Inhibition and Inactivation of Bovine Mammary and Liver UDP-galactose-4-epimerases*

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Bovine liver and mammary UDP-galactose-4-epimerases were investigated with respect to various inhibitors and inactivators. Uridine nucleotides and NADH are potent inhibitors with K_i values in the low micromolar range. The NAD^+ /NADH ratio may be an important physiological control mechanism for it affects markedly the activity of the enzyme with 50% inhibition occurring at a ratio of 20:1. In the presence of uridine nucleotides binding of NADH to the epimerases is enhanced. Consequently, the effect of changes in the NAD^+ /NADH ratio in vivo would not be immediately apparent as uridine nucleotides would slow down the displacement of NADH by NAD^+. Neither uridine nor galactose 1-phosphate inhibits the purified enzymes as previously reported with the impure liver enzyme. Uridine nucleotides provide almost total protection against the apparent first order inactivation of the epimerases by trypsin and allow determination of dissociation constants. NAD^+ partially protects against trypsin inactivation. Inactivation with various sulfhydryl reagents is complex and the results indicate that at least three sulfhydryl groups may be modified before total inactivation occurs. Partial inactivation occurs upon modification of the epimerases with 2-hydroxy-5-nitrobenzyl bromide. Some protection against this modification is provided by the combination of NAD^+ and UDP.

The interconversion of UDP-galactose and UDP-glucose is catalyzed by UDP-galactose-4-epimerase, EC 5.1.3.2, which is an important enzyme in sugar nucleotide metabolism. This enzyme not only allows galactose to enter glucose metabolic pathways, but also permits the conversion of glucose to galactose for synthesis of polysaccharides (1). Although other reports have indicated that the UDP-galactose-4-epimerase activity contained in crude preparations from mammalian sources is inhibited by uridine nucleotides and NADH (2-7), no systematic studies, other than that of Tsai et al. (2) with partially purified bovine mammary epimerase, have been reported. Preliminary studies of the inactivation of crude mammary epimerase preparations by different sulfhydryl reagents have indicated that this activity is susceptible (2, 3), but no kinetic studies aimed at determining the number of sulfhydryl groups involved have been published. Extensive inhibition and inactivation studies were undertaken with the highly purified, electrophoretically homogenous, bovine mammary and liver UDP-galactose-4-epimerase (8) in order to compare properties of the two enzymes, to gain insight into requirements for substrate and inhibitor binding, and to define possible metabolically significant effectors.

EXPERIMENTAL PROCEDURES

Materials — Uridine, UMP, UDP, UTP, NAD^+, NADH, ADP, galactose-1-P, galactose-6-P, glucose-1-P, glucose-6-P, trypsin, N-ethylmaleimide, showdomycin, cysteine, p-hydroxymercuribenzoate, 2-mercaptoethanol, dithiothreitol, 2-hydroxy-5-nitrobenzyl bromide type II, sodium pyruvate, and type III lactic dehydrogenase (EC 1.1.1.22) were purchased from Sigma Chemical Co. All other reagents were as described previously (8).

Methods — UDP-galactose 4-epimerase was isolated from bovine liver and mammary tissue as previously described (8). For the inhibition studies, DEAE-cellulose chromatography (8) was used to remove UMP remaining from the final purification step. The epimerases used in all of these experiments were in a buffer consisting of 100 mM potassium phosphate, pH 7.6, containing 1 mM mercaptoethanol and 20% glycerol. UDP-galactose 4-epimerases used in the sulphydryl inactivation studies were eluted from DEAE-cellulose (8) with buffer containing no mercaptoethanol and used immediately.

Epimerase activity was determined by both Assays I and II (8). The inhibition by uridine nucleotides was examined with Assay I. The coupling enzyme, UDP-glucose dehydrogenase (EC 1.1.1.22), was not appreciably affected by the concentrations of nucleotides employed, especially since a large excess of dehydrogenase was employed. Assay II was used to examine the inhibition by NADH. To investigate the effect of the NAD^+/NADH ratio on activity, the NAD^+ concentration was held constant (10 \muM) and the NADH concentration was varied. With the higher NADH concentrations significant inhibition of the UDP-glucose dehydrogenase was apparent in Assay II, necessitating oxidation of the NADH to NAD^+ before addition of this coupling enzyme. This was done by adding sodium pyruvate to a concentration of 5 mM and 10 \mug/ml of lactic dehydrogenase after stopping the epimerase reaction with heat. After a 30- min incubation at 24° the lactic dehydrogenase was inactivated by heating for 3 min at 100°. Then NAD^+ was added to a final concentration of 1 mM and the UDP-glucose dehydrogenase was added to determine the amount of UDP-glucose produced by measuring total NADH production.

Sulphydryl modification of the epimerases by either N-ethylmaleimide or showdomycin was stopped by the addition of excess cysteine (200 \muM of cysteine to inactivating agent) (2, 8) before determination of residual epimerase activity by Assay I. Enzyme treated with p-hydroxymercuribenzoate was assayed essentially by Assay II, with one modification. At least a 200-fold molar excess of mercaptoethanol was added to each assay after the epimerase reaction was stopped by heating but before addition of the UDP-glucose dehydrogenase.

Trypsin inactivation studies were at 24° in the potassium phos-
phate, mercaptoethanol, glycerol-stabilizing buffer (8). Aliquots were removed at appropriate times, and UDP-galactose-4-epimerase activity was determined by Assay I.

The effect on epimerase activity of 2-hydroxy-5-nitrobenzyl bromide as a fairly tryptophan-specific inactivating agent (10, 11) was investigated. Stock solutions of 2-hydroxy-5-nitrobenzyl bromide were prepared in absolute methanol immediately before use. Nine volumes of phosphate-buffered epimerase solution, pH 7.6, were treated with 1 volume of 2-hydroxy-5-nitrobenzyl bromide solution and shaken vigorously for 1 min and allowed to incubate at 4° for 5 additional min to effectively hydrolyze the unreacted reagent. The activity of the modified enzyme as determined by Assay I was compared with that of a control which had been treated in the same manner with only absolute methanol.

p-Hydroxymercuribenzoate, NAD+, NADH, and uridine nucleotide concentrations were determined by using the appropriate extinction coefficients. UDP-galactose concentrations were determined enzymatically with excess UDP-galactose-4-epimerase and UDP-glucose dehydrogenase. Only the dehydrogenase was used to determine UDP-glucose concentrations.

Data for determination of $K_n$ and $K_v$ values were fitted by linear regression (12) using a Texas Instruments SK-52 programmable calculator.

Circular dichroism spectra were measured at 24° in a Jasco J-20 Circular Dichroism Spectrometer with 2-mm path length cuvettes. All spectra were run in duplicate. The buffer for all determinations was 100 mM potassium phosphate containing 30% glycerol and 1 mM mercaptoethanol, pH 7.6. A mean residue molecular weight of 118 was assumed and protein concentrations were estimated from enzymatic activity using a specific activity of 65 units/mg for the liver epimerase (8). All spectra represent the difference between enzyme, buffer, and added substrates and solutions of buffer and added substrates.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was by the procedure of Weber and Osborn (13) while discontinuous polyacrylamide gel electrophoresis was by the procedure of Davis (14).

**RESULTS**

Data regarding the inhibition of the epimerase by the uridine nucleotides, NADH, and 2-hydroxy-5-nitrobenzyl bromide as well as the circular dichroism spectrum in the absence and presence of UDP-galactose are presented in a supplement following this paper.

In view of the report by Ray and Bhaduri (41), galactose-1-P, galactose-6-P, glucose-1-P and glucose-6-P were tested as possible effectors with both enzymes. Five glucose concentrations of the carbohydrate phosphates had no effect on epimerase activity in either Assay I or Assay II. There was also no effect when UDP-glucose was used as the substrate. These carbohydrate phosphates, however, appear to be substrates for activities present in the partially purified UDP-glucose dehydrogenase preparation; particularly glucose-1-P and glucose-6-P.

NADH is an excellent inhibitor of both the bovine liver and mammary epimerases. The extent of inhibition is dependent upon the NAD+/NADH ratio as shown in Fig. 3. A NAD+/NADH ratio of 20:1 results in approximately 60% inhibition which is independent of the initial NADH concentration since similar results to those presented in Fig. 3 were observed when the initial NADH concentration was 600 μM. Five hundred micromolar ADP did not inhibit with 10 μM NADH and 100 μM UDP-galactose as substrates in Assay II.

Fig. 4A shows the effect of preincubation of the liver epimerase with NADH, and with NADH and UDP-galactose on displacement of NADH by NAD+. Although the control enzyme and the enzyme preincubated with NADH have the same final concentrations of NADH and NADH after zero time, the NADH-preincubated enzyme always exhibited a lower, but linear, rate of catalysis. This small effect was dependent on the time of preincubation with NADH and was maximal at 4 min. The enzyme preincubated with UDP-galactose and NADH eventually approached the initial rate of the NADH-preincubated sample indicating slow displacement of NADH. Preincubation of liver epimerase with either UDP and NADH or UMP and NADH produced results similar to preincubation with UDP-galactose and NADH. Preincubation of the enzymes with either UDP-galactose or UMP had no effect. The retention of NADH by the enzyme is obviously potentiated by the presence of UDP galactose. Fig. 4B suggests the displacement of NADH by NADH, since the inhibition is competitive. Since the inhibition is immediate, rather than slow, it would appear that NADH is dissociating with each catalytic event. Fig. 4C illustrates the similar immediate effect on catalytic rate caused by addition of the inhibitor UDP. Repetition of the above experiments with the mammary epimerase produced similar results.

To further investigate the effect of inhibitors and substrates on the epimerases, inactivation by trypsin was employed. This inactivation was apparent first order with both enzymes. The half-time ($t_{1/2}$) of inactivation decreased linearly with trypsin concentrations up to 25 μg/ml. This inactivation could be almost totally prevented by UDP-galactose and other uridine nucleotides while only partial protection was afforded by NADH and NADH. In Table I, conditions are described under which maximal protection with each ligand was observed. The inactivation of liver UDP galactose-4-epimerase by trypsin in the presence of various concentrations of UDP-galactose is shown in Fig. 5. The inserted plot on Fig. 5 shows the determination of the $K_c$ for the dissociation of UDP-galactose from the UDP-galactose-enzyme complex (15). Although the protection by NADH was only partial, this protection was concentration dependent and plots of $t_{1/2}$ versus NADH concentration were linear. The $K_c$ values determined for the various ligands from plots of $t_{1/2}$ versus protecting ligand concentrations are summarized in Table I. An examination of liver UDP-galactose-4-epimerase by sodium dodecyl sulfate-polyacrylamide gel electrophoresis was by the procedure of Weber and Osborn (13) while discontinuous polyacrylamide gel electrophoresis was by the procedure of Davis (14).
Inhibition and Inactivation of Epimerases

Fig. 4. A, effect of preincubation of liver epimerases with NADH, and NADH and UDP-galactose. The enzyme was preincubated for 5 min at 24° in 100 mM glycylglycine, pH 8.5, with no additions (○), 1 μM NADH (●), and 1 μM NADH and 500 μM UDP-galactose (△). At zero time all solutions were made the same in concentrations of UDP-galactose and NADH and 500 μM NAD⁺ was added. All of these were added as a single addition. Aliquots were removed at the indicated times and UDP-glucose determined by Assay II. B, displacement of NAD⁺ from the liver epimerase by NADH. Enzyme was incubated with 100 mM glycylglycine, pH 8.5, 500 μM UDP-galactose and 1 μM NAD⁺ (○). At 4.5 min (●), 1 μM NADH was added to half the solution (△). Assay II was used to determine UDP-glucose production. No UDP-glucose was produced when enzyme was incubated with UDP-galactose and NADH, c, displacement of UDP-galactose from liver epimerase by UDP. Enzyme was incubated with 1 μM NAD⁺, 100 μM UDP was added. The lower curve (△) represents activity in the presence of 500 μM UDP-galactose added at zero time. UDP-glucose production was determined by Assay I with a 1-h incubation to negate inhibitory effects of UDP on UDP-glucose dehydrogenase. The same types of data were obtained for the mammary UDP-galactose-4-epimerase in all three cases.

**Table 1**
Protection against trypsin inactivation of mammary and liver UDP-galactose-4-epimerases by substrates and inhibitors

| Ligand       | Mammary % V/V₀ | Liver % V/V₀ |
|--------------|----------------|-------------|
| None         | 11             | 92          |
| 0.5 mM UMP   | 93             | 76          |
| 0.5 mM UDP   | 100            | 86          |
| 0.5 mM UTP   | 91             | 92          |
| 0.5 mM UDP-glucose | 95       | 94          |
| 0.5 mM UDP-galactose | 93       | 100         |
| 0.5 mM uridine | 11          | 32          |
| 1 mM NAD⁺    | 36             | 42          |

Electrophoresis after a 2-h incubation with trypsin in the presence of UDP-galactose revealed no apparent change in molecular weight while enzyme with only trypsin was almost totally degraded since no major staining fragments were apparent. Electrophoresis of the same enzyme preparations in the discontinuous system of Davis (14) revealed no apparent charge differences between enzyme incubated with trypsin and UDP-galactose and control enzyme. Similar experiments with the mammary enzyme resulted in almost identical electrophoretic patterns.

Studies of inactivation of the UDP-galactose-4-epimerases by sulphydryl reagents was complicated by the instability of the enzymes in the absence of reagents such as mercaptoethanol or dithiothreitol (8). Enzymes for use in such studies were eluted from a small DEAE-cellulose column (8) immediately before use with buffer containing no mercaptoethanol. Such preparations lost from 30 to 50% of their original activity during 1 h incubation at 24°. Accordingly, all experiments were limited to 10 min and at this time the enzyme retained over 90% of its initial activity. Incubation of the enzyme for about 5 to 6 h at 24° in 100 mM potassium phosphate containing 1 mM mercaptoethanol. Additions were none (○), 10 μg/ml of trypsin (■), trypsin and 0.25 μM UDP-galactose (△), trypsin and 0.50 μM UDP-galactose (▲), and trypsin and 0.75 μM UDP-galactose (□). Activity was determined at the indicated times by Assay I. V₀ is the initial catalytic rate and V is the rate at the indicated times. Inset, half-time of inactivation (t₁/₂) plotted against concentration of protecting ligand.
increasing showdomycin concentration. With 3 mM showdomycin, the residual level was reached in 5 min whereas in its absence it took 20 h. Plots of log \((V - V_o)/(V_M - V_o)\) versus time were nonlinear, and in all cases a very rapid initial loss of activity was observed. No substantial protection against inactivation by 3 mM showdomycin was observed with either 1 mM NAD\(^+\) or 0.05 mM UDP-galactose. Very similar results were obtained using N-ethylmaleimide as the inactivating agent with two exceptions. The very rapid initial decreases in activity were more pronounced than with showdomycin, and the enzyme was totally inactivated by concentrations of N-ethylmaleimide greater than 0.3 mM. Neither 0.05 mM UDP-galactose nor 1 mM NAD\(^+\) offered substantial protection against inactivation by 0.5 mM N-ethylmaleimide. With low concentrations of \(\rho\)-hydroxymercuribenzoate as the inactivating agent, the time course of the initial loss of activity could be followed whereas this was impractical to do with both N-ethylmaleimide and showdomycin. With concentrations of \(\rho\)-hydroxymercuribenzoate greater than 1 \(\mu\)M, no protection was afforded by substrates. Since the reaction with \(\rho\)-hydroxymercuribenzoate was somewhat temperature-dependent, protection by substrates was examined at 4° with 0.05 \(\mu\)M of the inactivating reagent. As shown in Fig. 6, the substrates NAD\(^+\) and UDP-galactose and the inhibitor UDP caused the semilogarithmic inactivation plots to become linear. Almost total protection was observed when both NAD\(^+\) and UDP were incubated with the enzyme. Enzyme totally inactivated by concentrations of \(\rho\)-hydroxymercuribenzoate greater than 1 \(\mu\)M were only partially reactivated (30 to 50%) by incubation with 50 \(\mu\)M mercaptoethanol or 50 \(\mu\)M dithiothreitol. Enzyme only partially inactivated (less than 50%) was totally reactivated by the same procedure. Inactivation studies with N-ethylmaleimide and \(\rho\)-hydroxymercuribenzoate with the highly purified mammary epimerase resulted in data very similar to that obtained for the liver enzyme.

**DISCUSSION**

Uridine nucleotides are very effective inhibitors of the electrotheretically homogenous bovine mammary and liver UDP-galactose-4-epimerases as determined by Assay I. This inhibition is competitive with respect to UDP-galactose and \(K_i\) values obtained are comparable to those reported by Tsai et al. (2) for the partially purified mammary enzyme. The absence of inhibition by uridine and galactose 1-phosphate and the competitive inhibition by UMP indicates the involvement of both the uridine and phosphate moieties for effective interaction with the enzymes. The lower \(K_i\) values observed for UDP as compared to those for UMP and UTP are consistent with the closer similarity of this nucleotide with the substrates UDP-glucose and UDP-galactose. ADP was not an inhibitor of either enzyme.

**TABLE II**

| Ligand     | Mammary | Liver |
|------------|---------|-------|
| UMP        | 0.48    | 0.28  |
| UDP        | 1.00    | 0.28  |
| UTP        | 0.45    | 0.28  |
| UDP galactose | 0.45    | 0.28  |
| UDP glucose| 1.40    | 1.40  |
| NAD\(^+\)  | 0.28    | 0.35  |

\(K_i\) values were determined as described for UDP-galactose from the UDP-galactose epimerase complex in Fig. 5.

![Fig. 6 Inactivation of bovine liver UDP-galactose-4-epimerase with \(\rho\)-hydroxymercuribenzoate, protection by substrates, and NAD\(^+\)](http://www.jbc.org/)

NADH is a much more effective inhibitor than the uridine nucleotides. The \(K_i\) values for UDP for the two enzymes are approximately twice the apparent \(K_i\) values for UDP-galactose (8) while the \(K_i\) values for NADH are approximately one-tenth the apparent \(K_i\) value for NAD\(^+\) (8). The effect of the NAD\(^+\)/NADH ratio on epimerase activity is very pronounced as shown in Fig. 3 and provides evidence for the importance of this ratio in controlling the metabolic flux of UDP-glucose and UDP-galactose. Robinson et al. (16) have shown that the NAD\(^+\)/NADH ratio and pH are very important in controlling the expression of epimerase activity in cell cultures and tumor cells with the effect of NADH being more pronounced at higher hydrogen ion concentrations. As illustrated in Fig. 4A, the effect of increasing the NAD\(^+\)/NADH ratio would not be immediately apparent because in the presence of uridine nucleotides, NADH is not rapidly displaced by NAD\(^+\). Experiments very similar to those shown in Fig. 4A and B, were reported earlier with partially purified bovine liver UDP-galactose-4-epimerase by Langer and Glaser (17). These workers reported that the NAD\(^+\) did not dissociate from the enzyme with each catalytic event as several minutes were required for complete inhibition by NADH. Data presented in this study indicate that the effect of NADH is immediate. Langer and Glaser (17) also observed no displacement on NADH by NAD\(^+\) in an experiment similar to that described by Fig. 4A, but this was due to the experimental procedure since they used only 5.7 times as much NAD\(^+\) as NADH. This ratio of NAD\(^+\)/NADH is sufficient to cause 70 to 80% inhibition so displacement would not be readily apparent. Langer and Glaser (17) also observed a 340 nm absorbance increase with time with NADH, epimerase preparation, and UDP-galactose under conditions similar to Assay I. This was never observed with either of the highly purified epimerases. The apparent loss of activity caused by preincubation with only NADH (Fig. 4A) could be due to subtle changes in the enzyme which result in a less active
form. An alternate explanation is that there is a small amount of enzyme-NADH formed which is inactive and dissociates slowly. The change caused by NADH required 4 min of incubation at 24°C of the liver epimerase with NADH for maximum decrease in apparent activity with no further loss observed after that length of time. This indicates that the loss of activity was not due to simple inactivation as this process would have continued. The linear nature of UDP-glucose production catalyzed by NADH-preincubated enzymes (Fig. 4A) shows that in the absence of uridine nucleotides, NADH is rapidly displaced by NAD+ under these assay conditions rather than slowly as reported by Langer and Glaser (17).

The inactivation studies with the sulfhydryl specific reagents were complex but indicate that more than one susceptible group was necessary for full activity in both the mammary and liver enzyme. The semilogarithmic plots of activity loss as a function of time show at least three different rates of inactivation suggesting the possible involvement of at least three sulfhydryl groups. The amount of activity lost during the initial rapid process was 35 to 45% of the original activity with either N-ethylmaleimide or p-hydroxymercuribenzoate as the inactivating reagent (18). With the two slower processes, the amount of activity lost varied with the inactivating reagent, but with both reagents total inactivation was the final result. The slowest process evidently does not occur with incubation of the enzyme in the absence of β-mercaptoethanol nor does it occur when showdomycin was the inactivating reagent. Only with low concentrations (0.5 μM) of p-hydroxymercuribenzoate could any protection by substrates against inactivation be observed. The observed protection by substrates to inactivation at low sulfhydryl reagent concentration could possibly be due to different susceptible sulfhydryl groups being shielded as a consequence of substrates binding to the enzyme. The sulfhydryl groups which are modified by these reagents are very reactive since low concentrations of reagents are required for total inactivation of the enzyme and since the enzyme is inactivated readily upon incubation in the absence of mercaptoethanol.

Only partial inactivation of the enzyme was observed when 2-hydroxy-5-nitrobenzyl bromide was the inactivating agent. Partial protection against this inactivation was observed only in the presence of both NAD+ and UDP. Interestingly, the 2-hydroxy-5-nitrobenzyl bromide-modified enzyme appeared to regain activity upon incubation with substrates. The time required for maximum regain of activity increased with increasing inactivating reagent concentration. This could conceivably be due to the substrates interacting with the enzyme and forcing reassumption of an active conformation from the inactive or less active perturbed form caused by reaction with 2-hydroxy-5-nitrobenzyl bromide.

The protection by micromolar concentrations of uridine nucleotides against trypsin inactivation of the epimerases could indicate that the binding domain for these nucleotides contains a particular peptide bond which serves as the initial site of attack for trypsin. Cleavage at this site could be necessary before further trypsinoysis could occur. This seems unlikely as trypsin specificity is directed toward lysyl and arginyl residues (19) and amino acid analyses (8) have indicated 49 potential trypsin cleavage sites for the liver enzyme and 44 for the mammary enzyme. Also, the sodium dodecyl sulfate-polyacrylamide gel patterns of enzyme incubated with trypsin in the absence of uridine nucleotides showed only low molecular weight fragments while in the presence of these nucleotides no fragmentation was observed.

A probable explanation for these results would be that upon binding of uridine nucleotides to the epimerases, the protein assumes a tighter, more trypsin resistant conformation. Preliminary evidence for such a structural change is indicated by the circular dichroism studies.

Both NAD+ and uridine nucleotides bind to the free enzyme, as shown by binding to both the affinity columns used for purification (8) and the protection against trypsinoysis by uridine nucleotides and NAD+. The necessity of uridine nucleotides for tight binding of NADH to these enzymes is especially interesting since the Kᵯ for dissociation of NAD+ from the free enzyme as determined by trypsinoysis is approximately 5-fold greater than the apparent Kᵯ for NAD+ for both enzymes. This could indicate that uridine nucleotides cause a change in these enzymes which enhances their affinity for NAD+.

Only small differences were observed when the Kᵯ and Kᵯ values of substrates and inhibitors were compared for the liver and mammary UDP-galactose-4-epimerase which provides additional evidence that the properties of these enzymes are similar. In general, all of the Kᵯ values obtained for UMP, UDP, and UTP as determined by trypsin inactivation were lower than the corresponding Kᵯ values determined by inhibition kinetics. It would be anticipated that these values would be more closely related since they reflect the dissociation of the ligand from the enzyme. Protection by inhibitors against trypsin inactivation suggests a conformational change in the enzyme. It may be possible that the reversal of this conformational change after dissociation of the inhibitor is slow and if the native form of the enzyme is the only form susceptible to trypsin as indicated by the linearity of the plots (Fig. 5) it may be possible to obtain spuriously low dissociation constants. Another possibility is that the inhibitor may bind to more than one site on the enzyme and a tight binding site affords protection against trypsin whereas the determination of Kᵯ reflects a less tight binding site.

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**Inhibition and Inactivation of Epimerases**

**Supplementary Material to**

Inhibition and Inactivation of Bovine Mammary and Liver UDP-Galactose-4-Epimerases

**Cellia R. Carmon, Lois M. Severson, and Kurt E. Ober**

**Inhibition of Epimerases by 2-Hydroxy-5-Nitrobenzyl Bromide:** UDP, UDP, and VTP were competitive inhibitors of the highly purified liver and mammary epimerases. Figure 1 shows, for the liver enzyme, that the inhibition by UDP is linear with UDP in the presence of constant substrate and adenine to determine the K values. The K values obtained for the liver epimerase were: 10 μM for UDP, 12 μM for UDP and 60 μM for VTP. The values for the mammary epimerase were: 20 μM for UDP, 15 μM for UDP, and 100 μM for VTP. One mM uridine did not inhibit the enzyme with 100 μM UDP-galactose as substrate.

**Figure 1.** Inhibition of highly purified bovine liver UDP-galactose-4-epimerase by UDP. Activity was determined by assay 1 and UDP concentrations were 10 μM, 40 μM, 100 μM, and 200 μM (a). (b) 0.6 μM UDP-galactose and various NAD concentrations. Insert: Secondary plot of slope versus NAD concentration.

**Inhibition of Liver UDP-Galactose-4-Epimerase with 2-Hydroxy-5-Nitrobenzyl Bromide:** Treatment of the highly purified liver epimerase with concentrated solutions of 2-hydroxy-5-nitrobenzyl bromide resulted in partial inactivation. Exposure of 0.5 mg of UDP-galactose for 0.5 h at 37°C individually provided any protection against inactivation by 2-hydroxy-5-nitrobenzyl bromide. The concentration of 1 mM UDP resulted in an 85 to 85% decrease in the amount of epimerase as compared to controls. Although assays were normally done 2 or 3 min after the addition of enzyme to the assay mixture, the time lapse before addition of enzyme to the assay mixture and achievement of linearity was plotted also in Figure 2. Figure 2 shows an increase of 40% in the activity of liver epimerase 15 min after the addition of 2-hydroxy-5-nitrobenzyl bromide. The inhibition of liver epimerase by 2-hydroxy-5-nitrobenzyl bromide as described in Experimental Procedure. V_0 is the activity of control enzyme which was treated with only ethyl alcohol. Control enzyme retained 98% of its activity.

**Figure 2.** Circular dichroist spectra of bovine liver UDP-galactose-4-epimerase in the absence and presence of 2-hydroxy-5-nitrobenzyl bromide. Spectra were obtained of enzyme (a), enzyme and 0.5 mM UDP-galactose (b), enzyme and 0.5 mg UDP-galactose (c), further addition of UDP-galactose and 200 mM (d). Further changes in the spectra. Addition of 10 mM UDP also had no effect.

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