Specific Tritium Labeling of Uridine Diphosphogalactose 4-Epimerase by D-[1-^{3}H]Galactose*

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ABSTRACT: The sugar product during the concerted stoichiometrical reduction of the bound DPN of uridine diphosphogalactose 4-epimerase (EC 5.1.3.2) by specific sugars and 5'UMP was identified as a sugar acid by means of: (1) thin-layer chromatography, (2) behavior on weak and strong basic anion-exchange resins, (3) character on charcoal column, and (4) specific transfer of tritium labeled on C-1 position of galactose to the bound DPN of the epimerase. The tritium labeling of the bound DPN was subject to an appreciable dilution.

Uridine diphosphate galactose 4-epimerase from yeast and *Escherichia coli* induced by galactose contain tightly bound DPN$^+$ (Maxwell *et al.*, 1958; Maxwell and Szulmajster, 1960; Wilson and Hogness, 1964).

Yeast and *E. coli* epimerases have other features in common. Both enzymes can be converted to highly fluorescent DPNH enzymes upon incubation with D-galactose, D-glucose, or L-arabinose, provided free 5'UMP is also present (Bhaduri *et al.*, 1965; Kalckar *et al.*, 1970; Bertland *et al.*, 1971). The specific substrate, UDP-galactose,[1] is also able to generate a fluorescent enzyme but only from the *E. coli* epimerase (Kalckar *et al.*, 1970; see also following paper).

In the present article it will be shown that the concerted reduction of epimerase brought about by addition of 5'UMP and the specific sugars, L-arabinose and D-galactose, is an oxidation–reduction between the sugar and the enzyme-bound DPN$^+$.

The C-1 hydrogen of sugars was transferred to the enzyme-bound DPN$^+$. This process gives, however, rise to an appreciable dilution of the tritium transferred.

Experimental Section

Enzymes. UDP-galactose 4-epimerase from *Saccharomyces fragilis* (*Candida pseudotropicalis*) was prepared according to the method of Darrow and Rodstrom (1968). Epimerase from *E. coli* was purified by a slight modification of the procedure of Wilson and Hogness (1964) (see following paper). In this procedure a particular *E. coli* K-12 strain W3092cy$^-$, the epimerase of which is endogenously induced, was grown in large scale (Kalckar *et al.*, 1970). Enzyme assays were carried out by measuring the DPNH production after the addition of UDG-galactose, in the presence of excess DPN$^+$ and UDP-glucose dehydrogenase (Darrow and Rodstrom, 1968). The properties of the *E. coli* 4-epimerase used will be described briefly in the following paper (Seyama and Kalckar, 1972).

Radiochemicals. L-[1-^{14}C]Arabinose, D-[1-^{14}C]galactose, and D-[1-^{3}H]galactose were obtained from New England Nuclear. Radioactivities were measured in a liquid scintillation counter, Packard Tri-Carb Model 3320. Double isotope counting was done with the isotope exclusion method (Kobayashi and Maudsley, 1970). Premixed scintillation counting solution, Aquasol, was purchased from New England Nuclear. Thin-layer plates were scanned in Packard scanner Model 7201. They were also measured in the liquid scintillation counter after scraping successive 5-mm wide bands of a developed plate with a razor blade.

Chemicals. Arabonic acid and D-arabonic acid δ-lactone were obtained from K & K Laboratories. Calcium D-galactonate and D-galactose-1,4-lactone were obtained from Pfanstiehl Laboratories. All other reagents were obtained from general commercial sources.

Thin-Layer Chromatography. Thin-layer chromatography was performed on a plate of Avicel-cellulose obtained from Brinkmann Instruments, Inc., using solvent system of ethyl acetate–pyridine–water (12:5:4, v/v) (Smith, 1958). This system was also used for preparative purposes, but the plates were washed with methanol (dipping in absolute methanol for 10 min) to minimize the contamination at the time of methanol extraction after development.

Ion-Exchange Resins. Amberlite CG4B, Amberlite CG50, and Dowex 1 (formate) were suspended in water and packed in disposable pipets (0.5 × 5 cm). The columns were eluted with water.

Charcoal Column Chromatography. Darco G-60 obtained from Matheson Coleman & Bell was washed with concentrated HCl and HCl was removed by extensive washing with distilled water. Disposable pipets (0.5 × 5 cm) were packed with 200 mg of charcoal, mixed with 200 mg of Celite 535 (Johns-Manville). Sample was applied with water and the column was eluted stepwise by increasing the ethanol concentration.

Sephadex Column Chromatography. Sephadex G-50 column (1.1 × 15 cm) was prepared with 0.001 M Tris-HCl buffer (pH 8.5) and eluted with the same buffer.

Other Methods. Protein was determined by absorption at 280 and 260 mμ (Layne, 1957). Fluorescence was measured with a Turner Model 210 Spectro absolute spectrofluorometer (Turner, 1964). Absorbance was obtained by a Zeiss PMQ II spectrophotometer.

Results

Chemical Modification of UDP-Galactose 4-Epimerase. UDP-galactose 4-epimerase (24 mg; 200 nmoles from yeast

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[1] Abbreviations used are: UDP-glucose 4-epimerase (EC 5.1.3.2), UDP-galactose 4-epimerase; uridine 5'-(α-D-galactopyranosyl pyrophosphate), UDP-galactose or UDPGal; 2-mercaptoethanol, 2-ME.

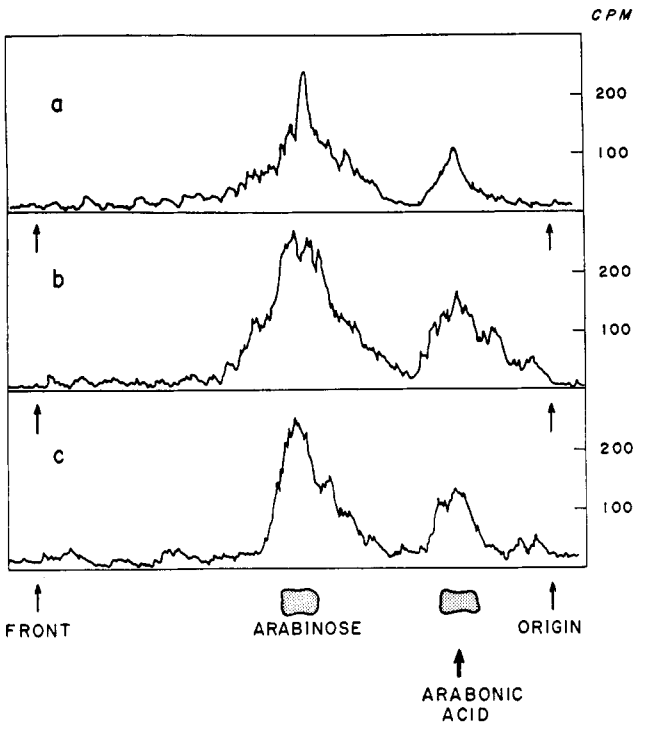

FIGURE 1: Scanning of radioactivities of thin-layer chromatograms of sugars after the incubation of UDP-galactose 4-epimerase with L-[1-^{14}C]arabinose and 5'UMP. (a) Sugars separated from enzyme by dialysis; (b) "a" passed through Amberlite CG4B column; (C) "b" passed through Amberlite CG50 column. Radioactivities were detected by Packard scanner Model 7201 under the following conditions: time constant, 30-sec, range, 300 cpm, scanning speed, 10 cm/hr; plate, Avicel-cellulose plate (20 × 20 cm), 0.1 mm thick, developing solvent; ethyl acetate-pyridine-water (12:5:4, v/v). Standards, arabinose, and arabonic acid were detected with alkaline silver nitrate spray.

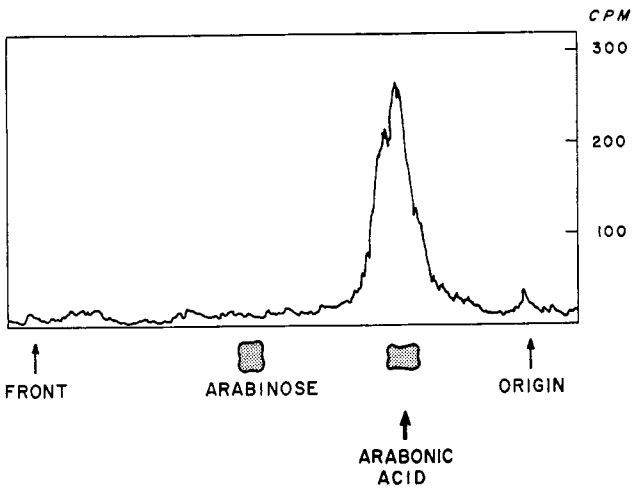

FIGURE 2: Scanning of radioactivities of thin-layer chromatogram of the substance obtained by preparative thin-layer chromatography. Conditions were the same as described in Figure 1.

was incubated with 400 nmoles of L-[1-^{14}C]arabinose (7×10^6 cpm) and 600 nmoles of 5'UMP in 6.3 ml of 0.1 M Tris-HCl buffer (pH 7.5), containing 10^{-3} M 2-ME and 10^{-4} M EDTA. The fluorescence emission at 450 mμ (excited at 350 mμ) due to reduction of bound DPN$^+$ increased 8.6-fold during the incubation at 4° for 4 days. The reaction mixture was dialyzed against distilled water in the flow-type dialysis cell (continuous dialysis), obtained from Bolab, Inc. The outer fluid was collected and concentrated with rotary evaporator.

Detection of Products from L-*Arabinose.* Thin-layer chromatography was used to examine the sugar components in the outer fluid. Sample was applied on Avicel-cellulose plate and developed with ethyl acetate–pyridine–water (12:5:4, v/v) (Figure 1a). There were two peaks, one is L-arabinose and the other is unknown substance. The R_F of the second peak was smaller than that of L-arabinose, and it corresponded to that of L-arabonic acid.

Removal of Salts by Ion-Exchange Resins. Since the incubations were done in 0.1 M Tris-HCl buffer, ion-exchange resins were used to remove interfering salts on thin-layer chromatography. Outer fluid was applied to Amberlite CG4B column first and eluted with water, which recovered 94.9% of radioactivity. The eluate was examined on Avicel-cellulose plate. The same pattern of radioactive peaks were obtained (Figure 1b). The water eluate from Amberlite CG4B was applied to Amerlite CG50 column and eluted with water. In this case, 99.1% of radioactivity was recovered. The eluate was examined again on Avicel-cellulose plate, and the pattern of radioactive peaks remained unchanged (Figure 1c).

Separation of Unknown Substances. To separate the unknown substances (with the R_F value of arabonic acid) from L-arabinose, preparative thin-layer chromatography was used. The water eluate from Amberlite CG50 column was applied on the prewashed plates, and developed with ethyl acetate–pyridine–water (12:5:4, v/v). The band corresponding to the unknown peak was scraped off from the plates and extracted with 70% methanol–water. Cellulose powder was removed by centrifugation and the methanol solution was concentrated to dryness and was reextracted with water. The substance obtained by preparative thin-layer chromatography showed single peak on Avicel-cellulose plate (Figure 2).

Dowex 1 Column Chromatography. The unknown substance corresponded to arabonic acid on thin-layer chromatography, but this is not the full proof of identification.

The unknown substance was not absorbed on Amberlite CG4B, so it is not a strong acid. The substance obtained by preparative thin-layer chromatography was applied to Dowex 1 (formate form) column. The column was eluted with water, and 10.5% of radioactivity was recovered with water. The substance obtained by preparative thin-layer chromatography was dissolved in 0.1 N NaOH and heated at 100°, 5 min. This solution was applied to above column and only 1.7% of radioactivity was eluted with water; 98.3% was absorbed on Dowex 1 resin.

Charcoal Column Chromatography. The substance obtained by preparative thin-layer chromatography was dissolved in water and applied to acid-washed Darco G60 column. L-Arabinose was not absorbed on charcoal, but the substance remained on column. The column was eluted with water containing stepwise increasing ethanol concentration. The substance was eluted with 15% ethanol–water (Figure 3). So it is possible to separate the substance by charcoal column instead of preparative thin-layer chromatography. Authentic L-arabonic acid was also tested on charcoal column and showed exactly the same behavior with the unknown substance.

Chemical Reduction of UDP-Galactose 4-Epimerase with D-[*1-*^{3}*H*]*Galactose and* D-[*1-*^{14}*C*]*Galactose.* UDP-Galactose 4-epimerase from *E. coli* (4.8 mg, (60 nmoles) was incubated with 154 nmoles of D-galactose containing D-[1-^{3}H]galactose (2.76×10^8 dpm) and D-[1-^{14}C]galactose (1.39×10^7 dpm) in the pres-

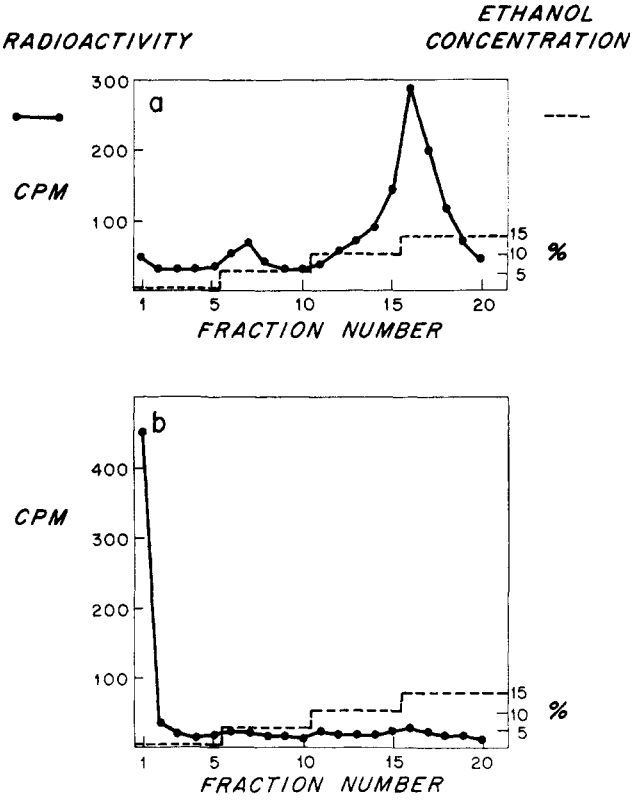

FIGURE 3: Charcoal column chromatography. (a) The substance obtained by preparative thin-layer chromatography; (b) L-[1-^{14}C]arabinose. Acid-washed Darco G-60 (200 mg), mixed with 200 mg of Celite 535, was packed in disposable pipets (0.5 × 5 cm). The column was eluted stepwise by increasing the ethanol concentration. Five milliliters of each fraction was collected; 0.5 ml was taken for radioactivity measurement.

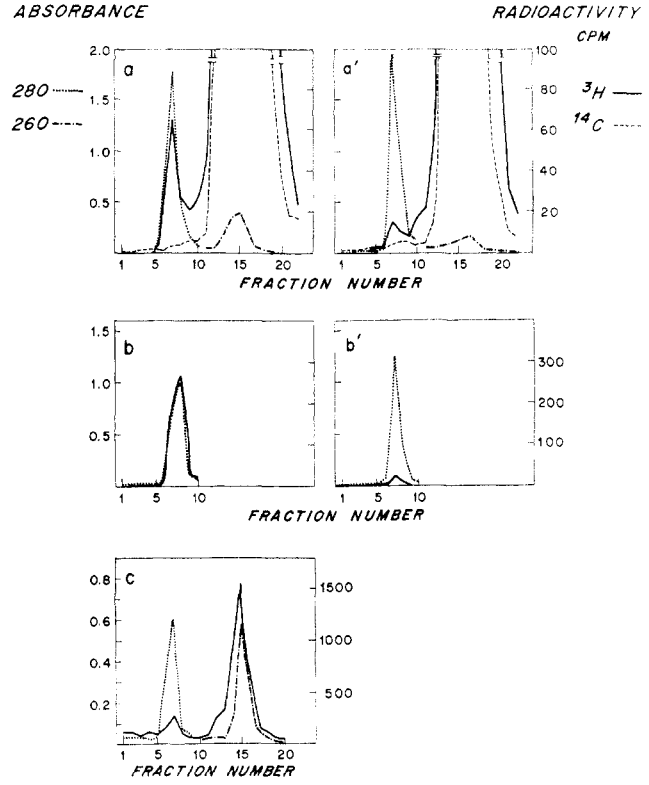

FIGURE 4: Sephadex G-50 column chromatography. (a) *E. coli* epimerase after incubation with D-galactose in the presence of 5'UMP; (a') *E. coli* epimerase after incubation with D-galactose in the absence of 5'UMP; (b) rechromatography of epimerase from column a. (b') rechromatography of epimerase from column a'; (c) epimerase from b after heating at 60°, 9 min in 1 mM Tris-HCl buffer (pH 8.5). Sephadex G-50 column (0.9 × 15 cm) was equilibrated with 1 mM Tris-HCl buffer (pH 8.5), and fractions of 1 ml were collected. 10 μl (a, a'), 50 μl (b, b'), and 500 μl (c) were taken for radioactivity measurement.

ence of 100 nmoles of 5'UMP in 1 ml of 0.1 M Tris-HCl buffer (pH 7.5) containing 10^{-3} M 2-ME and 10^{-4} M EDTA. The fluorescence increased 9.2-fold and the specific activity decreased to 41.8% during 3-hr incubation at room temperature. In the control mixture, which did not contain 5'UMP, there was no increase in fluorescence, and no decrease of specific activity. The reaction mixtures were applied to Sephadex G-50 column equilibrated with 0.001 M Tris-HCl buffer (pH 8.5) and eluted with the same buffer (Figure 4a). Tritium counts were detected at the modified epimerase peak, but no ^{14}C counts were detected on it. This tritium was firmly bound to epimerase since it was still on the epimerase after rechromatography (Figure 4b).

The fluorescent protein peak in Figure 4b corresponded to approximately 1 mg of *E. coli* epimerase and since the fluorescence increase indicated that close to 50% reduction had taken place, 5–6 nmoles of bound DPNH had been formed. The stoichiometry with respect to tritium showed the following relationships (Table I).

The tritiated fluorescent epimerase was heated at 60° for 9 min and then applied on Sephadex G-50 column (Figure 4c). The protein peak showed neither DPNH fluorescence nor tritium counts. As reported by Bertland and Bertland (1971), cofactor is separated from epimerase by heating in low ionic strength buffer. The substance separated from enzyme was examined on PEI-cellulose plate and developed with 0.3 M LiCl. There were two peaks corresponding to DPN^+ and DPNH, respectively (Figure 5).

Stoichiometry of the Reaction. UDP-Galactose 4-epimerase (2.40 mg) from *E. coli* of an approximate purity of 70% (Seyama and Kalckar, 1972) was incubated with 1.5 μmoles of L-[1-^{14}C]arabinose (15 μCi) in the presence of 10^{-4} M 5'UMP. The activity decreased to 15–20% and fluorescence increased about 15-fold in 2 hr at 25°. The ratio of L-arabinose to reduced enzyme was 90:1. Thin-layer chromatography revealed the ratio of L-arabinose to L-arabonic acid to be 73:1.

Discussion

Previous observations on the chemical modification of UDP-galactose 4-epimerase with specific sugars and 5'UMP have demonstrated a reduction of the bound DPN^+ complex to that of DPNH (Bhaduri *et al.*, 1965), while 5'UMP is bound, approximately 1 mole/mole of epimerase (Bertland and Kalckar, 1968). However, the nature of the sugar product formed in this stoichiometrical reaction remained to be identified. The present work deals with the identification of the sugar products formed in the concerted reduction and also with the fate of the tritium introduced in the 1 position of D-galactose in order to decide between the various reaction mechanisms in the oxidation–reduction process.

The product from sugars after the modification of enzyme was detected and successfully separated by means of thin-layer chromatography. Thr R_F value of the product on Avicel-cellulose plate corresponded to that of sugar acid (L-arabonic acid or D-galactonic acid). The product had the char-

TABLE I

	nmoles of Bound DPNH	cpm/nmole	$^3H:^{14}C$
Incubation mixture of [^{12}C,^{14}C,^3H]Gal		150,000	5
Newly reduced epimerase	5	15,000[a]	>50

[a] Assuming a mass effect of only 3; if the mass effect for ^3H were 3^3, the cpm per nmole of bound DPNH would be 135,000.

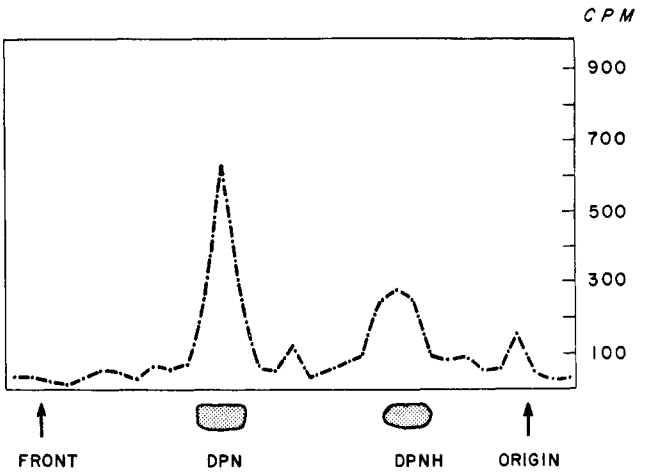

FIGURE 5: Thin-layer radiochromatogram of the substance separated from epimerase by heating at 60°, 9 min in 1 mM Tris-HCl buffer (pH 8.5). Radioactivities (^3H) were measured in a liquid scintillation counter after scraping successive 5-mm wide bands of a developed plate. Plate: PEI-cellulose plate (20 × 20 cm), 0.1 mm thickness, developing solvent: 0.3 M LiCl. Standards, DPN and DPNH were detected with an ultraviolet lamp.

acter of weak acid according to the behavior on ion-exchange resins. Amberlite CG4B (weak basic anion exchanger) could not absorb the product, but Dowex 1 (strongly basic anion exchanger) absorbed almost 90% of the product. After treatment of product with hot alkali, more than 98% of the product was absorbed on Dowex 1 column. This phenomenon may be explained if the product is a sugar acid, and some of the sugar acid exists as lactone in water. In this case, 90% of the product exists as sugar acid and 10% as lactone.

The assumption of sugar acid formation was confirmed by charcoal column chromatography. The product was adsorbed on acid-washed Darco G60, and was eluted with 15% ethanol–water. Neutral sugars were not absorbed on charcoal, but L-arabonic acid showed the same behavior with the product.

The active sugars are presumably oxidized through a non-catalytical stoichiometrical reaction with the enzyme (*cf.* Kalckar *et al.*, 1970). This presumption was ascertained by the fact that the ratio of L-arabinose:epimerase was close to that of L-arabinose:L-arabonic acid after modification.

During the reduction of UDP-galactose 4-epimerase, active sugars are oxidized to sugar acid, while enzyme-bound DPN is reduced to bound DPNH. Coupling of this oxidation–reduction reaction suggests the possibility of specific hydrogen transfer from the C-1 carbon of the free sugars to enzyme-bound DPN^+. Hence, if the C-1 position of these sugars is labeled with tritium, this tritium should be transferred to enzyme-bound DPN^+.

The experiment with D-[1-^3H]galactose confirmed this mechanism and enzyme-bound DPN was successfully labeled with tritium. This tritium transfer was observed only if 5'UMP was present. Hence, concerted oxidation–reduction differs strikingly from the otherwise related reaction catalyzed by galactose dehydrogenase. The tritium remained on the enzyme through Sephadex rechromatography, but was released from the enzyme by heating at 60° for 9 min, which also released the cofactor from the epimerase (Bertland and Bertland, 1971).

The basis for the dilution of tritium is obscure. Either the epimerase itself or traces of another enzyme catalyze a reaction in which the 1-H of galactose is exchanged with water.

This tritium-bearing substance split off from the enzyme appeared in the DPN^+ and DPNH peaks on Sephadex column or PEI-cellulose thin-layer chromatography. On thin-layer plate, 63% of radioactivity moved with the R_F value of DPN^+ and only 37% was detected as DPNH. The interpretation of the appearance of tritium on the DPN^+ spot is complicated by the fact that the heat treatment of the reduced epimerase in very dilute buffer generates a larger proportion of DPN^+ as compared with previous techniques (Bertland and Bertland, 1971). It is plausible that a combination of stereospecific reduction and stereounspecific reoxidation during the heating step is responsible for the appearance of ^3H in DPN^+ as well as DPNH. Even in the case of reduction of DPN^+ epimerase from *E. coli* by tritiated borohydride, Nelsestuen and Kirkwood could report stereospecificity (1970). It is, therefore, reasonable to assume that the reduction by galactose is the stereospecific reaction.

The transfer of the 1-^3H from galactose to the prosthetic group of epimerase can well be taken as another criteria for a dehydrogenation of the active sugar on the C-1 position forming the corresponding sugar acid and its lactone (galactonate and galactonolactone) in this concerted stoichiometrical oxido-reduction.

In a recent study of *E. coli* epimerase, using glucose deuterated in various positions (Davis and Glaser, 1971), tentative conclusions were presented in favor of a hydrogen transfer from the 3 position of this sugar. This stands in contrast to the present findings as well as to our previous findings that 2-deoxyglucose as well as 3-deoxyglucose are as active as glucose in the reduction of epimerase (Bertland *et al.*, 1971). The reaction mechanism demonstrated in the present paper, may play a role in the regulation of epimerase, but as will appear from the succeeding article, it is not a model reaction of the catalytic epimerization of glucose to galactose.

Acknowledgment

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Interaction between Uridine Diphosphate Galactose and Uridine Diphosphate Galactose 4-Epimerase from *Escherichia coli**

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ABSTRACT: Incubation of purified *Escherichia coli* UDPGal-4-epimerase in high concentrations with excess of its specific substrate, UDP-galactose gives rise to a marked increase of the blue fluorescence emission of the bound NADH formed. This type of reduction is reminiscent of the concerted reduction brought about by 5′UMP and free galactose. Both reactions can be characterized as reductive inhibitions, since the fluorescent enzymes show only a small fraction of the catalytical activity of the native epimerase. However, labeling of the 1-H of the galactose moiety of UDP-galactose with ^3H does not elicit a transfer of ^3H to the bound DPN^+ of epimerase as seen in the concerted reduction by free galatose labeled in the same position. Also, intermediates formed from UDP-hexoses are bound much stronger to reduced epimerase than UDP-hexoses. The intermediates or their derivatives can be released from epimerase by heating at 60° for 5 min. They are nonreducible by sodium borohydride.

It has been shown that addition of an excess of UDP-glucose[1] to *Escherichia coli* epimerase is able to generate DPNH epimerase in significant amount (Wilson and Hogness, 1964). Recently, we have shown that UDP-galactose added in excess to *E. coli* epimerase gives rise to a striking increase in fluorescence emission at 450 mμ (Kalckar *et al.*, 1970).

This reaction is very reminiscent of the reduction of *E. coli* epimerase by free D-galactose (or L-arabinose) and 5′UMP (Seyama and Kalckar, 1972). Epimerase isolated from yeast can also be reduced by these two sugars in the presence of 5′UMP (Bhaduri *et al.*, 1965; Kalckar *et al.*, 1970; Bertland *et al.*, 1971), but it does not form a fluorescent enzyme upon addition of UDP-galactose or UDP-glucose (Bhaduri *et al.*, 1965).

In the present paper we have first ascertained that the generation of fluorescent *E. coli* epimerase by UDP-galactose is due to the interaction with the nucleotide itself and not with hydrolysis products such as galactose or glucose and 5′UMP.

It was shown in the previous paper (Seyama and Kalckar, 1972) that the generation of fluorescent epimerase by D-[1-^3H]-galactose gives rise to a tritiated epimerase (enzyme-bound [^3H]DPN). It therefore was of interest whether a related reaction might also take place upon addition of UDP-galactose to epimerase, the nucleotide being labeled in the corresponding position of the galactose moiety.

Materials and Methods

Enzymes. UDP-Galactose 4-epimerase from *E. coli* K-12 strain, 3092cy$^-$ (Kalckar *et al.*, 1970), was purified according to the procedure of Wilson and Hogness (1964) with slight modification. The hydroxylapatite chromatography step gave very low recovery of activity, and it was possible to obtain the activity with somewhat improved recovery only when we used an elution gradient of 0.015–0.060 M potassium phosphate buffer (pH 6.5). The activity was measured by the method of Darrow and Rodstrom (1968). The purest enzyme preparation showed a catalytic activity of 5×10^3 to 6×10^3 μmoles per mg of protein per hr at 25°. Electrofocussing (courtesy of Dr. D. Stathakos of our department) revealed the presence of 20–30% of other protein, presumably inpurities. Large-scale preparation from 500-l. fermentation has been

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[1] Abbreviations used are: UDP-glucose 4-epimerase (EC 5.1.3.2.), UDP-galactose 4-epimerase; uridine 5′-(α-D-galactopyranosyl pyrophosphate), UDP-galactose and UDPGal; uridine 5′-(α-D-glucopyranosyl pyrophosphate), UDP-glucose and UDPGlc; 2-mercaptoethanol, 2-ME; 1-H, hydrogen in 1 position of sugar.