Purification and Characterization of UDP-galactose-4-epimerase from Bovine Tissues*

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Bovine liver and mammary UDP-galactose-4-epimerases have been purified to apparent electrophoretic homogeneity by a simple procedure involving the use of two affinity adsorbants, UDP-hexanolamine-Sepharose and NAD+-hexanolamine-Sepharose. The bovine thyroid epimerase has been partially purified by the same procedure. All three enzymes require NAD+ for activity, and all have a similar apparent molecular weight of approximately 40,000 as determined by gel filtration of the active enzymes. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the liver and mammary epimerases revealed similar molecular weights of 37,000 for both enzymes thus indicating that neither protein was dimeric. During development of the isolation procedure conditions were determined which would stabilize enzymatic activity in very dilute solutions. The liver and mammary enzymes, although similar in some respects, differed in amino acid composition and specific activity.

UDP-galactose-4-epimerase, EC 5.1.3.2, was first described by Leloir (1) and is believed to be essential for the direct interconversion of UDP-galactose and UDP-glucose in mammalian systems as well as in yeast, certain bacteria, and higher plants (2). This enzyme is important for the utilization of galactose as energy and for the production of the galactosyl moiety of lactose and more complex glycoproteins and glycolipids from glucose (3). UDP-galactose-4-epimerase has been highly purified from Escherichia coli (3, 4) and galactose-adapted yeast (5), but primarily due to the instability and low concentration of the enzyme, only partial purification has been achieved from bovine liver (6, 7), mammary gland (8), and thyroid (9).

An efficient procedure involving affinity chromatography is described for the isolation of highly purified UDP-galactose-4-epimerase from bovine liver, mammary, and thyroid tissue. The partial characterization of these enzymes is also reported.

**EXPERIMENTAL PROCEDURES**

**Materials**—Bovine livers, thyroids, and lactating mammary glands were obtained immediately after slaughter from Wilson Packing Co. Kansas City, Mo., and placed in ice. Liver and mammary acetone powders were prepared as described by Tsai et al. (8). Galactose-adapted Candida pseudotropicalis powder, UDP, NAD+, 2-mercaptoethanol, galactose 1-phosphate, glucose 1-phosphate, protein standards for molecular weight determinations, and dithiothreitol were purchased from Sigma. Reagents for sodium dodecyl sulfate and disc polyacrylamide gel electrophoresis, Bio-Gel A-0.5m and P-150 were from Bio-Rad. UDP-galactose was from Calbiochem, dialysis tubing was from Fisher, sodium glycoalycol (15,000 to 20,000 molecular weight) was from Matheson, Coleman and Bell, and DEAE-cellulose (DE232) was from Whatman. UDP-glucose dehydrogenase (UDP-glucose:NAD+ oxidoreductase; EC 1.1.1.22) was purified from calf liver through Step V by the procedure of Strominger et al. (10). UDP-hexanolamine-Sepharose containing approximately 4.5 μmol of UDP/ml of packed Sepharose was synthesized in the procedure of Barker et al. (11), and NAD+-hexanolamine-Sepharose containing 4 to 6 μmol of NAD+/ml of packed Sepharose was synthesized by the procedure of Mosbach et al. (12). All other reagents were of analytical reagent quality.

**Methods**—UDP-galactose-4-epimerase was assayed by two methods. Assay I was a direct spectrophotometric assay (6). Concentrations in the final assay mixture were 100 mM glycylglycine, 1 mM NAD+, 0.5 mM UDP-galactose, and 32 milliunits of UDP-glucose dehydrogenase in a total volume of 1 ml, pH 8.5. The change in absorbance at 340 nm was measured with a Gilford model 2400-2 automatic recording spectrophotometer at 24° and was proportional to enzyme concentration from 0 to 3.0 milliunits. A unit is defined as 1 μmol of UDP-glucose formed/min.

Assay II was a two-step assay (13) and was used when the effect of substrates and effectors was determined. Optimal concentrations in the final assay mixture were glycylglycine, 100 mM; NAD+, 1 mM; and UDP-galactose, 0.5 mM, in a total volume of 1 ml, pH 8.5. The reactions were run for either 4 or 10 min at 24° and then stopped by placing in a 100° water bath for 3 min. Sixty-four milliunits of UDP-glucose dehydrogenase were added to each assay and the UDP-glucose produced was determined by total change in absorbance at 340 nm after 90 min incubation at 24°. The change in absorbance was proportional to enzyme concentration from 0 to 8 milliunits of UDP-galactose-4-epimerase. When the NAD+ concentration was varied, NAD+ was added to make the final solution 1 mM after the reaction was stopped by heating and before addition of the UDP-glucose dehydrogenase.

**Types of blanks employed varied with the experiment, but basically consisted of substitution of buffer for the enzyme solution, heat denaturation (100°, 3 min) of the enzyme before adding substrates, or omission of UDP-galactose. The necessity of the latter blank will be shown later. Other blanks must be included in experiments with possible effectors or the UDP-glucose dehydrogenase preparation isolated and a commercial preparation from Sigma Chemical Co. contained additional enzymatic activities which utilized glucose 1-phosphate and glucose 6-phosphate as substrates.

Protein was determined by absorbance at 280 nm or by the method of Lowry et al. (14) using bovine serum albumin as a standard. Samples required extensive dialysis against distilled and deionized H2O before the latter method could be employed.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (15) and discontinuous polyacrylamide gel electrophoresis were done in a

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number of buffer systems and polyacrylamide concentrations (16–18). Gels were stained with Coomassie brilliant blue or, in some cases, with Schiff’s reagent (19) and scanned with a Gilford 2410-S linear transport on a Gilford 240 spectrophotometer. Samples for gel electrophoresis were concentrated by placing the enzymatic solution in dialysis tubing and covering it with polyethylene glycol which was replaced periodically. Solutions were concentrated 19- to 29-fold in 4 to 6 h.

Sephadex G-200 thin layer gel chromatography was done in 100 mM potassium phosphate buffer, pH 7.6, containing 6 mM guanidine HCl (20). Proteins were reduced and alkylated by the procedure of Mann and Fish (21).

Samples for amino acid analysis were dialyzed extensively against distilled, deionized H2O, lyophilized, and hydrolyzed in evacuated glass tubes with constant boiling HCl for 24 h at 106°C. Analyses were performed in duplicate on a Beckman model 121 Automatic Amino Acid Analyzer according to the method of Moore and Stein (22).

Sialic acid was determined by the method of Warren (23) after hydrolysis of samples with 0.1 N H2SO4 at 80°C for 1 h. Total hexoses were determined by the anthrone reaction (24).

A crude extract of C. pseudotropicalis powder containing 4.0 units/ml of UDP-galactose-4-epimerase was prepared as described by Darrow and Rodstrom (5). Eighty grams of acetone powder from liver or mammary tissue were extracted at 4°C with 1200 ml of 200 mM potassium phosphate, 1 mM mercaptoethanol, pH 7.6, by two 30-s full-speed bursts with a Sorvall Omnimixer. The homogenate was stirred for 30 min and centrifuged at 10,000 × g for 30 min at 4°C and the supernatant solution was filtered through a glass wool pad. Extracts of both liver and mammary powders contained approximately 200 units of epimerase. A 30 to 60% ammonium sulfate precipitate of the mammary acetone powder extract (8) was dissolved in 200 ml of 200 mM potassium phosphate, 1 mM mercaptoethanol, pH 7.6. Fresh calf thyroids were cooled on ice and freed from connective tissue and fat. Twenty grams of tissue were cut into small pieces and homogenized with 40 ml of 200 mM potassium phosphate, 1 mM mercaptoethanol, pH 7.6, by two 30-s full-speed bursts with a Sorvall Omnimixer. The homogenate was stirred for 30 min and centrifuged at 10,000 × g for 30 min at 4°C. The supernatant solution was brought to 65% saturation over a 30-min period with ammonium sulfate (430 g/liter) and stirred for an additional 30 min. This solution was centrifuged at 10,000 × g for 1 h and the precipitated material was dissolved in 20 ml of 200 mM potassium phosphate, 1 mM mercaptoethanol, pH 7.6, and contained 2.0 units of UDP-galactose-4-epimerase activity.

Unless otherwise indicated, all procedures were at 4°C. After use, affinity chromatography materials were removed from the column and gently stirred with several volumes of 500 mM potassium phosphate, 1 mM mercaptoethanol, pH 7.6, to a small NAD+-hexanolamine-Sepharose column equilibrated with the same buffer resulted in retention of the activity. The bound epimerase was eluted rapidly and with the highest specific activity at 40 mM potassium phosphate, pH 7.6. Other retained proteins must be eluted with 500 mM phosphate before the column can be re-equilibrated with the 1 mM phosphate buffer and reused. Application of the partially purified epimerase to the NAD+-hexanolamine-Sepharose in phosphate concentrations higher than 5 mM resulted in no binding of activity. Only 10 to 20% of the epimerase activity of a 1 mM phosphate-buffered extract of liver acetone powder was retained by the NAD+ column. Although the epimerase solu-

RESULTS

Acetone powders of bovine liver and mammary tissues were excellent sources of UDP-galactose-4-epimerase as they contained essentially all of the activity found in fresh tissue. The epimerase activity extracted from these powders was stable. Materials stored desiccated at −20°C for 5 years had activity comparable to that from freshly prepared powder. Application of extracts of fresh tissue to affinity columns resulted in a drastic reduction of flow rate while no such reduction was observed with extracts of the powders.

Chromatography on UDP-hexanolamine-Sepharose—This affinity material retained UDP-galactose-4-epimerase activity from the liver acetone powder extract, the 30 to 60% ammonium sulfate fraction of the mammary powder extract, and the 65% ammonium sulfate fraction of the thyroid tissue extract. Only a small fraction (2%) of the yeast extract was retained. The enzyme was retained on the column at buffer concentra-

![Fig. 1. Chromatography of liver acetone powder extract on UDP-hexanolamine-Sepharose.](http://www.jbc.org/fig1.png)

The extract of 80 g of bovine liver acetone powder was prepared as described under "Experimental Procedures" and applied to UDP-hexanolamine-Sepharose column (6.5 × 6.5 cm) previously equilibrated with 200 mM potassium phosphate, 1 mM mercaptoethanol, pH 7.6. The extract contained 238 units of UDP-galactose-4-epimerase activity (Assay I) and the specific activity was 0.014 unit/mg of proteins. Column flow rate was 140 ml/h. Extract application was complete at the fraction indicated by the first arrow and 900 ml of 200 mM phosphate, 1 mM mercaptoethanol, pH 7.6, were applied. The second arrow indicates the start of a 200-ml wash with 1 mM potassium phosphate, 1 mM mercaptoethanol, 20% glycerol, pH 7.6. The activity was eluted with 1 mM potassium phosphate, 1 mM mercaptoethanol, 20% glycerol, 0.5 mM UMP, pH 7.6, the start of which is indicated by Arrow 3. Recovery of activity was 85% with specific activity of 1.3.
tion obtained by UDP-hexanolamine-Sepharose chromatography contained 0.5 mM UMP, this nucleotide was not essential for nor did it prevent binding of the enzyme by the NAD⁹-hexanolamine-Sepharose. Enzymatic activity could also be eluted from the NAD⁹ column by 10 mM NAD⁹. The enzyme was not retained on a column equilibrated and eluted with buffer containing 1 mM NAD⁹. Hexanolamine-Sepharose did not bind the partially purified liver UDP-galactose-4-epimerase.

Fig. 2 illustrates a larger scale purification of partially purified liver epimerase on a 25-ml (1.5 × 14 cm) NAD⁹-

hexanolamine-Sepharose column. Chromatography of the partially purified mammary enzyme with this column produced similar results. Using a smaller column (0.5 × 6.5 cm), 48 ml of the thyroid enzyme partially purified by UDP-hexanol-
amine-Sepharose chromatography (0.012 unit/ml) was chroma-

tographed using a procedure similar to that described in Fig. 2. Recovery of activity was 75% and specific activity increased to 1.0.

**Purification of Liver and Mammary UDP-galactose-4-epi-
merase by Affinity Chromatography**—On the basis of the behavior of mammary and liver UDP-galactose-4-epimerase with the specific adsorbents described, it was possible to devise a purification procedure based solely on affinity chromatography. Table I summarizes the purification with the liver en-

zyme whereas Table II summarizes the purification of the mammary epimerase. The liver extract was applied directly to the UDP-hexanolamine-Sepharose column described in Fig. 1 whereas a 30 to 60% ammonium sulfate fraction of the mam-

mary extract was used for purification of this enzyme. The active fractions eluted from the UDP column were pooled and applied directly to the NAD⁹-hexanolamine-Sepharose column described in Fig. 2. The active fractions eluted by the 40 mm phosphate buffer were applied to a UDP-hexanolamine-

Sepharose column (1 × 5 cm) equilibrated with 40 mm potas-

sium phosphate, 1 mM mercaptoethanol, 20% glycerol, pH 7.6. The column was washed by varying potassium phosphate, pH 7.6, 40 mm (50 ml), 200 mm (100 ml), 1 mm (50 ml), and 40 mm (25 ml). The enzyme was eluted sharply with 40 mm potassium phosphate containing 0.5 mM UMP, pH 7.6. All the above buffers contained 1 mM mercaptoethanol and 20% glycerol. Similar results were obtained with both enzymes.

Fig. 3 shows scans of discontinuous polyacrylamide gel elec-

trophoresis patterns of the liver epimerase preparations after each of the three affinity chromatography steps. The purity of the enzyme obtained from the final UDP-hexanolamine-Sepharose step was also examined electrophoretically in the differ-

et pH discontinuous polyacrylamide systems of Williams and Reisfeld (17), Davis (16), and Reisfeld and Small (18). In all cases a single stained band was observed. Migration of the

![Fig. 2. Chromatography of partially purified liver UDP-galactose-4-epimerase on NAD⁹-hexanolamine-Sepharose. A 600-ml sample of liver epimerase partially purified by UDP-Sepharose chromatography (see Fig. 1) containing 198 units of epimerase activity, specific activity 1.5, was applied to a NAD⁹-hexanolamine-Sepharose column (1.5 × 14 cm). The arrows labeled 1 and 2 represent the start of a 1 mm potassium phosphate, 1 mM mercaptoethanol, 20% glycerol, pH 7.6, wash and elution with 40 mm potassium phosphate, 1 mM mercaptoethanol, 20% glycerol, pH 7.6. Recovery of activity was 90% with a 5.8-fold increase in specific activity.](image)

| Table I | Purification of liver UDP-galactose-4-epimerase by affinity chromatography |
|---------|--------------------------------|
| Step    | Volume | Protein concentration | Total activity | Yield | Purification |
|         | ml     | mg/ml | units | % | % | units/mg | -fold |
| 1. Extract of 80 g acetone powder | 1050 | 13.9 | 228 | 100 | 100 | 0.016 + 0.004 |
| 2. UDP-Sepharose | 660 | 0.16 | 185 | 81.1 | 81.1 | 1.8 ± 0.4 | 112.5 | 112.5 |
| 3. NAD-Sepharose | 250 | 0.078 | 170 | 74.6 | 91.9 | 8.7 ± 1.3 | 543.8 | 4.8 |
| 4. UDP-Sepharose | 140 | 0.014 | 133 | 58.3 | 78.2 | 66.0 ± 9.0 | 4125.0 | 7.6 |

**a** Protein was determined by the method of Lowry et al. (14).

| Table II | Purification of mammary UDP-galactose-4-epimerase by affinity chromatography |
|---------|--------------------------------|
| Step    | Volume | Concentration | Total activity | Yield | Purification |
|         | ml     | mg/ml | units | % | % | units/mg | -fold |
| 1. Extract of 80 g acetone powder | 1050 | 9.35 | 228 | 100 | 100 | 0.023 ± 0.005 |
| 2. 30 to 60% ammonium sulfate fraction | 226 | 17.9 | 215 | 95.1 | 95.1 | 0.053 ± 0.001 | 2.3 | 2.3 |
| 3. UDP-Sepharose | 574 | 0.065 | 205 | 90.7 | 96.3 | 5.5 ± 1.0 | 239.1 | 103.8 |
| 4. NAD-Sepharose | 322 | 0.039 | 164 | 68.1 | 75.1 | 11.8 ± 1.8 | 513.0 | 2.1 |
| 5. UDP-Sepharose | 172 | 0.0055 | 127 | 56.2 | 82.5 | 210 ± 45 | 9150.0 | 17.8 |

**a** Protein was determined by the method of Lowry et al. (14).
the absence of glycerol resulted in about 60% loss during each freezing at -20° and thawing to room temperature. The polyethylene glycol technique described under "Experimental Procedures" was very useful with small samples. Although type UM-10 Diallo membranes (Amicon) can be used to concentrate the highly purified epimerase solutions, a more rapid procedure involving DEAE-cellulose chromatography was devised. This latter procedure had an added advantage in that the UMP used for final elution was removed.

**DEAE-cellulose Chromatography**—Fourteen milliliters of purified mammary epimerase (6.0 units) were applied to a DEAE-cellulose column (0.5 x 6 cm) in 20 mM potassium phosphate, 0.5 mM mercaptoethanol, 0.25 mM UMP, 10% glycerol at 4°. The column was washed with 10 ml of 20 mM potassium phosphate, 1 mM mercaptoethanol followed by linear gradient formed by 50 ml of 20 mM potassium phosphate, 1 mM mercaptoethanol, and 50 ml of 300 mM potassium phosphate, 1 mM mercaptoethanol. All buffers were pH 7.6. Recovery of activity was 85%. Enzyme was not retained by this column if the potassium phosphate concentration was greater than 20 mM. Identical results were obtained with the liver enzyme. An aliquot of the same highly purified liver epimerase was chromatographed on a DEAE-cellulose column (0.5 x 6 cm) using the elution scheme of Ray and Bhaduri (25) which involved elution with successive buffers of increasing potassium phosphate concentration. Approximately 80% of the activity was eluted with 80 mM potassium phosphate whereas the remainder was eluted with 120 mM potassium phosphate. The effect of 2.0 mM glucose 1-phosphate and 100 mM galactose 1-phosphate on the UDP-galactose-4-epimerase activity of both of these peaks was negligible. The enzymes from both peaks were identical electrophoretically and are the consequence of the stepwise elution procedure and are not multiple forms of the enzyme. For removing UMP and concentrating the enzyme, an extremely small (0.50 ml) DEAE-cellulose column was used. Up to 15 units of activity were retained by this column. After sample application, the column was washed with 10 ml of 20 mM potassium phosphate, 1 mM mercaptoethanol, pH 7.6, and activity was eluted with 120 mM potassium phosphate. 1 mM mercaptoethanol, pH 7.6. This procedure routinely resulted in over 95% recovery of activity.

**Stabilization of Liver UDP-galactose-4-epimerase**—Enzyme purified with NAD+-hexanolamine-Sepharose was not stable and lost 50% of the apparent activity during a 2-h incubation at 24° in 20 mM phosphate, 20% glycerol, pH 7.6. The enzyme was stabilized equally well by 1% bovine serum albumin, 0.5 mM diethiothreitol or 1 mM mercaptoethanol with essentially no loss of activity during 2 h incubation at 24°. Mercaptoethanol concentrations as low as 0.1 mM were effective in stabilizing the enzyme and incubation for 8 h with 200 mM mercaptoethanol at 4° did not result in any inactivation. At 4°, 1 mM mercaptoethanol stabilized the enzyme in the absence of glycerol, but glycerol was required for stability to freezing and thawing. Dilute solutions (less than 0.1 unit/ml) of highly purified liver or mammary epimerase were very stable in the presence of 20 to 200 mM potassium phosphate, 20% glycerol, 1 mM mercaptoethanol, pH 7.6, at 4°. Such solutions were only slightly less stable at 24° as approximately 10% of the activity was lost during 4 h of incubation. Enzyme solutions stored at -20° for over 6 months still retained greater than 90% of their original activity.

**Molecular Weight Determinations**—Molecular weights of the liver and mammary epimerase were done with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (15). Plots of log molecular weight versus Rf were linear for the standard.
proteins which were beef liver catalase, ovalbumin, rabbit muscle glyceraldehyde-3-phosphate dehydrogenase, human γ-globulin, light chain and hen lysozyme. Five separate experiments which included duplicate gels of epimerase and quadruplicate gels of the standard proteins were done. The average values for the molecular weight of mammary and liver epimerases were 36,700 ± 1,900 (S.D.) and 37,100 ± 3,800 (S.D.), respectively.

Using a calibrated Bio-Gel A-0.5m column (1 × 95 cm) eluted with 50 mM potassium phosphate, 1 mM mercaptoethanol, pH 7.6, molecular weights were determined for the active highly purified mammary and liver epimerases and the thyroid epimerase purified through the NAD⁺-hexanolamine-Sepharose affinity chromatography step. In each case enzymatic activity was measured by Assay I. The standard proteins were bovine serum albumin, ovalbumin, β-lactoglobulin, and myoglobin. All the epimerase enzymes had apparent molecular weights of 40,000. Gel filtration of the liver epimerase was examined under a variety of conditions as a molecular weight of 70,000 had been previously reported (7). Chromatography of the crude liver extract and purified enzyme from UDP-hexanolamine-Sepharose on a Bio-Gel A-0.5m column gave a molecular weight of 40,000. Similar results were also obtained when chromatography was at either 4 or 24°C; in the presence or absence of added standard proteins, 500 mM KCl, 100 μM NAD⁺, or various combinations of these and with the enzyme in all stages of purity. A calibrated Bio-Gel P-150 column similar to that described by Langer and Glaser (7) was prepared and both highly purified and crude liver epimerase were chromatographed. The elution buffer was that described by Langer and Glaser (7), i.e. 10 mM glycylglycine, 0.5 mM diithiothreitol, 500 mM KCl, 10 μM NAD⁺, pH 7.0. The apparent molecular weight was 40,000 with both the impure (Step 1) and purified (Step 4) enzymatic preparations. An explanation for these apparent contradictory findings are as follows. The crude liver acetone powder extract contained an activity which produced in the presence of NAD⁺ an increase in 340 nm absorbance without any UDP-galactose and UDP-glucose dehydrogenase. The rate of increase of this activity was linear with time for at least 10 min. This activity accounted for 20 to 30% of the change in 340 nm absorbance observed by the liver acetone powder extract under the conditions of Assay I. The presence of the "blank" activity was determined during initial experiments with UDP-hexanolamine-Sepharose. With Assay I, 30% of the apparent activity of the extract was not retained by the affinity column. At first this unretained activity was thought to be the minor form of the two forms of UDP-galactose-4-epimerase reported by Ray and Bhaduri (25); but as the activity had no requirement for UDP-galactose, this was not the case. Fig. 4 illustrates the chromatography of the crude liver acetone powder extract in the presence of added known proteins on a calibrated Bio-Gel A-0.5m column. The activity requiring UDP-galactose had an apparent molecular weight of 40,000 whereas the activity which required only NAD⁺ had an apparent molecular weight of 71,000. This "blank" activity was more stable in the crude extract than the epimerase activity during prolonged storage at 4°C. In this report, all liver extract activities are corrected for this spurious change by determining the difference in rate of change of absorbance in the absence and presence of UDP-galactose under the conditions of Assay I. This was not necessary with the mammary extract as no "blank" rate was observed.

A molecular weight of approximately 33,000 was determined for the highly purified, reduced, and alkylated liver epimerase by Sephadex G-200 thin layer gel chromatography in guanidine HCl. This technique was not applied to the mammary enzyme because of the difficulty in obtaining milligram quantities of the highly purified epimerase.

NAD⁺ Requirement of UDP-galactose-4-epimerase—The NAD⁺ requirement of the highly purified liver and mammary enzymes and the partially purified thyroid enzyme were examined with Assay II. All three enzymes exhibited an absolute requirement for NAD⁺ for enzymatic activity after the NAD⁺-hexanolamine-Sepharose purification step. The apparent Kₐ values as determined by double-reciprocal plots were 0.27 ± 0.03 (S.D.), 0.28 ± 0.05, and 0.29 ± 0.05 μM for the liver, mammary, and thyroid enzymes, respectively. All data were fitted by the method of least squares, and the values represent averages of three or more experiments.

Initially, all three enzymes exhibited some activity in the absence of added NAD⁺. These results were similar to those reported by Tsai et al. (8) for partially purified bovine mammary UDP-galactose-4-epimerase. This partial activity was due to a slight NAD⁺ contamination of some UDP-galactose solutions.

One hour incubation of 1 unit of the purified liver, mammary, or thyroid enzyme with substrate but no UDP-glucose dehydrogenase (Assay I conditions) resulted in no net increase in 340 nm absorbance. Addition of UDP-glucose dehydrogenase after inactivating the epimerases with heat indicated that the enzymes were active.

Kₐ for UDP-galactose—Apparent Kₐ values were determined from double reciprocal plots for both the highly purified liver and mammary epimerases. Although similar results were obtained with both types of assays, Assay I was more convenient. Using Assay I, apparent Kₐ values for UDP-
Bovine UDP-galactose-4-epimerases

Amino acid composition of liver and mammary UDP-galactose-4-epimerase

| Amino acid | Liver | Mammary |
|------------|-------|---------|
| Alanine    | 30    | 29      |
| Aspartic acid | 31   | 34      |
| Cysteine   | 3     | 3       |
| Glutamic acid | 37   | 42      |
| Glycine    | 30    | 29      |
| Histidine  | 10    | 10      |
| Isoleucine | 12    | 8       |
| Leucine    | 98    | 19      |
| Lysine     | 26    | 32      |
| Methionine | 8     | 5       |
| Phenylalanine | 10  | 8       |
| Proline    | 14    | 12      |
| Serine     | 23    | 12      |
| Threonine  | 14    | 16      |
| Valine     | 20    | 16      |
| Tyrosine   | 12    | 8       |
| Arginine   | 23    | 12      |
| Total      | 341   | 356     |

* Half-cystine content was calculated as the sum of half-cystine and cysteic acid.

Samples were hydrolyzed and duplicate analyses were obtained as described under "Experimental Procedures." Analyses were based on 37,000 molecular weight for both enzymes. Tryptophan was not determined. All analyses revealed traces (less than 0.5 residue/molecule) of glucosamine and galactosamine.

DISCUSSION

The rationale for the purification procedure was based on the observation that uridine nucleotides were excellent competitive inhibitors of bovine mammary UDP-galactose-4-epimerase with $K_r$ values in the low micromolar range (8). The enzyme bound tightly to the UDP-hexanolamine-Sepharose column even in the presence of 200 mM buffer but could be selectively eluted with low concentrations (mM) of UMP. Although UDP does elute the enzyme, it is more expensive. The mammalian enzymes require NAD$^+$ for enzymatic activity (6, 8), but in contrast to the yeast (9) and Escherichia coli enzymes (3), the NAD$^+$ is dissociable, thus allowing use of a NAD$^+$-hexanolamine-Sepharose column.

The capacities of the three affinity columns used for routine purification were never exceeded. The capacity of the final small UDP-hexanolamine-Sepharose column was over 40 units of liver epimerase activity/ml of packed Sepharose. Eighty grams of acetone powder were chosen as the starting material as a compromise between recovery of activity and total amount of enzyme purified. Although the average overall recovery for the complete purification was approximately 60% (Tables I and II) with both mammary and liver enzymes, the entire purification has been completed with greater than 90% recovery. Several reports (26, 27) have indicated nonspecific binding of proteins to affinity materials prepared by the cyanogen bromide technique used in the preparation of NAD$^+$-hexanolamine-Sepharose (12). Hexanolamine-Sepharose was prepared as described by Mosbach et al. (12), but without completing the final NAD$^+$ coupling step. This material did not bind liver epimerase activity from a preparation partially purified by UDP-hexanolamine-Sepharose chromatography.

The specific activities of the mammary and liver UDP-galactose-4-epimerases of 66 and 210, respectively, are comparable to those of yeast, 40 to 80, (5) and E. coli, 230 (3). Based on yields of acetone powder, the average calf liver contains approximately 50 mg of this very active enzyme.

Extreme care was necessary to avoid NAD$^+$ contamination during investigation of the NAD$^+$ requirement as all three enzyme preparations have apparent $K_m$ values of less than 0.5 mM. The difference in the $K_m$ for the NAD$^+$ for the thyroid enzyme reported here, 0.29 ± 0.04 mM, and the 70 ± 0.04 mM reported by Rodriguez et al. (9) cannot be adequately explained at the present time.

The low apparent $K_m$ values for NAD$^+$, the absolute requirement for NAD$^+$ for enzymatic activity, and the absence of NADH production when large amounts of epimerases (1 unit/ml) were incubated with NAD$^+$ and UDP-galactose all indicate that the mechanism originally proposed by Maxwell for the liver enzyme (6) might also apply to the mammary and thyroid enzymes. This mechanism involves hydride abstraction from the substrate forming an enzymized oxidized substrate NADH complex and the NADH is not released from the enzyme. The NADH is then oxidized and the same hydrogen is used to form the epimeric product.

The $K_m$ values for UDP-galactose and the $K_m$ values reported in this work and those previously published (6, 8) agree fairly well. No substrate inhibition by UDP-galactose of liver epimerase was apparent although this was previously reported (6, 25) with crude enzyme preparations.

The DEAE-cellulose chromatography of the highly purified liver epimerase was examined in some detail since Ray and Bhaduri (25) have reported the separation of two distinct forms of bovine liver UDP-galactose-4-epimerase from an ammonium sulfate fraction of a tissue extract by this technique. Those workers made no attempt to purify these two forms further. Experiments reported here show that two peaks of activity can be generated by the stepwise elution. The activity of neither of these peaks was affected by galactose 1-phosphate or glucose 6-phosphate. Ray and Bhaduri (25) indicated that these carbohydrates were effectors of their minor form of epimerase. The effects of the carbohydrate phosphates observed by Ray and Bhaduri (25) can be attributed to other enzymes in their crude enzyme preparations which utilize these compounds.
An apparent molecular weight of 40,000 was determined for the active, highly purified bovine liver UDP-galactose-4-epimerase by gel filtration under a variety of conditions. Molecular weights of 36,700 ± 1,900 (S.D.) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and 33,000 by thin layer gel chromatography in guanidine HCl were determined for this same enzyme. These data indicate that the enzyme is not a dimer of 70,000 as reported by Langer and Glaser (7). The value of 70,000 was determined with a partially purified enzyme preparation. The "blank" activity of the liver acetone powder extract which required only NAD⁺ for activity had an apparent molecular weight of 71,000 by gel filtration and most likely is the activity reported by Langer and Glaser (7), particularly since it is more stable than the epimerase in crude preparations. This "blank" activity which is currently being further investigated may be the histochemically demonstrated "nothing dehydrogenase" (28) and appears to be related to liver alcohol dehydrogenase which has a molecular weight of about 70,000.

The liver and mammary epimerases appeared to be very similar in a number of characteristics. The only major differences were in amino acid compositions and in specific activities of the highly purified enzymes. The specific activities of these enzymes are high relative to most mammalian enzymes and as a consequence they are found in low concentrations in these tissues. In addition, they have low apparent Kₘ values for substrates which makes them efficient catalysts.

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