Some Kinetic Properties of Human-Milk Galactosyl Transferase

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Reactions catalyzed by human milk N-acetyl lactosamine synthetase in the presence and absence of α-lactalbumins have been investigated by steady-state kinetics. N-Acetyl lactosamine synthesis and lactose synthesis in the absence of α-lactalbumin appear to proceed by an ordered sequential reaction, with substrates attaching in the order: Mn\(^{2+}\), UDP-galactose and monosaccharide. Under the conditions used (pH 7.4, 37°C) the attachment of Mn\(^{2+}\) is not at thermodynamic equilibrium and it appears that the enzyme can accept either free UDP-galactose or its Mn\(^{2+}\) complex as substrate. Evidence is presented which suggests that the Mn\(^{2+}\) complex of UDP may be the final product released from the enzyme. Reactions in the presence of α-lactalbumin proceed by a similar ordered mechanism. Kinetic effects observed in the presence of human α-lactalbumin with three different monosaccharide acceptors, and in the presence of bovine α-lactalbumin with glucose, can be reasonably explained only by assuming that α-lactalbumin attaches to the enzyme immediately before monosaccharides, contrary to suggestions by other workers. It is proposed that α-lactalbumin attaches to an enzyme · Mn\(^{2+}\) · UDP-galactose complex at thermodynamic equilibrium, producing a new enzyme form with increased affinity for monosaccharides. The inhibitory effects of α-lactalbumin on N-acetyl lactosamine synthesis are attributed to inhibition resulting from attachment of the protein to a central complex in an alternative pathway in the reaction scheme. The kinetic effects of four α-lactalbumins with human galactosyl transferase are characterized and intrinsic differences shown to be independent of the source of galactosyl transferase. The function deduced for α-lactalbumin in the lactose synthetase system is discussed in relation to its structure and a procedure is indicated for quantitatively measuring activity differences in α-lactalbumins.

Lactose synthetase consists of two protein components [1]. One is a widely-distributed galactosyl transferase which in isolation catalyzes the synthesis of N-acetyl lactosamine:

\[
\text{UDP-galactose} + \text{GlcNAc} \rightarrow \text{N-acetyl lactosamine} + \text{UDP}.
\]

This enzyme can also utilize GlcNAc covalently bound to an oligosaccharide or a glycoprotein as substrate, and functions in the synthesis of glycoproteins in many tissues [2,3]. The second component is the milk protein α-lactalbumin [4] which regulates the monosaccharide-binding properties of the galactosyl transferase so that it will catalyze the synthesis of lactose:

\[
\text{UDP-galactose} + \text{glucose} \rightarrow \text{lactose} + \text{UDP}
\]

at physiological concentrations of glucose [2,3,5,6].

Abbreviations. GlcNAc, N-acetyl glucosamine; MnUDP, the Mn\(^{2+}\) complex of UDP; MnUDP-galactose, the Mn\(^{2+}\) complex of UDP-galactose.

Enzyme. Lactose synthetase or UDP-galactose : D-glucose β4-galactosyl transferase (EC 2.4.1.22).

α-Lactalbumin, which has been designated the "specifier protein" of lactose synthetase as it controls the organ-specific nature of lactose production [2], thus exerts a unique form of regulation in the enzyme system. It is therefore particularly important to determine the mechanism of action of the lactose synthetase system to help in understanding one aspect of the biosynthesis of the components of milk, in particular the nature of the regulation of lactose synthesis by α-lactalbumin [7]. Furthermore, α-lactalbumin is homologous with chicken egg-white lysozyme [8] and it is hoped that a better understanding of its mechanism of action will help to reveal the nature of any functional relationship with lysozyme.

As part of an investigation of this problem, we report here a steady-state kinetic study of the reactions catalyzed by pure human galactosyl transferase in the absence and presence of α-lactalbumin from human and other milks. For the system in the absence of regulatory protein, a mechanism is proposed which differs only slightly from one suggested by Morrison and Ebner [9] based on kinetic studies.
with the bovine enzyme under different conditions from those used here. We have observed certain kinetic effects in the presence of human α-lactalbumin with various monosaccharide acceptors which are not consistent with the mechanism put forward by Morrison and Ebner [10] for the bovine lactose synthetase system. An alternative scheme is presented here which is consistent with our observations and with the bovine enzyme under different conditions of other workers [5], in which a different role, of a biologically unique character, is assigned to α-lactalbumin. The effects in the system of α-lactalbumins from four different species are compared and a procedure for quantitating activity differences in α-lactalbumins suggested. A preliminary account of some of these findings has already been published [11].

**MATERIALS AND METHODS**

Cyanogen bromide and 2-mercaptoethanol were purchased from Koch-Light Laboratories Ltd and uridine, UMP, UDP, UTP, UDP-glucose, glucosamine hydrochloride and N-acetyl glucosamine from Sigma Chemical Co. Scintillation Grade 2,5-diphenyloxazole and naphthalene were supplied by Nuclear Enterprises Ltd and dioxane by Victor Blagden (Surrey).

UDP-[U-14C]galactose was obtained from the Radiochemical Centre (Amersham). All other chemicals were from B.D.H. Ltd.

Sephadex G-25 and G-150 and Sepharose 4B were purchased from Pharmacia (G.B.).

**α-Lactalbumins**

α-Lactalbumins were isolated from bovine, human, pig and guinea-pig milks as described previously [12-15]. The concentrations of these proteins were determined spectrophotometrically, using the following values for the absorbance at 280 nm of a 1% solution; bovine, 20 [16]; human, 18.8 [13]; pig, 20 (E. Kessler and K. Brew, unpublished observation); guinea-pig, 16.7 [17].

**Preparation of α-Lactalbumin-Sepharose**

Bovine α-lactalbumin was coupled with CNBr-activated Sepharose 4B as described by Cuatrecasas [18]. The extent of coupling was determined subtractively. The substituted gel, which contained 10-15 mg protein/ml was stored in 0.02% sodium azide at 4 °C.

**Preparation of N-Propionyl Glucosamine and N-Butyryl Glucosamine**

N-Propionyl and N-butryl glucosamines were prepared by acylation of glucosamine with the appropriate acid anhydrides [19]. The products were crystallized from water—ethanol (100:75, v/v) after the addition of a small volume of acetone. The compounds gave single spots on paper chromatography in n-propanol—water—ethyl acetate (7:2:1, v/v/v) with Rf values increasing with the length of the acyl group. Elemental analysis of the products gave reasonable agreement with calculated values for the expected products.

**Assay of Galactosyl Transferase Activities**

The rate of transfer of 14C from UDP-[14C]-galactose into neutral disaccharide was measured by a procedure essentially similar to that described previously [2]. All incubations were performed in 50 mM 3-(N-morpholino)-propanesulphonate buffer, pH 7.4. At this concentration no measurable chelation of Mn²⁺ by the buffer was observed by electron spin resonance spectroscopy. When not otherwise stated, reaction mixtures contained 10 mM MnCl₂, 20 mM monosaccharide (glucose or GlcNAc), 0.68 mM UDP-galactose (476 counts × min⁻¹ × nmol⁻¹) and 0.2 mg/ml bovine serum albumin, with 10 to 20 μl of enzyme preparation in a total volume of 100 μl. Assay mixtures were prepared in ice, and the reaction started by the addition of monosaccharide. After incubation (5 to 10 min at 37 °C) the reaction was terminated by the addition of 0.5 ml water containing an excess of EDTA over the Mn²⁺ ions present in the incubation mixture. Neutral sugars were then separated from unchanged UDP-galactose as described previously [2]. To each scintillation vial containing 2.1 ml of aqueous solution was added 10 ml dioxane containing 5 g 2,5-diphenyloxazole and 100 g naphthalene per litre. The samples were counted in a Beckman LS200 scintillation counter using a combined channel of 14C and ³H. In all experiments, appropriate blanks omitting monosaccharide acceptor were included to correct for hydrolysis of UDP-galactose. The relationship between the production of uncharged radioactive sugars and enzyme concentration, or time of reaction, was checked for linearity over a range of UDP-galactose concentrations. Linearity with time extended as far as conversion of 25% of the substrate to product, a proportion not exceeded in any of the subsequent experiments.

Rates are expressed in milli-units (mU), one unit being defined as 1 micromole of galactose transferred per minute.

**Analysis of Kinetic Data**

Initial velocity data were first inspected in the form of double-reciprocal plots to determine the appropriate rate law [20]. The data were then fitted to the corresponding rate equation using computer programmes written in Algol 60 for use in the ICL KDF9 and later 1906A computer installations at Leeds University. These programmes performed...
calculations based on the methods of Cleland [21].
Initial velocity data for varying concentrations of
two substrates conforming to a symmetrical sequen-
tial initial velocity pattern, an asymmetrical sequen-
tial initial velocity pattern or a ping-pong initial
velocity pattern, and for varying concentrations of an
inhibitor and a single substrate conforming to linear
competitive inhibition, linear uncompetitive inhibi-
tion and linear noncompetitive inhibition, were fitted
to Eqs. (1), (2), (3), (4), (5), and (6), respectively.

\[ v = VAB/(K_A + K_B + AB) = \frac{V_{max}(1 + I/K_i)}{1 + I/K_i} \]  
\[ v = VAB/(K_A + K_B + AB) = \frac{V_{max}A}{K_A + A(1 + I/K_i)} \]  
\[ v = VAB/(K_A + K_B + AB) = \frac{V_{max}B}{K_B + B(1 + I/K_i)} \]  
\[ v = VAB/(K_A + K_B + AB) = \frac{V_{max}AB}{K_A + K_B + AB} \]  

where \( A \) and \( B \) are concentrations of substrates
A and B and inhibitor, respectively.

For initial velocity patterns with pairs of sub-
strates, in the cases where fit to Eqs. (2) or (3) was
indicated by visual inspection, the data were also
tested for fit to Eqn (1). The rate equation giving the
best fit to the experimental data was chosen as that
with the least variance, calculated as the average
square of the difference between an experimental
velocity and the corresponding value derived from
this criterion leads to a choice of Eqn (1), (2), or (3).
However, where Eqn (2) or (3) is the choice, it seems justifiable
to assume that this equation is a true description of
the kinetic behaviour of the system.

A similar procedure was used in deciding the na-
ture of the inhibition by a particular compound, where
Eqs. (4) and (5) may be regarded as extreme cases of
Eqn (6).

Polyacrylamide-Gel Electrophoresis

Disc electrophoresis on polyacrylamide gels was
carried out by the method of Davis [22]. Electropho-
resis in the presence of sodium dodecylsulphates
was performed as described by Weber and Osborn
[23].

Preparation of Human-Milk Galactosyl Transferase

A simplified purification procedure was used for
the isolation of galactosyl transferase from human
milk. The most effective step in the procedure in-
volved the application of affinity chromatography with
\( \alpha \)-lactalbumin covalently attached to Sepharose 4B.
As reported by Trayer and Hill [24] and by Andrews
[25], galactosyl transferase is adsorbed by this gel
in the presence of glucose, or, more effectively,
GlcNAc. Before utilizing this procedure with milk,
it is necessary to remove the large quantities of \( \alpha 
-lactalbumin present in milk which would otherwise
compete with the column-bound protein for the
enzyme. The following procedure was accordingly
used.

a) Pooled human milk was adjusted to 20 mM with
respect to Tris-HCl buffer pH 7.4, 10 mM with
MnCl\(_2\) and 1 mM with mercaptoethanol. The fat,
together with some casein, was removed by centrifugation
at 15000 \( \times \) g for 30 min. The skimmed milk
was then concentrated to 1/5 of its original volume
by ultrafiltration using a Millipore PM30 membrane.
Samples of concentrated milk were stored at \(-15^\circ\) C.

b) 200-m1 samples of concentrated milk were
applied to a column of Sephadex G-150 (10 x 100 cm)
equilibrated with 20 mM Tris-HCl buffer pH 7.4
containing 10 mM MgCl\(_2\) and 1 mM mercaptoethanol.
The column was developed by upward flow at a flow
rate of 200 ml/h and the effluent collected in 50-m1
fractions. Galactosyl transferase emerged in the first
protein peak eluted from the column, well separated
from \( \alpha \)-lactalbumin.

c) Fractions containing the galactosyl transferase
were pooled, adjusted to 20 mM with respect to
GlcNAc and applied in 500-m1 batches to a column
of Sepharose 4B-\( \alpha \)-lactalbumin (1 x 15 cm) previously
equilibrated with 20 mM Tris-HCl pH 7.4 containing
20 mM GlcNAc and 1 mM mercaptoethanol. Under these
conditions all the galactosyl transferase was effectively adsorbed to the column. The column was washed with buffer containing
GlcNAc until the absorbance of the effluent at
280 nm fell below 0.3. Galactosyl transferase was then
eluted with buffer devoid of GlcNAc (20 mM Tris-
HCl pH 7.4 containing 10 mM MgCl\(_2\) and 1 mM mercaptoethanol). To obtain completely pure enzyme
the chromatography was repeated using a column
containing 5 m1 of Sepharose-\( \alpha \)-lactalbumin. The
product of the purification procedure migrated as a
diffuse band on polyacrylamide gel electrophoresis
at pH 8.9. On electrophoresis in the presence of
sodium dodecylsulphate, two protein components
were observed of molecular weights (determined from
the rate of migration relative to proteins of known
molecular weight) 50000 and 96000. After treatment
with 1% mercaptoethanol in 8 M urea at pH 5, a
single band of apparent molecular weight 51000
was present. The physical and chemical properties of
this enzyme are the subjects of further studies
which will be reported elsewhere.

As the starting material in the purification con-
tained large amounts of \( \alpha \)-lactalbumin which inhibits
N-acetyl lactosamine synthetase activity, it is dif-

difficult to estimate the overall yield of the purification procedure. However, if it is assumed that 100\% yield is obtained in the gel filtration step, it is estimated that an overall yield of 60 or 70\% is obtained in the purification procedure.

The enzyme preparation was concentrated, adjusted to 0.1\%, with respect to bovine serum albumin and passed through a column of Sephadex G-25 equilibrated with 50 mM 3-(N-morpholino)-propanesulphonate pH 7.4 containing 1 mM mercaptoethanol. This preparation, which was used in the kinetic studies, was stored frozen in aliquots at -15 °C.

Preparation of Bovine-Milk Galactosyl Transferase

Raw bovine milk was concentrated and fractionated with Sephadex G-150 as described for human milk. The enzyme was further purified by ion-exchange chromatography with CM-Sephadex C-50 as described previously [2]. Partially purified galactosyl transferase, which emerged from the column after the main protein peak on elution, was pooled, concentrated, dialyzed against 20 mM Tris-HCl buffer pH 7.4 containing 10 mM MgCl₂ and 1 mM mercaptoethanol and stored frozen in aliquots at -15 °C. No attempt was made to obtain completely pure enzyme from bovine milk as this was not necessary for the comparative experiments in which it was used.

It should be noted that although Mn²⁺ or Mg²⁺ ions were present at various stages during the purification of the enzymes used in this study, metal ions are not essential and the chromatographic separations can be performed equally well in the presence of EDTA.

RESULTS

General Properties of Galactosyl Transferase

Purified galactosyl transferase showed a pronounced tendency to aggregate, and it proved impossible to concentrate it to an absorbance greater than 0.4 at 280 nm. In the absence of metal ions, the enzyme can be stored frozen in 0.1 M 3-(N-morpholino)-propanesulphonate buffer pH 7.4 containing 0.1\% bovine serum albumin for periods of up to 12 months without significant loss of activity. All the studies reported here were carried out on purified preparation stored in this way.

The activity of the purified enzyme (devoid of serum albumin) was enhanced by the addition of serum albumin (Fig. 1). Similar enhancement was also obtained by the addition of Triton X-100 (1\%) or phospholipids, and at present it is impossible to attribute this effect to a specific mechanism. The non-specific nature of the effect suggests that it may result from protection of the enzyme from denaturation or aggregation. All assays reported below were therefore carried out in the presence of 0.1\% bovine serum albumin.

Metal-Ion Requirement

The purified enzyme showed low but significant activity in the absence of added metal ions. This activity was inhibited by EDTA, and titration studies showed that 32 \mu M EDTA in a 100-\mu l reaction mixture was just sufficient to completely eliminate all endogenous activity from 10 \mu l of enzyme preparation. The enzyme treated with this concentration of EDTA was completely dependent on Mn²⁺ ions for activity and was inactive in the presence of Mg²⁺ ions. As shown in Fig. 2, at pH 7.4, the enzyme activity is increased by Mn²⁺ up to a concentration of 50 mM above which only slight inhibition is observed. This is in contrast with the observation of Morrison and Ebner [9] on the bovine enzyme, that at pH 8, concentrations of Mn²⁺ exceeding 4 mM are inhibitory. The reciprocal plot of these data was found to be non-linear, showing activation beyond that expected on the basis of the Michaelis-Menten equation by concentrations of Mn²⁺ above 10 mM, (but below 100 mM) a result which suggests that the effects of Mn²⁺ are not as simple as previously suggested [9].

Kinetic Studies of Reactions Catalyzed by Human Galactosyl Transferase in the Absence of \alpha-Lactalbumin

In the absence of \alpha-lactalbumin, there are effectively three substrates to be considered in a kinetic

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Fig. 1. The effect of increasing concentrations of bovine serum albumin on the activity of human galactosyl transferase for N-acetyl-lactosamine synthesis. The preparation of enzyme used was free of serum albumin.
analysis: Mn$^{2+}$, UDP-galactose and monosaccharide. Initial velocity studies of the reactions were carried out by varying the concentration of individual substrates in the presence of a series of concentrations of a second substrate while maintaining the third substrate at a constant concentration. The most complete set of studies has been carried out using GlcNAc as the acceptor substrate. Some of these results are illustrated in Fig. 3–5. As all the results were analyzed by computer to obtain the best values for the kinetic constants, reciprocal plots and replots of their slopes and intercepts are not necessary for the quantitative analysis of the results. It should be noted that with Mn$^{2+}$ and UDP-galactose as variable substrates (Fig. 3), patterns of lines intersecting to the left of the vertical axis are obtained [i.e. best fit to Eqn (1)] in contrast with the results of Morrison and Ebner [9] who obtained an asymmetrical intersecting pattern [described by Eqn (2) with Mn$^{2+}$ as substrate A].

![Fig. 2. The effect of increasing Mn$^{2+}$ concentration on the activity of human galactosyl transferase for N-acetyl-lactosamine synthesis. The enzyme preparation was treated with EDTA corresponding to a concentration in the assay of 32 µM, which had been found to be the minimum required to remove endogenous activity in the absence of added Mn$^{2+}$.]

![Fig. 3. The effect of UDP-galactose concentrations on the rate of N-acetyl-lactosamine synthesis at different fixed concentrations of Mn$^{2+}$ and a fixed concentration of GlcNAc (20 mM). The concentrations of Mn$^{2+}$ were: (○) 0.2 mM, (△) 0.25 mM, (□) 0.33 mM, (●) 0.5 mM, (▲) 1.0 mM, (■) 10 mM.]

![Fig. 4. The effect of Mn$^{2+}$ concentrations on the rate of N-acetyl-lactosamine synthesis at different fixed concentrations of GlcNAc and a fixed concentration of UDP-galactose (0.25 mM). The concentrations of GlcNAc were: (△) 2.0 mM, (□) 2.5 mM, (●) 3.33 mM, (▲) 5.0 mM, (■) 10 mM.]

![Fig. 5. The effect of UDP-galactose concentrations on the rate of N-acetyl-lactosamine synthesis at different fixed concentrations of GlcNAc and a fixed concentration of Mn$^{2+}$ (10 mM). The concentrations of GlcNAc were: (○) 2.0 mM, (△) 2.5 mM, (□) 3.33 mM, (●) 5.0 mM, (▲) 10 mM.]

Kinetics of Human Galactosyl Transferase

Under our conditions, at higher Mn$^{2+}$ concentrations, reciprocal plots with Mn$^{2+}$ as variable substrate became non-linear, and data for this higher concentration range are not shown here. A pattern of lines intersecting to the left of the vertical axis [Eqn (1)] was also obtained with Mn$^{2+}$ and GlcNAc as variable substrates at a fixed concentration of UDP-galactose (Fig.4). With UDP-galactose and GlcNAc as variable substrates at 10 mM Mn$^{2+}$, reciprocal plots (Fig.5) were patterns of parallel lines consistent with Eqn (3). In the absence of α-lactalbumin, glucose will also act as an acceptor for galactose, when used at high concentrations. Under these conditions, kinetic patterns closely similar to those found with GlcNAc are obtained (Fig.6). Kinetic patterns of this type occur in cases of ping-pong mechanisms [26], but can more generally be interpreted to indicate the presence of an irreversible step between the attachment of the two substrates, i.e. release of a product at zero product concentration (ping-pong mechanism) or attachment of a third substrate at saturating concentration.

Inhibition Studies

Further information on the mechanism of the galactosyl transferase reaction was obtained from dead-end and product inhibition studies. UDP-glucose was an effective inhibitor of the system and gave linear inhibition patterns which were competitive with respect to UDP-galactose (Fig.7), non-competitive with respect to GlcNAc (Fig.8), and uncompetitive with respect to Mn$^{2+}$ (Fig.9). As this result would appear to preclude both explanations of the parallel lines in Fig.5 and 6, i.e. a ping-pong mechanism or addition of Mn$^{2+}$ at saturating

![Graph](image-url)

**Fig. 6.** The effect of UDP-galactose concentrations on the rate of lactose synthesis by galactosyl transferase in the absence of α-lactalbumin at different fixed concentrations of glucose and a fixed concentration of Mn$^{2+}$ (10 mM). The concentrations of glucose were: (○) 0.5 M, (△) 0.67 M, (□) 1.0 M, (●) 2.0 M.

**Fig. 7.** Linear competitive inhibition of N-acetyl-lactosamine synthesis by UDP-glucose with UDP-galactose as the variable substrate. Concentrations of Mn$^{2+}$ and GlcNAc were fixed at 10 mM and 20 mM, respectively. The concentrations of UDP-glucose were: (●) zero, (△) 0.25 mM, (●) 0.5 mM, (□) 0.75 mM, (△) 1.0 mM, (○) 1.25 mM.

**Fig. 8.** Linear noncompetitive inhibition of N-acetyl-lactosamine synthesis by UDP-glucose with GlcNAc as the variable substrate. The concentrations of Mn$^{2+}$ and UDP-galactose were 10 mM and 0.63 mM, respectively, while the concentrations of UDP-glucose were: (●) zero, (■) 0.25 mM, (△) 0.75 mM, (□) 1.0 mM.

**Fig. 9.** Linear uncompetitive inhibition of N-acetyl-lactosamine synthesis by UDP-glucose with Mn$^{2+}$ as the variable substrate. The concentrations of UDP-galactose and GlcNAc were 0.25 mM and 10 mM, respectively. The concentrations of UDP-glucose were: (●) zero, (■) 0.25 mM, (○) 0.5 mM, (△) 0.75 mM, (□) 1.0 mM.

concentration to the enzyme after UDP-galactose (see Discussion), it was considered possible that this result indicates that the binding of UDP-galactose is virtually irreversible. To test therefore to see if the results are consistent with a low but non-zero value for $K_{ia}$ in Eqn (1), the approach of Koster and Veeger was used [27]. The rate dependence on UDP-galactose and GlcNAc concentrations was examined in the presence of a fixed concentration of UDP-glucose, which being competitive with respect to UDP-galactose, will amplify $K_i$, in Eqn (1) by a factor of $(1 + I/K_i)$ and therefore magnify any tendency of the lines in reciprocal plots to intersect [Eqn (7)].

$$v = \frac{VAB}{K_{ia} K_b \left(1 + \frac{I}{K_1}\right) + K_b A + K_a \left(1 + \frac{I}{K_1}\right) B + AB}.$$  

From Fig. 10 it can be seen that in the presence of UDP-glucose, the lines quite clearly intersect showing that the system is described by Eqn (1) rather than Eqn (3).

No suitable inhibitory analogue of the monosaccharide substrate was found to extend the inhibition studies. Of the products of the reactions studied, N-acetyl-lactosamine was not obtained in sufficient quantities to permit study of its inhibitory characteristics, and lactose was a very poor inhibitor even at high concentrations. Inhibition studies could not be carried out with free UDP since UDP has a strong affinity for Mn$^{2+}$ (stability constant of 8700 M$^{-1}$ [28]) and the enzyme is dependent on Mn$^{2+}$ for activity. As it would therefore be difficult to examine the relative inhibitory properties of free and Mn$^{2+}$-complexed UDP, it was decided to study the effect of the Mn$^{2+}$ complex of UDP alone. This complex is a possible product in the reaction scheme. Inhibition with respect to Mn$^{2+}$ by MnUDP was investigated by varying Mn$^{2+}$ in the range of 2–10 mM, and using concentrations of UDP between 0.04 and 0.16 mM. In this concentration range, the ratio of free to complexed UDP will be between 0.05 and 0.01, and the effects of free UDP may be considered to be negligible. Under these conditions, MnUDP acted as a linear noncompetitive inhibitor with respect to Mn$^{2+}$ (Fig. 11). In studying the inhibition with respect to GlcNAc and UDP-galactose, UDP was used in a concentration range of 0 to 1 mM at a concentration of 10 mM Mn$^{2+}$, and an additional amount of

![Fig. 10](image1.png)

**Fig. 10.** The effect of UDP-galactose concentrations on the rate of N-acetyl-lactosamine synthesis at different fixed concentrations of GlcNAc and a fixed concentration of Mn$^{2+}$ (10 mM), in the presence of 0.25 mM UDP-glucose. The concentrations of GlcNAc were: (●) 2.0 mM, (○) 2.5 mM, (△) 3.33 mM, (■) 5.0 mM, (□) 10 mM

![Fig. 11](image2.png)

**Fig. 11.** Linear noncompetitive inhibition of N-acetyl-lactosamine synthesis by MnUDP with Mn$^{2+}$ as the variable substrate. The concentrations of UDP-galactose and GlcNAc were 0.25 mM and 20 mM, respectively, and the concentrations of MnUDP were: (●) zero, (■) 0.04 mM, (△) 0.08 mM, (□) 0.12 mM, (○) 0.16 mM

Fig. 12. **Linear competitive inhibition of N-acetyl-lactosamine synthesis by MnUDP with UDP-galactose as the variable substrate.** The concentrations of GlcNAc and Mn²⁺ were 20 mM and 10 mM, respectively, while the concentrations of MnUDP were: (■) zero, (▲) 0.25 mM, (●) 0.5 mM, (□) 0.75 mM, (△) 1.0 mM, (○) 1.25 mM.

Fig. 13. **Linear noncompetitive inhibition of N-acetyl-lactosamine synthesis by MnUDP with GlcNAc as the variable substrate.** The concentrations of Mn²⁺ and UDP-galactose were 10 mM and 0.6 mM respectively and the concentrations of MnUDP were: (●) zero, (□) 0.25 mM, (○) 0.5 mM, (△) 0.75 mM.

Mn²⁺ was added to balance that complexed by the UDP, assuming the value for the stability constant of the MnUDP complex given above. MnUDP acted as a linear competitive inhibitor with respect to UDP-galactose, and a linear non-competitive inhibitor with respect to GlcNAc (Fig. 12, 13).

A series of other uridine compounds was also tested as inhibitors: uridine, UMP and UTP were found to be competitive with respect to UDP-galactose. Inhibitor constants for all uridine compounds are compared below (Table 4, Discussion).

*N-Propionyl* glucosamine and *N*-butyryl glucosamine were tested for their effects upon the galactosyl transferase. Both were found to be active as substrates, and showed apparent $K_m$ values similar to that of GlcNAc. The maximum velocity at a single concentration of UDP-galactose and Mn²⁺ increased with the length of the acyl group (Fig. 14).

**Kinetics Studies of Galactosyl Transferase Reactions in the Presence of α-Lactalbumin**

**Glucose as Substrate.** For reactions catalyzed by the galactosyl transferase in the presence of α-lactalbumin, there are four substrates to be considered in a kinetic analysis: Mn²⁺, UDP-galactose, monosaccharide and α-lactalbumin. As a simplification, the investigation of the effect of the Mn²⁺ concentration was not repeated in the presence of α-lactalbumin, since its role in the reaction is unlikely to be affected by the regulatory protein, and indeed this study, and that of Morrison and Ebner [29] both indicate that a basically similar mechanism is found in the presence and absence of α-lactalbumin. The kinetic experiments were therefore all carried out at 10 mM Mn²⁺. At this high concentration of metal ion it appears that an essentially irreversible binding of UDP-galactose occurs in the reaction, a phenomenon that simplifies the interpretation of our results. Reciprocal plots for UDP-galactose as variable substrate at a series of concentrations of human α-lactalbumin and fixed concentrations of glucose and Mn²⁺ consist of a series of parallel lines (Fig. 15). A similar series of parallel lines is obtained for plots with UDP-galactose as variable substrate at a series of concentrations of glucose and fixed concentrations...
The effect of UDP-galactose concentrations on the rate of lactose synthesis at different fixed concentrations of human α-lactalbumin and fixed concentrations of Mn²⁺ (10 mM) and glucose (20 mM). The set of lines was not determined by computer. The concentrations of α-lactalbumin were: (○) 25 μg/ml, (□) 50 μg/ml, (●) 100 μg/ml, (■) 200 μg/ml.

The effect of different concentrations of human α-lactalbumin on the rate of lactose synthesis at different fixed concentrations of glucose. The concentrations of Mn²⁺ and UDP-galactose were fixed at 10 mM and 0.63 mM, respectively, and the concentrations of glucose were: (○) 1 mM, (▲) 2 mM, (□) 5 mM, (●) 10 mM, (△) 15 mM, (■) 20 mM.

A replot of the slopes of the lines from Fig. 17 against 1/[glucose].

A unique type of intersecting pattern is found for reciprocal plots with α-lactalbumin and glucose as variable substrates at fixed concentrations of Mn²⁺ and UDP-galactose. Computer analysis of the data showed a better fit by Eqn (2) than Eqn (1), provided α-lactalbumin is taken as substrate A and glucose as substrate B. This phenomenon is demonstrated graphically in Fig. 17—19. For reciprocal plots with α-lactalbumin as variable substrate at a series of glucose concentrations, a pattern of lines intersecting to the left of the vertical axis is obtained (Fig. 17). When the slopes of these lines are plotted against 1/[glucose], a straight line passing through the origin is generated (Fig. 18). Alternatively, it can be seen that with the same data, using glucose as the variable substrate at a series of concentrations of human α-lactalbumin, the lines produced intersect precisely on the vertical axis (Fig. 19). This is an effect similar to that observed with metal activators of some enzymes and the first-binding substrate [30] but contrasts with the result obtained by Morrison and Ehner [9] with bovine galactosyl transferase and bovine α-lactalbumin at pH 8 and 30 °C. These workers obtained symmetrical intersecting plots [Eqn (1)] in a corresponding experiment. We have found the same phenomenon when bovine α-lactalbumin is used in place of human α-lactalbumin, and also when other monosaccharide acceptors of the galactosyl moiety are used with human α-lactalbumin (see below). Inhibition of lactose synthesis in the presence of α-lactalbumin by UDP (Mn²⁺ complex) was found to be competitive with respect to UDP-galactose.

Fig. 19. The initial velocity pattern obtained by plotting the data from Fig. 17 with glucose as the variable substrate. The concentrations of α-lactalbumin were: (○) 25 μg/ml, (△) 50 μg/ml, (□) 100 μg/ml, (●) 200 μg/ml, (■) 400 μg/ml.

Fig. 20. Linear competitive inhibition of lactose synthesis by MnUDP with UDP-galactose as the variable substrate. Mn²⁺, α-lactalbumin and glucose were held constant at concentrations of 10 mM, 100 μg/ml and 20 mM, respectively. The concentrations of MnUDP were: (□) zero, (△) 1.0 mM, (○) 2.5 mM.

Fig. 21. Linear noncompetitive inhibition of lactose synthesis by MnUDP with glucose as the variable substrate. The concentrations of Mn²⁺, UDP-galactose and α-lactalbumin were held constant at 10 mM, 0.60 mM and 250 μg/ml, respectively, and the concentrations of MnUDP were: (●) zero, (○) 0.25 mM, (□) 0.5 mM, (△) 0.75 mM.

Fig. 22. Linear noncompetitive inhibition of lactose synthesis by MnUDP with α-lactalbumin as the variable substrate. The concentrations of Mn²⁺, UDP-galactose and glucose were fixed at 10 mM, 0.6 mM and 10 mM respectively, while the concentrations of MnUDP were: (□) zero, (△) 0.5 mM, (○) 1.0 mM.

Fig. 23. The effect of xylose concentrations on the rate of disaccharide synthesis at different fixed concentrations of human α-lactalbumin. The concentrations of UDP-galactose and Mn²⁺ were held constant at 0.63 mM and 10 mM, respectively, and the concentrations of α-lactalbumin were: (○) 50 μg/ml, (△) 63 μg/ml, (□) 83 μg/ml, (●) 125 μg/ml, (■) 250 μg/ml.

Fig. 24. A replot of the data from Fig. 23 with α-lactalbumin as the variable substrate. The concentrations of xylose were: (○) 0.10 M, (△) 0.125 M, (□) 0.17 M, (●) 0.25 M, (■) 0.50 M.


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547

35t
30
25
15
10
5t
0

-15
-20
-25

I

\text{[GlcNAc]} (\text{mM}^{-1})

0 4 8 12 16 20 24

1/\text{[GlcNAc]} (\text{mM}^{-1})

0 1 2 3 4 5

1/\nu (\text{mM}^{-1})

0 2 4 6 8 10 12 14

1/\text{[UDP-galactose]} (\text{mM}^{-1})

0 1 2 3 4 5

1/\nu (\text{mM}^{-1})

0 0.04 0.08 0.12 0.16 0.20 0.24 0.28 0.32 0.36 0.40

[\alpha\text{-Lactalbumin}] (\text{mg/ml})

0 1 2 3 4 5

Fig. 25. The effect of GlcNAc concentrations on the rate of N-acetyl-lactosamine synthesis at different fixed concentrations of human $\alpha$-lactalbumin. The set of lines was not determined by computer. The concentrations of $\text{Mn}^{2+}$ and UDP-galactose were held constant at 10 mM and 0.63 mM, respectively, and the concentrations of $\alpha$-lactalbumin were: (O) 25 $\mu$g/ml, (□) 50 $\mu$g/ml, (△) 100 $\mu$g/ml, (■) 250 $\mu$g/ml

Fig. 26. Non-linear uncompetitive inhibition of N-acetyl-lactosamine synthesis by $\alpha$-lactalbumin with UDP-galactose as the variable substrate. The set of lines was not determined by computer. The concentrations of $\text{Mn}^{2+}$ and GlcNAc were fixed at 10 mM and 20 mM, respectively, and the concentrations of $\alpha$-lactalbumin were: (O) zero, (△) 25 $\mu$g/ml, (□) 50 $\mu$g/ml, (△) 100 $\mu$g/ml, (■) 200 $\mu$g/ml, (■) 400 $\mu$g/ml

and non-competitive with respect to both glucose and $\alpha$-lactalbumin (Fig. 20-22).

D-Xylose and GlcNAc as Substrates. Because of its structural similarity with D-glucose, D-xylose was examined as a substrate with the galactosyl transferase. It was found to be an extremely poor substrate in the absence of $\alpha$-lactalbumin, with an apparent $K_m$ too high to be measured with any accuracy (about 30 M). In the presence of $\alpha$-lactalbumin, xylose became a reasonable substrate and double-reciprocal plots for both xylose and $\alpha$-lactalbumin as variable substrate at a series of concentrations of the other substrate were found to be qualitatively similar to those obtained with glucose (Fig. 23, 24). Again, the data give a better fit to Eqn (2) than (1). Although at most concentrations of GlcNAc, $\alpha$-lactalbumin acts as an inhibitor of N-acetyl-lactosamine synthesis, at low concentrations of GlcNAc, activation by $\alpha$-lactalbumin is observed [2]. When the effect of GlcNAc concentrations on the velocity was examined at a series of concentrations of $\alpha$-lactalbumin, the data were also found to fit best to an asymmetrical intersecting pattern [Eqn (2), Fig. 25].

$\alpha$-Lactalbumin as an Inhibitor of N-Acetyl-lactosamine Synthesis. In agreement with Andrews [6] and Morrison and Ebner [10] we find that the inhibition of N-acetyl-lactosamine synthesis by human $\alpha$-lactalbumin at high GlcNAc concentrations is uncompetitive with respect to UDP-galactose (Fig. 26). The inhibition is, however, clearly non-linear in nature and changes to activation at high concentrations of $\alpha$-lactalbumin, as shown by a replot of the intercepts of the above figure against $\alpha$-lactalbumin concentration in Fig. 27.

$\alpha$-Lactalbumins of Different Species. Effects exactly similar to those observed with human $\alpha$-lactalbumin were also observed with bovine $\alpha$-lactalbumin (Fig. 28). Data for pig $\alpha$-lactalbumin fitted marginally better to Eqn (1) than to Eqn (2) (Fig. 29). This could possibly be attributed to the greater number of adjustable parameters in Eqn (1), making it inherently easier to fit to it with any errors. With guinea-pig $\alpha$-lactalbumin, the data clearly fitted more easily to Eqn (1) and thus symmetrical intersecting plots are obtained with both glucose and $\alpha$-lactalbumin as variable substrates (Fig. 30).

Replots of the slopes of double-reciprocal plots for glucose as variable substrate at different concentrations of the various $\alpha$-lactalbumins (Fig. 31), show clearly the considerable quantitative difference between these $\alpha$-lactalbumins with respect to their effects on human galactosyl transferase. The lowest

Kinetics of Human Galactosyl Transferase

Fig. 28. The effect of glucose concentration on the rate of lactose synthesis at different fixed concentrations of bovine α-lactalbumin. The concentrations of Mn²⁺ and UDP-galactose were held constant at 10 mM and 0.63 mM, respectively, and the concentrations of bovine α-lactalbumin were: (⊙) 25 µg/ml, (△) 50 µg/ml, (□) 100 µg/ml, (●) 200 µg/ml, (■) 400 µg/ml

Fig. 29. The effect of glucose concentration on the rate of lactose synthesis at different fixed concentrations of pig α-lactalbumin. The concentrations of Mn²⁺ and UDP-galactose were held constant at 10 mM and 0.63 mM respectively, and the concentrations of pig α-lactalbumin were: (⊙) 25 µg/ml, (△) 50 µg/ml, (□) 100 µg/ml, (●) 200 µg/ml, (■) 400 µg/ml

Fig. 30. The effect of glucose concentration on the rate of lactose synthesis at different fixed concentrations of guinea-pig α-lactalbumin. The concentrations of Mn²⁺ and UDP-galactose were held constant at 10 mM and 0.63 mM respectively, and the concentrations of guinea-pig α-lactalbumin were: (⊙) 25 µg/ml, (△) 50 µg/ml, (□) 100 µg/ml, (●) 200 µg/ml, (■) 400 µg/ml

Fig. 31. A replot of the slopes of lines from Fig. 19, 28, 29, 30 against 1/[α-lactalbumin]. (⊙) Human α-lactalbumin, (□) bovine α-lactalbumin, (△) pig α-lactalbumin, (Ο) guinea-pig α-lactalbumin

Line (human α-lactalbumin) represents the highest activities at all glucose concentrations, and it was considered possible that this might reflect mutual adaptation between the lactose synthetase components from the same species. To investigate this possibility, the same four α-lactalbumins were tested at a series of concentrations for their effects on both human and bovine galactosyl transferases at fixed concentrations of glucose, UDP-galactose and Mn²⁺. The results are presented in Fig. 32 and 33. It is clear that with both galactosyl transferases a similar order of activities is found with the α-lactalbumins, i.e. human > bovine > pig > guinea-pig. Although this experiment is not appropriate for quantitatively measuring differences in activity among the α-lactalbumins, it is apparent that the differences observed using human galactosyl transferase reflect intrinsic differences in the biological activities of the α-lactalbumins from different species.

DISCUSSION

Reaction Mechanism in the Absence of α-Lactalbumin

The intersecting patterns of lines in double reciprocal plots given by every pair of the three substrates, Mn²⁺, UDP-galactose and monosaccharide (GlcNAc or glucose), indicate a sequential mechanism, that is, one in which all substrates must add to the enzyme before any product is released (Fig. 3—6). The alternative type of mechanism, pig-pong, would involve release of a product between the points of addition.
of two substrates, for example, the formation of a galactosyl-enzyme with release of UDP before addition of the monosaccharide galactosyl acceptor. Although at first sight the parallel patterns of Fig. 5 and 6 appear to be consistent with a ping-pong mechanism, the presence of UDP-glucose, a competitive inhibitor of UDP-galactose, converts the pattern to an intersecting one (Fig. 10), showing that the parallel lines are in fact intersecting at a large negative value of [substrate]$^{-3}$, corresponding to a value near zero for $K_{ia}$ in Eqn (1), where A is UDP-galactose.

The most general sequential three-substrate mechanism, a random one in which the substrates may add in any order, is excluded by the nature of the inhibition by UDP-glucose. Since UDP-glucose inhibits uncompetitively with respect to Mn$^{2+}$ (Fig. 9), it must react with an enzyme complex which is formed after the addition of Mn$^{2+}$; and therefore the addition of UDP-galactose, of which UDP-glucose is an analogue, must similarly follow Mn$^{2+}$. Scheme 1 shows the possible pathways of substrate addition.

The complete pattern of UDP-glucose inhibition, uncompetitive with respect to Mn$^{2+}$, competitive with respect to UDP-galactose (Fig. 7) and non-competitive with respect to GlcNAc (Fig. 8) is consistent with Scheme 1, with UDP-glucose acting as an analogue of UDP-galactose. It is also consistent with the following parts of Scheme 1: (a) addition of Mn$^{2+}$ and then either UDP-galactose or monosaccharide, and (b) obligatory order of addition, Mn$^{2+}$, UDP-galactose, monosaccharide; but is not consistent with obligatory orders of addition, monosaccharide, Mn$^{2+}$, UDP-galactose; or Mn$^{2+}$, monosaccharide, UDP-galactose.

For a mechanism such as Scheme 1, with alternative pathways, the finding that double-reciprocal

![Fig. 32. The effect of increasing concentrations of four different α-lactalbumins on the rate of lactose synthesis with galactosyl transferase from human milk. The concentrations of Mn$^{2+}$, UDP-galactose and glucose were fixed at 10 mM, 0.63 mM and 20 mM respectively. (a) Human α-lactalbumin, (B) bovine α-lactalbumin, (C) pig α-lactalbumin, (D) guinea-pig α-lactalbumin. The broken line shows the inhibition of N-acetyl-lactosamine synthesis at 20 mM GlcNAc by increasing concentrations of human α-lactalbumin](image)

![Fig. 33. The effect of increasing concentrations of four different α-lactalbumins on the rate of lactose synthesis with galactosyl transferase from bovine milk. The conditions and symbols are the same as in Fig. 32](image)

![Scheme 1](image)
plots are linear would indicate the absence of higher powers of substrate concentrations in the rate equation, a result expected if the rate-determining step in the mechanism is the transformation of the $E \cdot \text{Mn}^{2+} \cdot \text{UDP-galactose} \cdot \text{monosaccharide complex}$ to the corresponding enzyme-products complex. This restriction allows the alternative pathways to be considered as being in rapid equilibrium and enables a greatly simplified rate equation to be applied. On the other hand, Gulbinsky and Cleland [31] have carried out simulation experiments to show that random mechanisms where the rapid equilibrium assumption is not valid may still provide double-reciprocal plots with undetectable curvature.

A consequence of the addition of a substrate at equilibrium in a multisubstrate system is that its Michaelis constant has a magnitude comparable with the enzyme concentration, which is zero on the scale of substrate concentrations [30]. This removes the dependence of the maximum velocity for the next substrate on the concentration of the substrate adding at equilibrium, so that double-reciprocal plots with this next substrate varying intersect on the vertical axis. Our failure to observe this phenomenon in the absence of $\alpha$-lactalbumin excludes a rapid equilibrium version of Scheme 1 in which double-reciprocal plots for UDP-galactose at different $\text{Mn}^{2+}$ concentrations would be expected to intersect on the vertical axis, since UDP-galactose always follows $\text{Mn}^{2+}$, no matter which route is considered. Part (a) of Scheme 1 is similarly excluded, as here the GlcNAc-$\text{Mn}^{2+}$ pair would also be expected to show the phenomenon. Part (b), the steady-state ordered addition of $\text{Mn}^{2+}$, UDP-galactose, and finally monosaccharide, is alone consistent with the results.

A natural deduction, however, from the conclusion that $\text{Mn}^{2+}$ is the first substrate is that it will also be the last product, and in this case the metal ion addition to the enzyme must be at equilibrium in the steady state [30]. Once again our failure to observe lack of dependence on $\text{Mn}^{2+}$ concentration of the intercept on the vertical axis in double-reciprocal plots with UDP-galactose varying (Fig. 3) excludes such a mechanism and we must assume that $\text{Mn}^{2+}$ is not a product of the reaction but is released in some other form, for example as MnUDP. This ordered ter-ter mechanism is shown in Scheme 2 (below).

To confirm this mechanism, one should study the nature of the inhibition by a non-substrate analogue of the monosaccharide and by products of the reaction. As analogues of GlcNAc, we examined $N$-propionyl glucosamine and $N$-butyryl glucosamine but found them to be good substrates which could not be studied as inhibitors because our assay method measures total galactose transfer. In this connection, Hill and coworkers [32] have used glucose as an alternative substrate with GlcNAc and the bovine galactosyl transferase under assay conditions closely similar to ours. The products of the reaction, lactose and $N$-acetyl lactosamine, were separated by paper chromatography. Glucose inhibited competitively with respect to GlcNAc and uncompetitively with respect to UDP-galactose, a finding consistent only with Scheme 2. Morrison and Ebner [9] deduced the same order of substrate additions for the bovine enzyme at a higher pH (pH 8) from the nature of the dead-end inhibition by UDP-glucose and substrate inhibition by GlcNAc but were unable to study product inhibition because $N$-acetyl lactosamine was not available, and their measurement of velocity by the rate of UDP production precluded the use of UDP as an inhibitor. Whether or not one may expect species differences, the bovine and human galactosyl transferases seem to follow the same basic mechanism. In contrast to our results with the human enzyme, however, Morrison and Ebner [9] observed evidence for the equilibrium addition of $\text{Mn}^{2+}$ and so proposed an ordered ter-ter mechanism with $\text{Mn}^{2+}$ as the first substrate and last product.

As the non-availability of $N$-acetyl lactosamine prevented us from studying it as a product inhibitor, only the inhibition patterns expected for MnUDP are summarised in Table 1. The mechanisms considered in Table 1 include Scheme 1 and its part (a) which are completed by a rapid equilibrium random release of products. Whether there are two products (N-acetyl lactosamine and MnUDP) or three (N-acetyl lactosamine, UDP and Mn$^{2+}$) is immaterial here because in rapid equilibrium random mechanisms, all product inhibitions are competitive [30]. The high proportion of UDP present as MnUDP (99%) for 10 mM $\text{Mn}^{2+}$, 0.1 mM UDP) makes it difficult to distinguish between inhibition by UDP and MnUDP. We expect, however, that either of these can act as a UDP-galactose analogue and so the necessary dead-end inhibitions have been incorporated into the mechanisms in Table 1, converting certain inhibitions from competitive to noncompetitive. This modification is supported by the fact that uridine, UMP, UTP and UDP-glucose are all competitive inhibitors with respect to UDP-galactose. In addition, it has been reported [33] that MnUDP stabilizes the interaction between the galactosyl transferase and GlocNAc which has been covalently bound to Sepharose. In the ordered ter-ter mechanism with the last product ($\text{Mn}^{2+}$) identical with the first substrate, UDP already combines with the same enzyme complex as does UDP-galactose and so cannot be included as a dead-end inhibitor; however its dead-end combination with the free enzyme would convert the uncompetitive inhibition with respect to Mn$^{2+}$ to noncompetitive inhibition. In the ordered ter-ter mechanism (Scheme 2), dead-end inhibition by MnUDP as a UDP-galactose analogue alters the competitive inhibition with respect to Mn$^{2+}$ to noncompetitive. Although the inhibition pattern
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Table 1. Predicted inhibition patterns by product Q acting also as a dead-end inhibitor for the various mechanisms considered

<table>
<thead>
<tr>
<th>Variable substrate</th>
<th>Mechanism</th>
<th>Scheme 1 with rapid random product release</th>
<th>Scheme 1, simplification with rapid random product release</th>
<th>Ordered ter-ter mechanism with first substrate and last product same</th>
<th>Ordered ter-bi (Scheme 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>noncompetitive(^a)</td>
<td>noncompetitive(^a)</td>
<td>uncompetitive(^b)</td>
<td>noncompetitive(^a)</td>
<td>noncompetitive(^a)</td>
</tr>
<tr>
<td>B</td>
<td>competitive</td>
<td>competitive</td>
<td>competitive</td>
<td>noncompetitive</td>
<td>noncompetitive</td>
</tr>
<tr>
<td>C</td>
<td>noncompetitive(^a)</td>
<td>noncompetitive(^a)</td>
<td>noncompetitive(^a)</td>
<td>noncompetitive</td>
<td>noncompetitive</td>
</tr>
</tbody>
</table>

\(^a\) If Q does not act as a dead-end inhibitory B-analogue, these inhibitions are competitive.

\(^b\) Dead-end complex formation EQ converts this to noncompetitive.

---

obtained by experiment, noncompetitive with respect to Mn\(^{2+}\), competitive with respect to UDP-galactose and noncompetitive with respect to glucose, agrees with the two random schemes and with the modified ordered ter-ter mechanism, the absence of any indication of equilibrium steps as discussed previously causes us to reject these mechanisms and to consider that the competitive inhibition by MnUDP with respect to UDP-galactose which we have found may in fact be noncompetitive. Intersection of lines for different inhibitor concentrations on the vertical axis in a double-reciprocal plot can be interpreted as a limiting case of noncompetitive inhibition rather than true competitive inhibition. The range of variable substrate concentrations taken, and the particular values of the fixed substrate concentrations, may have caused the intersection point of the pattern of lines to lie indistinguishably close to the vertical axis. A consideration of the values for the kinetic constants supports this view (see below).

On the basis of the above arguments, we propose that human galactosyl transferase operates through an ordered mechanism as shown in Scheme 2, and that MnUDP can form a dead-end complex with E·Mn\(^{2+}\). The preference for a single product, MnUDP, rather than two separate products, Mn\(^{2+}\) and UDP, is based on the absence of indications of the equilibri um addition of Mn\(^{2+}\) to the enzyme. Both the ordered ter-ter and ordered ter-bi mechanisms will include a E·Mn\(^{2+}\)·UDP complex. If this complex involves an association between Mn\(^{2+}\) and UDP similar to that found in solution, then considerations of thermodynamic equilibrium demand that MnUDP will be the final product in the catalytic scheme, as its dissociation constant is 0.115 mM [28] while that for E·Mn\(^{2+}\) can be deduced to be 1.42 mM (Table 2). Nevertheless, in the steady-state catalytic mechanism, there may be no place for equilibrium thermodynamics, and therefore the question of the nature of the final product must to some extent remain open.

Arguments about the participation of MnUDP need also to be applied to MnUDP-galactose. The apparent dissociation constant for this complex is 7.5 mM (unpublished observation) and at 10 mM Mn\(^{2+}\) and 0.1 mM UDP-galactose, a little over half of the UDP-galactose will occur as the Mn\(^{2+}\) complex. Furthermore, concentrations of Mn\(^{2+}\) up to 50 mM continue to increase the catalytic velocity, and with some good of UDP-galactose now as the Mn\(^{2+}\) complex, it seems certain that the enzyme will accept MnUDP-galactose as a substrate. The fact that good fits were obtained for the velocity data to equations involving total UDP-galactose concentrations would appear to suggest that the enzyme does not discriminate between UDP-galactose and its Mn\(^{2+}\) complex. However, the augmented activation obtained at higher Mn\(^{2+}\) concentrations (see Results), suggests that either MnUDP-galactose is favoured or that a different mechanism operates at high Mn\(^{2+}\).

Kinetics of Human Galactosyl Transferase concentrations. On the basis of present evidence, we cannot tell whether the MnUDP-galactose complex will add to the free enzyme or to E·Mn²⁺.

The complete rate equation for the ordered ter-bi mechanism has been given in reverse form by Cleland [20]. If we let A, B, C and Q represent the concentrations of Mn²⁺, UDP-galactose, monosaccharide and MnUDP, respectively, and include dead-end inhibition by Q interacting with EA, Eqn (8) is obtained for zero concentration of the first product.

In Eqn (8), V is the maximum velocity in the forward direction of the reaction. $K_{1a}$, $K_{1b}$, $K_{1q}$, $K_I$ and $K_e$ are dissociation constants for addition of A to E, B to EA, Q to EA, I to EA and Q to E, respectively, while $K_a$ is the Michaelis constant for A, $K_b$ for B, and $K_C$ for C.

Rearrangement in reciprocal form with $A$ or $B$ or $C$ varying shows the dependence of slopes and intercepts on the concentrations of the other two substrates or the mixed product and dead-end inhibitor MnUDP (Q). If the $(1 + Q/K_{1q})$ terms are replaced by $(1 + Q/K_{1q} + I/K_I)$, the influence of the dead-end inhibitor UDP-glucose (I) can be seen [Eqsns (9) to (11)].

With $Q$ and $I$ equal to zero, Eqsns (9) to (11) show that both slopes and intercepts are linear functions of the concentrations of the other two substrates. For the slope dependence on $C$ in Eqn (10) and on $B$ in Eqn (11) to be apparently absent, $K_{1b}$ should be close to zero. Introduction of a constant concentration of I (UDP-glucose) has the effect of raising the value of one of the terms containing $K_{1b}$ by multiplication with the factor $(1 + I/K_I)$ to a level where the term can no longer be considered as zero.

With I equal to zero, Eqsns (9) to (11) show that $Q$ is a noncompetitive inhibitor with respect to all three substrates. In order that the inhibition should appear competitive with respect to B(UDP-galactose), the value of $K_a/A$ in the intercept term of Eqn (10) must be small. A value for $K_a$ of 0.083 mM is deduced later so that at 10 mM Mn²⁺ (A), $K_a/A$ does have a relatively small value, 0.008.

Eqsns (9) to (11) were used to derive the relationships between the true values of the kinetic constants and the apparent values given by the computer fitting of rate data to Eqsns (1), (3), (4), (5) and (6). These values are listed in Tables 2 and 3. There is a reasonable consistency among the values for a particular constant calculated from different sets of experimental data, which lends support to the correctness of the assumed mechanism.

The various uridine derivatives, which act as competitive inhibitors with respect to UDP-galactose, are listed in Table 4 with their dissociation constants from the E·Mn²⁺·I complex ($K_I$ values). The corresponding dissociation constant for UDP-galactose, $K_{1b}$, is included for comparison. Considered as such, or as standard free energy changes, the values
Table 2. Values for the kinetic constants associated with substrates of the galactosyl transferase reaction in the absence of α-lactalbumin

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Kinetic constant</th>
<th>Apparent value</th>
<th>True value</th>
<th>Obtained from Fig. No.</th>
<th>Mean</th>
<th>Literature value [9]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mn²⁺ (A)</td>
<td>$K_a$</td>
<td>0.058</td>
<td>0.083</td>
<td>3</td>
<td>0.083</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>$K_{1a}$</td>
<td>1.42</td>
<td>1.42</td>
<td>3</td>
<td>1.42</td>
<td>1.35</td>
</tr>
<tr>
<td>UDP-galactose (B)</td>
<td>$K_{fb}$</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>0.0245</td>
<td>0.065</td>
</tr>
<tr>
<td></td>
<td>$K_{ib}$</td>
<td>0.0260</td>
<td>0.0245</td>
<td>5</td>
<td>0.072</td>
<td>0.060</td>
</tr>
<tr>
<td></td>
<td>$K_{eb}$</td>
<td>0</td>
<td>0</td>
<td>6</td>
<td>0.024</td>
<td>0.026</td>
</tr>
<tr>
<td></td>
<td>$K_{eb}$</td>
<td>0.15</td>
<td>0.067</td>
<td>5</td>
<td>0.072</td>
<td>0.060</td>
</tr>
<tr>
<td>GlcNAc (C)</td>
<td>$K_c$</td>
<td>8.3</td>
<td>8.7</td>
<td>5</td>
<td>7.41</td>
<td>5.8</td>
</tr>
<tr>
<td></td>
<td>$K_e$</td>
<td>5.17</td>
<td>0.12</td>
<td>4</td>
<td>7.41</td>
<td>5.8</td>
</tr>
</tbody>
</table>

*Values calculated from an experiment in which the initial velocity was measured as a function of UDP-galactose concentration at various set concentrations of GlcNAc and a fixed concentration of 1 mM Mn²⁺.

Table 3. Apparent and true inhibition constants for UDP-glucose as inhibitor of the galactosyl transferase reaction

<table>
<thead>
<tr>
<th>Variable substrate</th>
<th>Type of inhibition</th>
<th>Apparent $K_i$</th>
<th>True $K_i$</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mn²⁺</td>
<td>Uncompetitive</td>
<td>$K_{1i}$ 0.69</td>
<td>0.130</td>
<td>0.081</td>
</tr>
<tr>
<td>UDP-galactose</td>
<td>Competitive</td>
<td>$K_{1i}$ 0.081</td>
<td>0.079</td>
<td>0.074</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>Noncompetitive</td>
<td>$K_{1i}$ 1.15</td>
<td>0.043</td>
<td>0.074</td>
</tr>
</tbody>
</table>

Table 4. $K_i$ values for various uridine derivatives with human galactosyl transferase

These true values were obtained from the apparent values by using the relationship:

$\text{True } K_i = \text{Apparent } K_i \left(\frac{A}{(K_{1a} + A)}\right)$,

where $A$ is the Mn²⁺ concentration and $K_{1a}$ is the dissociation constant of E·Mn²⁺ complex

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>$K_i$</th>
<th>mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uridine</td>
<td>13.8</td>
<td></td>
</tr>
<tr>
<td>UMP</td>
<td>0.82</td>
<td></td>
</tr>
<tr>
<td>MnUTP</td>
<td>0.57</td>
<td></td>
</tr>
<tr>
<td>UDP-glucose</td>
<td>0.079</td>
<td></td>
</tr>
<tr>
<td>MnUDP</td>
<td>0.043</td>
<td></td>
</tr>
<tr>
<td>UDP-galactose (K₁b)</td>
<td>0.0245</td>
<td></td>
</tr>
</tbody>
</table>

indicate that the UDP moiety of the substrate makes the major contribution to the stabilization of the E·Mn²⁺·UDP-galactose complex.

**Reaction Mechanism in the Presence of α-Lactalbumin**

The kinetic behaviour of the enzyme system in the presence of α-lactalbumin is entirely consistent with the view that reactions catalyzed by the galactosyl transferase proceed through similar sequential ordered mechanisms in the presence and absence of the regulatory protein. A similar conclusion was reached by Morrison and Ebner [29] from their studies with the bovine enzyme. The parallel lines obtained in double-reciprocal plots with UDP-galactose as variable substrate at a series of concentrations of either glucose or α-lactalbumin (Fig. 15, 16) are similar to those obtained at different concentrations of either GlcNAc or glucose in the absence of α-lactalbumin (Fig. 5 and 6). As discussed previously, such a result can be interpreted as indicating that under the experimental conditions used, the dissociation constant of UDP-galactose from the appropriate enzyme complex is close to zero, and that both glucose and α-lactalbumin attach to the enzyme after UDP-galactose. The inhibition patterns obtained with MnUDP, which were competitive with respect to UDP-galactose and noncompetitive with respect to both glucose and α-lactalbumin (Fig. 20 to 22), also suggest that these substrates bind after UDP-galactose. Thus, in agreement with Morrison and Ebner [10,29] we conclude that in the catalytic mechanism, UDP-galactose is the obligatory first-binding substrate to the enzyme·Mn²⁺ complex, and that α-lactalbumin and glucose attach at some later stage. Morrison and Ebner argue that as ordered binding of Mn²⁺, UDP-galactose and monosaccharide is unaffected by α-lactalbumin, and attachment

of α-lactalbumin occurs after UDP-galactose, α-lactalbumin must bind after all substrates have added to the enzyme, i.e. after glucose. They do not, however, present any experimental evidence for their proposed order of binding of glucose and α-lactalbumin to the enzyme. We do not accept the validity of this argument, as their results are equally consistent with the reverse order of binding of α-lactalbumin and glucose, or even random binding.

Under our experimental conditions in the presence of α-lactalbumin, we find a very specific kinetic interaction between α-lactalbumin and monosaccharides. The asymmetrical intersecting patterns obtained with human α-lactalbumin and three different monosaccharide acceptors of galactose (glucose, GlcNAc and xylose: Fig. 19, 25, 23) as well as with bovine α-lactalbumin and glucose (Fig.28) can best be interpreted as indicating that the binding of α-lactalbumin is a rapid-equilibrium step which precedes the binding of monosaccharide within the ordered steady-state mechanism. As α-lactalbumin and glucose bind after UDP-galactose, the following reactions must be present in the reaction mechanism:

\[
\begin{align*}
E \cdot Mn^{2+} \cdot UDP-galactose + \alpha LA & \Rightarrow E \cdot \alpha LA \cdot Mn^{2+} \\
E \cdot Mn^{2+} \cdot UDP-galactose + \alpha LA + glucose & \Rightarrow E \cdot Mn^{2+} \cdot UDP-galactose \cdot \alpha LA \cdot glucose
\end{align*}
\]

where \(\alpha LA = \alpha\)-lactalbumin.

If the first of these reactions is at thermodynamic equilibrium, then at infinitely high concentrations of glucose, the reaction rate will be effectively independent of the concentration of α-lactalbumin, because only one mole of α-lactalbumin will be required per mole of enzyme to obtain a maximum velocity at saturating glucose concentrations. This amount of α-lactalbumin is negligible compared with the total amount present and the Michaelis constant for α-lactalbumin thus appears to be zero. Double-reciprocal plots for glucose or other monosaccharides as variable substrate at different concentrations of α-lactalbumin will intersect on the vertical axis, as is observed. This phenomenon is similar to that often observed with metal activators of enzymes, where double-reciprocal plots for the first substrate at different fixed concentrations of metal ion consist of lines intersecting on the vertical axis [30].

The kinetic effects observed with the galactosyl transferase in the presence and absence of α-lactalbumin can be explained in terms of the general mechanism given in Scheme 3. The introduction of alternative pathways in this scheme (and in Scheme 4) would lead us to except non-linear reciprocal plots in principle. In most circumstances, however, the reaction flux will be directed through one pathway only, and in any case, the results of Gulbinsky and Cleland [31], already referred to, show that in real cases linear plots are most likely to be found.

The upper part of this scheme is the same as the mechanism deduced for the galactosyl transferase alone (Scheme 2). In the lower pathway α-lactalbumin binds to an enzyme \(\cdot Mn^{2+} \cdot UDP-galactose\) complex prior to monosaccharide to produce an enzyme form with a higher affinity for monosaccharides. With this scheme the kinetic effects observed in the enzyme system with glucose, xylose and GlcNAc as acceptors can be explained. Thus, at low concentrations of glucose and at all concentrations of xylose, there will be very low flux through the upper pathway as the galactosyl transferase has a low affinity for these monosaccharides. The prior attachment of α-lactalbumin to the enzyme to produce an enzyme form which has a greatly increased affinity for monosaccharide produces the characteristic activation effects observed. Similarly, at very low concentrations of GlcNAc (i.e. compared with the Michaelis constant for GlcNAc in the absence of α-lactalbumin) there will be a low flux through the upper pathway, and binding of α-lactalbumin before GlcNAc will
occur in a substantial proportion of catalytic cycles, thus generating the observed type of activation. The analogy between these activation effects and those found with metal activators of certain enzymes (i.e., rapid equilibrium ordered addition of metal and first substrate with \( K_a \) effects [30]) suggests that \( \alpha \)-lactalbumin is best described as an activator of monosaccharide binding to the galactosyl transferase. At higher concentrations of GlcNAc the steady-state concentrations of the central complexes in the upper pathway will be increased (E\( \cdot \)Mn\(^{2+}\)\cdot UDP-galactose\cdot GlcNAc and E\( \cdot \)MnUDP\cdot N-acetyl-lactosamine). It is proposed that \( \alpha \)-lactalbumin then acts as a dead-end inhibitor by forming complexes with one or both of these intermediate forms, giving inhibition patterns which are uncompetitive with respect to UDP-galactose and Mn\(^{2+}\) [10] (Fig.26). The non-linear nature of this inhibition (Fig.27) is consistent with the presence of alternative pathways for \( \alpha \)-lactalbumin in the reaction scheme. It is interesting to note that Andrews has observed that the activation and inhibition of \( N \)-acetyl lactosamine synthesis by \( \alpha \)-lactalbumin is extremely sensitive to temperature [34]. At 25 °C, inhibition but not activation by \( \alpha \)-lactalbumin is observed, which appears to be uncompetitive with respect to GlcNAc as well as to the other substrates. At 37 °C, activation or inhibition effects are observed which are dependent on the concentration of GlcNAc as reported here. This phenomenon can be explained on the basis of our proposed mechanism, if it is assumed that \( \alpha \)-lactalbumin can attach to and be released from E\( \cdot \)Mn\(^{2+}\)\cdot UDP-galactose at rates which are differently affected by temperature. If the rate of attachment of \( \alpha \)-lactalbumin to this complex is slow at the lower temperature, reaction flux through the lower pathway will be negligible compared with that through the upper pathway, and activation will not be observed. This hypothesis is quite reasonable as the interaction of \( \alpha \)-lactalbumin with the galactosyl transferase and with the complex involving GlcNAc may very well be different in nature, as suggested by the stabilisation of \( \alpha \)-lactalbumin galactosyl transferase complexes by GlcNAc [24,25]. The uncompetitive nature of the inhibition by \( \alpha \)-lactalbumin with respect to GlcNAc is not consistent with the scheme of Morrison and Ebner [10] who propose that inhibition by \( \alpha \)-lactalbumin results from its attachment to enzyme-substrate complexes other than E\( \cdot \)Mn\(^{2+}\)\cdot UDP-galactose\cdot monosaccharide. In the above scheme, dead-end inhibition resulting from attachment to the central complexes of the upper pathway would be predicted to be uncompetitive with respect to all substrates, as is indeed observed [10,25,34] (Fig.26). Although Scheme 3 is therefore consistent with the observed kinetic effects, a feature that is not entirely satisfactory is the postulation of the existence of two types of complex containing \( \alpha \)-lactalbumin and all the substrates. One of these complexes is productive and on the lower reaction pathway, whereas the other is a dead-end-inhibitory complex. Although it is conceivable that with an enzyme such as the galactosyl transferase possessing several binding sites to which substrates attach in an obligatory sequence, more than one type of complex containing the same components might exist, it should be noted that the effects of \( \alpha \)-lactalbumin are also consistent with a similar scheme (Scheme 4) which does not postulate the existence of two such complexes. In this scheme, besides acting as an activator as before, \( \alpha \)-lactalbumin can also attach to either or both of the central complexes containing all substrates or products (E\( \cdot \)Mn\(^{2+}\)\cdot UDP-galactose\cdot GlcNAc or E\( \cdot \)MnUDP\cdot N-acetyl-lactosamine), producing a new link between the alternative pathways. Activation effects will then be observed only when the attachment of \( \alpha \)-lactalbumin to E\( \cdot \)Mn\(^{2+}\)\cdot UDP-galactose is greater than that of monosaccharide, since the activation by \( \alpha \)-lactalbumin results from its increasing the affinity for monosaccharide. With glucose and xylose as substrates and with GlcNAc at very low concentrations (i.e., relative to the Michaelis constant), the predominant pathway is the unbranched lower one,

\[ \text{Scheme 4} \]

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and activation by α-lactalbumin is observed. At higher concentrations of GlcNAc, the concentration of E·Mn$^{2+}$·UDP-galactose·GlcNAc becomes much greater than that of E·Mn$^{2+}$·UDP-galactose·α-
lactalbumin. α-Lactalbumin and galactosyl trans-
ferase form tight complexes in the presence of
GlcNAc [24,25] and so it can be postulated that
α-lactalbumin will have a high affinity for the E·Mn$^{2+}$
·UDP-galactose·GlcNAc complex. The predominant
pathway in the general mechanism in Scheme 4 will
then be that given in Scheme 5. As α-lactalbumin will
no longer activate the forward reaction in the
sense of increasing the affinity for GlcNAc, its effects
will be those of a product inhibition. The release of
α-lactalbumin occurs between two irreversible steps
in the reaction pathway (release of N-acetyl lactos-
amine and MnUDP at zero concentrations), and so
it will act as an uncompetitive inhibitor with respect
to all substrates. It should be noted that if the inter-
conversion of the central complexes in the scheme is
essentially irreversible, uncompetitive inhibition
would be given by α-lactalbumin even if the order
of release of N-acetyl lactosamine and α-lactalbumin
were reversed. Activation and inhibition by α-lact-
albumin will depend on its rates of attachment to
and detachment from E·Mn$^{2+}$·UDP-galactose rela-
tive to those of GlcNAc, and it is therefore not sur-
prising that at high α-lactalbumin concentrations,
inhibition starts to reserve (Fig.26 and 27). The
disappearance of the activation with respect to Glc-
NAc at temperatures below 25°C [34] can be attrib-
uted as before to different effects of temperature on
the attachment of α-lactalbumin and GlcNAc to the
enzyme complexes.

It is clear from the effects of substrates and
products on the stabilisation of complexes between
galactosyl transferase and α-lactalbumin-Sepharose,
that a range of complexes can be formed between
the two proteins [24,25,35]. The observation [10] that
α-lactalbumin increases the substrate inhibition by
glucose and GlcNAc can be attributed to the forma-
tion of dead-end complexes such as E·MnUDP·
GlcNAc·α-lactalbumin, and this feature is included
in Scheme 3.

Although we propose two different though related
explanations for the inhibitory effects of α-lactalbumin
with GlcNAc as substrate, the activation by
α-lactalbumin with three monosaccharide acceptors
can be reasonably explained only on the basis of its
attachment to the enzyme before monosaccharides.

For lactose synthesis in the presence of α-lact-
albumin, an insignificant proportion of reaction flux
proceeds through the upper pathway, and the rate
equation for the system can be derived by considering
it as an ordered quad-ter system (Scheme 6) in which
the attachment of α-lactalbumin to both the EAB
and ER complexes is at thermodynamic equilibrium.
The rate equation for this mechanism was derived
by the method of King and Altman [36], and is given
in Eqn (12). Twelve terms are missing from the
denominator because of the restriction that the inter-
actions of C with EAB and ER are at equilibrium.

The constants in Eqn (12) are defined in terms
of the rate constants of Scheme 6 (see page 557).

\[ V \equiv \frac{1}{K_{1a} K_{1b} K_{1c} K_{d} + \frac{K_{1b} K_{1c} K_{d}}{V_{c} K_{d}} + \frac{K_{1c} K_{d}}{V_{c} K_{d}}} \]

\[ + \frac{K_{1a} K_{b}}{V_{1} V_{c}} + \frac{K_{b}}{V_{1} V_{c}} + \frac{K_{a}}{V_{1} V_{c}} + \frac{1}{V_{1}} \]  

If values from Table 2 are taken such that $K_{1b} = 1.42 \text{mM}, K_{1c} = 0$ and $K_{a}/D = 0.0083$
then the rate equation for the system in the absence
of products at $A = 10 \text{mM (Mn}^{2+})$ becomes

\[ V = \frac{K_{1c} K_{d}}{V_{1} V_{c}} + \frac{1.14 K_{b}}{K_{d} B} + \frac{K_{d}}{D} + 1 \]  

True values for the kinetic constants together with
their experimentally determined apparent values can
then be extracted from the following equations.

For variation of $B$ and $C$,

\[ \text{apparent } K_{b} = \frac{1.14 K_{b}}{1 + K_{d}/D} \]

\[ \text{apparent } K_{d} = K_{1c} K_{d} (D + K_{d}) \]

For variation of $B$ and $D$,

\[ \text{apparent } K_{b} = 1.14 K_{b} \]

\[ \text{apparent } K_{d} = K_{d} (1 + K_{1c}/C) \]

\[ v = \frac{V_1 (ABCD - PQR/K_{eq})}{K_{1a}K_{1b}K_{1c}K_{d} + K_{1a}K_{1b}K_{2}A + K_{1a}K_{2}AB + K_{1a}K_{1b}K_{2}CD + K_{1a}K_{2}ABC + K_{1b}ACD + K_{1b}BACD + ABCD + K_{1a}K_{b}ODR/K_{tr} + K_{1a}K_{b}BCDR/K_{tr} + K_{1a}K_{1b}K_{1c}BPQR} \]

\[ + \frac{K_{1a}K_{b}CDQR}{K_{1a}K_{tr}} + \frac{K_{b}ABCD}{K_{1a}K_{1b}K_{1c}} + \frac{K_{1a}K_{b}CDQR}{K_{1a}K_{tr}} + \frac{K_{1a}K_{b}CDPQR}{K_{1a}K_{tr}} + \frac{K_{1a}K_{b}ABCPQ}{K_{1a}K_{1b}K_{1c}} + \frac{K_{1a}K_{b}ABCD}{K_{1a}K_{1b}K_{1c}K_{tr} + K_{1a}K_{1b}K_{1c}K_{tr}} + \frac{K_{1a}K_{1b}K_{1c}BPQR}{K_{1a}K_{1b}K_{1c}K_{tr}} \]

\[ + \frac{V_1}{V_2 K_{eq}} \left( K_{1a}K_{tr} + K_{1b}PQ + K_{2}QR + PQR + K_{tr}APQ/K_{1a} + K_{tr}ABPQ/K_{1a}K_{1b} + K_{tr}ABCDPQ/K_{1a}K_{1b}K_{1c}K_{tr} + K_{tr}ABCDPQ/K_{1a}K_{1b}K_{1c}K_{tr} + K_{tr}ABCDPQ/K_{1a}K_{1b}K_{1c}K_{tr} + K_{tr}ABCDPQ/K_{1a}K_{1b}K_{1c}K_{tr} \right). \]
Table 5. Apparent and true values for kinetic constants associated with human lactose synthetase

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Kinetic constant</th>
<th>Pair of substrates varied</th>
<th>Apparent value</th>
<th>True value</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mn²⁺</td>
<td>( K_{i\text{a}} )</td>
<td>B and C</td>
<td>0.14</td>
<td>0.135</td>
<td>0.16</td>
</tr>
<tr>
<td>UDP-galactose</td>
<td>( K_{i\text{b}} )</td>
<td>B and D</td>
<td>0.21</td>
<td>0.184</td>
<td>0.16</td>
</tr>
<tr>
<td>( \alpha )-Lactalbumin</td>
<td>( K_{i\text{c}} )</td>
<td>C and D (glucose or xylose)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Glucose</td>
<td>( K_{i\text{d}} )</td>
<td>B and D</td>
<td>14.9</td>
<td>2.31</td>
<td>2.1</td>
</tr>
<tr>
<td>Xylose</td>
<td>( K_{i\text{d}} )</td>
<td>C and D</td>
<td>1.4</td>
<td>1.87</td>
<td>2.1</td>
</tr>
<tr>
<td>NleNAc</td>
<td>( K_{i\text{d}} )</td>
<td>C and D</td>
<td>81</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\( a \) As this is the dissociation constant of the \( E \cdot Mn²⁺ \) complex, it is assumed to be the same in the presence and absence of \( \alpha \)-lactalbumin.

\( b \) At 10 mM Mn²⁺.

For variation of \( C \) and \( D \),

\[
\text{apparent } K_{i\text{c}} = K_{i\text{c}}/(1 + 1.14 K_{i\text{b}}/B).
\]

The true and apparent values for these kinetic constants are given in Table 5.

Although good evidence is available for the order of attachment of the substrates to the enzyme, and the inhibition patterns indicate that MnUDP is the last substrate to be released in the catalytic cycle, no evidence is available on the order of release of \( \alpha \)-lactalbumin and disaccharide from the enzyme. They have been provisionally ordered in our scheme to be symmetrical with the substrate attachment, but could equally well be released in a reverse or random order.

Our scheme contrasts with that of Morrison and Ebner [10] who carried out their kinetic studies under conditions where asymmetrical intersecting reciprocal plots are not obtained with \( \alpha \)-lactalbumin and disaccharides from the enzyme. As the kinetic effects of \( \alpha \)-lactalbumin are best interpreted in terms of rapid equilibrium binding, it would seem that the increased affinity for monosaccharides following the attachment of \( \alpha \)-lactalbumin, probably does not result from a conformational change in the galactosyl transferase. Such a change would be expected to be slow relative to other steps in the reaction mechanism and would not appear as a rapid equilibrium process. Instead, it can be suggested that the role fulfilled by \( \alpha \)-lactalbumin is to contribute directly to a binding site for monosaccharides in the enzyme \( \cdot Mn²⁺ \cdot UDP-galactose \cdot \alpha \)-lactalbumin complex. The structural similarity of \( \alpha \)-lactalbumin to lysozyme [8], an enzyme which possesses binding sites for monosaccharides in a surface cleft region, suggests a way in which this can come about. The attachment of \( \alpha \)-lactalbumin to the enzyme \( \cdot Mn²⁺ \cdot UDP-galactose complex, perhaps in a manner

similar to the binding of glycoprotein substrates, could bring a surface area of $\alpha$-lactalbumin into proximity with the monosaccharide-binding site of the enzyme. If such a region on $\alpha$-lactalbumin can provide extra interactions for glucose and other monosaccharides, the observed effects would logically follow. Comparative sequence studies of $\alpha$-lactalbumins have provided indirect evidence for a role in a region corresponding to the lysozyme cleft

**Interspecies Differences in the Activity of $\alpha$-Lactalbumin**

From the kinetic data, evaluated using the rate equation derived for the lactose synthetase system, kinetic parameters associated with the effects of $\alpha$-lactalbumin can be determined. These are $K_{ic}$, defined as the dissociation constant of $\alpha$-lactalbumin from the $E \cdot Mn^{2+} \cdot UDP$-galactose $\cdot \alpha$-lactalbumin complex, $K_e$, the Michaelis constant for $\alpha$-lactalbumin (zero for human and bovine $\alpha$-lactalbumin) and $K_d$, the Michaelis constant for glucose in the presence of saturating levels of $\alpha$-lactalbumin. Table 6 summarises the values of these parameters. The value of $K_d$ shows little variation with $\alpha$-lactalbumin from different species, whereas $K_{ic}$ (which can be determined directly from Fig.31) shows a much greater degree of interspecies variation. The value of this parameter for human $\alpha$-lactalbumin is not much changed when xylose replaces glucose as substrate, confirming our evaluation of the system (see Table 5). Guinea-pig $\alpha$-lactalbumin has a somewhat higher value for this parameter than the other $\alpha$-lactalbumins (Table 6), and a finite value for $K_e$ is obtained with the guinea-pig and pig proteins, suggesting that the attachment of these $\alpha$-lactalbumins to the intermediate enzyme-substrate complex is not at thermodynamic equilibrium.

Although detailed studies were not carried out with bovine galactosyl transferase, it appears that the differences between the $\alpha$-lactalbumins with respect to activity with human galactosyl transferase are also found with the bovine enzyme. It is reasonable to suggest therefore that the differences do not reflect mutual adaptation between the lactose synthetase components in a particular species, but instead are a measure of intrinsic differences in the activities of the different $\alpha$-lactalbumins.

The apparent maximum velocity obtained for the different $\alpha$-lactalbumins with human galactosyl transferase, cannot at present be corrected to true values as this would necessitate determining the kinetic constants for $Mn^{2+}$ and UDP-galactose with each $\alpha$-lactalbumin. However the value of this parameter appears to be related to the lactose content of the milk from which the protein is derived (Table 6). The concentrations of the $\alpha$-lactalbumins in milk (and presumably also in the Golgi region of the cell which is the site of lactose synthesis [7]) are much greater than their $K_{ie}$ values (e.g. human $\alpha$-lactalbumin is present at a concentration of 1.3 mg/ml in milk and has a $K_{ie}$ value of 0.415 mg/ml; guinea pig $\alpha$-lactalbumin is present in a concentration of 5 mg/ml and has a $K_{ie}$ value of 1.1 mg/ml). As previously discussed, near-zero values of the Michaelis constant will mean that the enzyme is saturated with respect to $\alpha$-lactalbumin at the site of lactose synthesis. It appears possible therefore that the rates of lactose synthesis in different species may to some extent be controlled by the nature of the $\alpha$-lactalbumin which they produce although this probably does not apply to species with little or no lactose where a low $\alpha$-lactalbumin concentration appears to be the controlling factor [39].

Measurement of the value of $K_{ie}$ under different conditions should give some indication of the physical nature of the interaction between the galactosyl transferase and $\alpha$-lactalbumin in the catalytic mechanism. Such studies are in progress in this laboratory.

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**Table 6. A comparison of the kinetic constants associated with different $\alpha$-lactalbumins for lactose synthesis with human galactosyl transferase**

The kinetic parameters are all apparent values obtained from computer analyses of the data presented in Fig. 19, 23, 28, 29, 30. As the concentrations of the fixed substrates, $Mn^{2+}$ and UDP-galactose, are close to saturating, the corrections to true values will be very small. This particularly applies to the parameter $K_{ic}$ (see text)

<table>
<thead>
<tr>
<th>$\alpha$-Lactalbumin</th>
<th>$K_e$</th>
<th>$K_{ic}$</th>
<th>$K_d$</th>
<th>$V_i$</th>
<th>Lactose in milk [38]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>0</td>
<td>0.029</td>
<td>1.4</td>
<td>6.29</td>
<td>7.0</td>
</tr>
<tr>
<td>Bovine</td>
<td>0</td>
<td>0.036</td>
<td>0.89</td>
<td>4.49</td>
<td>4.4</td>
</tr>
<tr>
<td>Pig</td>
<td>0.0004</td>
<td>0.034</td>
<td>0.87</td>
<td>4.23</td>
<td>3.8</td>
</tr>
<tr>
<td>Guinea-pig</td>
<td>0.0026</td>
<td>0.076</td>
<td>0.71</td>
<td>3.32</td>
<td>2.8</td>
</tr>
</tbody>
</table>

* Obtained from an experiment with xylose as substrate (Fig.23).
REFERENCES