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The Enzymes of the Galactose Operon in *Escherichia coli*

I. PURIFICATION AND CHARACTERIZATION OF URIDINE DIPHOSPHOGALACTOSE 4-EPIMERASE*

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Uridine diphosphogalactose 4-epimerase catalyzes the terminal reaction in the following sequence, which functions as the major pathway of galactose metabolism in *Escherichia coli* as well as in many other organisms (1).

$$\text{D-Galactose} + \text{ATP} \xrightarrow{\text{galactokinase}} \text{galactose-1-P} + \text{ADP}$$

$$\text{Galactose-1-P} + \text{UDP-glucose} \underset{\longleftarrow}{\xrightarrow{\text{galactose-1-P uridyltransferase}}} \text{UDP-galactose} + \text{glucose-1-P}$$

$$\text{UDP-galactose} \underset{\longleftarrow}{\xrightarrow{\text{UDP-galactose 4-epimerase}}} \text{UDP-glucose}$$

$$\text{Sum:} \quad \text{D-Galactose} + \text{ATP} \longrightarrow \text{glucose-1-P} + \text{ADP}$$

Our interest in the *E. coli* UDP-galactose 4-epimerase is 2-fold. On the one hand, it is a member of the above set of enzymes which, with their respective genes, have particular advantages for the development of a system *in vitro* in which protein synthesis and its control can be analyzed. On the other hand, this enzyme catalyzes a reaction of intrinsic interest which has been only partially elucidated.

The biosyntheses of these three enzymes are coordinately induced by either D-galactose or D-fucose (2). This coordinate induction evidently results from the fact that the genes which individually control each of these enzymes form a closely linked group, or operon, which functions as a single unit within the *E. coli* chromosome (2, 3). One advantageous characteristic of this galactose operon is that deoxyribonucleic acid molecules which contain it can be isolated from a smaller, more specific source than the *E. coli* cell. This alternative source is the λdg bacteriophage, a defective variant of coliphage λ in which the above operon has been inserted into the DNA molecule of the phage, this insertion being accompanied by a loss of some of the normal λ phage genes (4). Because of the difference in DNA content between the λdg and *E. coli* genomes, the ratio of galactose operons to DNA is about 100-fold greater for DNA isolated from λdg than for DNA isolated from *E. coli*. This operon has been further purified by midpoint breakage of complete λdg DNA molecules and isolation of the half-molecule that contains the galactose operon (5). Thus, at the initiating end of the pathway of synthesis of these enzymes, we have available a DNA solution that is highly specific for the corresponding genes.

A preliminary step necessary for exploiting this DNA in a system of protein synthesis is the purification and characterization of the end products, *i.e.* the three enzymes determined by the galactose operon. Galactokinase has been extensively purified, although its physical and chemical characteristics have as yet received little attention (6). The galactose-1-P uridyltransferase has been partially purified, but no significant characterization of the protein has been done (7). There appear to be no reports in the literature concerning any appreciable purification of the *E. coli* UDP-galactose 4-epimerase. Hence all three enzymes require more study before even relatively crude parameters of structure can be specified. In this initial paper, we report the purification of UDP-galactose 4-epimerase and its characterization with regard to homogeneity, molecular weight, and other physicochemical characteristics.

The second aspect of our interest in the UDP-galactose 4-epimerase centers about the mechanism of the epimerization reaction which results in an inversion of the hydrogen and hydroxyl group on carbon 4 of the hexose. The reaction has been studied with partially purified enzymes derived from calf liver (8), yeast (9), and *Lactobacillus bulgaricus* (10). In each of these cases, no incorporation of either ^{18}O or tritium from water into the hexose residue could be observed, tending to eliminate mechanisms involving dehydration-hydration or hydroxyl ion-catalyzed inversion. The involvement of DPN as a necessary added cofactor for the liver enzyme (8) and as a tightly bound moiety in the yeast enzyme (9), has led to the supposition that the epimerization mechanism includes a reversible oxidation-reduction between diphosphopyridine nucleotide and the hexose residue. However, no incorporation of tritium from free DPN or DPNH into the hexose residue was observed during the epimerization catalyzed by either the liver (8) or the yeast enzyme (9). Furthermore, it has recently been found that carbon-bound tritium in position 4 of UDP-glucose is retained in the UDP-galactose formed with the yeast enzyme (11). Thus the experiments in the literature yield no direct evidence that DPN is a reactant in the epimerization reaction.

We report here results that invoke enzyme-bound DPN as a reactant in the epimerization reaction catalyzed by the *E. coli* enzyme.

EXPERIMENTAL PROCEDURE

Materials

Bacteria—E. coli K12 *gal*$^+$ (λdg)[1] was generously supplied by

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[1] In designating the bacterial type, *gal*$^+$ indicates that all genes in the galactose operon are wild-type; (λdg) indicates that the strain is lysogenic for phage λdg. If no parenthesis terminates the designation, the strain is not of any known lysogenic type.

TABLE I

Purification of UDP-galactose 4-epimerase

Fraction and step	Units		Protein	Specific activity
	$ml^{-1} \times 10^{-3}$	Total $\times 10^{-6}$		
			mg/ml	*units/mg* $\times 10^{-2}$
I. Extract	1.2	1.7	24	0.50
II. Streptomycin supernatant	1.0	1.6		
III. First ammonium sulfate fractionation	5.6	1.5	32	1.7
IV. Second ammonium sulfate fractionation	14	1.4	40	3.5
V. First potassium phosphate fractionation	19	1.2	23	8.3
VI. Hydroxylapatite chromatography	18	0.89	3.2	56
VII. DEAE-Sephadex chromatography	32	0.70	3.2	100
VIII. Second potassium phosphate fractionation	125	0.61	9.4	130

Dr. E. Horowitz (12). It is a homogenote (4) that is singly lysogenic for bacteriophage λdg and was chosen as a source of enzyme since the induced synthesis of enzymes of the galactose operon occurs at a 3-fold greater rate in this strain than in the nonlysogenic *E. coli* K12 *gal*$^+$ (strain W3110 of Dr. E. Lederberg). With respect to this characteristic, the specific activity of UDP-galactose 4-epimerase in crude extracts of the K12 *gal*$^+$ (λdg) and K12 *gal*$^+$ strains harvested during exponential growth in inducing medium is 50 and 17 units per mg of protein, respectively.

The bacteria were grown with maximal aeration at 37° in a Biogen apparatus (American Sterilizer Corporation) containing 100 liters of inducing medium (0.033 M KH_2PO_4, 0.067 M K_2HPO_4, 0.015 M $(NH_4)_2SO_4$, 1.8×10^{-6} M $FeSO_4$, 1.0×10^{-3} M $MgSO_4$, 0.11 M glycerol, 1.1% Bacto-casamino acids (Difco Laboratories, Inc.), and 5×10^{-4} M D-fucose). Under these conditions, the growth is exponential (40 minutes doubling time) throughout the growth period, and increasing the D-fucose concentration does not further increase the rate of UDP-galactose 4-epimerase synthesis. The suspension was chilled to 10–15° when the bacterial density reached 1×10^{10} viable cells per ml. The cells were then harvested at 29,000 × *g* in a Spinco model 170 continuous flow centrifuge, the temperature never exceeding 15°. The cell pellets were frozen in liquid nitrogen and stored at −20°.

Enzymes—The UDP-glucose dehydrogenase used in the assay of UDP-galactose 4-epimerase was Fraction V of a purification procedure recently developed in this laboratory (13). It had a specific activity of 2×10^4 units per mg (14) and contained less than 0.004 unit of UDP-galactose 4-epimerase per mg.

Reagents—The DPN, D-fucose, UDP-glucose, and UDP-galactose were commercial preparations from Sigma Chemical Company. The UDP-glucose had a molar extinction coefficient at 260 mμ (ϵ_{260}) of 9.8×10^3 M^{-1} cm^{-1} when molarity was determined by DPNH formation under the conditions of the UDP-glucose dehydrogenase assay (14). The ϵ_{260} for UDP-galactose was 9.6×10^3 M^{-1} cm^{-1} when molarity was determined by the same procedure used for UDP-glucose with the exception that purified UDP-galactose 4-epimerase was added to convert UDP-galactose to UDP-glucose. The UDP-galactose contained less than 0.1% UDP-glucose when tested with UDP-glucose dehydrogenase, and on acid degradation 1 mole of UDP-galactose (from ϵ_{260} value) yielded 1.97 moles of inorganic phosphate.

Hydroxylapatite was Hypatite C from Clarkson Chemical Company. DEAE-Sephadex A-50 was obtained from Pharmacia Laboratories. Glass beads of 200-μ average diameter (Superbrite 100, from the Minnesota Mining and Manufacturing Company) were acid-washed. Streptomycin sulfate was a gift from Merck Sharp and Dohme, Inc., and the hydrolyzed starch used for gel electrophoresis was purchased from Connaught Laboratories, Toronto.

Methods

Assay of UDP-galactose 4-Epimerase—The method of assay is a modification of that developed by Kalckar (15). The UDP-galactose 4-epimerase is added to 0.05 μmole of UDP-galactose, 0.25 μmole of DPN, 440 units of UDP-glucose dehydrogenase, and 20 μmoles of glycine-NaOH buffer, pH 8.5, yielding a final volume of 0.20 ml contained in a microcuvette with quartz windows 1 cm apart and sides separated by 0.25 cm. The solution temperature is 27.0° and the optical density at 340 mμ is read every 30 seconds in a Zeiss PMQ-II spectrophotometer whose cell compartment is maintained at 27.0° by a water jacket. The change in optical density per minute is constant with time over a total change of 0.12 for a given enzyme concentration and is proportional to enzyme concentration up to a change of 0.070 per minute. The change in optical density per minute is converted to micromoles of UDP-glucose per minute by using an ϵ_{340} for DPNH of 6.2×10^3 M^{-1} cm^{-1} (16), 2 μmoles of DPNH being formed per μmole of UDP-glucose. One unit of enzyme is defined as that amount which catalyzes the formation of 1 μmole of UDP-glucose per hour in this assay. The rate of DPNH formation is not increased by increasing the UDP-glucose dehydrogenase concentration in the assay solution.

Other Assays—The activity of UDP-glucose dehydrogenase was assayed by the procedure of Strominger *et al.* (14). The method of Chen *et al.* as modified by Ames and Dubin (17) was used to measure total phosphate in UDP-galactose.

Protein, during purification, was measured by the method of Lowry *et al.* (18), with crystalline bovine serum albumin as a standard. Protein concentrations in the sedimentation experiments were calculated from the refractive index determined in the Spinco model E ultracentrifuge by using a double sector boundary-forming cell (19) and assuming a refractive index increment of 1.86×10^{-4} ml per mg (20). With the purified UDP-galactose 4-epimerase, 1 mg of protein calculated from the refractive index equals 0.86 mg of protein determined by the Lowry method.

A Spinco model E ultracentrifuge equipped with temperature control, schlieren phase plate, and Rayleigh interference optics was used. Eastman Spectroscopic IIG plates were used to photograph schlieren patterns, which were read on a Gaertner M 2001 RS two-dimensional microcomparator.

RESULTS

Purification of Enzyme

The following procedure for the purification of UDP-galactose 4-epimerase from *E. coli* K12 *gal*$^+$ (λdg) induced with D-fucose results in a 260-fold purification with a 35% yield. The details are given below and the results in Table I. Unless otherwise

stated, manipulations were at 0–5° and centrifugations at 14,000 × *g* for 20 minutes, and all buffers contained 0.001 M EDTA.

Preparation of Extract—Frozen cells, 150 g, were partially thawed and added to a Waring Blendor along with 60 ml of 0.01 M potassium phosphate buffer, pH 7.0. This mixture was blended at low speed to apparent homogeneity and then 390 g of glass beads were slowly added. The blendor was turned to high speed for 10 minutes (maximum temperature, 15°), after which 300 ml of the above buffer were mixed in at low speed. The beads were allowed to settle and the extract was decanted. The beads were successively washed with 210 and 180 ml of buffer, and these washings combined with the extract. The material was then centrifuged and the supernatant collected to yield Fraction I.

Streptomycin Precipitation—Two of the above extracts were combined to yield a total volume of 1420 ml. To this were added, with stirring, 360 ml of a 10% streptomycin sulfate solution. After standing for 15 minutes, the suspension was centrifuged and the supernatant collected (Fraction II).

First Ammonium Sulfate Fractionation—To the 1570 ml of Fraction II were added, with stirring, 335 g of solid $(NH_4)_2SO_4$. The suspension was allowed to stand for 20 minutes after the $(NH_4)_2SO_4$ had dissolved, and was then centrifuged. To the supernatant were added, with stirring, 210 g of $(NH_4)_2SO_4$, and the suspension was centrifuged after standing for 20 minutes as before. The precipitate was dissolved in 180 ml of 0.10 M sodium carbonate buffer, pH 10 (Fraction III).

Second Ammonium Sulfate Fractionation—To the 258 ml of Fraction III were added, with stirring, 232 ml of an alkaline $(NH_4)_2SO_4$ solution, made by adding 3 ml of concentrated ammonia to 250 ml of 3.2 M $(NH_4)_2SO_4$ just before use. After standing for 45 minutes, the suspension was centrifuged. The precipitate was dissolved in 75 ml of 0.01 M K_2HPO_4 and dialyzed overnight against 6 liters of the same solution (Fraction IV).

First Potassium Phosphate Fractionation—Fraction IV was diluted to 5,600 units per ml (final volume, 214 ml) and 69.5 ml of a 4 M K_2HPO_4 solution were added with stirring. The suspension was allowed to stand for 40 minutes and then centrifuged. To the supernatant were added, with stirring, 38.5 ml of 4 M K_2HPO_4 solution; the suspension was allowed to stand for 60 minutes and then centrifuged for 30 minutes at 25,000 × *g*. The precipitate was dissolved in 50 ml of 0.007 M potassium phosphate buffer, pH 6.5, and dialyzed overnight against 6 liters of the same buffer (Fraction V).

Hydroxylapatite Chromatography—Two columns (14 × 3.3 cm) were prepared and each was washed with 4-column volumes of 0.007 M potassium phosphate buffer, pH 6.5. Then 42 ml of Fraction V were loaded on each column at a flow rate of 1.5 ml per minute. A constant elution gradient of 0.007 to 0.020 M potassium phosphate buffer, pH 6.5, was applied. The total gradient volume was 1 liter. Fractions of 16 ml were collected at a flow rate of 1.5 ml per minute. The fractions having a specific activity greater than 2000 units per mg were combined (Fractions 17 to 42 on one column and 17 to 40 on another). Protein determinations on individual fractions were calculated from the optical density at 280 mμ with a 1-cm light path on the assumption that 1 mg of protein per ml (Lowry method) is equal to an optical density of 1.05, which was found to be valid at this stage of purification. To the 828 ml of the combined fractions were added, with stirring, 328 g of solid $(NH_4)_2SO_4$. The suspension was allowed to stand for 45 minutes and was then centrifuged. The precipitate was dissolved in 45 ml of 0.02 M K_2HPO_4 and dialyzed overnight against 6 liters of the same solution (Fraction VI).

DEAE-Sephadex A-50 Chromatography—A column, 14 × 2.2 cm, was prepared and washed with several column volumes of 0.02 M K_2HPO_4. Fraction VI (50 ml) was loaded on the column at a flow rate of 1.5 ml per minute. A constant gradient of 0.02 to 0.30 M K_2HPO_4 (total volume of 2 liters) was applied and 16-ml fractions were collected at a flow rate of 1.5 ml per minute. Fractions with a specific activity greater than 5000 units per mg were combined (Fractions 49 to 70) to yield a total volume of 430 ml. To the combined fractions were added, with stirring, 172 g of $(NH_4)_2SO_4$. After standing for 45 minutes, the suspension was centrifuged. The precipitate was dissolved in 10 ml of 0.01 M K_2HPO_4 and dialyzed against 4 liters of the same solution (Fraction VII).

Second Potassium Phosphate Fractionation—Fraction VII was diluted to 10,000 units per ml (final volume, 71 ml) and 36 ml of a 4.0 M K_2HPO_4 solution were added with stirring. The suspension was allowed to stand for 60 minutes and then was centrifuged at 25,000 × *g* for 20 minutes. The precipitate was dissolved in 4 ml of 0.01 M K_2HPO_4 and dialyzed against 2 liters of the same solution (Fraction VIII).

Tests for Homogeneity

Analysis of Fraction VIII—Further attempts to purify Fraction VIII did not increase the specific activity by more than 10%. Such increases are on the borderline of significance relative to the accuracy of the activity and protein assays. Consequently, other criteria of purity were applied to Fraction VIII: starch gel electrophoresis, sedimentation analysis, and DEAE-Sephadex chromatography.

The results of starch gel electrophoresis according to the method of Smithies (21) are given in Fig. 1. After electrophoresis, the gel was sliced in a plane parallel to its surface to yield two halves, one of which was stained with diamido black (Fig. 1*a*), and the other analyzed for UDP-galactose 4-epimerase activity (Fig. 1*b*). The staining reveals the presence of one major and several minor components, while the enzyme analysis indicates that the activity is associated with the major component.

A small degree of heterogeneity in Fraction VIII is also indicated in the schlieren pattern during sedimentation of its components (Fig. 2*a*). One peak is visible, but this contains a small shoulder at its leading edge. If it is assumed that this pattern results from the additive effect of one major component with a symmetrical peak whose trailing edge is that of the observed peak, combined with faster sedimenting minor components, then the major component accounts for 92% of the total area under the observed peak.

Finally, chromatography of Fraction VIII on a DEAE-Sephadex column gives an elution distribution of activity and of material absorbing at 280 mμ that also indicates a low level of impurities (Fig. 3). Thus, two inactive minor components are visible which account for 7% of the total material absorbing at 280 mμ eluted from the column. The major peak is associated with the UDP-galactose 4-epimerase activity. This peak is quite homogeneous in that the activity to optical density (280 mμ) ratio is constant to ±14% for column Fractions 33 to 46, which include 94% of the eluted activity.

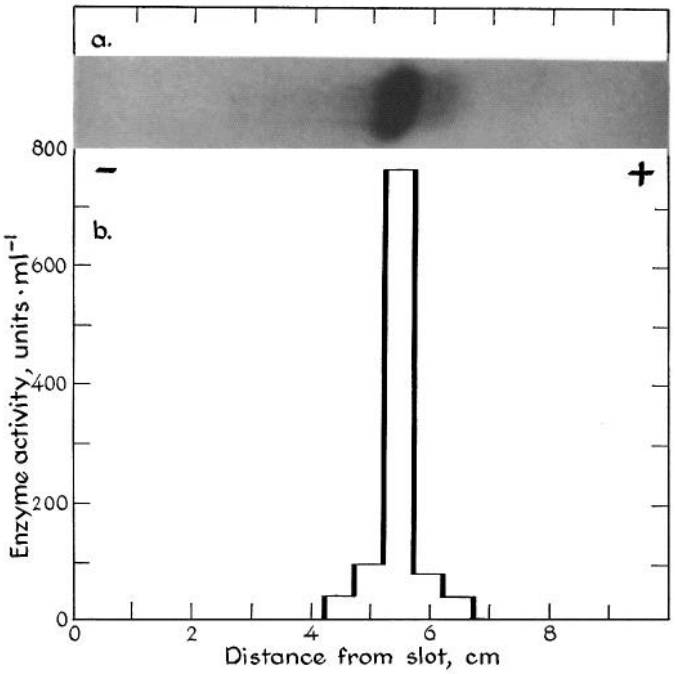

FIG. 1. Starch gel electrophoresis of Fraction VIII. Gels were made in 0.026 M Tris-citrate buffer, pH 8.7. After the slot was loaded with 0.06 ml of Fraction VIII, 175 volts were applied for 16 hours across electrodes which were immersed in reservoirs containing 0.076 M sodium borate buffer, pH 8.7. After electrophoresis, the gel was sliced in half. One half (*a*) was stained with diamido black and then washed according to Smithies (21). The other half (*b*) was cut, perpendicular to the path of migration, at 0.5-cm intervals along this path. Each of the resulting gel sections was eluted with 2 ml of 0.01 M potassium phosphate buffer, pH 7.0, at 2–4° for 18 hours. The eluate was assayed for UDP-galactose 4-epimerase activity by the standard method.

These three criteria for purity indicate that Fraction VIII contains minor impurities which appear to account for no more than 20% of the total protein, assuming the impurities detected by the three methods are identical.

Preparation and Analysis of Fraction IX—In order to eliminate the impurities detectable by DEAE-Sephadex chromatography, column Fractions 33 through 46 of the preceding chromatography (Fig. 3) were combined and concentrated by the same method used in the purification procedure for obtaining Fraction VIII from the eluate of the first DEAE-Sephadex column. The yield in this concentration procedure was 72%. The concentrated enzyme (Fraction IX) had a specific activity of 14×10^3 units per mg.

A portion of Fraction IX was divided into two subfractions: IXa, which was the precipitate resulting from raising the K_2HPO_4 concentration to 1.0 M; and IXb, the precipitate arising after the concentration of K_2HPO_4 in the preceding supernatant was further raised to 1.36 M. Both of these were subjected to starch gel electrophoresis with the results given in Fig. 4. The number of contaminating bands resulting from Fraction IX has been reduced to two, of which Fraction IXa contains one and Fraction IXb contains both.

The schlieren pattern of the sedimenting components of Fraction IXa is given in Fig. 2*b*. The pattern is similar to that given by Fraction VIII except that the shoulder on the leading edge has been reduced. Thus, 95% of the total area under the observed curve is associated with a single major component by the same calculation employed for the curve given by Fraction VIII (Fig. 2*a*).

These data do not allow a precise determination of the levels of impurities in Fraction IX. However, under the reasonable assumption that the faster sedimenting material causing the shoulder in the schlieren pattern and the single minor band in starch gel electrophoresis of Fraction IXa are the same, we estimate from the relative intensities of the minor bands shown in Fig. 4 that the level of impurities in Fraction IX is less than 10% of the total protein. No further attempt was made to eliminate these detectable impurities.

Molecular Weight of Enzyme

The previous data indicate that the enzyme preparations are sufficiently homogeneous with respect to molecular weight to

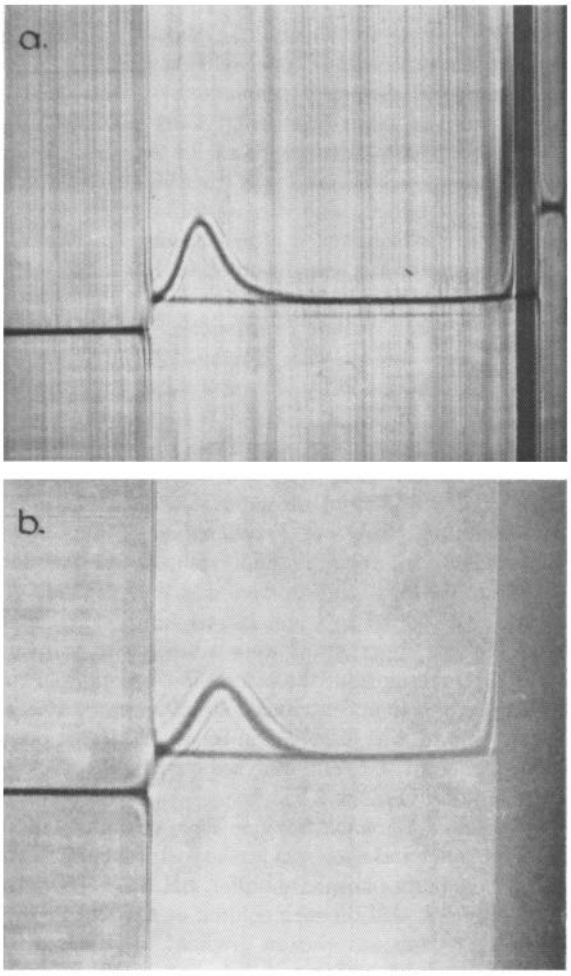

FIG. 2. Sedimentation of Fractions VIII and IXa. *a*, schlieren pattern of Fraction VIII having an initial concentration of 10.9 mg per ml in 0.10 M KCl-0.01 M potassium phosphate buffer, pH 7.0, contained in a 2.5°, 12-mm double sector cell at 25.0°. The photograph was taken 24 minutes after reaching a speed of 50,740 r.p.m., with a phase plate angle of 80°. *b*, schlieren pattern of Fraction IXa under the above conditions except that the protein concentration was 5.0 mg per ml and the photograph was taken 45 minutes after reaching a speed of 42,040 r.p.m., with a phase plate angle of 70°. In both cases, photographs taken before and after those shown here gave no indication of additional sedimenting components.

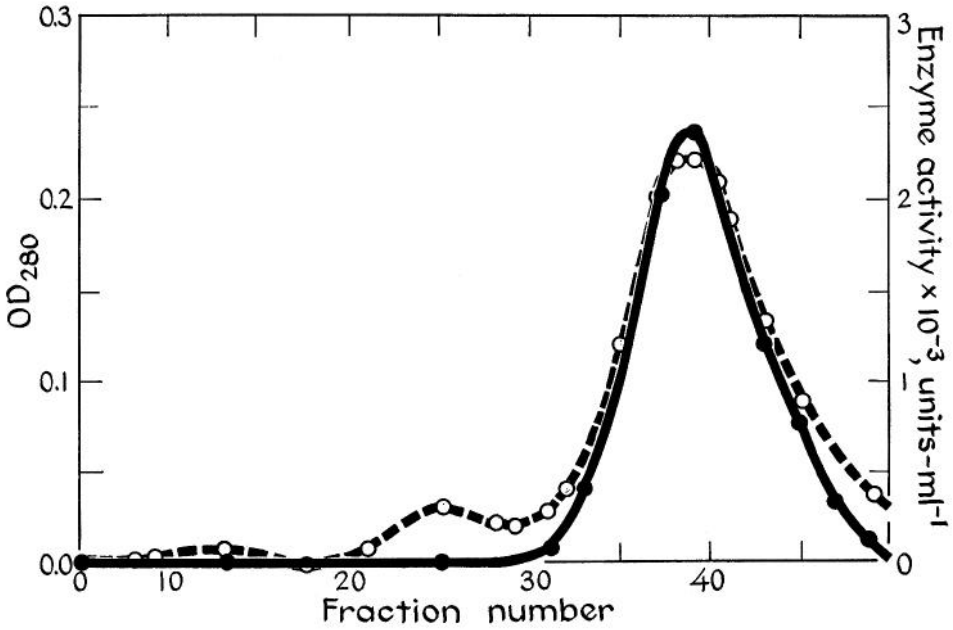

FIG. 3. Chromatography of Fraction VIII on DEAE-Sephadex A-50. A column, 5.5 × 2.2 cm, was prepared and loaded with 3 ml of Fraction VIII at a flow rate of 1 ml per minute. A constant gradient from 0.02 to 0.30 M K_2HPO_4 (total volume of 1 liter) was applied at a flow rate of 1.5 ml per minute, and 15-ml fractions were collected. These fractions were assayed for UDP-galactose 4-epimerase activity (●) and their optical density at 280 mμ was determined (○).

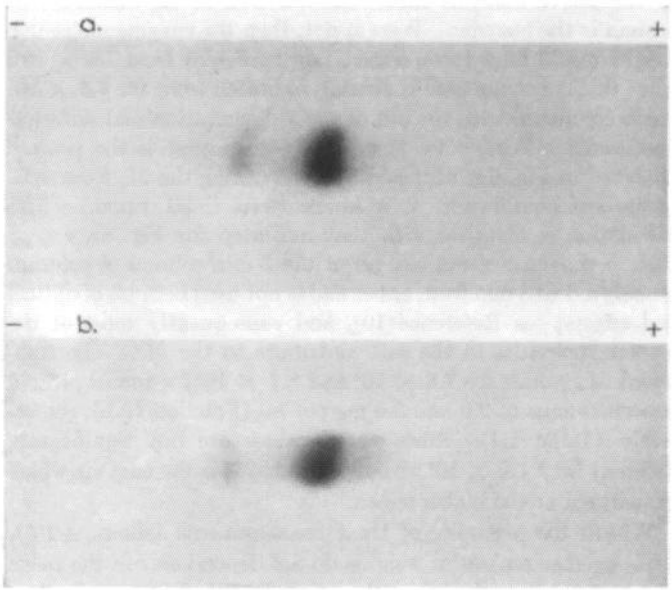

FIG. 4. Starch gel electrophoresis of Fractions IXa and IXb. The conditions of electrophoresis and staining were those given in Fig. 1, except that in *a*, the slot contained 0.06 ml of Fraction IXa at a concentration of 5.0 mg per ml; and in *b*, the slot contained 0.06 ml of Fraction IXb at a concentration of 4.5 mg per ml.

warrant its measurement. This molecular weight was computed from the sedimentation and diffusion coefficients as well as from distributions attained after centrifugation to sedimentation equilibrium.

Sedimentation and Diffusion Coefficients—The sedimentation coefficient (s) was measured at different protein concentrations (c), the conditions and results being given in Table II. The lack

TABLE II

Sedimentation coefficient of UDP-galactose 4-epimerase

Fraction VIII was dialyzed against 0.10 M potassium chloride-0.01 M potassium phosphate buffer, pH 7.0, prior to centrifugation in a 12-mm, 4° Epon cell at a speed of 50,740 r.p.m., and a temperature of 25.0°. The sedimentation coefficients were evaluated by the least squares method from plots of log r_{max} *versus* time, about 15 points being used per evaluation and r_{max} being the radial distance to the maximum in the schlieren pattern.

Initial concentration	$s_{20,w}$
mg/ml	*S*
10.9	4.95
6.6	5.07
3.9	5.02
2.0	5.02
Average	5.02

of any significant (±1%) dependence of s on c is surprising and has not been satisfactorily explained. It may result from aggregation phenomena, which could also be invoked to explain the shoulder on the leading edge of the sedimentation velocity patterns (Fig. 2). If so, the tendency to aggregate must be small, as there is no apparent variation (±2%) of molecular weight with protein concentration (see next section and Table III). We use the average value of $s_{20,w}$ = 5.0 S for c ranging from 0 to 10 mg per ml.

The diffusion coefficient, D, was computed according to the method of Sophianopoulos *et al.* (22), which utilizes the schlieren patterns of the transient states prior to sedimentation equilibrium as well as the equilibrium pattern itself. The method and data are summarized in Fig. 5, from which a value of 6.1 × 10^{-7} cm^2 sec^{-1} for the $D_{20,w}$ was calculated. When this value is

TABLE III

Molecular weight of UDP-galactose 4-epimerase

Method	Angular velocity	c_0	Molecular weight
	r.p.m.	*mg/ml*	$\times 10^{-4}$
Equilibrium centrifugation*			
Weight average (M_w)†	24,640	5.0	7.9
z-Average (M_z)†	24,640	5.0	7.9
	9,341	5.0	8.2
	9,341	2.0	7.8
Sedimentation-diffusion‡			7.5

* The conditions and specific methods of calculation are those given in Fig. 6 except as indicated here. The term c_0 stands for the initial protein concentration.

† See the text for further explanation.

‡ $s_{20,w} = 5.0$ S; $D_{20,w} = 6.1 \times 10^{7}$ cm^2 sec^{-1}.

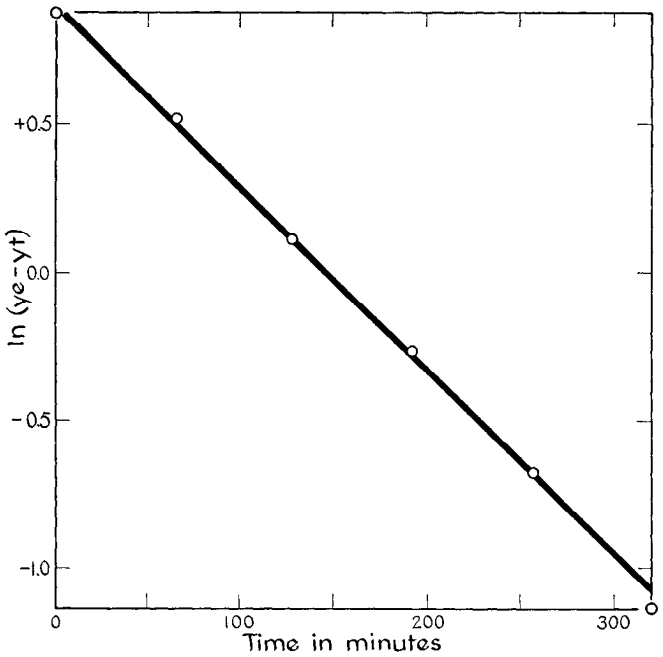

FIG. 5. The determination of the diffusion coefficient. After dialysis against 0.10 M potassium chloride-0.01 M potassium phosphate buffer, pH 7.0, Fraction IXa (c = 5.0 mg per ml) was centrifuged at 9341 r.p.m. for 20 hours in a 6-mm, 2° double sector Epon cell, with use of a 3-mm column of solution at 24.0°. The schlieren patterns during the approach to equilibrium as well as at equilibrium were photographed and the vertical displacement from the base-line at $(b + m)/2$ was measured, where b and m refer to the radial distances to the bottom and meniscus of the liquid column, respectively. At equilibrium this vertical displacement is referred to as y_e, while at other times, t, it is referred to as y_t. The diffusion coefficient can then be calculated from the equation (22),

$$D = \frac{(b-m)^2}{\pi^2}\frac{d \ln (y_e - y_t)}{dt} \Bigg/ \left[1 + \frac{(b-m)^2}{\pi^2}\left(\frac{\omega^2 M(1-\bar{v}\rho)}{2RT}\right)\left(\frac{b+m}{2}\right)^2\right]\left[1 + \frac{3}{\pi^2}\left(\frac{b-m}{b+m}\right)^2\right]$$

since the slope of the above curve is equal to $d \ln (y_e - y_t)/dt$. The terms in brackets are small correction terms having a value of 0.937 under the conditions used here.

combined with the above $s_{20,w}$ value in the Svedberg equation (utilizing a value of 0.73 cm^3 per g for the partial specific volume, $\bar{v}$, and neglecting a possible activity coefficient correction), a molecular weight of 7.5×10^4 is computed (Table III).

Sedimentation Equilibrium—Centrifugation of the enzyme to sedimentation equilibrium in a short column of solution (3 mm) at 24,640 r.p.m. (Fig. 6) yields a distribution in which the protein concentration at the meniscus (c_m) is zero. This condition has the advantage of allowing the calculation of the weight average molecular weight,[2] M_w (23), as well as the z-average,[2] M_z (19), from a single equilibrium schlieren pattern.[3] A further advantage of this condition is that it selectively counteracts the effects of higher molecular weight contaminants in the calculation of the averages. A coupled disadvantage is that only a fraction (in this case, one-eighth) of the total protein added to the centrifuge cell is analyzed in the schlieren pattern, the remainder being in a region of too high a dc/dr value to be registered.

When the M_m and M_z are computed under this condition (Fig. 6), both computations yield the value 7.9×10^4 (Table III). Since M_m and M_z are differently sensitive to molecular weight heterogeneity,[2] the agreement between these two averages indicates homogeneity with respect to molecular weight for that fraction of the protein population being analyzed. The lack of curvature in both of the plots given in Fig. 6 is a further indication of such homogeneity (19).

The fact that only one-eighth of the total protein is subject to the above analysis raises the question as to whether this protein is the enzyme. Were it not, then the enzyme molecular weight would have to be considerably different from 7.9×10^4. That this is not the case is already indicated from the 7.5×10^4 value computed with the aid of the sedimentation and diffusion coefficients. Further evidence that the enzyme is the protein analyzed in Fig. 6 is obtained by determining the M_z from sedimentation equilibrium at a lower speed (9341 r.p.m.). The calculation is identical with that indicated for Fig. 6*b*, except that in this case essentially all of the 3-mm column of solution is analyzed (0.3 mm from either end is not used because of optical end effects; see Reference 19), and consequently most of the protein molecules in the cell contribute to the M_z. The computed M_z values are 7.8×10^4 and 8.2×10^4 for initial protein concentrations of 2.0 and 5.0 mg per ml (Fraction IXb), respectively (Table III). Since these values are not significantly different from 7.9×10^4 we conclude that it is the enzyme which is analyzed at the higher speed.

Within the accuracy of these measurements (about ±2%), these average molecular weights do not depend on c in the range of 1 to 5 mg per ml. Thus the points in Fig. 6 fit straight lines over the concentration range of 0.4 to 3.5 mg per ml. (Note that although the initial concentration in this experiment was 5.0 mg per ml, the analyzable region, because of the high speed,

[2] $M_w = \sum_{i=1}^{n} c_i M_i / \sum_{i=1}^{n} c_i$; and $M_z = \sum_{i=1}^{n} c_i M_i^2 / \sum_{i=1}^{n} c_i M_i$, where c_i and M_i are the concentration, in units of weight per volume, and molecular weight of the ith component, respectively.

[3] At lower speeds (9,341 r.p.m.), the equilibrium c_m is not zero and must be evaluated for the M_w calculation, though not for that of M_z. Since this evaluation introduces an error in M_w not inherent in M_z, we have presented only the M_z value for the lower speed. At 24,640 r.p.m. there is no such difference in error between M_w and M_z, and both are given.

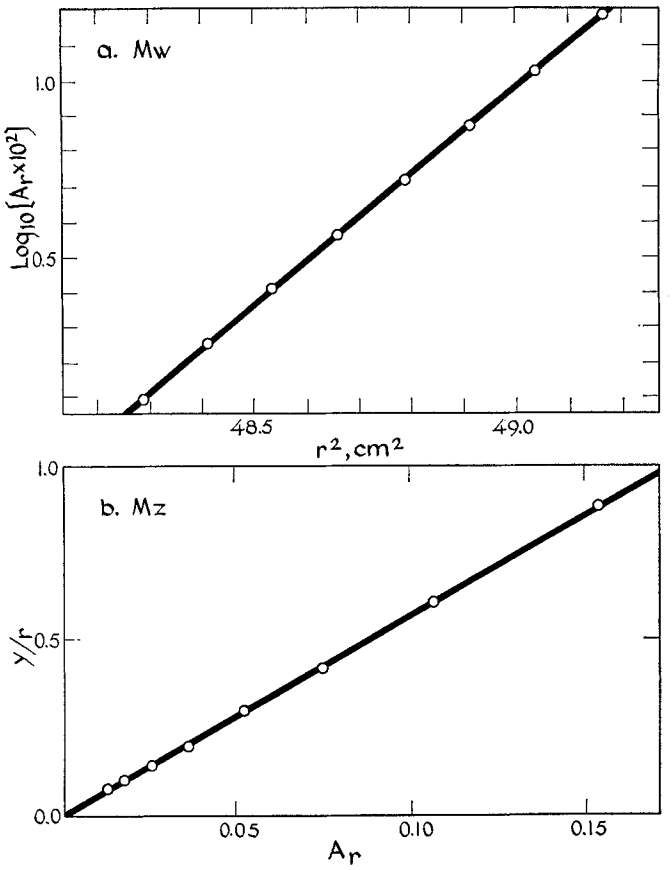

FIG. 6. Determination of M_w and M_z from equilibrium data at 24,630 r.p.m. The other conditions of centrifugation were identical with those given in Fig. 5. The schlieren pattern of the equilibrium distribution was photographed and analyzed to give the data in *a* and *b*, above. *a*, the M_w can be calculated from the equation (23),

$$M_w = \frac{2RT}{(1 - \bar{v}\rho)\omega^2} \cdot \frac{d(\ln c_r)}{d(r^2)}$$

where, in addition to the symbols defined in the text, R = gas constant, T = temperature in degrees Kelvin, ρ is the density of the solution, and ω is the angular velocity. Under the above conditions,

$$\frac{d(\ln c_r)}{d(r^2)} = (2.303)\left(\frac{d \log_{10} A_r}{d(r^2)}\right)$$

the latter term being evaluated from the slope of *Curve a*. The term A_r is determined by numerical integration of $\int_m^r y dr$, where m refers to the meniscus, and y is in arbitrary optical units determined by the vertical displacement of the schlieren pattern from the base-line. *b*, the M_z is calculable from the equation (19),

$$M_z = \frac{RT}{(1 - \bar{v}\rho)\omega^2} \cdot \frac{d\left(\frac{1}{r}\cdot\frac{dc_r}{dr}\right)}{dc_r}$$

Under the above conditions,

$$\frac{d\left(\frac{1}{r}\cdot\frac{dc_r}{dr}\right)}{dc_r} = \frac{d\left(\frac{y}{r}\right)}{dA_r}$$

which can be evaluated from the slope of *Curve b*.

includes only these concentrations.) At 9341 r.p.m. the concentration ranges analyzed were 0.9 to 2.6 and 2.4 to 6.7 mg per ml for initial concentrations of 2.0 and 5.0 mg per ml, respectively.

The preferred value for the molecular weight of UDP-galactose 4-epimerase is 7.9×10^4, on the assumption that $\bar{v}$ is equal to 0.73 cm³ per g. This is not so much because it is the average of the values given in Table III, but because it is the common value for both M_m and M_z computed at 24,630 r.p.m., at which speed the effective concentration was low, and the effect of higher molecular weight contaminants or of enzyme aggregates minimized.

The assumption of $\bar{v}$ = 0.73 cm³ per g may introduce the largest error into the molecular weight. Thus the molecular weights will be in error by 3.7% for every 0.01 cm³ per g by which the actual $\bar{v}$ differs from 0.73 cm³ per g. Since the $\bar{v}$ of simple proteins generally lies within the range 0.70 to 0.75 cm³ per g, it is unlikely that the error introduced by this assumption is greater than 10%. Thus we consider the molecular weight of UDP-galactose 4-epimerase to be $7.9 (\pm 0.8) \times 10^4$.

Parameters of Enzyme-catalyzed Reaction

Requirements—Superficially the catalysis of the epimerization reaction by the *E. coli* enzyme appears to be of the simplest type since the necessary components are limited to the purified enzyme, a single substrate (either UDP-galactose or UDP-glucose), and an aqueous solvent having a pH within a restricted range.

The data in Table IV indicate that neither DPN nor heavy metal ions need be added for catalysis. In order to test for the effect of added DPN, a two-step assay was used (see Table IV, Assay I). It is clear that added DPN neither is required for nor increases the rate of the catalysis.

Since neither Mg^{2+}, Mn^{2+}, nor EDTA affects the UDP-glucose dehydrogenase activity, the standard assay was used to test their effects on the epimerization reaction. The results (Table

TABLE IV

Requirements for UDP-galactose 4-epimerase

Assay I is a two-step assay in which the reaction mixture of the first step is 5×10^{-4} M UDP-galactose, 0.05 M glycine-NaOH buffer, pH 8.5, 0.13 unit of Fraction IX per ml, and, when present, 2.5×10^{-3} M DPN. At 0, 3, 6, and 9 minutes, 0.1 ml of this mixture was placed in a boiling water bath for 3 minutes. After cooling to 27°, 440 units of UDP-glucose dehydrogenase and 0.25 μmole of DPN were added to give a total volume of 0.2 ml, and the optical density of this mixture at 340 mμ was determined after it had attained its maximum value. Assay II is the standard assay described in "Experimental Procedure," except for the indicated additions. Fraction IX was used.

Assay	Additions		Rate of UDP-glucose formation
	Compound	Concentration	
		M × 10³	mμM/min
I	None		0.25
	DPN	2.5	0.23
II	None		0.50
	$MgCl_2$	0.25	0.48
	$MnCl_2$	0.25	0.48
	EDTA	1.0	0.48

IV) indicate that neither Mg^{2+}, Mn^{2+}, nor the heavy metal ions that complex strongly with EDTA at 8.5 (24) are required for catalysis of the epimerization.

The effective pH range for UDP-galactose 4-epimerase is large. Thus at pH 6.35, 7.15, 8.00, 8.50, and 9.64 the relative activities at 27° were 0.68, 0.90, 1.00, 1.00, and 0.79, respectively. The two-step assay used for these measurements was like Assay I of Table IV except that the buffer was varied. Potassium phosphate was used below pH 8.5, glycine was used at pH 8.5, and sodium carbonate was used at pH 9.64.

Stoichiometry and Equilibrium—A mixture of UDP-glucose and UDP-galactose can be assayed for each component by the successive use of UDP-glucose dehydrogenase and UDP-galactose epimerase. Thus the addition of DPN and the dehydrogenase to the mixture results in the formation of 2 moles of DPNH per mole of UDP-glucose present, and the further addition of the epimerase results in an additional 2 moles of DPNH per mole of UDP-galactose present. The stoichiometry and equilibrium constant of the epimerization reaction can therefore be determined simply and simultaneously.

The initial reaction mixture contains either UDP-galactose or UDP-glucose, and the purified UDP-galactose 4-epimerase. Aliquots are taken at various times and heated to inactivate the enzyme, and the resulting mixture is assayed by the above method. The details of the method and the results are given in Table V. The fact that the final ratio of UDP-glucose to UDP-galactose concentration attains a value of 3.5 ± 0.1 whether the starting substrate is UDP-glucose or UDP-galactose indicates that this is the equilibrium ratio. The fact that the sum of the UDP-glucose and UDP-galactose concentrations is equivalent to the starting substrate concentration indicates that this ratio is not affected by preferential loss of either component and defines the 1:1 stoichiometry of the epimerization reaction. As might be expected from the structure of the substrates, this equilibrium ratio exhibited little, if any, significant change when the pH changed from 7.1 to 8.5. Assuming activity coefficients of unity at these low substrate concentrations, the ΔF^0 for the UDP-galactose to UDP-glucose reaction is 0.75 ± 0.02 kcal at 27°.

TABLE V

Stoichiometry and equilibrium

Samples containing either UDP-glucose or UDP-galactose and 3×10^{-9} M epimerase (Fraction VIII) in 0.05 M buffer (potassium phosphate for pH 7.1, glycine for pH 8.7) were incubated at 27.0°. Aliquots of 0.100 ml were removed and boiled for 3 minutes, and the UDP-glucose was assayed as indicated in Assay I of Table IV. The UDP-galactose was subsequently assayed by addition of 2×10^{-12} mole of the epimerase (Fraction VIII) and the further increase in optical density at 340 mμ recorded. Aliquots were assayed in this manner until no further significant change was observed.

pH	Initial conditions		Final conditions			Recovery*
	UDP-glucose	UDP-galactose	UDP-glucose	UDP-galactose	UDP-glucose/UDP-galactose	
	μM		μM			
7.1	62.0	0	46.7	13.6	3.43	0.97
7.1	0	64.0	47.4	13.6	3.49	0.95
8.7	62.0	0	48.4	13.4	3.61	1.00
8.7	0	64.0	50.0	14.0	3.57	1.00
Average					3.52	0.98

* $(\Sigma\text{UDP-hexose})_{final}/(\Sigma\text{UDP-hexose})_{initial}$.

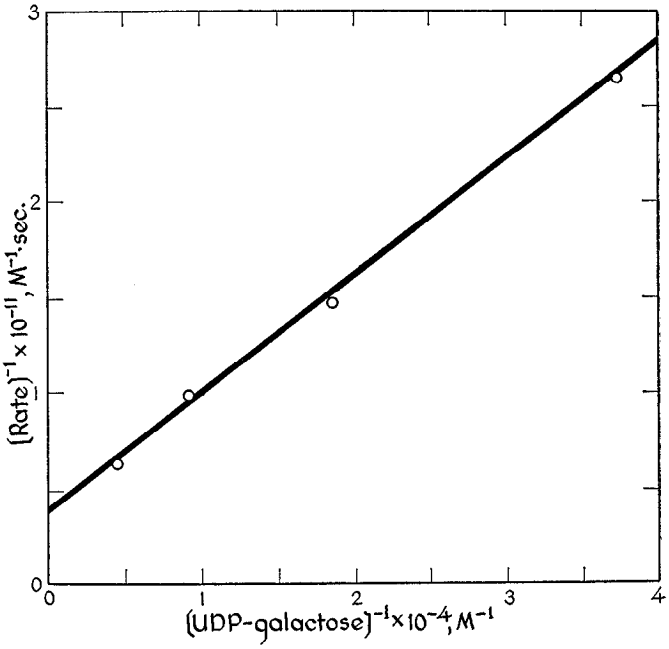

FIG. 7. Lineweaver-Burk plot for UDP-galactose. The standard assay was used and the amount of UDP-galactose varied. The concentration of enzyme (Fraction VIII) was 2.7×10^{-10} M.

Kinetic Constants—The rate of the UDP-galactose to UDP-glucose reaction can be determined independent of the reverse reaction if the UDP-glucose concentration is maintained at very low levels. This condition exists throughout the time intervals employed for the standard assay as a result of the presence of DPN and UDP-glucose dehydrogenase. However, in the two-step assay, the condition exists only at very early times. Consequently, the K_m and k_3 (turnover number) for UDP-galactose were determined within the framework of the standard assay conditions.

Fig. 7 indicates that a plot of the reciprocal of the rate against the reciprocal of UDP-galactose concentration is linear and consequently that under these conditions the kinetics of the reaction is consistent with the classical Michaelis-Menten model. From these data, the K_m and the k_3 for UDP-galactose are 1.6×10^{-4} M and 5.0×10^2 sec^{-1}, respectively, at 27.0° and pH 8.5. It is seen from these values that the standard assay defining the unit of enzyme employs a UDP-galactose concentration (2.5×10^{-4} M) at which the rate is only 61% of the maximum. This was done for reasons of economy, UDP-galactose not being easily obtainable in large amounts at the beginning of this work.

The effect of temperature on the reaction rate is indicated in Fig. 8, in which the logarithm of the rate of UDP-glucose formation is plotted against the reciprocal of the absolute temperature. The linearity of this plot and its slope indicate that the apparent activation energy has a constant value of 10.5 kcal per mole over the temperature range considered (10–25°). This amounts to an increase of 1.85-fold per 10° increase in temperature.

Enzyme-bound DPN—By analogy to the yeast UDP-galactose 4-epimerase (9), one might suppose that the absence of a requirement for added DPN indicates the *E. coli* enzyme contains DPN so tightly bound that it survives the purification procedure.

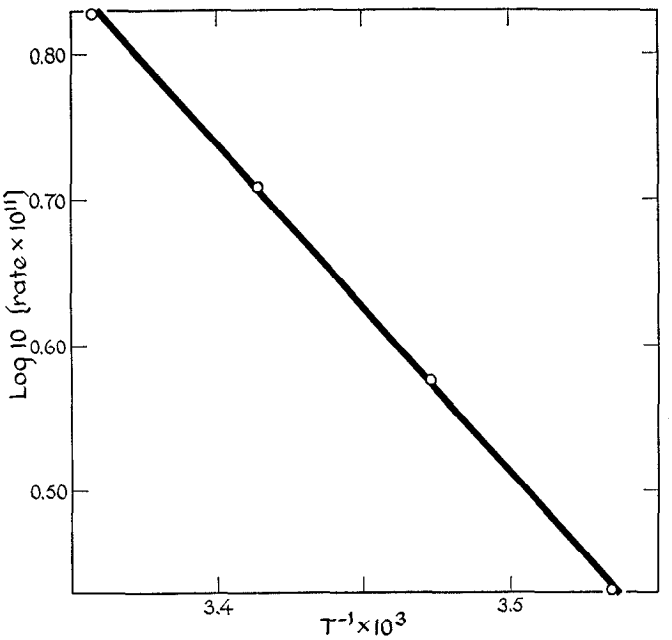

FIG. 8. Effect of temperature on the rate. The rates were determined by the Assay I procedure (Table IV) and are expressed in units of molar concentration per second. The temperature, T, is expressed in degrees Kelvin. The enzyme (Fraction VIII) concentration was 1.9×10^{-10} M.

Consequently, Fraction IX was tested for the presence of DPN by several techniques.

The first test was a direct assay of Fraction IX by the methyl ethyl ketone procedure, which is specific for *N*-substituted nicotinamide derivatives (25). With DPN as standard, this assay indicated the presence of 1 mole of DPN per 7.2 ($\pm$0.7) $\times$ 10^4 g of protein. A commercial preparation of rabbit muscle glyceraldehyde-3-P dehydrogenase (Sigma Chemical Company) was employed as a control in this assay. This preparation had an $O.D._{280}$:$O.D._{260}$ ratio of 1.19 and yielded the same amount of DPN per g (1.6×10^{-5} mole of DPN per g of protein) as that reported by Fox and Dandliker (26) for a preparation with the same ratio of optical densities.

In order to identify further the residue attached to epimerase, attempts were made to separate it from the protein. Treatment with Norit at pH 7.0 (0.01 M potassium phosphate buffer) neither caused inactivation nor altered the $O.D._{280}$:$O.D._{260}$ ratio, which is 1.44. In contrast, acid treatment did effect the separation, as is indicated in the following detailed description.

Perchloric acid (35%), 0.1 volume, was mixed with Fraction VIII (7.1 mg per ml), 0.9 volume, at 0°. A precipitate formed and the suspension was allowed to stand for 20 minutes at this temperature before being centrifuged at 6000 $\times$ g for 5 minutes. After removal of the supernatant, the precipitate was dissolved in 0.1 M sodium hydroxide and analyzed by the methyl ethyl ketone procedure described above. It contained less than 2% of the reactive material present before this acid treatment.

Analysis of the supernatant indicated the presence of DPN. Thus the $O.D._{250}$:$O.D._{260}$ and the $O.D._{280}$:$O.D._{260}$ ratios of the supernatant were 0.85 and 0.29, respectively, while authentic DPN in 3.5% perchloric acid yielded corresponding values of 0.85 and 0.26. A more specific assay for DPN was performed by testing for DPNH formation resulting from the oxidation of UDP-glucose in the presence of UDP-glucose dehydrogenase. Thus 0.15 ml of supernatant was mixed with 0.040 ml of 1.0 M glycine-NaOH buffer, pH 8.5, and 0.011 ml of 7.0 M potassium hydroxide. The resulting mixture has a pH of about 8 and contains a dense precipitate of potassium perchlorate which was removed by centrifugation. An aliquot of the supernatant was brought to 5×10^{-4} M UDP-glucose and 5×10^3 units of UDP-glucose dehydrogenase per ml, after which an absorption at 340 mμ appeared and was followed until it no longer increased. Authentic DPN in 3.5% perchloric acid, put through the same procedure, was used as a standard and indicated a 95% recovery. The increase in the 340 mμ absorption resulting from the supernatant aliquot indicated that 1 mole of DPNH could be formed from 7.2×10^4 g of protein having a specific activity of 14×10^3 units per mg. This is in agreement with the previous methyl ethyl ketone assay performed on Fraction IX, both assays indicating a minimum molecular weight of 7.2×10^4, with a possible error of about $\pm$10%.

When these results are compared with the molecular weight determined from sedimentation data, it is clear that an average value of 1 DPN residue per enzyme molecule satisfies both sets of data.

Effect of Substrate on Enzyme-bound DPN—The absorption spectrum of the purified UDP-galactose 4-epimerase has no peak in the 300 to 400 mμ range, indicating that in the absence of substrate the bound dinucleotide is in the oxidized form. However if this DPN functions in the epimerization reaction by oxidation-reduction, then in the presence of substrate a certain fraction of the bound dinucleotide would exist in reduced form and exhibit an absorption maximum in the 300 to 400 mμ range. One model depicting this possibility is the following

$$DPN_{ox}\text{-}E + S_1 \rightleftharpoons DPN_{ox}\text{-}E\text{—}S_1 \rightleftharpoons [DPN_{red}\text{-}E\text{---}S_{ox}] \rightleftharpoons$$

$$DPN_{ox}\text{-}E\text{—}S_2 \rightleftharpoons DPN_{ox}\text{-}E + S_2$$

where S_1 and S_2 represent the substrates UDP-glucose and UDP-galactose, respectively, the substance in *brackets* is the speculated intermediate in which the UDP-hexose moieties are oxidized (—S_{ox}) and the DPN reduced (DPN_{red}) to a structure absorbing in the 300 to 400 mμ range, and the substances on either side of the speculated intermediate are enzyme-substrate complexes of the classical type.

This concept was tested by measuring the difference spectrum between the enzyme with substrate and the enzyme without substrate. The details of the method used and the results are given in Fig. 9. The absorption due to the mixture of enzyme and substrate is very much like that of free DPNH, but displaced 5 to 10 mμ toward the higher wave lengths. It forms immediately (less than 1 minute) after addition of the substrate to the enzyme, although there is a subsequent very slow, small increase in the absorption which does not alter the shape of the absorption curve. This slow reaction has not been studied further, but it may reflect an inactivation of the speculated intermediate, thus removing it from the above equilibria. It should be noted that the amount of enzyme employed in this experiment is so great that the equilibrium ratio of UDP-glucose to UDP-galactose should be attained in much less than 1 minute.

The extent of the immediate absorption at the maximum of the band (345 mμ) as a function of substrate concentration was also examined. The raw data and the technique of this experiment are presented in Fig. 10*a*. To show that these data are

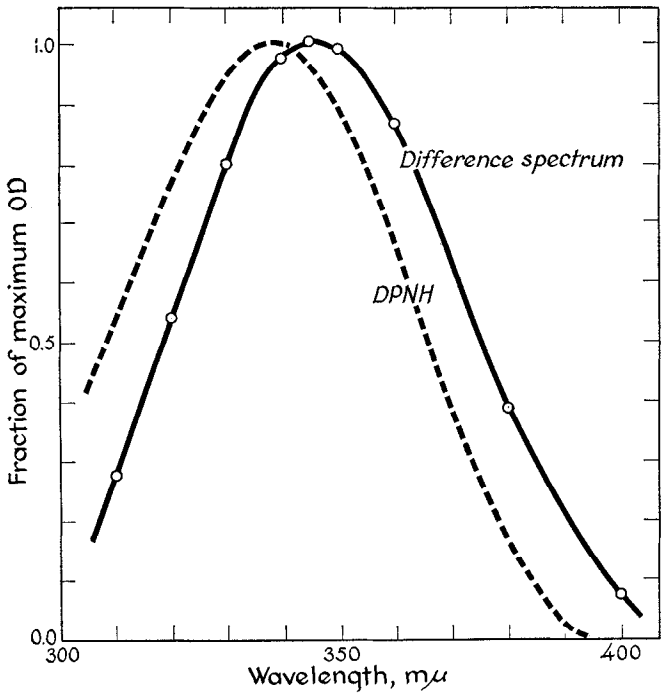

FIG. 9. Difference spectrum between enzyme plus substrate and enzyme alone. Each of two cuvettes contained 1.2×10^{-4} M UDP-galactose 4-epimerase (Fraction VIII) in 0.01 M K_2HPO_4, and one, but not the other, contained 1.67×10^{-3} M total UDP-hexose, added as UDP-glucose. The cuvette with the UDP-hexoses was read against the other at each of the indicated wave lengths in a Zeiss PMQ II spectrophotometer, all components being equilibrated to 27.0°. The maximum optical density was at 345 mμ, this being 0.127 a few minutes after mixing the components. It increased very slowly (0.010 per hour) thereafter.

consistent with the previous model we first derive the relationship between the optical density at 345 mμ and the substrate concentrations that is predicted by the model.

To shorten the symbolism, let the model be represented by

$$e + s_1 \underset{}{\overset{K_1}{\rightleftharpoons}} c_1 \overset{K_1'}{\rightleftharpoons} i \overset{K_2'}{\rightleftharpoons} c_2 \overset{K_2}{\rightleftharpoons} e + s_2$$

where e = DPN_{ox}-E, $s_1 = S_1$, c_1 = DPN_{ox}-E—S_1, i = [DPN_{red}-E—S_{ox}], etc. The K's are defined as $K_1 = e \cdot s_1/c_1$, $K_1' = c_1/i$, $K_2' = c_2/i$, and $K_2 = e \cdot s_2/c_2$, molar concentrations being implied. The equilibrium ratio, $s_1/s_2 = 3.5$, is equivalent to K_1K_1'/K_2K_2' in this model, and is termed K_{eq} here. Letting $E = e + c_1 + i + c_2$, the total added enzyme concentration, then from the above we have

$$E = K_1 \cdot \frac{c_1}{s_1} + i(1 + K_1' + K_2') = i\left[1 + K_1' + K_2' + \frac{K_1K_1'}{s_1}\right]$$

or

$$\frac{1}{i} = \frac{1 + K_1' + K_2'}{E} + \frac{K_1K_1'}{E} \cdot \frac{1}{s_1} \qquad (1)$$

Two other equivalent forms of Equation 1 are

$$\frac{1}{i} = \frac{1 + K_1' + K_2'}{E} + \frac{K_2K_2'}{E} \cdot \frac{1}{s_2} \qquad (2)$$

and

$$\frac{1}{i} = \frac{1 + K_1' + K_2'}{E} + \frac{K_2K_2'(1 + K_{eq})}{E} \cdot \frac{1}{s_1 + s_2} \qquad (3)$$

Equation 3 is used here and can be rewritten to contain the observed optical density at 345 mμ and to eliminate K_{eq} by substitution according to $O.D._{345} = \epsilon \cdot i$ and $K_{eq} = 3.5$, where ϵ is the molar extinction coefficient of i at 345 mμ and 1-cm light path. Thus

$$\frac{1}{O.D._{345}} = \frac{1 + K_1' + K_2'}{\epsilon \cdot E} + \frac{4.5K_2K_2'}{\epsilon \cdot E} \cdot \frac{1}{s_1 + s_2} \qquad (4)$$

which predicts that a plot of $(O.D._{345})^{-1}$ *versus* $(s_1 + s_2)^{-1}$ will be linear with a positive intercept $(1 + K_1' + K_2')/\epsilon \cdot E$ and slope $4.5\ K_2K_2'/\epsilon \cdot E$.

It should be noted that s_1 and s_2 are the concentrations of UDP-glucose and UDP-galactose, respectively, which are free, not bound to the enzyme. Their sum $(s_1 + s_2)$ thus differs

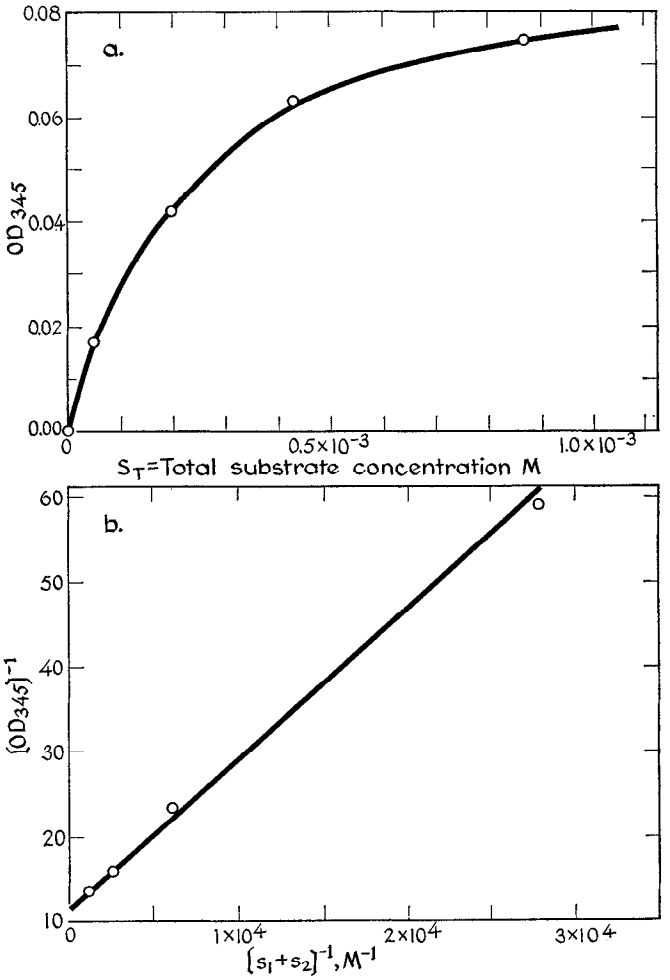

FIG. 10. The effect of substrate concentration on the optical density at 345 mμ, read as a difference value by the procedure given in Fig. 9. The total substrate concentration, S_T, is equivalent to the UDP-glucose initially added. The enzyme (Fraction VIII) concentration was 7.6×10^{-6} M in the solvent given in Fig. 9 and the temperature was 27.0°. The sum of the free UDP-glucose and UDP-galactose concentrations $(s_1 + s_2)$ was calculated by the method given in the text.

from the total added substrate (S_T in Fig. 10*a*) by the amount of substrate that is bound to the enzyme (*i.e.* $c_1 + i + c_2$). At the two higher S_T values of 4.3×10^{-4} and 8.7×10^{-4}, S_T forms a good first approximation for $s_1 + s_2$ since the total enzyme concentration (7.6×10^{-5} M) is only one-sixth the lesser of these two values. Substituting these two values of S_T for $s_1 + s_2$ and the corresponding observed O.D.$_{345}$ values in Equation 4, a first approximation to the intercept $(1 + K_1' + K_2')/\epsilon \cdot E$ of 10.9 is obtained. The reciprocal of this value, 0.092, represents the O.D.$_{345}$ obtained when the enzyme is saturated with substrate and i is maximum (i_{max}). Thus the ratio O.D.$_{345}$/0.092 equals i/i_{max}, and since $(c_1 + i + c_2)/i = 1 + K_1' + K_2'$, a constant, then $(c_1 + i + c_2)/(c_1 + i + c_2)_{max}$, or $(c_1 + i + c_2)/E$, also is equal to O.D.$_{345}$/0.092. Since $E = 7.6 \times 10^{-5}$ M, then $(c_1 + i + c_2)$ can be evaluated for each O.D.$_{345}$, and subtracting it from the corresponding S_T will yield the desired $s_1 + s_2$ for all conditions. This was done, and the reciprocal of the O.D.$_{345}$ was then plotted against the reciprocal of the resulting $s_1 + s_2$ values (Fig. 10*b*). The fit to a straight line is good and indicates that the data are consistent with the model.

If ϵ is given the same value as that for DPNH (6.2×10^3 M^{-1} cm^{-1}), then from this curve, i_{max} has the value $0.090/(6.2 \times 10^3) = 1.45 \times 10^{-5}$ M. Since the total enzyme concentration is 7.6×10^{-5} M, this assumption and the previous conclusion that there is 1 DPN residue per enzyme molecule lead to the conclusion that when the enzyme is saturated with substrate, 19% of the enzyme-bound DPN is in the reduced form.

From the slope and intercept found in Fig. 10*b*, and assuming $\epsilon = 6.2 \times 10^3$ M^{-1} cm^{-1}, one can calculate that $K_2K_2' = 1.87 \times 10^{-4}$ M; $K_1' + K_2' = 4.2$; and $K_1K_1' = 6.6 \times 10^{-4}$ M. However, the individual values cannot be calculated without additional information. If one makes the common but generally unsupported assumption that the K_m for UDP-galactose is a good approximation for the dissociation constant of the enzyme-UDP-galactose complex, K_2, then one can solve for all of the constants: $K_2 = K_m = 1.6 \times 10^{-4}$ M; $K_2' = 1.2$; $K_1' = 3.0$; and $K_1 = 2.2 \times 10^{-4}$ M. Since we have no particular basis for supporting this last assumption, the values of the four equilibrium constants must be considered as only of speculative value.

DISCUSSION

The induced synthesis of the three enzymes associated with the *E. coli* galactose operon are coordinated in the sense that ratios of their activities, in extracts of a given genotype, are invariant to conditions of growth which drastically alter their individual specific activities (see Buttin (2), footnote 4). This is the typical result for enzymes associated with a single operon (27–30). From it and data yielding the genetic definition of the operon, Jacob and Monod (29) evolved a working hypothesis to explain such coordinated syntheses and their regulation; a hypothesis that places the coordination or linkage of the syntheses at the level of messenger RNA formation.

Thus if n different polypeptides (P_i) are determined by an operon, it is supposed that the syntheses of the corresponding n different messenger RNA base sequences (M_i) are either all "on" or all "off." It is further supposed that although the rates of synthesis of the various M_i may vary, they remain in constant ratio, one to another, as do their cytoplasmic concentrations. (The most common assumption here is that all the M_i are contained in a single messenger RNA molecule and consequently that these ratios equal unity.) The final arguments of the hypothesis are that the rates of synthesis of the P_i are proportional to the M_i concentrations; that therefore the numbers of the P_i in the cytoplasm will be in constant ratio; and that this will yield the constant ratios that are found among the activities of the extracted enzymes.

It is the actual values of these ratios which are at present left out of the argument, and which are critical for its extension to specific molecular models. As a first step in these evaluations, it is of interest to calculate the molecular ratios of the extracted enzymes associated with the galactose operon. To calculate the molecular ratio from the activity ratio, the specific activity of the pure enzymes as well as their molecular weights must be known. The data in this paper yield these two values for UDP-galactose 4-epimerase, if one accepts the arguments of the preceding sections regarding purity. These data for *E. coli* galactokinase can be taken from a recent paper by Sherman and Adler (6), in which this enzyme has been purified to yield a preparation that is homogeneous to chromatography on DEAE-cellulose and that gives a single sedimentation boundary with a coefficient of 2 S. The authors estimate a molecular weight of about 2×10^4 for this protein. On the assumption that this preparation represents pure galactokinase, we can combine the two sets of data to calculate the molecular ratio of galactokinase to UDP-galactose 4-epimerase in the crude extracts.

Using the same galactokinase assay as Sherman and Adler (6), we find that the ratio of galactokinase activity to epimerase activity in extracts of fully induced K12 *gal*$^+$ or K12 *gal*$^+$ (λdg) strains (see "Materials") is 0.32 ± 0.05.[4] Since the specific activities of the purified kinase and epimerase are 0.84×10^3 (6) and 14×10^3 units per mg, respectively, we calculate from these values and the molecular weights that the ratio of galactokinase molecules to UDP-galactose 4-epimerase molecules in the extracts is 21. It is possible that the larger epimerase molecule may be divisible into identical polypeptide chains of approximately the same size as galactokinase. Consequently, it is also of interest to calculate the ratio of the weight of galactokinase to that of the epimerase in the extracts. This ratio has a value of 5.

Not only are these values surprisingly different from unity, but they bear a reciprocal relationship to the nonunity ratios found for enzymes of the lactose (30) and histidine (28) operons. In these cases the ratios indicate that the proteins whose structural genes are closest to the operator gene are synthesized at the greater rate. The opposite relation is indicated by the above ratios for the galactose operon. Thus the operator gene lies closest to the structural gene for the epimerase and farthest from that for the kinase; the structural gene for galactose-1-P uridyltransferase lies in between these two (2, 3).

In view of this conflict, the critical nature of the purity assumption should be emphasized here. Thus the value of the galacto-

[4] This ratio has been found both for exponentially growing cells and for cells starved on their carbon source with the medium given in "Materials." In both strains (K12 *gal*$^+$ and K12 *gal*$^+$ (λdg)) the specific activities are about 2-fold greater in the extracts of starved cells than of exponentially growing cells. (This finding was noted too recently to be of use in the purification procedure.) Since the K12 *gal*$^+$ (λdg) activities are about 3-fold more than the K12 *gal*$^+$ activities (see "Materials" and Reference 12), the ratio has been found constant (±12%) over a 6-fold variation in specific activities of the extracts.

kinase to epimerase ratio will be decreased if our assumption of galactokinase purity is not valid, and increased by the lack of validity of this assumption for the epimerase. We have presented the arguments that make us confident that the purified epimerase contains only a small percentage of contaminating proteins. The arguments for purity of the galactokinase are less rigorous (6), and it is conceivable that the level of contaminating proteins in the "purified" galactokinase is sufficient to reduce the weight ratio from 5 to unity or less. We are presently investigating this possibility.[5]

To complete the determination of the ratios among all the P_n of the galactose operon, it will be necessary to determine the nature of the polypeptide chain, or chains, in each enzyme. We are now in a position to commence such determinations for the epimerase, but must wait upon the establishment of purity criteria for the galactokinase and galactose-1-P uridyltransferase.

That portion of our results which contribute novel information relevant to the mechanism of epimerization is restricted to data concerning enzyme-bound DPN. The significant findings are: (*a*) there is only 1 mole of *N*-substituted nicotinamide derivative per mole of enzyme and this, when dissociated from the protein, is DPN; (*b*) in the absence of substrate the isolated enzyme exhibits no absorption band in the 300 to 400 mμ region; (*c*) the presence of substrate causes the appearance of an absorption band at 345 mμ; and (*d*) the absorption at 345 mμ is a saturating function of substrate concentration, *i.e.* $[O.D._{345}]^{-1} = [O.D._{345}]^{-1}_{max} + K[(S_2) + (S_2)]^{-1}$, where $[O.D._{345}]_{max}$ is the saturating value, K is a constant, and (S_1) and (S_2) are the concentrations of free substrates.

We suggest that the substrate-induced appearance of the absorption band at 345 mμ is due to the reduction of enzyme-bound DPN to enzyme-bound DPNH. The absorption band is very similar in shape to that of free DPNH, the only marked difference being that the wave length of the maximum is about 5 mμ greater than that for DPNH. While the binding of DPNH to most proteins (dehydrogenases) causes a shift of the maximum toward lower wave lengths (31), a shift to a higher wave length (351 mμ) has been observed for malic dehydrogenase (32). (Another interesting corollary between this malic dehydrogenase and *E. coli* UDP-galactose 4-epimerase is that both bind only 1 mole of pyridine nucleotide per mole of enzyme (32), whereas the majority of dehydrogenases examined contain more than one coenzyme site per molecule (31).)

The extinction coefficient of the known DPNH-protein complexes at the maximum of their 300 to 400 mμ absorption band is only slightly (about 10%) less than the value 6.2×10^3 M^{-1} cm^{-1} for DPNH (31, 33). Using the 6.2×10^3 M^{-1} cm^{-1} value for the extinction coefficients of the component absorbing at 345 mμ, we find 0.19 mole of this component per mole of substrate-saturated enzyme. This value is compatible with the 345 mμ-absorbing component being enzyme-bound DPNH since there is 1 molecule of pyridine nucleotide available per molecule of enzyme.

[5] *Note*—Since submission of this article we have purified the galactokinase of *E. coli* to a specific activity 4- to 5-fold greater than that obtained by the procedure of Sherman and Adler (6). This preparation of galactokinase is of comparable homogeneity to the purified UDP-galactose 4-epimerase characterized above. Its molecular weight is 3.8×10^4. As a result of these new data the weight ratio of galactokinase to UDP-galactose 4-epimerase must be corrected from 5 to 1.

These data then represent the first direct evidence (beyond the observation of a DPN requirement (8) or its presence (9)) that the epimerization mechanism involves enzyme-bound DPN as a reactant which undergoes a change in structure of its pyridine ring, and strongly suggests that this change is the formation of enzyme-bound DPNH. Consequently the proposition that the epimerization mechanism includes a reversible oxidation at carbon 4 of the hexose residue (34) is rendered more probable, although direct evidence concerning the structure of the intermediate derived from the UDP-hexoses is still lacking. It should be noted that the present data restrict the possible mechanisms to those involving only 1 pyridine nucleotide molecule, always assuming that the population of enzyme molecules in the isolated preparation is homogeneous with respect to DPN content.

The mechanism that comes first to mind is the oxidation by the DPN on the enzyme of the hexose residue at carbon 4 to yield a ketone which becomes symmetrically placed relative to the DPNH so that subsequent reduction of the ketone by the DPNH can yield either a galactose or a glucose residue. The equilibrium ratio of DPN-containing complexes to DPNH-containing complexes is 4 when the *E. coli* enzyme is saturated with substrates at about pH 9 (see the last section of "Results"). This is not inconsistent with a DPN-DPNH, alcohol-ketone (or aldehyde)-coupled oxidation-reduction on the enzyme surface. For example, Theorell and Yonetani (35), working with liver alcohol dehydrogenase at 23.5° and pH 7.0, found that the equilibrium ratio of the ternary complexes, *E*-DPN-alcohol to *E*-DPNH-aldehyde, is about 1, although the $(DPN)_{free}$ $(alcohol)_{free}$:$(DPNH)_{free}$ $(aldehyde)_{free}$ equilibrium ratio is 1.1×10^4.

This type of model is consistent with the failure of tritium in DPN, DPNH, or water to exchange with UDP-hexoses in the presence of other UDP-galactose 4-epimerases (8–10), provided that one makes the assumption that free DPNH and enzyme-bound DPNH do not exchange—an assumption that is made reasonable by the extreme stability of the DPN-enzyme complex of the *E. coli* and yeast (9) enzymes, the low K_m for DPN with the liver enzyme ($K_m = 2 \times 10^{-7}$ M; see Reference 8), and the general observation that DPNH binds much more strongly to dehydrogenases than does DPN (36).

Such stable complexes between enzyme and intermediates would necessitate the use of large amounts of purified enzyme for the determination of the structure of the intermediates. At present it appears that the *E. coli* enzyme has the advantage in this regard. This is not only because it is the only UDP-galactose 4-epimerase for which some definition of purity has been established, but also because the maximum specific activity reported here is about 300-fold greater than the maximum reported for the liver enzyme (8), and about 60-fold greater than the maximum reported for the yeast enzyme (9), although both of these have been purified about 200-fold. In connection with future uses of the *E. coli* enzyme to establish the mechanism of epimerization, one should note that it would make an ideal subject for temperature jump methods (37), since perturbation of the equilibrium condition could be specifically detected by the change in absorption at 345 mμ.

SUMMARY

Escherichia coli uridine diphosphogalactose 4-epimerase was purified from cultures induced for its biosynthesis. The purified preparation contains only small amounts of impurities (no more

than about 10%) as analyzed by column chromatography, starch gel electrophoresis, and velocity and equilibrium sedimentation. The latter two methods were also used to determine a molecular weight of 7.9 (± 0.8) $\times 10^4$. Comparison of these data with those for galactokinase (6), another enzyme of the galactose operon, indicates that the rate of synthesis of the kinase is greater than that of the epimerase.

Although the purified enzyme exhibits no requirements for added cofactors, 1 mole of diphosphopyridine nucleotide was found tightly bound to each mole of enzyme. This DPN-protein complex exhibited no absorption band in the region of 300 to 400 mμ until substrate was added; this addition caused the appearance of a band at 345 mμ whose shape was almost identical with that of free DPNH. The intensity of absorption at 345 mμ was dependent on substrate concentration in a manner consistent with the model,

$$S_1 + E\text{-DPN} \rightleftharpoons E\text{-DPN},S_1 \rightleftharpoons E\text{-DPNH},S_{ox} \rightleftharpoons E\text{-DPN},S_2 \rightleftharpoons E\text{-DPN} + S_2$$

where E-DPN is the purified enzyme, S_1 is uridine diphosphoglucose, S_2 is uridine diphosphogalactose, and S_{ox} is a hypothetical derivative of either uridine diphosphohexose oxidized at carbon 4 of the hexose residue.

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