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Purification and Properties of Uridine Diphosphate Galactose 4-Epimerase from Yeast*

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ABSTRACT: Uridine diphosphate galactose 4-epimerase has been isolated from galactose-adapted yeast, by a method which facilitated the preparation of large amounts of the protein. The specific catalytic activity of the final product from five different preparations varied between 41.4 and 87.4 μmoles of substrate converted into product per min per mg of protein, at 23°. The enzyme was free of contaminating protein as judged by chromatography on CM-cellulose, DEAE-cellulose, and gel filtration media. It contained, per mg of protein, 8 mμmoles of bound diphosphopyridine nucleotide and 116 mμmoles of SH titratable with *p*-mercuribenzoate. The preparation migrated as a single boundary upon sedimentation in the ultracentrifuge in buffers of low ionic strength. In 0.01 M Tris-HCl (pH 7.5) a molecular weight of 125,000 ± 5600 was calculated from data obtained by low-speed equilibrium centrifugation, high-speed equilibrium centrifugation, and by combination of sedimentation and diffusion data. The minimum molecular weight calculated from the amount of diphosphopyridine nucleotide bound to the protein was 125,000. The fluorescence of yeast epimerase, with excitation at 350 mμ and emission at 435 mμ, has previously been related to the presence of reduced diphosphopyridine nucleotide (Creveling, C. R., Bhaduri, A., Christensen, A., and Kalckar, H. M. (1965), *Biochem. Biophys. Res. Commun. 15*, 182). In the present studies, the fluorescence decreased markedly on treatment with guanidine hydrochloride, suggesting a strong dependence upon an ordered protein structure.

We report here a new method of preparing UDP galactose 4-epimerase[1] from yeast which has advantages of simplicity, speed, and applicability to large-scale operation. The final product has a specific activity of up to ten times that recorded previously for this enzyme (Maxwell and de Robichon-Szulmajster, 1960) and contains three times as much bound DPN.

The purified protein has a molecular weight of approximately 125,000 as determined by sedimentation studies under conditions of low ionic strength (0.01 M). At higher ionic strengths (0.1 M), the enzyme can be separated into two or more catalytically active components either by ultracentrifugation (Darrow and Rodstrom, 1966) or by chromatography on DEAE-cellulose (R. A. Darrow and R. Rodstrom, unpublished data).

The fluorescence of native epimerase from yeast has been shown to be associated with the presence of DPNH (Creveling *et al.*, 1965) and to be influenced by a number of sugars and uridine nucleotides (Bertland *et al.*, 1966). Studies of the loss of fluorescence in urea and guanidine solutions reported in this paper indicate that the fluorescence is dependent upon a highly ordered protein structure.

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: UDP glucose 4-epimerase (EC 5.1.3.2), UDP galactose 4-epimerase; uridine 5′-(α-D-galactopyranosyl pyrophosphate), UDP galactose; UDP glucose:NAD oxidoreductase (EC 1.1.1.22), UDP glucose dehydrogenase; uridine 5′-monophosphate, UMP; *p*-mercuribenzoate, PMB.

Materials and Methods

Source of Chemicals. Partially hydrolyzed starch, glycylglycine, and iodoacetic acid were from the Mann Laboratories. CM-, P-, and DEAE-cellulose were from Bio-Rad Laboratories. The DEAE-cellulose had an exchange capacity of 0.7 mequiv/g. Guanidine·HCl and 2-mercaptoethanol were products of the Eastman Chemical Co.; EDTA came from the Matheson Co.; DPN, DPNH, Tris, and several samples of galactose-adapted yeast were purchased from the Sigma Chemical Co. Other chemicals were of reagent grade unless otherwise stated, and were obtained from commercial sources.

Amino Acid Analysis. The amino acid composition of epimerase was determined according to the method of Spackman *et al.* (1958), on a reduced, carboxymethylated sample which was prepared according to the procedure of Crestfield *et al.* (1963). The protein was denatured with 5 M guanidine·HCl instead of 8 M urea, and 0.2 M $(NH_4)HCO_3$ was used instead of 50% acetic acid as a solvent for the removal of excess iodoacetic acid. The protein was hydrolyzed *in vacuo* at 110° in 6 M HCl for 24 and 48 hr. One determination was made for each time of hydrolysis. Values of serine and threonine were determined by extrapolating the observed values to zero time. The 48-hr value for valine and isoleucine was taken as the most reliable. Tryptophan was determined colorimetrically on a separate sample (Spies and Chambers, 1949).

Other Methods and Instruments. Sedimentation analyses were performed with a Spinco Model E analytical ultracentrifuge. Amino acid analyses were done with a Spinco 120B analyzer. Fluorescence was measured with an Aminco-Bowman spectrophotofluorometer and qualitatively located among fractions, or on columns, with a Blak-Ray ULV-22 lamp. Substituted cellulose chromatography was done according to the general precepts of Peterson and Sober (1962). Starch gel electrophoresis was done according to Smithies (1955), and polyacrylamide gel electrophoresis according to Davis (1964). Enzyme-bound DPN was estimated from the fluorescence resulting from the direct addition of alkali to the enzyme, by the method of Lowry *et al.* (1957). Protein was determined either by the biuret reaction or calculated from the absorption at 280 and 260 mμ (Layne, 1957).

Organism. The enzyme was isolated from *Candida pseudotropicalis* (*Saccharomyces fragilis*), American Type Culture Collection, no. 10022. The same strain was used previously (Maxwell and de Robichon-Szulmajster, 1960). Stocks of yeast were maintained at 15° on slants of the growth medium described below, containing 2% agar, and glucose in place of galactose. Transfers were made twice yearly.

Growth Conditions. The medium was a mixture of two volumes of salt solution (15 g of Difco yeast extract, 1.8 g of $(NH_4)_2SO_4$, and 15 g of KH_2PO_4 per l.) and one volume of galactose solution (150 g of technical grade galactose/l.), each autoclaved separately. The organism was transferred from the stock slant to a roller tube containing 10 ml of medium and allowed to grow for 8–20 hr at 37°. The contents of the tube were then transferred to a 4-l. flask containing 750 ml of medium which was shaken at 37° for about 16 hr. The resulting culture was then transferred to an 18-l. carboy, equipped for vigorous aeration, containing 12 l. of medium at 30° to which 0.5 ml of Dow-Corning Antifoam A (or F) had been added. Growth was continued with vigorous aeration for 24 hr in the 37° room. During this period the temperature of the medium remained near 30° because of evaporation. After 24 hr the turbidity was no longer increasing, and always exceeded an optical density of 0.400 at 650 mμ when measured in a 1-cm cell in a Beckman DU spectrophotometer. The yeast cells were collected in a refrigerated Sharples supercentrifuge and washed with about ten times the wet volume of cold water. The washed cell paste was then frozen, freeze dried, and stored at −10°. It could be kept for at least 6 months before use. Satisfactory preparations of enzyme have also been made from yeast obtained in 150-gal. fermentations.[2]

Assay of Enzymic Activity. Epimerase activity was determined from the rate of increase in optical density at 340 mμ, after the addition of UDP galactose to a solution containing epimerase and an excess of DPN and UDP glucose dehydrogenase (Maxwell, 1957). As previously reported (Darrow and Creveling, 1964), the rate of the epimerase reaction depends upon the nature of the buffering ion employed. For this reason, and because (a) the enzyme is subject to inactivation at high dilution and (b) the assay is not performed at saturating substrate concentrations, it was necessary to adhere strictly to the following procedure to obtain comparable results.

Enzyme dilutions were made in 0.05 M Tris-maleate buffer (pH 6.5) (Gomori, 1955), containing 0.1% albumin (amorphous, Armour Pharmaceuticals Co., fraction V from bovine plasma, kept as a 5% stock solution in 1% NaCl). Diluted enzyme (0.1 ml) was added to a spectrophotometer cell having a 1-cm light path and containing 0.1 ml of 1.0 M sodium glycylglycinate (pH 8.9), 0.1 ml of 0.01 M DPN, 0.05 ml of UDP glucose dehydrogenase, purified through step V as defined by Strominger *et al.* (1957), containing 400 units of activity, and 0.64 ml of H_2O. The optical density at 340 mμ was observed in the absence of UDP galactose to check for spurious reduction of DPN with crude enzyme samples. When there was no further change in optical density, the reaction was started by the addition of 0.01 ml of 0.01 M disodium UDP galactose. The rate of increase in optical density at 340 mμ was proportional to epimerase concentration up to a value of 0.05/min. The temperature of the assay was approximately 23°. During the early stages of purification, epimerase activity is underestimated by this assay, since crude yeast extracts bring about a slow decrease in the absorption of light at 340 mμ after the addition of DPNH to the reaction mixture.

Enzyme units are reported as international units, one unit transforming 1 μmole of substrate/min. The ob-

[2] Large-scale preparations from 150-gal fermentations have been made at the Tufts New England Enzyme Center.

served velocity was converted to maximum velocity with the Michaelis–Menten equation using a value of 1.1×10^{-4} M for the K_m of UDP galactose (Darrow and Creveling, 1964).

Purification Procedure. Unless stated otherwise, all manipulations in the following procedure are at 0–4°, and all centrifugations are performed on the Servall RC-2 refrigerated centrifuge, using the GSA rotor, at 9000 rpm for 30 min. All measurements of pH are made at room temperature.

CRUDE EXTRACT. Suspend 375 g of freeze-dried yeast cells in 1.125 l. of 0.1 M sodium phosphate (pH 7.0) at room temperature. Bring the paste to 34° in a 35° water bath, add 150 ml of toluene, and stir slowly for 90 min. Dilute the suspension with 3 l. of 0.1 M sodium phosphate (pH 7.0), 4°. Add 3 ml of β-mercaptoethanol and stir gently overnight. Centrifuge, save the supernatant solution, and suspend the residue with 3 l. of 0.1 M sodium phosphate (pH 7.0) containing 3 ml of β-mercaptoethanol. Stir for 90 min, centrifuge, discard the residue, and combine the supernatant solution with the previous supernatant solution to give 6625 ml of crude extract (fraction I).

FIRST AMMONIUM SULFATE STEP. Warm the crude extract in a 35° water bath and allow it to remain in the bath for 1 hr after the temperature of the extract reaches 30°. Cool rapidly to 4° in an ice bath, add 2075 g of $(NH_4)_2SO_4$ (313 g/l.), and stir gently for at least 2 hr (up to 24 hr). Centrifuge, discard the supernatant, and resuspend the residue (A) in water to a final volume of 1.5 l. Add 208.5 g of $(NH_4)_2SO_4$ (139 g/l.) and assay the suspension. Stir for 2 hr, centrifuge, retain the residue (B), and assay the supernatant solution. If this contains more than 20% of the activity of the suspension, add more $(NH_4)_2SO_4$ (127 g/l.), stir, and centrifuge as before, to obtain residue C.[3] Suspend residue B (or C) in 2.0 l. of succinate buffer at 4°, which is a fivefold dilution of a stock solution containing, per l., 27.0 g of disodium succinate·$6H_2O$, 5.8 g of NaCl, 25 ml of 2 N HCl, and 0.5 ml of β-mercaptoethanol. Stir the suspension for 20 min, centrifuge, and keep the supernatant (1.93 l., fraction II).

SECOND AMMONIUM SULFATE STEP. Add 996 g of $(NH_4)_2SO_4$ (516 g/l.) to fraction II. Note the volume of the suspension, centrifuge, and measure the volume of the supernatant solution, obtaining the volume of the residue by difference (250 ml). Suspend the residue in ten times its volume (2.5 l.) of 75% saturated $(NH_4)_2SO_4$ containing per liter of final solution: (a) 750 ml of $(NH_4)_2SO_4$, saturated at 4°; (b) 200 ml of succinate buffer stock solution described in the previous section; (c) 10 ml of 10^{-2} M EDTA; and (d) H_2O to final volume. Stir for 20 min and centrifuge, again evaluating the volume of the residue (160 ml). Carry out analogous washings

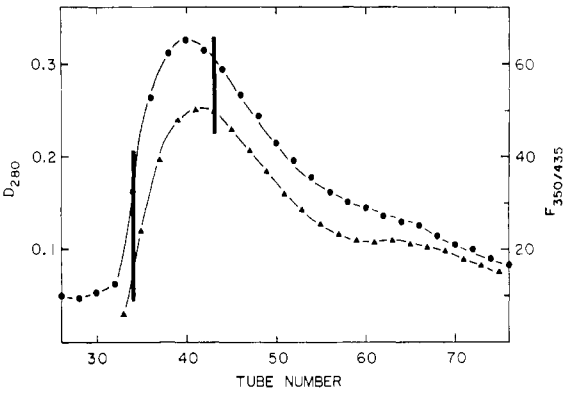

FIGURE 1: Elution of UDP galactose 4-epimerase from DEAE-cellulose. A column of DEAE-cellulose (2.5 × 30 cm) was equilibrated with a solution containing 0.1 M NaCl, 0.02 M Tris-HCl, and 2×10^{-4} M EDTA (pH 7.5). It was charged with 50 mg of preparation d, Table I, and developed at 4°, at 1 ml/min, with 1 l. of 0.02 M Tris-HCl and 2×10^{-4} M EDTA (pH 7.5) containing a linear gradient between 0.1 M NaCl and 0.5 M NaCl. Circles show absorbance at 280 mμ; the curve for catalytic activity was superimposable. Triangles show fluorescence with excitation at 350 mμ and emission at 435 mμ, in arbitrary units.

with ten residue volumes (1.6 l.) of 35% saturated $(NH_4)_2SO_4$, 30% saturated $(NH_4)_2SO_4$, and finally with a solution containing only succinate buffer and EDTA, without $(NH_4)_2SO_4$ (0% $(NH_4)_2SO_4$). Immediately add 346 g of $(NH_4)_2SO_4$/l. of the final supernatant solution (0% $(NH_4)_2SO_4$), which is fraction III.

FRACTIONAL SOLUBILIZATION. Centrifuge fraction III, estimate the volume of the residue approximately (30 ml), and discard the supernatant. Add to the residue a measured volume (15 ml) of 0.1 M Tris-HCl–10^{-3} M EDTA (pH 7.5) containing 0.007 M β-mercaptoethanol. Measure the volume of the resulting suspension and calculate the exact volume (25 ml) of the residue by difference. Bring to twice this calculated volume (50 ml) by the further addition of the above Tris buffer. Homogenize the suspension thoroughly by hand, with a tissue grinder having a Teflon pestle. Centrifuge for 1 hr at 40,000 rpm in the 40 rotor of the Spinco Model L in stainless-steel tubes, with the refrigeration set to give a compartment temperature of 5°. Decant and save the supernatant, and resuspend the translucent gel-like pellet to the same final volume as before (50 ml), using the same Tris buffer. Homogenize thoroughly and repeat the centrifugation, continuing as many times as are necessary to solubilize all the precipitate. The $(NH_4)_2SO_4$ saturation of the supernatant from the first, second, and third resuspensions should be approximately 30, 3–6, and 0%, respectively, as determined by conductivity measurements. The supernatant from the second resuspension normally contains the bulk of the enzymatic activity and is stored at −90° as fraction IV.

DEAE-cellulose Chromatography. Chromatography on DEAE-cellulose did not lead to any increase in specific activity or reveal the presence of any contaminating inert protein in fraction IV. It was undertaken because of the presence of a second epimerase component whose sedimentation properties differed from those of the major component with respect to conversion into a more

[3] Over 80% of the activity was in residue C in some preparations (see Table I). In these cases subsequent purification steps led to final preparations identical with those in which the activity was precipitated in residue B with respect to electrophoretic pattern, SH content, and micromoles of bound DPN per milligram of protein, although the resulting specific activity was lower.

TABLE I: Purification of UDP Galactose 4-Epimerase.

Fraction	Date of Prepn	ml	Protein (g)	Protein (units/mg)	DPN (mμmoles)/ Protein (mg)	Yield (%)
I		6625	68.2	0.8	0.34	(57)
II		1930	6.9	13.7	2.0	(97)
III		1257	2.1	45.7	6.0	100
IV	(a) 4/65	35	0.36	87.4[a]	8.7	31
	(b) 7/65			79.0[a]	7.2	
	(c) 11/65			57.5[b]	9.0	
	(d) 1/66			47.5[b,c]	7.7	
	(e) 11/64			41.4[b]	7.3	

[a] Activity was in residue B in the first ammonium sulfate step. [b] Activity was in residue C in the first ammonium sulfate step. [c] Prepared at the Tufts New England Enzyme Center.

rapidly sedimenting species (10.8 S) at high ionic strength (0.1 M). The details of this observation will be included in a separate study (R. A. Darrow and R. Rodstrom, unpublished data). Figure 1 shows the elution diagram of fluorescence and optical density at 280 mμ obtained upon chromatography of preparation d, Table I, on DEAE-cellulose. The presence of at least two components is clear. The fractions between the bold, vertical lines were combined and used for the studies reported in this paper related to sedimentation or diffusion, and for the amino acid analysis.

Results

Purity and Homogeneity. Samples of purified epimerase gave a single diffuse component as visualized with Amido Black, after starch gel electrophoresis either in 0.06 M sodium borate at pH 9.0, 0.06 M sodium phosphate at pH 7.0, or 0.035 M sodium acetate at pH 5.6. A single component was also observed when 6 M urea was incorporated into the starch gel made in 0.035 M sodium acetate (pH 5.6). Sedimentation in a sucrose gradient at protein concentrations of 1–2 mg/ml gave symmetrical peaks in which the activity coincided with the absorption at 280 mμ (Darrow and Rodstrom, 1966). No further purification as judged by specific activity and no resolution of *inactive* protein components was obtained by fractionation with organic solvents or ammonium sulfate at various values of pH, on ion-exchange chromatography with DEAE- or CM-cellulose or on gel filtration with Sephadex G-100 or G-200.

Several of these methods of fractionation have revealed, however, that the purified enzyme can be separated into more than one catalytically *active* component. As reported earlier (Darrow and Rodstrom, 1966), two poorly resolved, active peaks occur on sucrose density gradient centrifugation at low ionic strength at protein concentrations of 10–20 μg/ml. A similar result has been obtained with Sephadex G-200 (R. A. Darrow and R. Rodstrom, unpublished data). In both of these cases, the difference in position between the principal peak and the shoulder corresponded to less than a whole multiple of the lower molecular weight. In addition, partial resolution of two active peaks has been achieved with DEAE-cellulose chromatography, as depicted in Figure 1.

Polyacrylamide Gel Electrophoresis. Electrophoresis on polyacrylamide gel according to the method of Davis (1964) gave the patterns reproduced in Figure 2. The rate of migration was inversely proportional to acrylamide concentration. In addition to the main band there were several minor components which became more apparent at the higher concentrations of acrylamide. The inclusion of 4 M urea in 7.5% acrylamide gel made the main component more diffuse but did not resolve it into more than one band.

Attempts at elution of active enzyme from the polyacrylamide gels after electrophoresis have resulted in little or no recovery of activity. Attempts at locating the bands with phenazine methosulfate, nitro blue tetrazolium, UDP galactose, DPN, and UDP glucose dehydrogenase led to colored bands corresponding with those produced with protein stain. The emergence of these bands did not depend upon the addition of UDP galactose, however, and hence it is questionable whether a true enzymatic activity was being measured.

In an attempt to understand the multiple banding illustrated in Figure 2, a freshly run, unstained gel (7.5% acrylamide) was cut longitudinally, in the region where the protein bands were expected. One of the half-cylinders thus obtained was stained with Amido Black according to the usual procedure, while the other half-cylinder was divided into six equal portions, each of which was cast in a second "sample" gel, and run in the usual way. Each of the regions in the first gel yielded a complete set of bands in the second gel, the most prominent being the band from which the sample was originally taken. From this observation we have inferred that the multiple banding arises from some type of equilibrium between different forms of the enzyme which is slowly established under the conditions of the application of the sample, after which the components of the

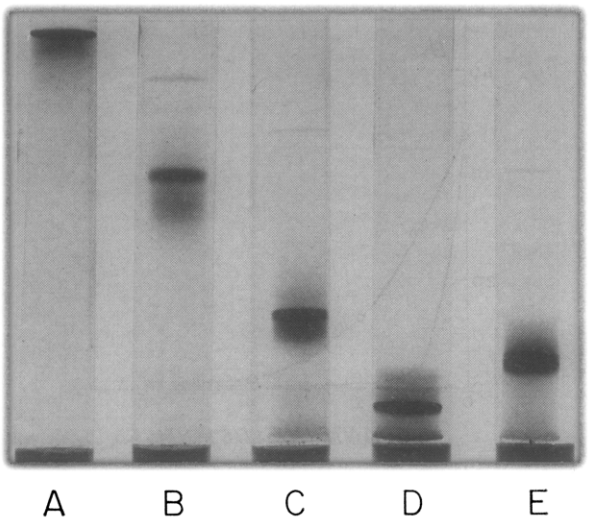

FIGURE 2: Polyacrylamide gel electrophoresis of UDP galactose 4-epimerase. All gels contained 122 μg of fraction IV, preparation b, Table I. Migration is from bottom (cathode) to top (anode) for 90 min at 2.5 mA/tube. Staining is with amido black 10B. Gels contained the following percentages of acrylamide: (a) 3%, (b) 5%, (c) 7.5%, (d) 15%, and (e) 7.5% acrylamide, 4 M urea.

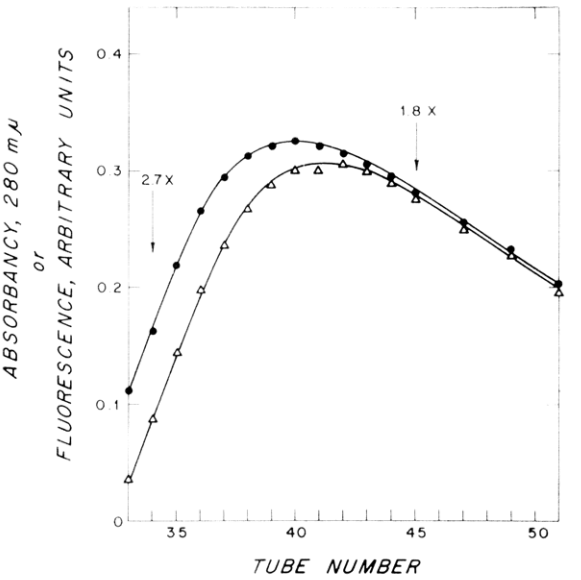

FIGURE 3: Elution of UDP galactose 4-epimerase from DEAE-cellulose. Experimental conditions are the same as for Figure 1. The fractions shown represent that portion of the gradient between 0.17 M NaCl and 0.2 M NaCl. Data points are absorbance at 280 mμ (●), which gave a curve which was superimposable with that for catalytic activity, and fluorescence in arbitrary units, with excitation at 350 mμ and emission at 435 mμ (△). Arrows indicate the fractions tested for the enhancement of fluorescence by adding UMP (0.1 μmole/ml) and D-galactose (10 μmoles/ml) directly to the column effluent (tube 34 and 45; 2.7- and 1.8-fold enhancement, respectively).

equilibrium are separated out by sieving through the more concentrated gels.

Catalytic Activity. The specific activity at the various stages of purification for the preparation in which the highest final specific activity was achieved is given in Table I. The purification was approximately 50-fold, after correcting for the underestimation of the activity in the crude extract. Also shown in Table I are the specific activities of four other preparations of enzyme, which varied over a range of approximately twofold. Bound DPN (Maxwell and de Robichon-Szulmajster, 1960) was also determined, employing for this purpose the method of Lowry *et al.* (1957). As shown in Table I, the values for DPN per milligram of protein rose during the purification and reached a final value of about 8 mμmoles/mg of protein, which is the average figure of the five preparations shown. Each preparation was also tested by PMB titration (Boyer, 1954), in which values close to 116 mμmoles of SH/mg of protein were obtained, and by polyacrylamide gel electrophoresis. These techniques gave the same result regardless of the specific activity.

Stability. Purified epimerase (fraction IV) was routinely kept frozen at −90°. Repeated freezing and thawing was avoided. The preparations with the higher values of specific activity shown in Table I were unstable, losing from one-third to one-half of their activity within 1 week. The preparations with the lower values tended to be more stable. More than 80% of the activity of fraction IV was lost by freeze drying. Storage for longer than 24 hr at 4° at neutral pH has caused on one occasion a 50–90% *decrease* in total activity, with the loss inversely proportional to protein concentration between 0.5 and 10 mg/ml. On another occasion, however, up to a 50% *increase* in total activity was observed under similar conditions. Storage at 4° above pH 8.5 or below pH 6 led to an apparently irreversible loss of activity, accompanied by precipitation below pH 6. No effect has been seen of cations on the stability of the enzyme, although it is known that their presence greatly stimulates the rate of the epimerase reaction (Darrow and Creveling, 1964). At protein concentrations between 0.1 and 1.0 μg/ml, such as were encountered in diluting the enzyme for measurement of catalytic activity, 0.1% bovine serum albumin was added as a preservative. Mercaptoethanol (0.007 M) could partially substitute for serum albumin under these conditions, but had no additional effect when present together with albumin.

Fluorescence. The pyridine nucleotide-like fluorescence of yeast epimerase (excitation, 350 mμ; emission, 435 mμ) has been studied by Kalckar and coworkers (Maxwell *et al.*, 1958; Creveling *et al.*, 1965; Bhaduri *et al.*, 1965; Bertland *et al.*, 1966). In the present work, we have found that this fluorescence remained with the protein following dialysis in a number of aqueous buffers at pH values between 6 and 9, and on electrodialysis in 0.05 M Tris-glycinate buffer at pH 8.4. It migrated with the enzyme on starch gel electrophoresis, during chromatography on Sephadex G-25, G-100, and G-200, and in sucrose density gradient centrifugation. Upon chromatography on DEAE-cellulose, as shown previously by Maxwell *et al.* (1958), and as illustrated in Figure 1, the fluorescence was eluted with the enzyme. As shown in Figure 3, however, the curves for fluorescence and specific activity did not coincide exactly. We have observed a similar phenomenon in starch gel elec-

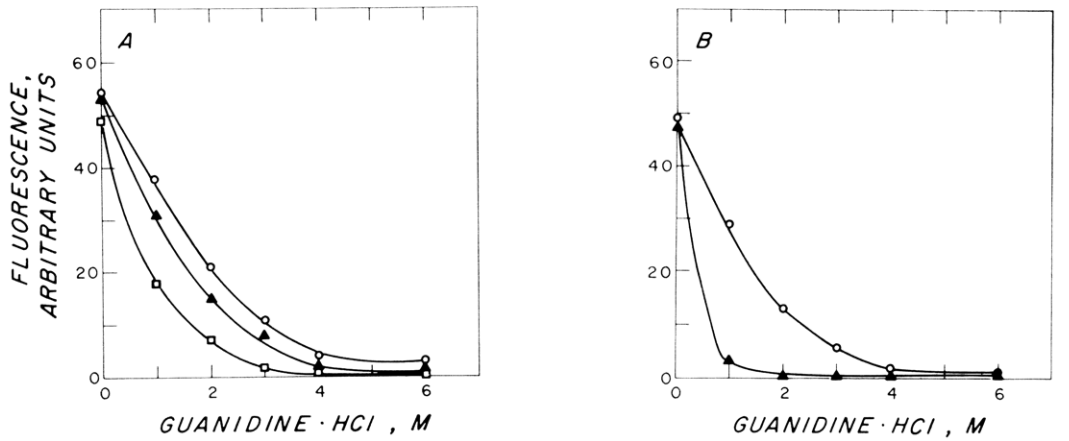

FIGURE 4: The destruction of fluorescence and catalytic activity of UDP galactose 4-epimerase by guanidine·HCl. Preparation a (25 μl), Table I, was added at zero time to 500 μl of 0.1 M Tris-HCl, 10^{-3} M EDTA, 7×10^{-3} M β-mercaptoethanol (pH 7.6) containing varying concentrations of guanidine·HCl. (A) Fluorescence from excitation at 350 mμ with emission at 435 mμ was measured at 1 min (○), 5 min (▲), and 60 min (□). (B) Fluorescence was measured in the same manner as in A at 110 min (○); activity was measured as described in the Experimental Section after 1:40 dilution into 0.05 M Tris-maleate buffer (pH 6.5) containing 0.1% bovine serum albumin, at 95 min (▲).

trophoresis in 0.06 M sodium borate at pH 8.9, where fluorescence appeared most intense nearest the anode, at the forward edge of the region stained for protein. In Figure 3, the difference between the leading and trailing samples of the DEAE-cellulose column effluent, in fluorescence per milligram of protein, could be partially redressed by treating with UMP and galactose (Bertland *et al.*, 1966). Such treatment lowered the ratio of fluorescence per milligram of protein between tube 45 and tube 34 from 1.8 to 1.2. This procedure has been shown by Bertland *et al.* (1966) to increase the fluorescence of purified epimerase samples as much as threefold, with a concomitant increase in optical density in the absorption region of DPNH.

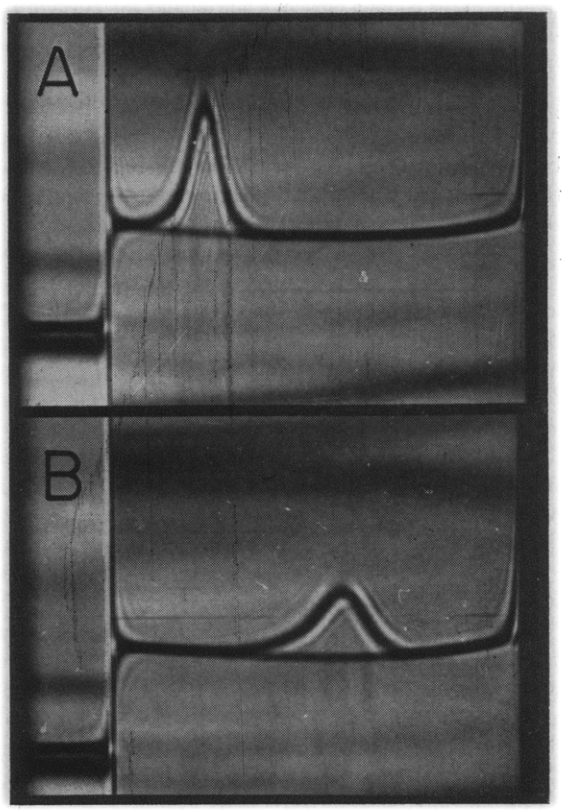

FIGURE 5: Schlieren patterns showing sedimentation of yeast epimerase (preparation d, Table I) at 48 min (A), 112 min (B) after attaining top speed. Rotor speed 59,780 rpm, bar angle 65°, protein concentration 4.7 mg/ml; temperature 3.9°; solvent, 0.1 M sodium glycinate, 2×10^{-4} M EDTA (pH 8.7).

The fluorescence of epimerase in 0.06 M glycylglycine buffer (pH 8.0), 0.2 M in NaCl, did not vary significantly between temperatures of 10.5 and 27.5°, whereas the fluorescence of a quinine standard decreased by 50% over the same temperature interval.

The effect of guanidine hydrochloride upon the fluorescence of epimerase is illustrated in Figure 4. Figure 4A shows the time course of decrease in fluorescence over a range of guanidine·HCl concentrations. It should be noted that 6 M guanidine·HCl did not quench the fluorescence of free DPNH. The decrease in fluorescence was proportional to guanidine·HCl concentration, and was essentially complete in 6 M guanidine·HCl after 60 min. Figure 4B compares the relative enzymatic activity and fluorescence of epimerase 95 and 110 min after the addition of varying concentrations of guanidine·HCl. It can be seen that enzymatic activity was much more sensitive to quanidine·HCl than fluorescence, 1 M guanidine·HCl reducing fluorescence to 60% and activity to 7% of its initial value. A similar result has been obtained with urea, and also by simply incubating the enzyme at 4° in 0.1 M glycine buffer between pH 8.5 and 10.5, for several days. No recovery of either activity or fluorescence after treatment with guanidine or urea has been observed upon removal of the denaturant by either dilution or dialysis.

Crystallization. Attempts at crystallization of epimerase, both at low ionic strength (<0.001 M) and in $(NH_4)_2SO_4$ (~0.5 M), have yielded irregularly formed needles. Without exception, losses in total activity have

TABLE II: Amino Acid Composition[a] of UDP Galactose 4-Epimerase.

	Residues/10^5 g of Protein		
	24-hr Hydrolysis	48-hr Hydrolysis	Av or Extrapolated Value
Lysine	64.1	67.3	65.7
Histidine	17.2	18.5	17.8
NH_3	102	109	106
Arginine	26.3	26.8	26.6
Aspartic acid	116	115	116
Threonine	64.0	59.9	68.3
Serine	46.6	39.5	54.9
Glutamic acid	77.3	77.3	77.3
Proline	51.0	52.6	51.8
Glycine	69.6	70.7	70.1
Alanine	57.9	58.7	58.3
Valine	52.9	65.7	56.7
Methionine	8.8	9.3	9.0
Isoleucine	42.8	47.6	47.6
Leucine	79.0	80.3	79.6
Tyrosine	39.9	40.6	40.2
Phenylalanine	45.1	45.0	45.1
S-Carboxymethylcysteine	17.2	17.7	17.4
Tryptophan			3.4

[a] Amino acid analyses were performed on preparation d, Table I, as described in the Experimental Section.

occurred, and strands of denatured protein have appeared during the formation of these crystals which have proved to be insoluble in all buffers tested.

Amino Acid Analysis. Table II gives the amino acid composition of purified epimerase. The average or extrapolated values were used to calculate the partial specific volume of the protein, according to the method of Cohn and Edsall (1943). The value of 0.74 was obtained and used in all other calculations.

Molecular Weight. Figure 5 shows the schlieren pattern obtained with yeast epimerase at low ionic strength at a high centrifugal field. In the run illustrated in Figure 5, the solvent for the enzyme was 0.1 M sodium glycinate (pH 8.7) which is approximately 0.01 M with respect to sodium ions. The same buffer was used previously in studies of the sedimentation of epimerase in a sucrose density gradient (Darrow and Rodstrom, 1966). The rest of the experiments involving sedimentation reported in this paper were done in 0.01 M Tris-HCl, at pH 7.5, where the enzyme is more stable. As shown most clearly in picture B of Figure 5, taken after 112 min at 59,780 rpm, the boundary was not symmetrical. However, calculation of the apparent diffusion coefficient from the ratio of the area under, to the maximum height of the gradient curve (Schachman, 1957), gave a value

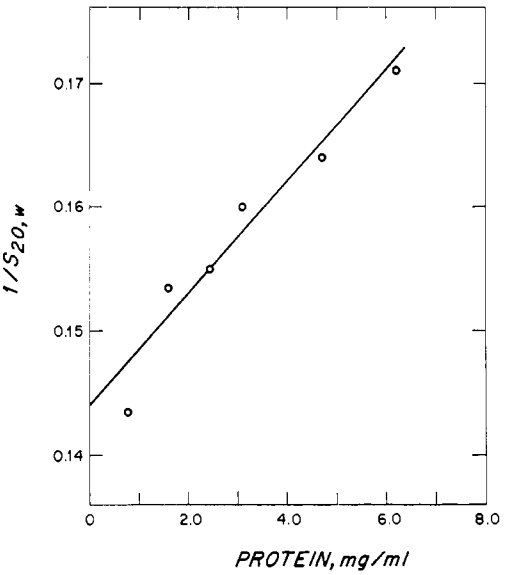

FIGURE 6: Dependence of sedimentation coefficient of yeast epimerase on protein concentration. Epimerase, preparation d, Table I; rotor speed 59,780 rpm; temperature 2°; solvent, 0.01 M Tris·HCl and 10^{-3} M EDTA (pH 7.5). The line was calculated from the experimental points by the method of least squares.

which was constant with time, indicating a low degree of artificial sharpening or heterogeneity. Consequently, it was felt of significance to make further efforts to determine the molecular weight in spite of the heterogeneity apparent under certain conditions, and in spite of the necessity of working at low ionic strength at a region of pH removed from the isoelectric point.

The dependence of the sedimentation coefficient upon protein concentration is shown in Figure 6. The line calculated from the data points by the method of least squares, and extrapolated to zero protein concentration, gives a value, plus and minus one standard error of estimate, of $s_{20,w} = 6.95 \pm 0.10$ S. The average value obtained in sucrose gradient centrifugation in low ionic strength was 6.49 S (Darrow and Rodstrom, 1966).

Figure 7 shows the concentration dependence of the apparent diffusion coefficient, obtained from the same photographic plates from which the sedimentation data plotted in Figure 6 were derived. The data in Figure 7 give a value of 3.0×10^{-7} cm^2 sec^{-1} for the apparent diffusion coefficient, extrapolated to zero protein concentration under the conditions of the experiment, and 5.1×10^{-7} cm^2 sec^{-1} for $D^0_{20,w}$.

In Figure 8 are plotted the values for apparent molecular weight obtained by low-speed equilibrium centrifugation of epimerase using interference optics. The data points represent eleven samples of enzyme at eight different protein concentrations. The concentration of protein for each sample was obtained in fringe numbers by the use of a synthetic boundary cell. The methods employed were essentially those used by Harrington and his coworkers in the determination of the molecular weight of myosin (Woods *et al.*, 1963). The data in Figure 8 were derived from plots of ln C *vs.* r^2 which were perfectly linear. However, the small number of fringes involved limits the value of this linearity as evidence of

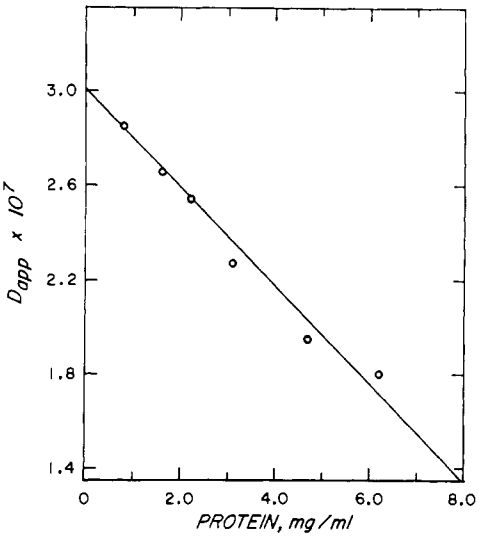

FIGURE 7: Dependence of diffusion coefficient (D_{app}) of yeast epimerase on protein concentration. Same experimental conditions as Figure 6. D_{app} was obtained from heights and areas of schlieren peaks during sedimentation and corrected to standard conditions as outlined by Schachman (1957).

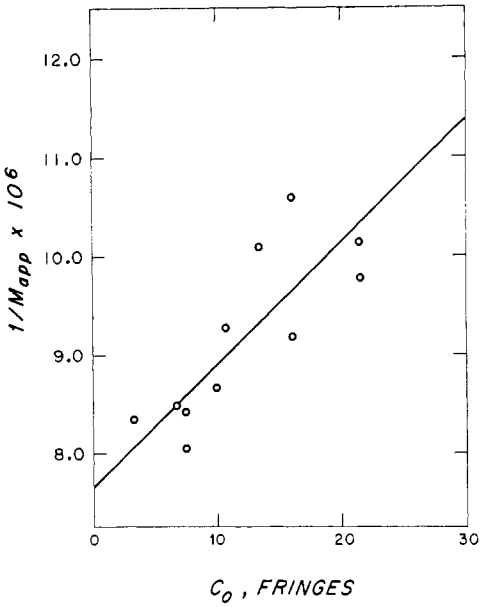

FIGURE 8: Dependence of the apparent weight-average molecular weight of yeast epimerase on protein concentration, low-speed equilibrium. Epimerase, preparation d, Table I; rotor speed 5563, 5784, or 6166 rpm, depending on protein concentration, which ranges between 1.0 and 6.2 mg/ml; temperature 2°; solvent 0.01 M Tris·HCl and 10^{-3} M EDTA (pH 7.5); duration of runs, 18 hr. The line was calculated from the data points by the method of least squares.

homogeneity. The line calculated from the data points in Figure 8 by the method of least squares, and extrapolated to zero protein concentration, gave a value of 125,000 ± 5600 for the weight-average molecular weight, plus and minus one standard error of estimate.

This value was confirmed by the meniscus depletion technique of Yphantis (1964). The data from a single run are given in Figure 9 in which the reciprocal of both weight- and number-average molecular weights was plotted at various concentrations of protein throughout the cell, at an equilibrium speed of 17,980 rpm. Values for fringe displacement less than 0.3 mm are subject to considerable error (Yphantis, 1964). Excluding these points, the weight average throughout the cell was between 122,000 and 126,500, with an extrapolated value of 125,000, and the number average between 117,000 and 124,000, with an extrapolated value of 121,000.

Data obtained by several independent methods of estimation of molecular weight are summarized in Table III. The value of 125,000 ± 5600, obtained by low-speed equilibrium centrifugation, is the best estimate of the molecular weight of UDP galactose 4-epimerase from yeast. It represents the largest body of experimental data and is consistent with the values obtained by the other methods listed in Table III, with the exception of chromatography on Sephadex G-200, which gave a value of 159,000.

TABLE III: Molecular Weight of Yeast UDP Galactose 4-Epimerase Obtained by Various Methods.

Method	Mol Wt
Low-speed equilibrium[a]	M_w 125,000 ± 5600
High-speed equilibrium[b]	M_w 124,000
	M_n 121,000
DPN content[c]	M_w 125,000
Sedimentation and diffusion[d]	M_n 121,000
Chromatography on Sephadex G-200[e]	M 159,000

[a] Data from Figure 8. [b] Data from Figure 9. [c] Based on the average of the five values listed in Table I. [d] Obtained from the data in Figure 6 and 7, using the Svedberg equation. [e] Estimated according to the method of Andrews (1964), using yeast alcohol dehydrogenase and hemoglobin as markers, in 0.1 M sodium glycinate (pH 8.7) containing 10^{-4} M EDTA, at 3°.

Discussion

UDP galactose 4-epimerase of a high degree of purity has been prepared from *Escherichia coli* 12 by Wilson and Hogness (1964). Like the yeast enzyme, the enzyme from *E. coli* was shown to contain 1 mole of DPN/mole of protein. The actual figure obtained for the molecular weight of the enzyme from *E. coli* was 79,000, and the catalytic efficiency can be calculated from the data of Wilson and Hogness (1964) as 30,250 moles of substrate transformed/mole of enzyme per min. The values for molecular weight and catalytic efficiency, respectively, for the yeast enzyme are 125,000 and 10,900. There has been no report of either association and dissociation of subunits or pyridine nucleotide-like fluorescence in the enzyme from *E. coli* although both phenomena are prominent features of the enzyme from yeast (Darrow and Rodstrom, 1966; Bertland *et al.*, 1966).

Experiments of Kalckar and his coworkers have shown that the amount of fluorescence of yeast epimerase is dependent upon the degree of reduction of the bound pyridine nucleotide (Creveling *et al.*, 1965). The most probable explanation for the displacement of the curve for fluorescence behind that for absorption at 280 mμ in Figure 3 is a partial resolution of enzyme bearing DPN from that bearing DPNH. Enzyme molecules bearing DPNH would have one less positive charge than those bearing the oxidized coenzyme, and would thus be more tightly bound to an anion exchanger such as DEAE-cellulose. The same explanation would apply to the displacement of fluorescence ahead of the general protein stain during migration to the anode in starch gel electrophoresis, described earlier in the text.

The high quantum yield of epimerase fluorescence compared with that of free DPNH (Bhaduri *et al.*, 1965) would suggest that the enzyme-bound DPNH is highly restricted by the surrounding protein in the ways in which it can lose energy after once being excited by light. The insensitivity reported here of the fluorescence to temperature changes, within a range in which the enzyme is stable, is consistent with this view, as is the marked effect of guanidine hydrochloride on the fluorescence seen in Figure 4.

More information is needed than is now available to provide a satisfactory explanation for the variations in specific catalytic activity observed in Table I and the instability of the fractions with the highest specific activity. Work presently in progress (A. Bertland and H. M. Kalckar, unpublished data) suggests that specific activities equal to the highest shown on Table I (85 units/mg) can be obtained merely by incubating dilute solutions of the purified enzyme at 4° for several days. Until a method is obtained to stabilize catalytic activity in a reproducible way, other tests such as analysis for bound DPN, SH titration, and polyacrylamide gel electrophoresis must be used in assessing the purity of a given preparation.

Creveling *et al.* (1965) have reported that the percentage of DPNH may be as high as 25% of the total enzyme-bound pyridine nucleotide in some preparations of yeast epimerase. Since the fluorescence of free DPNH in alkali is only one-quarter of that of DPN (Lowry *et al.*, 1957), differences in the percentage of DPNH could have led to the variation of ±11% among the five preparations listed in Table I. Thus, the metnod of Lowry *et al.* (1957) as employed by us may give a somewhat inaccurate estimation of total enzyme-bound pyridine nucleotide, although it is useful as a convenient method of standardizing preparations of enzyme.

The multiple banding of yeast epimerase seen after polyacrylamide gel electrophoresis occurred because of the formation of the components during the process of electrophoresis itself, since reelectrophoresis of individual bands gave a spectrum of bands rather than a single band. Pertinent to this observation may be the recent report by Fantes and Turminger (1967) that ammonium persulfate, which was used as a catalyst for gel formation in our experiments, caused alteration in the electrophoretic properties of a number of proteins. These authors recommend photopolymerization of polyacrylamide gel.

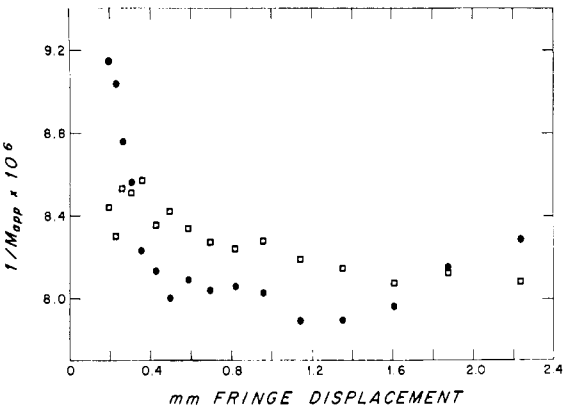

FIGURE 9: Dependence of the apparent molecular weight of yeast epimerase on concentration, high-speed equilibrium. Epimerase preparation d, Table I: protein concentration, 0.9 mg/ml; temperature 4°; solvent, 0.01 M Tris·HCl and 10^{-3} M EDTA (pH 7.5); duration of run, 18 hr. Weight-average molecular weight (M_w), calculated at each concentration from the least-squares slope of the five nearest points (●); number-average molecular weight (M_n) (□), obtained as described by Yphantis (1964).

The conditions of low ionic strength and neutral pH at which the present sedimentation studies were performed gave the best approximation to a single protein species of any of the conditions tested, and yielded an estimate of molecular weight which is adequate for many purposes. In a previous report (Darrow and Rodstrom, 1966), UDP galactose 4-epimerase from yeast was shown to exhibit sedimentation coefficients in a sucrose density gradient of either 4.3, 6.5, or 10.8 S, depending upon the concentration of cation in the solvent and the titration of the SH groups on the protein with PMB. The ability to interconvert these differently sedimenting species, together with confirming evidence from gel filtration, was taken as evidence for a binary subunit structure of the enzyme. Such a subunit structure might explain the apparent heterogeneity of yeast epimerase which can be seen in the schlieren patterns in Figure 5 and which is indicated by the difference between the weight-average molecular weight of 125,000 and the number-average molecular weight of 121,000 listed in Table III. For example, these data are compatible with the existence of an equilibrium composed of 90% of a species with a molecular weight of 130,000 and 10% of a species with a molecular weight of 65,000, whose rate of interconversion is slow with respect to the rate of transport during sedimentation. It may be that PMB, which converts epimerase from a species sedimenting at 6.5 S to a species sedimenting at 4.3 S (Darrow and Rodstrom 1966), acts by stabilizing the monomer in such an equilibrium.

The figure of 159,000 for molecular weight obtained by gel filtration (Table III) is anomalous, and suggests a departure from spherical shape. Calculation of the frictional ratio, f/f_0, according to Edsall (1953), from $D_{20,w}$ obtained from Figure 6 and M_w obtained from Figure 7, gave a value of 1.26. This value is typical of most globular proteins. An inaccurate value for molec-

ular weight might be obtained by gel filtration if yeast epimerase should contain significant amounts of carbohydrate, since glycoproteins are disproportionately excluded from dextran gels (Whitaker, 1963; Kawasaki *et al.*, 1966). It is of interest in this regard that Bertland *et al.* (1966) have reported the incorporation of [^{14}C]-galactose into yeast epimerase and its conversion into an unknown product which is still bound to protein after digestion with Pronase.

Acknowledgments

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