On the Interaction of \( \alpha \)-Lactalbumin and Galactosyltransferase during Lactose Synthesis*

(Received for publication, February 3, 1975)

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The regulatory effect of \( \alpha \)-lactalbumin in the lactose synthase system has been ascribed to its reversible association with a complex of galactosyltransferase with \( \text{Mn}^{2+} \) and UDP-galactose, prior to the binding of monosaccharides; the resulting complex has a higher affinity for various monosaccharides. Two steps in the postulated catalytic cycle have been investigated; UDP-galactose binding to enzyme \( \cdot \text{Mn}^{2+} \) by equilibrium dialysis and \( \alpha \)-lactalbumin binding to enzyme \( \cdot \text{Mn}^{2+} \cdot \text{UDP-galactose} \) by sedimentation velocity and kinetics.

There is a single binding site for UDP-galactose on the enzyme \( \cdot \text{Mn}^{2+} \) complex, and the dissociation constant for UDP-galactose from enzyme \( \cdot \text{Mn}^{2+} \cdot \text{UDP-galactose} \) was found to be 72 \( \mu \text{M} \) at 37°. The formation of a complex between galactosyltransferase and \( \alpha \)-lactalbumin in the presence of \( \text{Mn}^{2+} \) and UDP-galactose was observed as an increase in sedimentation coefficient \( s_{w} \) from 3.25 \pm 0.03 in the absence of \( \alpha \)-lactalbumin to 4.22 \pm 0.03 at saturating concentrations of \( \alpha \)-lactalbumin, a value closely similar to that of a cross-linked 1:1 complex of the proteins under the same conditions (4.35 \pm 0.03). No interaction was observed in the absence of substrates or with UDP-galactose and EDTA.

From the ultracentrifuge data and steady state kinetics, dissociation constants for \( \alpha \)-lactalbumin from the enzyme \( \cdot \text{Mn}^{2+} \cdot \text{UDP-galactose} \cdot \alpha \)-lactalbumin complex were determined at several temperatures and salt concentrations. These showed good internal agreement. The free energy change \( \Delta G^{\circ} \) for the association of the two proteins is calculated, and the results are discussed in relation to the nature of the interaction.

Interaction of the two components of lactose synthase (EC 2.4.1.22), \( \alpha \)-lactalbumin (1) and galactosyltransferase (2), is required for significant catalysis of lactose synthesis at physiological concentrations of glucose. In isolation, the galactosyltransferase catalyzes the transfer of galactose from UDP-galactose to free or protein-bound N-acetylglucosamine (2). Transfer to free GlcNAc is inhibited by \( \alpha \)-lactalbumin at monosaccharide concentrations above 4 mM (2). The mechanism of the reactions catalyzed by galactosyltransferase in the absence of \( \alpha \)-lactalbumin, appears to involve an ordered sequential addition of the substrates \( \text{Mn}^{2+} \), UDP-galactose, and monosaccharide, and an ordered release of products: disaccharide and MnUDP (3, 4). Considerable evidence indicates that the binding of \( \alpha \)-lactalbumin to the galactosyltransferase is consequent on the prior attachment of a substrate to the latter, for example \( \alpha \)-lactalbumin can bind to galactosyltransferase in the presence of 0.1 mM glucose, millimolar concentrations of GlcNAc, or micromolar concentrations of UDP-galactose in the presence of \( \text{Mn}^{2+} \) (5–9). Whereas it was previously suggested that during the course of lactose synthesis, \( \alpha \)-lactalbumin attaches to a complex of galactosyltransferase with all substrates (i.e. \( E \cdot \text{Mn}^{2+} \cdot \text{UDP-galactose} \cdot \text{glucose} \)) (10), more recent kinetic evidence indicates that \( \alpha \)-lactalbumin attaches to a complex of galactosyltransferase with \( \text{Mn}^{2+} \) and UDP-galactose alone (i.e. \( E \cdot \text{Mn}^{2+} \cdot \text{UDP-galactose} \)) (4). The effect of the attachment of \( \alpha \)-lactalbumin is to increase the affinity of this complex for monosaccharides (4), a proposal that is supported by the kinetic properties of a 1:1 complex of galactosyltransferase and \( \alpha \)-lactalbumin covalently cross-linked with dimethyl pimelimidate (11). Inhibition by \( \alpha \)-lactalbumin of galactose transfer to free GlcNAc appears to result from the combination of \( \alpha \)-lactalbumin with an \( E \cdot \text{Mn}^{2+} \cdot \text{UDP-galactose-GlcNAc} \) or an \( E \cdot \text{MnUDP-N-acetylactosamine} \) complex (4).

The attachment of \( \alpha \)-lactalbumin to the galactosyltransferase \( \cdot \text{Mn}^{2+} \cdot \text{UDP-galactose} \) complex appears to be at thermodynamic equilibrium during the catalytic action of the enzyme system (4, 12), and from the steady state kinetic data an equilibrium dissociation constant for the reaction

\*The use of \( E \) in an enzyme-substrate complex refers to galactosyltransferase.
can be derived. The value of this parameter at pH 7.4 and 37° is 25.5 μM for the bovine colostrum galactosyltransferase-bovine α-lactalbumin system (12). The values for other α-lactalbunins with human galactosyltransferase have also been determined (4).

We report here the investigation of two thermodynamic equilibria involved in the lactose synthase catalytic cycle, using homogenous bovine colostrum galactosyltransferase. The binding of UDP-galactose, at near saturating concentrations of Mn²⁺, to galactosyltransferase was determined by equilibrium dialysis. The binding of bovine α-lactalbumin to the complex of enzyme with Mn²⁺ and UDP-galactose has been studied by sedimentation velocity experiments as well as by steady state kinetics. The effect of ionic strength on the latter reaction is reported, and the nature of the interaction of the two proteins is discussed.

**EXPERIMENTAL PROCEDURE**

**Materials**—Unpasteurized bovine milk was obtained from a local dairy and bovine colostrum from the Department of Agriculture, University of Florida, Gainesville. UDP-[¹⁴C]galactose was purchased from Amerham/Searle, Arlington Heights, Illinois; UDP-galactose, GlcNAc, and sodium cacydate from Sigma, and AG X-8 from Bio-Rad Laboratories. All other reagents, of the best available grade, were purchased from J. T. Baker Chemical Co. and Mallinckrodt Chemical Works.

α-Lactalbumin was prepared from bovine milk, and galactosyltransferase from bovine colostrum as previously described (12). The purity of both proteins was checked by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

**Equilibrium Dialysis**—Equilibrium dialysis studies were performed in screw-cap tubes (20 ml) attached to a rotating wheel. Enzyme (0.2 mg in 1 ml) was dialyzed against 15 ml of 0.1 M NaCl containing 25 mM cacydate (pH 7.4), 10 mM MnCl₂, and eight concentrations of UDP-[¹⁴C]galactose (20 to 80 μM), specific activity 5 × 10⁸ cpm/μmol, for 24 hours. UDP-galactose concentrations were determined in triplicate by scintillation counting of 0.2-ml aliquots. Enzyme concentrations were measured at the beginning and termination of dialysis by assay.

**Ultracentrifugation**—Sedimentation coefficients were determined using a separation cell in the An-H rotor of a Beckman model E analytical ultracentrifuge. Centrifugation was carried out at speeds of 56,000 and 40,000 × g for periods of 10 min to 1 hour. Both α-lactalbumin and enzyme solutions were previously dialyzed against 0.1 M NaCl buffered with 25 mM sodium cacydate, pH 7.4, the former protein being contained in acetylated dialysis tubing to minimize losses. Concentrations of the proteins were determined by absorbance at 280 nm and enzyme activity, respectively.

After centrifugation, the cell was removed from the rotor and material on both sides of the barrier, as well as the original solution, were assayed for lactose synthase in the presence of additional α-lactalbumin (see "Results and Discussion"). Initial velocities were measured as described previously (4), except that 20 mM sodium cacydate, pH 7.4, was substituted for 3-(N-morpholino)-propane sulfonate buffer. These assays were performed in triplicate to give a maximum error of 1.5%, and sedimentation coefficients were calculated from an average of three separate ultracentrifugations (for different times and rotor speeds) and reported as s₂₀ₑₓ.

**Preparation of Cross-Linked Lactose Synthase Complex**—Covalent cross-linking of colostrum galactosyltransferase and α-lactalbumin (bovine) was performed essentially as previously described (11) except that dimethylsuberimidate was substituted for the pimelimidate as the bifunctional reagent, and 0.1 M borate, pH 8.1, replaced triethanolamine as the buffer. Cross-linking was allowed to proceed for 2 hours at 22° in the presence of 3 mM MnCl₂, 0.3 mM UDP-galactose, and 105 μM α-lactalbumin. The reaction was terminated by the addition of 0.2 volume of 0.5 M glucose, and the cross-linked lactose synthase was isolated by gel filtration on a column of Bio-Gel P150 (1.5 × 87 cm) followed by affinity chromatography with α-lactalbumin-Sepharose (11).

**RESULTS AND DISCUSSION**

**Binding of UDP-galactose**

According to previous kinetic studies of galactosyltransferase, Mn²⁺ is an obligatory first binding substrate to the enzyme, whose attachment directly precedes that of UDP-galactose (4, 19). For the homogenous galactosyltransferase from human milk or bovine colostrum, with which these studies were performed, the role of Mn²⁺ in the reaction appears from steady state kinetics not to be as simple as was initially suggested (10, 13), and there is a possibility that two manganese ions may attach to the enzyme during the catalytic cycle. While studies are currently in progress to clarify this, we have chosen for the purpose of the current study to work at a concentration of Mn²⁺ sufficient to saturate most of the enzyme. The dissociation constant for the Mn²⁺ complex of bovine colostrum galactosyltransferase is 0.95 mM (12), and the concentration of Mn²⁺ used (10 mM) is sufficient to give more than 90% saturation. The interpretation of the results must take this into consideration, as well as the fact that the dissociation constant for MnUDP-galactose is 7.5 mM (4), although the enzyme may not discriminate between free UDP-galactose and its Mn²⁺ complex as substrates (see Ref. 4 for discussion). The studies of UDP-galactose binding are obviously a prerequisite to the studies with ligands binding after UDP-galactose in the proposed reaction mechanism.

The results of the equilibrium dialysis studies of the binding of UDP-[¹⁴C]galactose to galactosyltransferase (0.2 mg/ml) in 25 mM sodium cacydate buffer, pH 7.4, containing 10 mM MnCl₂ and 0.1 M NaCl at 4°, 20°, and 37° are summarized in Table I. A Van’t Hoff plot yields the additional information to be obtained from the experiments which is a value of 4.9 kca/mol for the free energy change for the dissociation of UDP-galactose from E·Mn²⁺·UDP-galactose (Fig. 1). Also, at each temperature a Scatchard plot for the binding of UDP-galactose to E·Mn²⁺ can be constructed (Fig. 2) to give a value for the number of binding sites for the substrate per molecule of enzyme. The results (0.85 at 4°, 0.78 at 20°, and 0.75 at 37°) are only consistent with the presence of a single binding site, and the rather low values may be attributed to the inaccuracies inherent in the measurements at the relatively low enzyme concentrations used, and also perhaps to incomplete saturation of the enzyme with Mn²⁺.

**Binding of α-Lactalbumin to E·Mn²⁺·UDP-galactose**

**From Kinetics**—Initial velocity data at varying concentrations of glucose and α-lactalbumin can be interpreted to yield a dissociation constant for α-lactalbumin from the E·Mn²⁺·UDP-galactose α-lactalbumin complex. The value previously obtained with bovine colostrum galactosyltransferase at 37° was 25.5 μM (12). Similar initial velocity experiments were performed at three lower temperatures (27°, 17.5°, and 10.7°), and as the same characteristic kinetic patterns were obtained (i.e., the presence of slope but not intersect effects in double reciprocal plots of initial velocity and glucose concentration at a series of fixed concentrations of α-lactalbumin) they were interpreted in the same way, to give the corresponding dissociation constants at the lower temperatures (Table II). As the binding of UDP-galactose is tighter at these lower temperatures, it is valid to assume that the dissociation constant for α-lactalbumin need not be corrected for UDP-galactose concentration. That such a correction is unnecessary at 37° has been previously demonstrated (4). The plot of logarithm of the
TABLE I

Values for dissociation constant of UDP-galactose from enzyme-Mn\(^{2+}\)-UDP-galactose complex

| Temperature | Dissociation constant (μM) | Kinetics |
|-------------|--------------------------|---------|
| 28°         | 22                       | N.D.*  |
| 20°         | 43                       | N.D.*  |
| 37°         | 72                       | 0°      |

* N.D., not determined.

 Conditions were similar to the equilibrium dialysis experiments, except for the absence of NaCl. The zero value comes from an observation of parallel lines in a double reciprocal plot for initial velocity and UDP-galactose concentration at different fixed concentrations of GlcNAc (12).

Fig. 1. Van’t Hoff plot for the binding of UDP-galactose to bovine colostrum galactosyltransferase, at 10 mM MnCl\(_2\). Data taken from Table I.

Fig. 2. Scatchard plot for the binding of UDP-galactose to bovine galactosyltransferase at 37°. Data obtained by equilibrium dialysis in the presence of 10 mM MnCl\(_2\), 0.1 M NaCl, and 25 mM cacodylate buffer (pH 7.4).

dissociation constant \(K_d\) (kinetic parameter \(K_{ic}\) (4, 12)) against \(1/T\) (Fig. 3) with a value of ~6.5 kcal/mol for the free energy change, \(\Delta G^\circ\), of the association of \(\alpha\)-lactalbumin with the \(E\cdot\text{Mn}^{2+}\cdot\text{UDP-galactose}\) complex.

**From Sedimentation Velocity Studies**—Protein-protein interactions may be studied by a variety of physicochemical techniques, provided that the interacting system is at equilibrium and that the technique used does not cause a deformation of equilibrium position. Thus sedimentation velocity experiments may be used to study the interaction of galactosyltransferase and \(\alpha\)-lactalbumin, shown below.

**Fig. 3.** Van’t Hoff plots for the binding of bovine \(\alpha\)-lactalbumin to bovine colostrum galactosyltransferase, in the presence of 10 mM MnCl\(_2\) and 0.3 mM UDP-galactose. ○ results from kinetics; ● results from sedimentation velocity experiments in the presence of 0.1 M NaCl.

\[ E\cdot\text{Mn}^{2+}\cdot\text{UDP-galactose} + \alpha\text{-lactalbumin} \]

\[ \Rightarrow E\cdot\text{Mn}^{2+}\cdot\text{UDP-galactose} \cdot \alpha\text{-lactalbumin} \]

In such experiments, the equilibrium may be perturbed by concentration and pressure effects. The first of these may be eliminated by the use of very low concentrations of enzyme in a separation cell, and the significance of the second, which is concomitant with a change of molar volume of the complex with respect to reactants, may be determined by the method used to evaluate the sedimentation coefficient, i.e. variation of the average rotor speed during the sedimentation run. In the present case, pressure effects proved insignificant.

The use of the separation cell has been described by Yphantis and Waugh (14). The calculation of sedimentation coefficients rests on the distribution of enzyme above the partition after centrifugation compared with the original enzyme solution and the integral of the square of the angular velocity time profile.

\[ s = \frac{-1}{2a^2 t} \ln \left[ \frac{x_0^2}{x_p^2} + \frac{v_C}{C_0} \left(1 - \frac{x_0^2}{x_p^2}\right) \right] \] (1)
where $x_p$ and $x_0$ refer to the distances from the center of the rotor to the partition and meniscus, respectively; $C_s$ and $C_o$ are the concentrations of enzyme of the initial solution and the upper solution at the conclusion of the run.

The concentration of galactosyltransferase required for accurate assay is 1 to 2 µg/ml, permitting the use of concentrations in the range 2 to 5 µg/ml in the centrifuge cell. Several runs are made for the determination of each sedimentation coefficient; these may be made at different speeds or for different periods of time. If the equilibrium being studied is pressure-dependent, i.e. has a molar volume change, the sedimentation coefficient at a fixed concentration of reactants would vary. (For different length runs this arises because the ratio of run time to deceleration time varies.)

**Assay Conditions**

Unfortunately it is not possible to determine free galactosyltransferase separately by its ability to utilize GlcNAc as a substrate, since the synthesis of N-acetyllactosamine is inhibited by α-lactalbumin at most GlcNAc concentrations. Enzyme concentration was therefore determined by lactose synthase activity. The concentration of α-lactalbumin in the upper solution in the centrifuge cell will have diminished compared to the original solution. The low sedimentation coefficient of bovine α-lactalbumin 1.78 S at pH 7.4 (cf. Ref 15) results in a much smaller change in α-lactalbumin concentration, $C_t/C_o 0.85$ to 0.95, compared to lactose synthase activity, $C_t/C_s 0.5$ to 0.8. Aliquots of 20 µl of enzyme solution were assayed for lactose synthase in the presence of added α-lactalbumin at 400 µg/ml; also added were MnCl$_2$ to 10 mM, UDP-galactose to 0.3 mM, glucose to 10 mM, and bovine serum albumin to 0.1%. The extremes of α-lactalbumin concentration used in the sedimentation velocity experiments, 50 µg/ml to 1600 µg/ml, result in concentration variations of α-lactalbumin of 1.5 in 410 µg/ml and 50 in 1920 µg/ml, respectively. The plot of lactose synthase activity against α-lactalbumin concentration (Fig. 4) shows that these concentration variations will give errors of less than 1% in the determination of lactose synthase activity.

The sedimentation coefficient of free galactosyltransferase $S_{st}$ may be determined in the absence of α-lactalbumin. The value of the sedimentation coefficient of the α-lactalbumin complex $S_{ct}$ can be determined by extrapolation of the graph of $S_{app}$ against α-lactalbumin concentration; although, as is evident from Fig. 5, virtually all of the galactosyltransferase is complexed at an α-lactalbumin concentration of 800 µg/ml at 16°.

Thus at an intermediate concentration of α-lactalbumin $S_{st}$ and $S_{ct}$ may be substituted in Equation 1 to yield theoretical values of the concentration factors $C_t$ and $C_o$. If the equilibrium is described correctly as

$$E.	ext{Mn}^{2+}.	ext{UDP-galactose} + \alpha\text{-lactalbumin}$$

The dissociation constant $K_d$ of the complex is given by

$$K_d = \frac{[\alpha\text{-lac}] [C_t/C_o - C_{15}]}{C_t - C_t/C_o}$$

The evaluation of an invariant $K_d$ at different concentrations of α-lactalbumin verifies that Equation 2 is an accurate description of the equilibrium, and yields the data for the corroborating Scatchard plot shown in Fig. 6.

$S_{st}$ and $S_{ct}$ may be measured at any temperature accessible to the ultracentrifuge, permitting the evaluation of $K_d$ at different temperatures, and the composition of a Van’t Hoff plot to yield a value for $\Delta G^\circ$. Since accessible temperatures are in the range of 0-25° the plot has to be extrapolated to obtain $K_d$ at 37°, and this dissociation constant can then be compared with that obtained from kinetic studies.

Although our sedimentation studies were performed at a high concentration of UDP-galactose relative to its dissociation constant from $E.	ext{Mn}^{2+}.	ext{UDP-galactose}$ (300 µM:43 FM for $K_d$ at 20°), the enzyme cannot be regarded as being at saturation with respect to this substrate, and the measured association constant for α-lactalbumin must be corrected as shown below.

For the ordered binding of UDP-galactose (A) and α-lact-
bimun (B) to enzyme saturated with Mn2+ (EM), the following two equilibria must be considered:

\[ \text{EM} + A \rightleftharpoons \text{EMA} \quad (3) \]

and

\[ \text{EMA} + B \rightleftharpoons \text{EMAB} \quad (4) \]

where \( K_d^A \) and \( K_d^B \), the respective dissociation constants, are defined as:

\[ K_d^A = \frac{[\text{EM}][A]}{[\text{EMA}]} \quad \text{and} \quad K_d^B = \frac{[\text{EMA}][B]}{[\text{EMAB}]} \]

The apparent dissociation constant for \( \alpha \)-lactalbumin (\( K_d^{\text{app}} \)) determined from sedimentation velocity studies at nonsaturating concentrations of \( \alpha \)-lactalbumin will be

\[ K_d^{\text{app}} = \frac{[\text{EM}][A]}{[\text{EMA}][B]} \]

Now \( [\text{EMA}] = K_d^{B} [\text{EMAB}]/[B] \)

and \( [\text{EM}] = K_d^{A} [\text{EMA}][A] = K_d^{A} B [\text{EMAB}][A]/[A] \)

so \( K_d^{\text{app}} = \frac{[B]}{[\text{EMA}] K_d^{A} + K_d^{B} [\text{EMAB}] + K_d^{B} [\text{EMAB}]} \)

\[ = K_d^{B} \left( \frac{K_d^{A}}{[A]} + 1 \right) \]

or \( K_d^{B} = \frac{K_d^{\text{app}} K_d^{A}}{[A] + 1} \)

Sedimentation velocity ultracentrifugation, using an ultraviolet scanner, has been used previously to demonstrate complex formation between galactosyltransferase and \( \alpha \)-lactalbumin, in the presence of either UDP-galactose or GlcNAc (8). This previous study utilized a multicomponent galactosyltransferase from bovine milk, and while a progressive increase in sedimentation coefficient of enzyme with increasing concentration of \( \alpha \)-lactalbumin was observed in the presence of GlcNAc, no quantitative data with respect to dissociation constants were presented.

Our previous ultracentrifuge studies, performed in the presence of 5 mM GlcNAc, gave a value of 3.3 for the sedimentation coefficient (\( s_{20,w} \)) of bovine colostrum galactosyltransferase. The sedimentation coefficient determined in the presence of 10 mM MnCl2 and 0.3 mM UDP-galactose in the present work is very similar \( s_{20,w} 3.25 \). The sedimentation coefficient of the enzyme, determined as lactose synthase activity, increases hyperbolically with \( \alpha \)-lactalbumin concentration to reach a plateau at 400 \( \mu \)g/ml of \( s_{20,w} 4.22 \). The \( s_{20,w} \) was not changed significantly at 1600 \( \mu \)g/ml. The saturation curve (Fig. 5) was determined at a constant temperature of 16°C. Sedimentation coefficients for the free enzyme (zero concentration of \( \alpha \)-lactalbumin) and enzyme \( \alpha \)-lactalbumin complex (800 \( \mu \)g of \( \alpha \)-lactalbumin/ml) were also determined as \( s_{20,w} \) at a series of temperatures (23.3°C, 20°C, 12°C, and 8°C), and were in all cases 3.25 ± 0.03 and 4.22 ± 0.03, respectively. As a check on the validity of these determinations, a sedimentation velocity experiment was performed under the same conditions (0.3 mM 1UDP-galactose, 400 \( \mu \)g/ml of \( \alpha \)-lactalbumin) except that 20 mM EDTA was substituted for MnCl2, and a value of 3.27 was obtained for \( s_{20,w} \). This is an interesting confirmation of the ordered binding of Mn2+ and substrates to the enzyme, and strengthens the validity of our methods.

A 1:1 cross-linked complex of \( \alpha \)-lactalbumin and galactosyltransferase was prepared using dimethylsuberimidate in the presence of Mn2+ and UDP-galactose at pH 8.1 and isolated by gel filtration and affinity chromatography as described under “Experimental Procedure.” The complex showed the properties expected from previous studies (11) including high activity for lactose synthesis at low concentrations of glucose and on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate migrated as a single band with an apparent molecular weight of 67,000. The molecular weight of bovine galactosyltransferase determined by sedimentation equilibrium ultracentrifugation is 50,000 (12), but it migrates on sodium dodecyl sulfate gels with an apparent molecular weight of 53,000 (probably as the result of the presence of a considerable proportion of carbohydrate). The apparent molecular weight of the cross-linked complex therefore corresponds very well with the expected value (53,000 + 14,000 = 67,000). The sedimentation coefficient of this complex in the presence of MnCl2 and UDP-galactose was found to be 4.02 ± 0.03 \( s \), in reasonable agreement with the value for galactosyltransferase at saturating concentrations of \( \alpha \)-lactalbumin (4.22 ± 0.03 \( s \)). It is possible that the difference may be due to the introduction of several dimethylsuberimidate molecules (residue weight 138) during the cross-linking procedure, increasing the molecular weight and the sedimentation coefficient. A slight difference in sedimentation coefficient is observed for the cross-linked derivative in the absence of substrates that may relate to a change in symmetry of the complex on Mn2+ and UDP-galactose binding. It seems possible from the results summarized in Table III, that the covalently cross-linked \( \alpha \)-lactalbumin-galactosyltransferase may be rather more symmetrical than the galactosyltransferase alone (if \( f_{\text{min}} \) of 1.34 and 1.5, respectively).

From these results \( s_{20,w} \) values of 3.25 and 4.22 may be
The dissociation constant of \( \alpha \)-lactalbumin from the \( E \cdot Mn^{2+} \cdot UDP \)-galactose complex with ionic strength. Result at lowest ionic strength from kinetics; other points from sedimentation velocity experiments.

**CONCLUSIONS AS TO MECHANISMS OF LACTOSE SYNTHESIS**

Previous studies of the binding of galactosyltransferase to \( \alpha \)-lactalbumin-Sepharose (5, 6) and to free \( \alpha \)-lactalbumin (by ultracentrifugation) (8, 9) gave rise to the hypothesis that \( \alpha \)-lactalbumin attaches to galactosyltransferase only after the binding of monosaccharides. From this evidence, together with that from kinetic studies, which indicated an obligatory ordered sequential binding of \( Mn^{2+}, UDP \)-galactose, and monosaccharide to the galactosyltransferase during the catalytic cycle, Morrison and Ebner (10) proposed a kinetic scheme whereby \( \alpha \)-lactalbumin attaches to an \( E \cdot Mn^{2+} \cdot UDP \)-galactose-monosaccharide complex. The recent kinetic evidence (4, 12) suggests that the obligatory order of sequential binding is \( Mn^{2+}, UDP \)-galactose, \( \alpha \)-lactalbumin, and monosaccharide. This kinetic evidence further suggests that the binding of \( \alpha \)-lactalbumin to the \( E \cdot Mn^{2+} \cdot UDP \)-galactose complex enhanced monosaccharide binding.

The evidence here presented shows first that galactosyltransferase has a single UDP-galactose binding site. Second, the formation of a 1:1 complex of galactosyltransferase and \( \alpha \)-lactalbumin, in the presence of \( Mn^{2+} \) and UDP-galactose, is demonstrated by sedimentation velocity experiments, and this confirms the proposal that \( \alpha \)-lactalbumin attaches to \( E \cdot Mn^{2+} \). UDP-galactose prior to monosaccharide binding. Furthermore the covalent cross-linking of galactosyltransferase to \( \alpha \)-lactalbumin (to form a 1:1 complex) occurs in the presence of \( Mn^{2+} \) and UDP, or \( Mn^{2+} \) and UDP-galactose (11, and this paper). The obligatory prior binding of \( Mn^{2+} \); in the lactose synthase catalytic cycle, is confirmed by the evidence that \( \alpha \)-lactalbumin does not attach to galactosyltransferase in the presence of EDTA and UDP-galactose.

The dissociation constant, \( K_d \), and free energy change, \( \Delta G \), for \( \alpha \)-lactalbumin binding (with an \( E \cdot Mn^{2+} \) -UDP-galactose complex) determined by sedimentation velocity and steady state kinetic experiments are in close agreement. It is difficult to draw pertinent conclusions as to the nature of the interaction of galactosyltransferase and \( \alpha \)-lactalbumin. Both the magnitude of the free energy change and the dependence of the association

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**Table III**

| Preparation and Conditions | \( s_{ax} \) | Molecular weight \( \times 10^{-3} \) | \( /min \) |
|---------------------------|-------------|-----------------------------------|---------|
| Galactosyltransferase      |             |                                   |         |
| 5 mM GlcNAC               | 3.30        | 50,000                            | 1.48    |
| 10 mM MnCl₂, 0.3 mM UDP   | 3.25±0.03   | 50,000                            | 1.50    |
| galactose                 |             |                                   |         |
| 10 mM MnCl₂, 0.3 mM UDP   | 4.22±0.03   | 64,200                            | 1.37    |
| galactose, 400 µg/ml \( \alpha \) lactalbumin |            |                                   |         |
| 20 mM EDTA, 0.3 mM UDP    | 3.27        |                                   |         |
| galactose, 400 µg/ml \( \alpha \) lactalbumin |            |                                   |         |
| Lactose synthase cross-linked |             |                                   |         |
| complex                   |             |                                   |         |
| No substrates             | 4.51±0.03   | 64,200                            | 1.28    |
| 10 mM MnCl₂, 0.3 mM UDP   | 4.32±0.03   | 64,200                            | 1.34    |

* Taken from Powell and Brew (12).

* Calculated as the sum of the molecular weights of galactosyltransferase and \( \alpha \)-lactalbumin (12).

significant effects on the \( \Delta G \) may be considered to eliminate the possibility of a simple electrostatic interaction between the two components.

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**Effect of NaCl Concentration**

The dissociation constant of \( \alpha \)-lactalbumin from the \( E \cdot Mn^{2+} \) -UDP-galactose-\( \alpha \)-lactalbumin complex was also determined at 0.2 and 0.4 M NaCl. A plot of \( \ln K_d \) against ionic strength is shown in Fig. 7, which demonstrates that the values determined by ultracentrifugation (0.1, 0.2, and 0.4 M NaCl) show colinearity with the value determined from kinetics, in the absence of NaCl. This validates the analysis of the kinetics in terms of an association of \( \alpha \)-lactalbumin with the \( E \cdot Mn^{2+} \) -UDP-galactose complex. Although the effect of increasing ionic strength on the association of the two proteins (decreasing \( K_d \) with increasing salt concentration) might superficially be interpreted in terms of a hydrophobic interaction, the free energy change, \( \Delta G \), for the association shows little difference in the presence and absence of 0.1 M NaCl. The variation of \( K_d \) with ionic strength may reflect salt-dependent changes in either or both macromolecules, while the absence of

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**Fig. 7.** The variation of the dissociation constant, \( K_d \), of bovine \( \alpha \)-lactalbumin from enzyme \( Mn^{2+} \) -UDP-galactose complex with ionic strength. Result at lowest ionic strength from kinetics; other points from sedimentation velocity experiments.

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**Preparation and Conditions**

- Galactosyltransferase
- 5 mM GlcNAC
- 10 mM MnCl₂, 0.3 mM UDP-galactose
- 10 mM MnCl₂, 0.3 mM UDP-galactose, 400 µg/ml \( \alpha \) lactalbumin
- 20 mM EDTA, 0.3 mM UDP-galactose, 400 µg/ml \( \alpha \)-lactalbumin
- Lactose synthase cross-linked complex
- No substrates
- 10 mM MnCl₂, 0.3 mM UDP-galactose

**Molecular Weight**

- 50,000
- 64,200
- 64,200
- 64,200
- 64,200
- 64,200
- 64,200
- 64,200

**Ionic Strength**

- 0.1
- 0.2
- 0.3
- 0.4

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**Preparation and Conditions**

- Galactosyltransferase
- 5 mM GlcNAC
- 10 mM MnCl₂, 0.3 mM UDP-galactose
- 10 mM MnCl₂, 0.3 mM UDP-galactose, 400 µg/ml \( \alpha \) lactalbumin
- 20 mM EDTA, 0.3 mM UDP-galactose, 400 µg/ml \( \alpha \)-lactalbumin
- Lactose synthase cross-linked complex
- No substrates
- 10 mM MnCl₂, 0.3 mM UDP-galactose

**Molecular Weight**

- 50,000
- 64,200
- 50,000
- 64,200
- 64,200
- 64,200
- 64,200
- 64,200

---

**Table III**

| Preparation and Conditions | \( s_{ax} \) | Molecular weight \( \times 10^{-3} \) | \( /min \) |
|---------------------------|-------------|-----------------------------------|---------|
| Galactosyltransferase      |             |                                   |         |
| 5 mM GlcNAC               | 3.30        | 50,000                            | 1.48    |
| 10 mM MnCl₂, 0.3 mM UDP   | 3.25±0.03   | 50,000                            | 1.50    |
| galactose                 |             |                                   |         |
| 10 mM MnCl₂, 0.3 mM UDP   | 4.22±0.03   | 64,200                            | 1.37    |
| galactose, 400 µg/ml \( \alpha \) lactalbumin |            |                                   |         |
| 20 mM EDTA, 0.3 mM UDP    | 3.27        |                                   |         |
| galactose, 400 µg/ml \( \alpha \) lactalbumin |            |                                   |         |
| Lactose synthase cross-linked |             |                                   |         |
| complex                   |             |                                   |         |
| No substrates             | 4.51±0.03   | 64,200                            | 1.28    |
| 10 mM MnCl₂, 0.3 mM UDP   | 4.32±0.03   | 64,200                            | 1.34    |

* Taken from Powell and Brew (12).

* Calculated as the sum of the molecular weights of galactosyltransferase and \( \alpha \)-lactalbumin (12).
on ionic strength preclude a simple electrostatic interaction. More probably the interaction is partly hydrophobic, as previously suggested (16).

Small but distinct changes observed in sedimentation coefficient of galactosyltransferase in the presence of GlcNAc, 3.3 S (12), and in the presence of Mn²⁺, UDP-galactose, 3.25 S, may reflect conformational changes upon the binding of those substrates. A more pronounced change is observed with cross-linked galactosyltransferase-α-lactalbumin in the presence of Mn²⁺ and UDP-galactose, 4.35 S and absence of substrates, 4.51 S.

Two further aspects of the attachment of substrates during the lactose synthase catalytic cycle remain to be investigated; the first, the nature of manganese binding, being difficult to investigate. The second is the nature of the attachment of monosaccharides, and how this is modified by α-lactalbumin. The present work provides a basis for the study of this second aspect which is currently in progress.

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