Porcine A Blood Group-specific
N-Acetylgalactosaminyltransferase

II. ENZYMATIC PROPERTIES

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Porcine A blood group-specific N-acetylgalactosaminyltransferase required either Mn**+, Cd**, or Zn** for activity and 2'-O-α-fucosylgalactosides as acceptor substrates. The presence of detergent stabilizes the enzyme but is not essential for catalysis. To obtain information about the kinetic mechanism of the transferase reaction, initial rate parameters have been determined using 2'-fucosyllactose or A-mucin as acceptors, and Mn**, Cd**, or Zn** as cosubstrates. 2'-Fucosyllactose is a competitive inhibitor with respect to A-mucin and a non-competitive inhibitor with respect to UDP-N-acetylgalactosamine. UDP inhibits non-competitively with respect to acceptor; thus UDP-N-acetylgalactosamine or acceptor can bind to the transferase via an equilibrium random pathway.

The transferase converts human O blood type erythrocytes of A blood type. After exhaustive glycosylation, 3 x 10^6 N-acetylgalactosaminyl residues were incorporated per cell. Gel electrophoretic analysis of the labeled erythrocyte membranes indicates that glycoproteins with apparent molecular weights from 30,000 to 100,000 have been glycosylated; glycolipids account for only 15% of the labeled material, although pure H-glycolipid is a good acceptor. The transferase, with its strict acceptor specificity, can thus be used as a tool to study the biosynthesis and function of glycolipids and glycoproteins.

EXPERIMENTAL PROCEDURES

Materials

The following materials were obtained as gifts: H-glycolipid,1 A-

1 The trivial names used are: H-glycolipid (type 2), Gal(2 → 1αFuc)β1 → 4GlcNAcβ1 → 3Galβ1 → 4GlcCer; 2'-fucosyllactose, galactosyltransferase (EC 2.4.1.38) from porcine submaxillary glands was described. The enzymatic properties of the transferase are reported here, including kinetic studies that point to similarities between the mechanism of the transferase and that of galactosyltransferase (EC 2.4.1.38) from bovine milk (2). In addition, the pure transferase has been found to efficiently convert human O blood type erythrocytes to type A.

Methods

METHODS

In the preceding paper (1), the purification of fucosylgalactose acetylgalactosaminyltransferase (EC 2.4.1.40) from porcine submaxillary glands was described. The enzymatic properties of the transferase are reported here, including kinetic studies that point to similarities between the mechanism of the transferase and that of galactosyltransferase (EC 2.4.1.38) from bovine milk (2). In addition, the pure transferase has been found to efficiently convert human O blood type erythrocytes to type A.

METHODS

Methods 1 and 2 have been described in the preceding paper (1). Method 3—The incubation mixture (50 μ1) contained the same components as given in Method 1, except that 0.15 μmol of 2'-fucosyllactose was substituted for asialo-PSM A**. After incubation at 37° for 10 min, the reaction mixture was diluted with 1 ml of water that studies the biosynthesis and function of glycolipids and glycoproteins.

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Methods 2—The incubation mixture (50 μl) contained the same components as given in Method 1, except that 0.15 μmol of 2'-fucosyllactose was substituted for asialo-PSM A**. After incubation at 37° for 10 min, the reaction mixture was diluted with 1 ml of water that studies the biosynthesis and function of glycolipids and glycoproteins.

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Determination of Parameters

Preparation of amount of erythrocyte membranes). Transferase, and erythrocytes as a 67% suspension (or an equivalent tained 6 nmol of UDP-GalNAc (88,000 cpm/nmol), 5 milliunits of transferase as outlined above, except that the incubation mixture con- the erythrocytes prior to disruption, were incubated with the trans- by centrifugation in a Sorvall HB-4 rotor for 30 min at 11,000 rpm, trolyl)lbenzene, and 6 g of benzoic acid in 1 liter of toluene) were then room temperature in 0.5 ml of Protosol (New England Nu- slice was incubated in a plastic vial for 24 h at 37" and then 24 h at room temperature. gels were frozen after electrophoresis and cut into 2-mm slices. Each 

Use of Glycolipids as Acceptors

Reaction mixtures were as given in Method 1, except that asialo-PSM A − was replaced by glycolipids, which were first dissolved in chloroform:methanol (2:1, v/v), mixed with the detergent, and dried at room temperature under a stream of N2 before adding the other components. In the experiments described here, transferase of GalNAc was determined by Method 1 since the labeled glycolipids appeared in the void volume of Sephadex G-50. Similar results were obtained with Method 3 or electrophoresis on Whatman No. 3MM paper in 1% tetraborate.

Hemaggutination Inhibition

These tests were carried out in phosphate-buffered saline (0.073 m NaCl, 0.018 m KH2PO4, 0.057 m Na2HPO4, pH 7.2). Appropriately diluted antisera (100 μl) were added to serial dilutions (100 μl: dilution factor = 1/2) of the mucin to be tested. After allowing the mixture to stand for 45 min at room temperature, 100 μl of a 1% suspension of the erythrocytes were added. The degree of agglutination was read after allowing the erythrocytes to settle for 2 h at room temperature.

Glycosylation of Erythrocytes

The incubation mixture (100 μl) contained 13 μmol of NaCl, 1 μmol of imidazole-HCl, pH 7.0, 1 μmol of MnCl2, 0.1 mg of bovine serum albumin, 10 nmol of UDP-[1-14C]GalNAc (4000 cpm/nmol), 4 milliunits of UDP-GalNAc:Gal transferase (in control tubes inacti- vated 4 min at 100°), and erythrocytes (blood type O or A) as a 50% suspension. After incubation at 37° for 1 h, the cells were washed three times with 5 ml 0.15 m NaCl and used for hemaggutination tests. To determine the extent of [1-14C]GalNAc transfer, cells, were counted using an internal standard to correct for quenching.

To determine the blood type of the erythrocytes a 1% suspension of the erythrocytes was treated (100 μl) and 100 μl of 0.15 m NaCl were added to serial dilutions (100 μl: dilution factor = 1/2) of antiserum. The degree of agglutination was read after allowing the erythrocytes to settle for 2 h at room temperature.

Preparation of [1-14C]GalNAc-labeled Erythrocyte Membranes

One volume of packed erythrocytes was mixed rapidly with 40 volumes of cold 10 m Tris/HCl, pH 7.5, 10 m sodium dodecyl sulfate, and 100 m sucrose (v/v). The mixture was centrifuged at 1000 g for 10 min. The supernatant was aspirated, the membranes were washed with 10 volumes of Tris buffer, and then resuspended in 1 volume of Tris buffer. These membranes, or the erythrocytes prior to disruption, were incubated with the trans- ferase as outlined above, except that the incubation mixture con- tained 6 nmol of UDP-GalNAc (88,000 cpm/nmol), 5 milliunits of transferase, and erythrocytes as a 67% suspension (or an equivalent amount of erythrocyte membranes).

Gel Electrophoresis of Labeled Membranes

Membranes were dissociated in sodium dodecyl sulfate-mercaptoethanol and subjected to polyacrylamide gel electrophoresis as described earlier (1). To determine the distribution of radioactive, gels were frozen after electrophoresis and cut into 3-mm slices. Each slice was incubated in a 5% TCA solution for 4 h at 37° and then 24 h at 4°C. The effluents was determined as in Method 1. Appropriate controls were included to correct for hydrolysis of UDP-GalNAc.

RESULTS

Requirements for Enzymatic Activity of UDP-GalNAc:Gal Transferase

Donor — The results given in Table I show that UDP- GalNAc is the only glycosyl donor used efficiently by the en- zyme. UDP-GlcNAc and UDP-Gal had poor but measurable donor activity; the other nucleotide sugars tested were inac- tive.

Acceptor — The acceptor specificity of the enzyme is given in Table II. The absence of added acceptor, no radioactivity was detected using the gel filtration assay (Method 1); when the ion exchange assay (Method 3) was used, however, a low level of enzyme-catalyzed conversion of UDP-GalNAc into a neutral compound (presumably GalNAc) was discerned. All good ac- ceptors were 2'-O-fucosyl-l-galactosides and either oligosaccharides (e.g. 2'-fucosyllactose), glycoproteins (PSM A −), or glycolipids (H-glycolipid). Substances with blood group A or Leb activity were poor acceptors, although they contain the Fuca1 → 2Gal grouping. Substances lacking Gal(2 → 1αFuc) had negligible acceptor activity.

Enzyme — The transferase was inactivated at 60° with a half-time of about 3 min, and at 65° with a half-time of less than a min (Fig. 1). The activity for hydrolysis of UDP-GalNAc was inactivated in parallel with loss of transferase activity, sug- gesting that the transferase rather than a small amount of contaminating enzyme hydrolyzes UDP-GalNAc. In standard assays (Method 1), GalNAc transfer to asialo-PSM A − was proportional to the amount of enzyme present in the assay mixture in the range of 0 to 50 microunits (not shown).

Incubation Time — The amount of GalNAc incorporated into asialo-PSM A − increased linearly with time for at least 4 h (Fig. 2). In this experiment, as well as in all kinetic experiments to follow, conditions were chosen that consumed less than 10% of the UDP-GalNAc, since after more than 10%...
transfer, rates decreased significantly due to product inhibition by UDP. Prolonged incubation of the transferase under standard assay conditions, however, in the absence of substrates, led to a slow decrease in enzyme activity with a half-time of about 4 days (data not shown). Thus the enzyme could be expected to support a reaction for a considerable length of time if product inhibition is circumvented.

Buffer – As seen in Fig. 3, transferase activity was largely unaffected by the type and pH of the buffer employed between pH 5 and 8. Transferase activity decreased below pH 5, and appeared to increase above pH 7.5. In Tris buffer, however, oxidation and precipitation of Mn²⁺ occurred, and use of Mes buffer at pH 6 was therefore considered more suitable for standard assays.

Metallo – No transferase activity was observed when Mn²⁺ was omitted from the standard assay mixture, but several other cations, notably Cd²⁺ and Zn²⁺, supported enzymic activity (Table III). Among salts not listed, LiCl, NaCl, and the hydrochlorides of trimethylamine, spermine, and spermidine could not substitute for MnCl₂. When two cations were used in the same incubation mixture (Table IV), inhibition of transferase activity resulted in most cases. Among the cations tested, Cu²⁺ was the most inhibitory. Only Cd²⁺ and Zn²⁺, used at low concentration, helped to increase transferase activity over the level seen with nonsaturating Mn²⁺ alone.
Porcine A Blood Group N-Acetylgalactosaminyltransferase

Time of heat treatment (min) vs pH of buffer

Fig. 1 (left). Heat inactivation of UDP-GalNAc:Gal transferase. Enzyme (0.5 μg in 40 μl of H2O) solutions were incubated in a water bath at 60°C (Δ, ○) or 65°C (▲, ●). After the time indicated, the solutions were placed in ice and assayed for transferase activity by Method 1 (Δ, ▲; left scale) and by Method 3 in the absence of acceptor, i.e. no 2'-fucosyllactose (○, ●; right scale).

Fig. 2 (center). Time course of transfer of N-acetylgalactosamine. The incubation mixture (200 μl) contained 25 nmol of UDP-[1-14C]GalNAc, 16,600 cpm/nmol, 0.08 mg of asialo-PSM A-, 32 units of UDP-GalNAc:Gal transferase, and the concentrations of other components as given in Method 1. At the times indicated, samples 25 μl were withdrawn, mixed with 5 μl of 0.5 M EDTA, and GalNAc transfer was determined as described in Method 1.

Fig. 3 (right). UDP-GalNAc:Gal transferase activity as a function of pH. Assay conditions are as given in Method 1, except that incubation mixtures contained 10 nmol of UDP-14CGalNAc, 740 cpm/nmol, lacked bovine serum albumin, and were incubated for 30 min. Furthermore, 5 μmol of the following buffers were substituted for the standard buffer, acetate-Na+ (●), Mes-Na+ (▲), imidazole-HCl (●), Tris/HCl (○). The abscissa represents pH of these buffers, measured at 22°C, and at a concentration of 0.1 M, rather than actual pH in the assay mixture.

Table III

Cation requirement of UDP-GalNAc:Gal transferase

Assays were carried out by Method 3, except that MnCl2 was omitted or replaced by ZnSO4, FeSO4, NiSO4, or the chlorides of other metals where appropriate. A background activity of 0.5 pmol/min/mg obtained in the absence of 2'-fucosyllactose, and which was independent of added metal, was subtracted from the measured values. Activities obtained with Mn2+ alone at each of the listed concentrations were used as the basis for calculating relative rate.

| Cation added | Concentration | Transferase activity | Relative rate |
|--------------|---------------|----------------------|---------------|
| None         | mM            | μmol/min/mg          | %             |
| Mn2+         | 0.3           | 12.4                 | 100           |
| Mn2+         | 5             | 30.4                 | 100           |
| Mn2+         | 20            | 33.6                 | 100           |
| Mn2+         | 40            | 33.6                 | 100           |
| Cd2+         | 0.3           | 14.9                 | 115           |
| Cd2+         | 20            | 15.1                 | 45            |
| Zn2+         | 0.1           | 5.0                  | 83            |
| Zn2+         | 0.3           | 8.2                  | 66            |
| Co2+         | 0.3           | 3.5                  | 28            |
| Co2+         | 5             | 11.9                 | 39            |
| Fe2+         | 0.3           | 3.0                  | 30            |
| Fe2+         | 0.3           | 0.3                  | 3             |
| Cu2+         | 0.3           | 0.1                  | 1             |
| Cu2+         | 0.3           | 0.2                  | 2             |
| Mg2+         | 0.3           | 3.1                  | 9             |
| Mg2+         | 40            | 3.1                  | 9             |
| Ca2+         | 0.3           | 0                    | 0             |
| Ca2+         | 5             | 0.5                  | 2             |

the glycosylation product constitutes PSM A+, containing the terminal GalNAc bound in α1 → 3 glycosidic linkage to the penultimate galactose residue.

Glycolipid Acceptors—H-glycolipid from canine intestine could be completely glycosylated by the transferase; all of the reaction product was found in the organic phase after extraction with chloroform:methanol:H2O (65:35:8, v/v/v). Kinetic analysis performed at saturating UDP-GalNAc and Mn2+ concentrations gave a Michaelis constant of 0.8 mM for H-glycolipid and a maximal velocity of 15 pmol of GalNAc/min/mg of enzyme.

Conversion of O-Erythrocytes into A-Erythrocytes—Human O-erythrocytes and, to a lesser extent, A-erythrocytes served as acceptors for the porcine UDP-GalNAc:Gal transferase, as seen in Table VII. From the GalNAc transfer observed and the estimated number (7 x 10^8) of cells per incubation mixture, O-
erythrocytes were calculated to have about 10^6 acceptor sites per cell. When tested for hemagglutination by A-antisem, O-erythrocytes previously incubated with inactivated transferase did not agglutinate. However, O-erythrocytes previously incubated with active transferase agglutinated with the same antiserum titer as authentic A-erythrocytes, indicating that their blood group specificity had indeed been changed from O to A.

TABLE V
Effect of Triton X-100 and bovine serum albumin on UDP GalNAc:Gal transferase activity

| Additions                      | Transferase activity (12 min) µmol/µg | Relative rate | Transferase activity (7 h) µmol/µg | Relative rate |
|--------------------------------|---------------------------------------|---------------|------------------------------------|---------------|
| None                           | 0.103                                 | 1             | 2.57                               | 1             |
| Bovine serum albumin           | 0.107                                 | 1.0           | 6.97                               | 2.3           |
| Triton X-100                   | 0.277                                 | 2.7           | 7.68                               | 3.0           |
| Bovine serum albumin and Triton X-100 | 0.310                                 | 3.0           | 8.38                               | 3.3           |

Fig. 4. Effect of temperature on the UDP-GalNAc:Gal transferase activity. Assays were performed by Method 1, with the following modifications. The temperature of incubation was varied between 0° and 65°. Reactions were started by the addition of an appropriate amount of transferase (between 12 and 1200 microunits) and incubated for a convenient time (between 4 and 200 min) to obtain incorporation between 400 and 2000 cpm at each temperature. As a check on linearity, one assay mixture at each temperature contained 4 times less enzyme than the other and was incubated 4 times longer.

Fig. 5. Stabilization of UDP-GalNAc:Gal transferase by detergent and protein. UDP-GalNAc:Gal transferase (12.6 mg/assay tube) was preincubated at 37° in mixtures containing only part of the components listed under Method 1, namely: Δ, Mes (in 15 µl); S, Mes, MnCl₂, asialo-PSM A⁻ (in 30 µl); O, Mes, MnCl₂, asialo-PSM A⁺, Triton X-100, bovine serum albumin (in 45 µl). After the times indicated, prewarmed mixtures of the missing components were added, and enzyme reactions were allowed to proceed for 1 min under standard assay conditions.
Incubation of A- and O-erythrocytes with UDP-GalNAc:Gal transferase

The experiment was carried out as indicated under "Methods." The change in blood group specificity upon incubation was assessed by determining the lowest titer of A-antiseraum causing detectable agglutination.

| Type of cells before incubation | Transferase | GalNAc transferase activity | A-antiseraum titer | Type of cells after incubation |
|-------------------------------|-------------|----------------------------|--------------------|-------------------------------|
| A Inactivated                 | 0.005       | 1:211                      | A                  |
| O Inactivated                 | 0.01        | >1:22                      | O                  |
| A Active                      | 0.27        | 1:211                      | A                  |
| O Active                      | 1.17        | 1:211                      | A                  |

Fig. 8. Dependence of GalNAc transfer on Mn²⁺ concentration. Assays were carried out by Method 3, except that the Mn²⁺ concentration was varied and two types of buffer were employed. △, Mes-Na⁺, pH 6.0; ○, imidazole-HCl, pH 7.5.

Fig. 9. Dependence of GalNAc transfer on Cd²⁺ concentration. As described in Fig. 8 except that Cd²⁺ was substituted for Mn²⁺.

Kinetic Studies

Metal Dependence of GalNAc Transfer — The dependence of GalNAc transfer on the Mn²⁺ concentration is shown in Fig. 8. At pH 6, the standard pH for most of the experiments described here, 20 mM Mn²⁺ was sufficient to saturate the reaction, whereas 50 mM Mn²⁺ was required at pH 7.5. In the range of 0.2 to 1 mM Mn²⁺, a linear relationship between e/Vₐ and 1/Mn²⁺ was obtained, and apparent Michaelis constants of 0.3 mM at pH 6 and 0.4 mM at pH 7.5 were calculated. However, toward higher Mn²⁺ concentrations, the lines were deflected downward (substrate activation), suggesting that the Mn²⁺ might serve more than one purpose in catalysis. Substituting Cd²⁺ for Mn²⁺, a strikingly different cation dependence was observed (Fig. 9). Above 4 mM Cd²⁺, strong inhibition occurred at pH 6, but not at pH 7.5. The data obtained below 4 mM Cd²⁺ were also strongly affected by pH as seen in the double reciprocal plot. The calculated apparent Michaelis constants were 0.1 mM at pH 6 and 1.2 mM at pH 7.5.

Determination of Initial Rate Parameters — Initial rates of GalNAc transfer were determined at pH 6 and saturating Mn²⁺ using six concentrations of UDP-GalNAc ranging from 5 to 125 μM and six concentrations of asialo-PSM A⁻ ranging from 0.4 to 7.5 mg/ml (180 to 3375 μM with respect to acceptor sites; see Fig. 6). Primary and secondary double reciprocal plots of the data are shown in Figs. 11 and 12, respectively, of the supplementary material, which immediately follows the text and references. A similar experiment was performed using the alternative substrate 2'-fucosyllactose at concentrations ranging from 30 to 750 μM, and the resulting primary and secondary plots are given in Figs. 13 and 14 of the supplementary material. From these plots, initial rate parameters (see "Methods") which are listed in Table VIII were obtained.

Initial rates of GalNAc transfer were also determined at

3 Figs. 11 to 18 are presented in a miniprint format on p. 2355. These data may be procured in the form of 10 pages of full size photocopies, upon request, as JBC Document No. 76M-1392. In ordering, the title, authors, and reference to this paper and the JBC Document number must be specified, as well as the number of copies desired. Order should be addressed to the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014, and must be accompanied by a remittance to the order of the Journal, in the amount of $1.50 per set of photocopies.
Initial rate parameters for UDP-GalNAc:Gal transferase obtained at saturating Mn$^{2+}$

**Rate equation:**

\[
\frac{e}{V_0} = \frac{\Phi_I}{\Phi_{I0}} + \frac{\Phi_A}{\Phi_{A0}} + \frac{\Phi_M}{\Phi_{M0}} + \frac{\Phi_{III}}{\Phi_{III0}}
\]

| Varied substrates | Asialo-PSM A$^-$ | UDP$^-$ | 2'-Fucosyllactose | UDP-GalNAc | Acceptor | UDP-GalNAc | Acceptor |
|------------------|----------------|---------|------------------|------------|----------|------------|----------|
| $\Phi_I$ (min)   |              | 0.632 ± 0.001 | 0.018 ± 0.006 |           |          |            |          |
| $\Phi_{I0}$ (min) |              | 0.29 ± 0.03 | 0.37 ± 0.09 |           |          |            |          |
| $\Phi_A$ (µM·min) | 12.5 ± 0.5 | 7.4 ± 0.4 |           |          |          |            |          |
| $\Phi_{A0}$ (µM·min) | 218 ± 11 | 108 ± 6 |           |          |          |            |          |

**DISCUSSION**

The enzyme described here is a blood group A-specific UDP-GalNAc:Gal transferase as evidenced by the following facts. It occurs only in submaxillary glands of blood group A-positive animals. Potential acceptors must carry the grouping Gal(3→6)GalNAc to be effective substrates. The same acceptor specificity has been established for the partially purified blood group A-specific transferases from human submaxillary glands (11), human milk (14), and human serum (13). The linkage formed by the human milk enzyme has been demonstrated to be Gal(3\(\rightarrow\)1αGalNAc) (12); other A-specific transferases form in all likelihood the same linkage, giving rise to the blood group A determinant.

The transferase is capable of converting blood group A-negative into A-positive structures. As demonstrated by hemagglutination inhibition assays, the transferase caused A-negative mucin to acquire the immunochemical characteristics of A-positive mucin. The fact that authentic A-positive mucin exhibited stronger A activity than the transferase reaction product may be due to the different origin and treatment of the two samples, or incomplete glycosylation, or simply experimental error. The slight A activity of the starting material might be caused by the presence of some contaminating A-positive mucin, or by cross-reaction of A antiserum with GalNAc residues bound to the polypeptide core.

The porcine transferase also converts human 0-erythrocytes into cells that are indistinguishable from A-erythrocytes, as judged by agglutination with A-antiserum. In this respect, the enzyme is similar to A-specific transferases from human gastric mucosa (14) and human serum. After exhaustive glycosylation by the transferase, 3 × 10$^6$ GalNAc molecules are

\[^1\] J. D. Whiting, and B. Kaufman, unpublished.
linked to each cell. This is in fair agreement with the known number (about 10^6) of immunoreactive A-type sites per human erythrocyte (15, 16). The transferase glycosylates a variety of components of the erythrocyte membrane. No attempt has been made to identify the labeled components, but it appears that the label is predominantly associated with glycoproteins, in contrast to the view (17, 18) that glycoproteins carry only a minor portion of the blood group A activity of human erythrocytes. In a recent report (19), glycoproteins isolated from human O-erythrocytes were glycosylated by a partially purified A-specific transferase from human milk (12); the gel electrophoretic pattern was similar to the one obtained here. Isolated glycolipids are good substrates for the transferase (Table II), and glycolipids of the isolated erythrocyte membrane are glycosylated more extensively than those of intact erythrocytes, suggesting that the latter may not be easily accessible to the enzyme.

The N-acetylgalactosaminyltransferase reaction involves three substrates (cation, UDP-GalNAc, and acceptor). It has been shown that many three-substrate mechanisms can be distinguished on the basis of complete initial rate studies (8). Therefore, the kinetic studies presented here were undertaken, and the following conclusions drawn.

The transferase reaction does not proceed by an enzyme-substituted (ping-pong) mechanism since the binary initial rate parameters, $\Phi_m$ and $\Phi_4$, are clearly present in the rate equation (Tables VIII and IX), whereas Dalziel has shown (8) that all enzyme-substituted mechanisms lack at least two of the three binary $\Phi$ parameters. Thus the transferase reaction involves a quaternary enzyme complex.

Five types of quaternary complex mechanisms may be considered: random addition of all three substrates (a), partly random, partly compulsory addition where one of the substrates must bind first (b), second (c), or third (d), and compulsory order of addition of all three substrates (e). All mechanisms of type b and most mechanisms of type e lack $\Phi_m$ or $\Phi_4$, or in their equation (8) and can therefore be eliminated.

The choice is narrowed further by inhibition studies. Compulsory addition of UDP-GalNAc followed by acceptor is not consistent with the finding that 2'-fucosyllactose is a competitive inhibitor with respect to UDP, a competitive inhibitor with respect to UDP-GalNAc, and a noncompetitive inhibitor with respect to UDP-GalNAc. Likewise, compulsory addition of acceptor followed by UDP-GalNAc can be excluded because UDP, a competitive inhibitor with respect to UDP-GalNAc, is found to be a noncompetitive inhibitor with respect to acceptor. This eliminates all mechanisms of type b and e as well as two mechanisms of type d.

At present, the remaining closely related mechanisms cannot be distinguished from one another. They all involve random order of addition of UDP-GalNAc and acceptor, but differ in the order of cation binding, which is either compulsory (in second or third place only), or random. The latter mechanism, which is perhaps the most likely, is shown in Scheme I. The left half of the scheme indicates that cation, donor, and acceptor bind to the enzyme via a random order equilibrium pathway to form a quaternary complex, which then enters the product phase in a rate-limited step. Pathways involving alternative substrates are shown in the right half of the scheme.

For a random order equilibrium mechanism, all equilibrium constants for the dissociation of substrates from enzyme-substrate complexes can be calculated from initial rate parameters (8). Dissociation constants calculated from initial rate parameters determined at saturating Mn^{2+} or saturating Mn^{2+} are given in Tables X and XI, respectively. The values for $K_{31}$, $K_{32}$, and $K_{33}$ ($=K_{312}$) were obtained from two different experiments and their close agreement serves to confirm that the model is internally consistent. Similarly the dissociation constants for 2'-fucosyllactose, $K'_{31}$ and $K'_{131}$, were obtained either directly or from the inhibition experiment described above and agree within the limits of experimental error.

It may be noted that in the proposed mechanism, the dissociation constants involving the quaternary complex are identical with the classical Michaelis constants. Binding of cation and UDP-GalNAc to the enzyme is synergistic. For example, the presence of UDP-GalNAc in an enzyme complex facilitates the binding of Mn^{2+}, since $K_{31}$ is an order of magnitude smaller than the corresponding $K_{31}$, Moreover, the presence of Mn^{2+} facilitates the binding of UDP-GalNAc. The synergistic effect is even stronger when Cd^{2+} is substituted for Mn^{2+}.

The kinetic mechanism of galactosyltransferase from bovine milk, recently investigated in this laboratory (2), is very similar to the mechanism proposed here for N-acetylgalactosaminyltransferase in that both enzymes bind donor and acceptor in random order. The two mechanisms differ, however, in the
order of cation binding. Galactosyltransferase must bind the cation first before donor and acceptor can bind. The order of cation binding of N-acetylgalactosaminyltransferase has not been established, but the data reported here are inconsistent with a mechanism where the cation binds as a compulsory first substrate.

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M Schwyzer and R L Hill

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