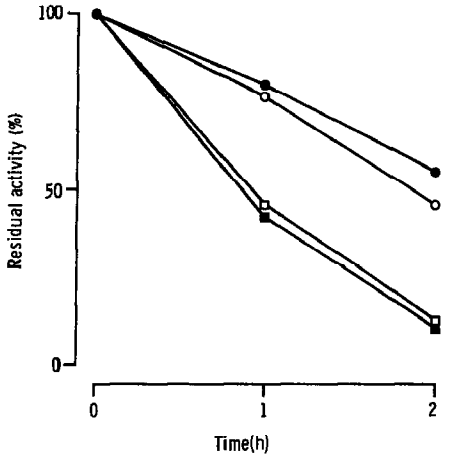

Fig. 5. The effect of heating on the α-(1→3)-*N*-acetyl-D-galactosaminyltransferase activity of group B and group A serum samples. One volume of group A_1 and A_2 serum samples were each diluted with 30 volumes of group O serum. Aliquots (1 mL) of diluted A_1 and A_2 serum and undiluted B serum were heated in stoppered tubes for periods of 1 and 2 h at 60°. At the end of the heating period, the tubes were cooled in ice and 20-μL samples were assayed for α-D-galactosyl and α-*N*-acetyl-D-galactosaminyltransferase activity as described in the legend to Fig. 4, except that mixtures containing UDP-D-[^{14}C]galactose were incubated for 16 h at 37°: (—●—●—) α-*N*-acetyl-D-galactosaminyltransferase activity of B serum; (—○—○—) α-D-galactosyltransferase activity of B serum; (—□—□—) α-*N*-acetyl-D-galactosaminyltransferase activity of A_1 serum; and (—■—■—) α-*N*-acetyl-D-galactosaminyltransferase activity of A_2 serum.

transfer of *N*-acetyl-D-galactosamine to 2'-fucosyllactose roughly comparable with that expected at pH 8 for the transfer of this sugar by the enzyme in group B serum. After heating for 1 h at 60°, the α-(1→3)-*N*-acetyl-D-galactosaminyltransferase activities in both the diluted A_1 and A_2 serum samples were reduced by more than 50%, and after 2 h only 12% remained (Fig. 5). The α-(1→3)-*N*-acetyl-D-galactosaminyltransferase activity in the B serum samples was more resistant to heat and 50% of the original activity was retained after 2 h at 60°. The reduction in the capacity of the group B serum to transfer *N*-acetyl-D-galactosamine fell in parallel with the drop in α-(1→3)-D-galactosyltransferase activity.

DISCUSSION

Under normal physiological conditions *in vivo* there is no apparent overlap in the functions of the blood group *A* and *B* genes. However, the observation that serum from group B individuals had the capacity to transfer small amounts of *N*-acetyl-D-galactosamine from UDP-*N*-acetyl-D-galactosamine to *H*-active acceptor molecules, whereas group O sera tested under the same conditions fail to exhibit this activity[8], led us to suspect that the *B* gene-associated transferase had the potential to synthesise blood group A-active structures. In the present investigation, a further 48 group B serum samples were found to have this property (Fig. 4),

and an equal number of group O samples, when tested under identical conditions, again failed to show any α-(1→3)-*N*-acetyl-D-galactosaminyltransferase activity. Thus, serum samples from nearly 100 individuals carrying the blood group *B* gene have now been shown to have the innate capacity to make blood group A structures, and no sample from individuals with the B phenotype has lacked this function.

The acceptor-substrate specificity of the enzyme in B serum catalysing the transfer of *N*-acetyl-D-galactosamine from UDP-*N*-acetyl-D-galactosamine to both oligosaccharide and macromolecular acceptor substrates parallels the acceptor specificity of the α-(1→3)-D-galactosyltransferase in the same serum (Tables I and II), and the concentration of manganese ions required for activation of *N*-acetyl-D-galactosaminyl transfer similarly parallels the requirement for activation of D-galactosyl transfer. However, the optimum pH values for the two activities differ; transfer of D-galactose is optimal at pH 6.5 (ref. 20) whereas the transfer of *N*-acetyl-D-galactosamine is optimal at pH 8.0 (Fig. 2). Thus, if the same enzyme is catalysing the transfer of both D-galactose and *N*-acetyl-D-galactosamine, some change in the conformation of the enzyme molecule may be required to accommodate the bulkier UDP-*N*-acetyl-D-galactosamine substrate in the active site or to effect the catalytic transfer of the sugar. The apparent K_m value for UDP-*N*-acetyl-D-galactosamine found for the enzyme in B serum is approximately 25 times greater than the apparent K_m value for UDP-D-galactose, but the V_{max} for the transfer of the two sugars is approximately the same (Fig. 3). Hence, although the affinity for UDP-*N*-acetyl-D-galactosamine is much lower, once the donor substrate has bound to the active site of the enzyme, the catalytic transfer of either sugar appears to proceed at the same rate. Neither UDP-D-glucose nor UDP-*N*-acetyl-D-glucosamine function as donor substrates under the conditions in which UDP-*N*-acetyl-D-galactosamine can be utilised; results indicating that a change in the configuration at C-4 in the hexose ring is incompatible with enzyme activity.

Blood group A has two major subgroups, A_1 and A_2 (ref. 3); the enzyme specified by the A^1 gene is characterised by a pH optimum of 6 whereas that specified by the A^2 gene has a broader pH optimum from 7.5 to 8 (ref. 30). Thus, in this respect, the enzyme in group B serum catalysing the transfer of *N*-acetyl-D-galactosamine resembles the A^2 gene-associated enzyme although its optimum is sharper than that normally observed for A^2 transferases. The transferase in untreated group A_2 serum samples brings about very little, if any, conversion of blood group O into A-active cells[31,32], and even purified, concentrated A_2 enzyme preparations are far less efficient at conversion than A_1 enzymes[33]. In contrast, the very weak A-like enzyme activity in B serum was relatively easy to detect by its ability to convert O into A-active cells[10,34]. Moreover, the enzyme in group B serum has an apparent K_m for UDP-*N*-acetyl-D-galactosamine (285 μM) which is considerably higher than that found for either the A^2 (K_m 35 μM) or A^1 (K_m 14 μM) gene-associated transferases when tested under the same experimental conditions. If, therefore, the capacity of group B serum samples to utilise UDP-*N*-acetyl-D-

galactosamine as a substrate is attributable to the presence of an α-(1→3)-*N*-acetyl-D-galactosaminyltransferase which is independent of the *B* gene-associated α-(1→3)-D-galactosyltransferase, this separate enzyme does not closely resemble normal A^1 or A^2 gene-associated glycosyltransferases.

Two measures that would normally be taken to determine whether one or two enzymes are involved in certain reactions are (a) competition experiments with the two substrates and (b) purification to homogeneity of the enzyme, or enzymes, catalysing the reactions. Competition experiments are complicated in the case of the *B* gene-associated α-(1→3)-D-galactosyltransferase by the fact that the putative, alternative donor substrate, UDP-*N*-acetyl-D-galactosamine, is known to be a weak competitive inhibitor of the transfer of D-galactose from UDP-D-galactose by this enzyme[20]. Some decrease in the amount of D-galactose transferred in the presence of UDP-*N*-acetyl-D-galactosamine would therefore result from the action of the nucleotide sugar as a competitive inhibitor of the α-(1→3)-D-galactosyltransferase, and a separate α-(1→3)-*N*-acetyl-D-galactosaminyltransferase could still be the cause of the transfer of *N*-acetyl-D-galactosamine to H acceptor substrates. The logical step of purifying the α-(1→3)-D-galactosyltransferase to homogeneity and then testing the pure enzyme for its capacity to transfer *N*-acetyl-D-galactosamine has not yet proved possible because of the low abundance of the transferase in human tissue fluids and the difficulty of obtaining highly purified preparations with sufficient amounts of protein to establish homogeneity. The capacity to transfer *N*-acetyl-D-galactosamine was retained in an α-(1→3)-D-galactosyltransferase preparation purified 100 000-fold[8]; although this preparation was not homogenous, it had been made by affinity chromatography on group O red cell membranes and the experiments described in this paper demonstrate that the *A* transferases in group A_1 or A_2 serum samples do not bind to these membranes under the conditions used to adsorb the *B* gene-associated transferase (Table III). The observation that the ratio of D-galactose to *N*-acetyl-D-galactosamine transferred by an enzyme preparation from group B serum remained constant when the enzyme was bound, eluted, and rebound to fresh red cell membranes (Table V) and the close correlations between levels of activity with UDP-D-galactose and UDP-*N*-acetyl-D-galactosamine (Fig. 4) supports the idea of one enzyme with a dual function. The failure to remove the weak α-(1→3)-*N*-acetyl-D-galactosaminyl-transferase activity from group B serum by treatment with Sepharose 4B, under conditions which bind the *A* transferases in A_1 or A_2 serum samples[27] and weaker subgroups of A (ref. 35), is also consistent with the interpretation that this activity is a property of the *B* transferase and does not result from the presence in the serum of a low level of an independent *A* transferase. Further, the loss of the capacity to utilise UDP-*N*-acetyl-D-galactosamine as a substrate on heat treatment of B serum closely corresponds to the loss of α-D-galactosyl-transferring activity, and these two functions are more resistant to heat than are the *A* gene-associated transferases in either A_1 or A_2 serum samples (Fig. 5).

Incontrovertible proof that the α-(1→3)-D-galactosyltransferase can utilise

UDP-*N*-acetyl-D-galactosamine as a donor substrate must probably await purification of the enzyme to homogeneity, or expression of the glycosyltransferase product of the cloned blood group *B* gene. Nevertheless, the weight of evidence strongly indicates that the *B* transferase has the potential to synthesise blood group A-active structures. From an enzymological point of view, this is not surprising since other glycosyltransferases have been reported to utilise more than one donor substrate. Bovine β-(1→4)-D-galactosyltransferase elicits disaccharide biosynthesis with *N*-acetyl-D-glucosamine as acceptor and UDP-D-glucose as the donor sugar in place of UDP-D-galactose, although the relative rate is only 0.3% of that for *N*-acetyllactosamine biosynthesis[36]. Similarly, although UDP-D-glucose is the normal donor substrate for glycogen synthetase, this enzyme is able to substitute UDP-D-glucosamine and to incorporate the amino sugar into glycogen[37]. The recognition by other enzymes of both D-galactose and *N*-acetyl-D-galactosamine as substrate molecules, or components of substrates, is also well known. The exoglycosidase of human liver previously designated "α-D-galactosidase B" is now considered to be primarily an α-*N*-acetyl-D-galactosaminidase[38], and both D-galactose and *N*-acetyl-D-galactosamine are substrates for the enzyme galactose oxidase isolated from *Dactylium dendroides*[39]. However, because individuals who inherit blood group *B* genes in the absence of blood group *A* genes normally show no trace of blood group A antigen, and because blood groups A and B are serologically and genetically so distinct, it is conceptually more difficult to accept that the product of the *B* gene can biosynthesise, albeit to only a very limited extent, A antigenic determinants. Under normal physiological conditions, it must be assumed that the weak capacity of the *B* transferase to synthesise A-active structures is either not manifested or that the structures are immediately neutralised by the antibodies directed towards A determinants which are always present in the blood of group B individuals[3]. The potential of the *B* transferase to make A determinants may, however, have relevance to the atypical expression of blood group A activity which has been reported to occur in certain malignant tumours in group B individuals[40–43]. Such expression challenges the conventional view of the inheritance of ABO groups and led Boettcher[4] to suggest that *A* and *B* genes function, not as specifiers of the glycosyltransferases, but as repressors of ubiquitous genes which code for the transferases and are derepressed in the malignant cells. The work described herein does not disprove this hypothesis but offers a possible alternative explanation for the appearance of blood group activity apparently incompatible with the individual's ABO phenotype.

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