The fact that the methyl groups incorporated into protein become volatile on mild hydrolysis gives an important clue to the probability that the linkage between the methyl and carboxyl groups is an ester bond (Liss *et al.*, 1969; Kim and Paik, 1970). Therefore, modification of free carboxyl groups by introduction of glycine methyl ester through the reaction with a water-soluble carbodiimide should be useful for investigating the possible participation of carboxyl groups as methylation sites, since this modification of ribonuclease exclusively involves free carboxyl groups (Hoare and Koshland, 1967). It has been clearly demonstrated that methyl acceptor capability of ribonuclease decreased to about 15% of the control when modified by glycine methyl ester formation (Table I). Furthermore, when the methyl group in the RNase–glycine methyl ester was removed and the carboxyl groups of RNase–glycine became free, RNase recovered full activity as substrate. These results together with the results in Table III strongly suggest that the internal carboxyl groups in ribonuclease are the sites of methylation. However, it is still possible that the C-terminal carboxyl groups of lysine, arginine, and aromatic amino acids which are available by the action of trypsin and chymotrypsin are the only C-terminal carboxyl groups which do not accept the methyl group.

Oxidation of proteins with performic acid affects only two amino acids: cystine is changed to cysteic acid and methionine to the sulfone (Hirs, 1956). Since substrate activities of the reduced or oxidized ribonuclease toward protein methylase II are about equal within experimental error (Figure 1), it can be said that cystine and methionine residues of RNase are not involved in the methyl transferase reaction.

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Ionic Strength Dependent Dissociation and Association of Yeast Uridine Diphosphate Galactose 4-Epimerase Subunits*

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ABSTRACT: It has been determined by equilibrium ultracentrifugation and gel filtration chromatography in 6 M guanidine hydrochloride that the subunits of yeast uridine diphosphate galactose 4-epimerase are of 60,000 molecular weight. Dissociation of the native enzyme without loss of cofactor occurred in buffers of very low ionic strength at neutral pH. In 0.001 M Tris-HCl (pH 7.4) the enzyme sedimented as an equal mixture of 3S and 4S components; sedimentation equilibrium centrifugation indicated only a single component with 60,000 molecular weight. The apoenzyme sedimented as a single 3.7S protein. When the Tris concentration was increased to 0.1 M, the subunits reassociated to dimers but not to tetramers. Gel filtration chromatography in 0.001 M Tris-HCl buffer (pH 7.4) yielded partial fractionation into components differing in specific activity and fluorescence. The reactivation and reassociation to dimers and tetramers of the apoenzyme subunits were found to be dependent on the presence of pyridine nucleotide.

Uridine diphosphate galactose 4-epimerase (EC 5.1.3.2.) from yeast catalyzes the reversible epimerization of UDP-galactose[1] to UDP-glucose. The enzyme contains 1 mole of tightly bound pyridine nucleotide cofactor per 120,000 grams of protein (Darrow and Rodstrom, 1968). The apo enzyme is formed by removal of the cofactor by *p*-chloromercuribenzoate titration which results in total loss of catalytic activity. The addition of NAD partially restores activity to the enzyme (Maxwell *et al.*, 1958); however, the cofactor removed from the native enzyme is more effective in restoring catalytic activity than is NAD.

Darrow and Rodstrom (1966) have demonstrated that the enzyme can assume three distinct states of aggregation. In buffers of low cation concentration such as 0.01 M Tris-HCl (pH 7.5) the enzyme has a molecular weight of 125,000 (Darrow and Rodstrom, 1968) and a sedimentation coefficient of 6.5 S. In buffers of higher cation concentration, such as 0.1 M Tris-HCl (pH 7.5), the sedimentation coefficient increases

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[1] Abbreviations used are: UDP-galactose 4-epimerase, UDP-glucose 4-epimerase (EC 5.1.3.2); UDP-galactose, uridine 5′-(α-D-galactopyranosyl pyrophosphate); 5′-UMP, uridine 5′-monophosphate; ME, 2-mercaptoethanol; PMB *p*-chloromercuribenzoic acid; DTT, dithiothreitol; Gdn·HCl, guanidine hydrochloride.

to 10.9 S and the molecular weight to 250,000 (Darrow and Rodstrom, 1970). This aggregation is accompanied by activation of the epimerization reaction (Darrow and Creveling, 1964; Darrow and Rodstrom, 1966, 1970).

Reduction of the enzyme-bound NAD to NADH does not alter the molecular weight although total inactivation of the enzyme and extensive changes in the polypeptide configuration occur (Bertland and Kalckar, 1968; Bertland *et al.*, 1966). Removal of the coenzyme by PMB titration causes dissociation of the enzyme into subunits of 60,000 molecular weight and a sedimentation coefficient of 4 S (Darrow and Rodstrom, 1966, 1970).

In addition to the dissociation of the enzyme by removal of the cofactor, we have found that dissociation also occurs in buffers of very low ionic strength without loss of cofactor and in guanidine hydrochloride and urea. This paper presents an investigation of the dissociation of galactose 4-epimerase and of the conditions necessary for the reassociation of the subunits after dissociation by either *p*-chloromercuribenzoate or low ionic strength.

Experimental Section

Materials

UDP-glucose dehydrogenase, NAD, NADH, and 5′-UMP were purchased from Sigma Chemical Co. L-Arabinose, UDP-galactose, and dithiothreitol were from Calibiochem. Bio-Gel A-5M, 50–100 mesh, was from Bio-Rad Laboratories. Gdn·HCl, urea, and *p*-chloromercuribenzoate were from Mann Research Laboratories. The Gdn·HCl and urea were the Ultra Pure grade and were used without recrystallization. All other reagents were obtained from general commercial sources.

Methods

UDP-galactose 4-epimerase was prepared from *Candida pseudotropicalis* (*Saccharomyces fragilis*), American Type Culture Collection No. 10022, as described previously (Darrow and Rodstrom, 1968). Large-scale preparations from 150-gal. fermentations have been made at the New England Enzyme Center. Epimerase activity was determined from the rate of increase in optical density of 340 mμ, after the addition of UDP-galactose to a solution containing epimerase and an excess of DPN and UDP-glucose dehydrogenase. The exact conditions have been reported by Darrow and Rodstrom (1968). Enzyme units are reported as international units, 1 unit transforming 1 μmole of substrate/min. Protein was determined by absorption at 280 and 260 mμ, or by the biuret reaction.

Preparation of the Apoepimerase. The holo (NAD) or reduced (NADH) enzyme is treated in one step for 30 min at 25°, with 24–25 equiv of PMB/mole of enzyme, in the absence of EDTA or mercaptoethanol. The cofactor is immediately removed by chromatography on a Sephadex G-50 column (1 × 16 cm), at 4 or 25°, equilibrated with the desired concentration of Tris-HCl buffer (pH 7.5) containing 10^{-3} M mercaptoethanol.

The apoepimerase is essentially 100% resolved from the cofactor as measured by enzymatic activity and NAD or NADH content measured by the alkali fluorescence method (Lowry *et al.*, 1957).

The apoenzyme can be reactivated with NAD or NADH at 10- to 50-fold excess equiv/mole of enzyme in the presence of 0.1 M 2-mercaptoethanol. This addition must be done rapidly after the removal of the cofactor or denaturation of the apoenzyme occurs.

Reduction. Reduction of native UDP-galactose 4-epimerase was performed in 0.1 M Tris-HCl buffer (pH 7.5). For the development of the reduction, the optimum concentration of L-arabinose is 10^{-2} M and the 5′-UMP concentration is between 10^{-3} and 10^{-4} M. The reaction mixture is incubated at 27° for a minimum of 4 hr or overnight at 4° (Bertland *et al.*, 1971).

Fluorescence. Emission and excitation spectra were measured with a Turner Model 210 Spectro absolute spectrofluorometer (Bertland, 1970). The instrument and methods of correction are described by Turner (1964). All measurements were performed at 25°.

Starch gel electrophoresis was performed by the method of Fine and Costello (1963). The gels were stained for protein with 1% Amido Black.

Ultracentrifugation. A Spinco Model E analytical ultracentrifuge equipped with a Rayleigh interference optical system was used for all sedimentation equilibrium experiments. Unless otherwise indicated, all experiments were conducted at 4° and with a protein concentration of 0.5 mg/ml. A 12-mm double-sector filled epon cell with sapphire windows was used. The sample compartment contained 0.11 ml of the enzyme solution; the reference compartment contained 0.112 ml of reference buffer. All solutions were brought to a concentration of 1% sucrose when the buffer conductivity was lower than 500 μmhos to minimize convective effects.

All sedimentation-equilibrium experiments were conducted by the method of Yphantis (1964). The speed was chosen so that $\sigma = \omega^2 M(1 - \bar{v}\rho)/RT = 5$ cm^{-2}. All runs were carried out for a period of 18 hr except for those in urea and Gdn·HCl which were for 24 hr. These time periods were found to be adequate for the attainment of equilibrium.

The photographic plates were measured with a Nikkon microcomparator. The vertical displacement of three black fringes was measured at 100-μ intervals. Centrifugation of buffer alone indicated a negligible contribution to the vertical displacement from window distortion. Values for the vertical displacement, j, were obtained by subtraction of the measured j displacement from the j_0 value determined from the best straight line drawn for the initial readings of j displacements in the meniscus region of the cell. The molecular weight was determined from the slope of the best straight line or lines of a plot of $\ln j$ *vs.* x, where x was the horizontal distance along the fringe. The value of $\sigma_{w(r)}$ was determined from these slopes by the relation $\sigma_{w(r)} = \mathrm{d} \ln j/(\mathrm{d}r^2/2)$, where $r = 5.7 + x/m$, where m is the magnification factor. The weight-average molecular weight was determined from the relation: $(M_{w(r)} = \sigma_{w(r)} \cdot RT/\omega^2(1 - \bar{v}\rho)$. The partial specific volume, $\bar{v}$, was taken to be 0.74 ml/g as determined by Darrow and Rodstrom (1968).

Sedimentation-velocity measurements were conducted with a Spinco Model E ultracentrifuge equipped with a schlieren optical system. Unless otherwise noted, all measurements were at 4° and at a speed of 50,740 rpm. The sedimentation coefficients were determined from the slope of the best straight line of the measured displacements of the protein peak. The relative viscosities and densities of the buffer were assumed to be the same as water, and the sedimentation coefficient was corrected to a temperature of 20°. The values used for the viscosity and density of urea and guanidine solutions were those of Kawahara and Tanford (1966).

Equilibration of the enzyme with the buffers for the ultracentrifuge experiments was carried out on Sephadex G-50 columns or dialysis. The column dimensions were 1 × 16 cm. Enzyme solution (1 ml) was applied and 1-ml fractions were collected.

TABLE I: Sedimentation Properties of Yeast UDP-galactose 4-Epimerase in 0.1 M Tris-HCl Buffer (pH 7.4)–1.4×10^{-2} M ME–1×10^{-3} M EDTA at 4°.

	$s_{20,w}$ (S)[a]	Protein Concn (mg/ml)	Sp Act. (U/mg)
1. NAD-epimerase	10.63 (6.3)	4	68
2. NAD-epimerase[b]	10.4 (6.2)	4	68
3. Apoepimerase with bound PMB[b]	4 + 5.8		
4. Apoepimerase after PMB removal	6.6 (4)	4	
5. Apoepimerase + 40 moles/mole of NAD	5.8	3.32	6.8
6. Apoepimerase + 1 mole/mole of cofactor	6.7	1.8	6.8

[a] Figures in parentheses refer to a minor component constituting less than 20% of the total protein. [b] This sample did not contain EDTA and mercaptoethanol.

TABLE II: Sedimentation Properties of Yeast UDP-galactose 4-Epimerase in 0.01 M Tris-HCl Buffer (pH 7.4) at 4°.

	$s_{20,w}$ (S)	Protein Concn (mg/ml)	Sp Act. (U/mg)
1. NAD-epimerase	6.3	4	59.5
2. Apoepimerase with bound PMB	4.3	4	
3. Apoepimerase after PMB removal	4.4	3	
4. Apoepimerase in 0.1 M Tris-HCl (pH 7.4)	4.7	2.73	
5. Apoepimerase + 1 mole/mole of native cofactor	6.7	3.35	35.7
6. Apoepimerase + 40 moles/mole of NADH	6.1	2.75	28.9
7. Apoepimerase + 20 moles/mole of NAD	6.5	2.75	33.2
8. NADH-reconstituted epimerase in 0.1 M Tris-HCl buffer (pH 7.4)	8.5 + 11.1	2.5	
9. Cofactor-reconstituted epimerase in 0.1 M Tris-HCl buffer (pH 7.4)	6.1 + 11.4	3.05	28.9

Solutions of 6 M Gdn·HCl were prepared by adding 8 M Gdn·HCl in the appropriate buffer to the protein solution in the same buffer; solutions in 8 M urea were prepared by the addition of a 10 M urea solution to the protein solution. All centrifugations in Gdn·HCl and urea were at 20°.

Results

Reassociation of the Apoenzyme Subunits. These experiments were conducted to determine if the apoenzyme prepared by PMB titration could be reactivated and reassociated to tetramers in the presence of pyridine nucleotide and high ionic strength. When the dissociation was carried out in 0.1 M Tris-HCl buffer (pH 7.4), an inactive dimer was formed which did not reassociate to tetramers (Table I). When the dissociation was carried out in 0.01 M Tris-HCl (pH 7.4) (Table II), dimer formation and partial reactivation occurred after the addition of pyridine nucleotide, and in 0.1 M Tris-HCl, approximately half of the reassociated enzyme was in the tetrameric form.

Molecular Weight in Very Low Ionic Strength Buffers. The ionic strength dependent dissociation of the epimerase led us to investigate the sedimentation properties of the enzyme in buffers of very low ionic strength. The NAD epimerase in 0.001 M Tris-HCl (pH 7.35) (conductivity of 50 μmhos) sedimented as two components in apparently equal amounts (Figure 1). The slower sedimented with $s_{20,w}$ values ranging from 2.8 to 3.6 S; the faster sedimented with $s_{20,w}$ values of 4.0–4.7 S (Table III). High-speed sedimentation-equilibrium ultracentrifugation yielded a linear plot of ln j *vs.* x (Figure 2). The average molecular weight determined by this method was 56,600.

The pyridine nucleotide cofactor remained bound to the enzyme since the epimerase retained 60–95% of its initial activity after equilibration with the 0.001 M Tris-HCl, and no free NAD was detected spectrophotometrically in the effluent of the columns used for the equilibration. Reduction of the bound NAD to NADH did not alter the sedimentation properties. The apoenzyme, containing bound PMB, sedimented as a single component with an $s_{20,w}$ value of 3.7 S and a molecular weight of 61,000.

Reassociation of the Low Ionic Strength Subunits. These experiments were conducted to determine whether the sub-

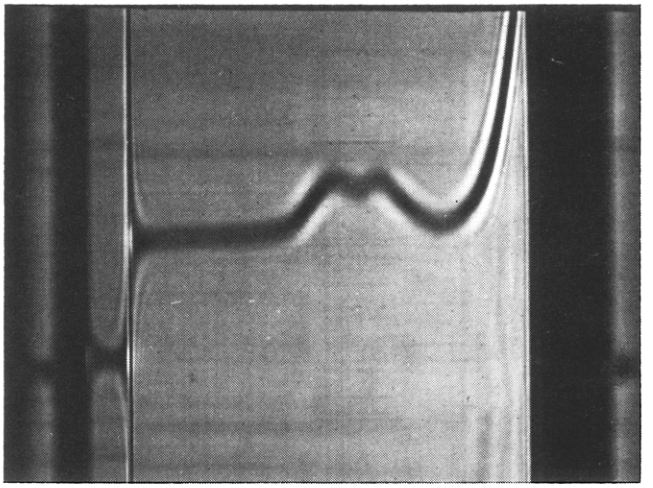

FIGURE 1: Ultracentrifugation of UDP-Gal-4-epimerase in 0.003 M Tris-HCl buffer (pH 8.1). The buffer conductivity was 28 μmhos. The protein concentration was 4.6 mg/ml. The photograph was taken 277 min after the rotor reached the final speed of 50,740 rpm. The direction of sedimentation is from left to right. Temperature 4°.

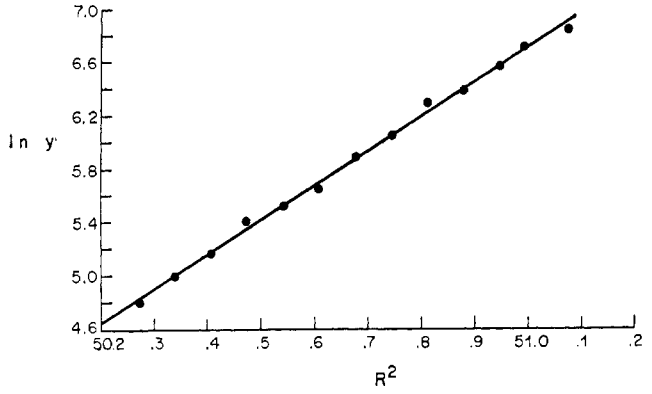

FIGURE 2: High-speed sedimentation equilibrium data for UDP-Gal-4-epimerase (0.5 mg/ml) in 0.001 M Tris-HCl buffer (pH 7.5) (conductivity of 50 μmhos) at 25,980 rpm and 4°.

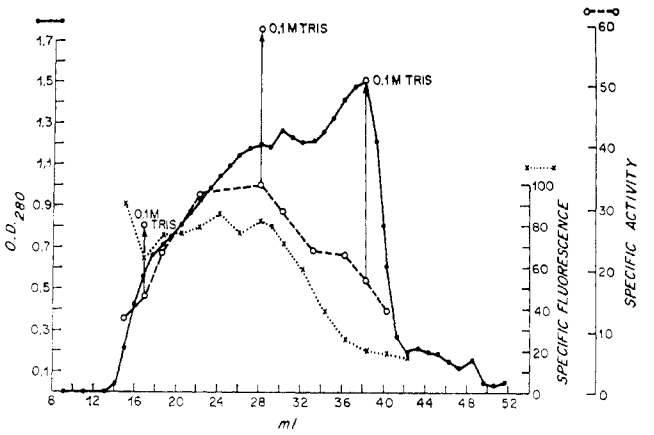

FIGURE 3: Sephadex G-150 chromatography of UDP-Gal-4-epimerase in 0.001 M Tris-HCl buffer (pH 8.08)–0.002 M DTT. Enzyme (35.7 mg) was applied to a column of 1 × 57 cm. (—) Optical density at 280 mμ. (---) Specific activity. (. . . . .) Specific fluorescence. The arrows indicate enhancement of activity when the Tris concentration in the enzyme solution was increased to 0.1 M and reassayed.

units in 0.001 M Tris-HCl buffer (pH 8.08) (conductivity o 16 μmhos) would reassociate to produce dimers and tetramers when the concentration of Tris in the solution was increased. The Tris concentration was raised by the addition of either 1 or 2 M Tris-HCl buffer (pH 8.4); all steps were carried out at 4°. When the initial protein concentration was 8 mg/ml, dimer formation occurred, but no tetramer was observed (Table IV) by sedimentation velocity centrifugation. The addition of a tenfold excess of NAD to the epimerase in 0.001 M Tris-HCl resulted in the disappearance of the slower peak which could reflect either a change in the conductivity of the solution or a specific effect of NAD on this form of the enzyme.

In contrast to the experiments at high protein concentrations, when the enzyme was equilibrated with the 0.001 M Tris-HCl at a protein concentration of 0.5 mg/ml, the specific activity fell to 5% of its initial amount (Table IVB). The reason for this decrease is not known. The specific activity did increase fourfold when the Tris concentration was raised to 0.1 M, although no form of the enzyme larger than the dimer was observed.

Chromatography on G-150 at Low Ionic Strength. Chromatography on Sephadex G-150 was used as a method for the separation of the 3S and 4S subunits observed in the analytical ultracentrifuge. To a column of Sephadex G-150 (1 × 57 cm) was applied 35.7 mg of epimerase (specific activity 39.1) which had been equilibrated by dialysis with 0.001 M Tris-HCl buffer (pH 8.08)–0.002 M DTT (conductivity of 19 μmhos). The elution pattern is illustrated in Figure 3.

TABLE III: Sedimentation Properties of Yeast UDP-galactose 4-Epimerase in 0.001 M Tris-HCl Buffer (pH 7.4) (Conductivity of 50 μmhos) at 4°.

	Protein Concn (mg/ml)	$s_{20,w}$ (S)	Equil Mol Wt (0.5 mg/ml)
1. NAD-epimerase	5.3	2.8 + 4.3	52,700
NAD-epimerase	6.7	2.9 + 4.2	60,500
NAD-epimerase + 5×10^{-4} M DTT	5.1	3.4 + 4.4	
NAD-epimerase + 2×10^{-3} M DTT	8.0	3.6 + 4.7	
2. Apoepimerase with bound PMB	4.0	3.7	61,000
3. Apoepimerase after PMB removal			54,800
4. NADH-epimerase + 1×10^{-3} M ME		3.2 + 4.5	53,300

TABLE IV: Reassociation of Low Ionic Strength Subunits.

Buffer	Sp Act. (U/mg)	$s_{20,w}$ (S)	Protein Concn (mg/ml)
A. Reassociation at High Protein Concentrations			
0.001 M Tris-HCl (pH 8.08)–2×10^{-3} M DTT	39.1	3.6 + 4.7	8.00
0.001 M Tris-HCl–2×10^{-3} M DTT–6.66×10^{-4} M NAD	39.1	4.95	7.64
0.1 M Tris-HCl–2×10^{-3} M DTT–6.66×10^{-4} M NAD	26.8	6.02	7.27
0.01 M Tris-HCl–2×10^{-3} M DTT	43	5.66	7.92
0.1 M Tris-HCl–2×10^{-3} M DTT	34	5.85	7.52
0.1 M Tris-HCl–2×10^{-3} M DTT–1×10^{-3} M EDTA	30.8	5.39	7.3
		Mol Wt (Wt Av)	Speed (rpm)
B. Reassociation at Low Protein Concentration(0.5 mg/ml)			
0.001 M Tris-HCl (pH 8.05)	4.93	57,300	25,900
0.001 M Tris-HCl (pH 8.05)–2×10^{-3} M DTT	2.72	61,500	25,900
0.01 M Tris-HCl	8.00	76,400	17,980
0.01 M Tris-HCl–2×10^{-3} M DTT	4.93	86,700	17,980
0.1 M Tris-HCl	16.3	118,000	17,980
0.1 M Tris-HCl–2×10^{-3} M DTT	10.7	115,000	17,980

Although two distinct peaks were not obtained, the elution pattern was quite broad, and there was fractionation of the protein based on measurements of specific activity and specific fluorescence. Equilibrium centrifugation of the front, middle, and rear fractions showed a molecular weight of 60,000. The starch gel patterns of all fractions were identical. The specific activities of the front (15.7 U/mg) and back (18.4 U/mg) were lower than that of the middle fractions (34 U/mg). The specific activities of all fractions could be increased by increasing the Tris concentration in the enzyme solution from 0.001 to 0.1 M. The enhancement of the front fraction was twofold to 27.2 U/mg while the middle fractions increased 1.4-fold to 59.5 U/mg. The specific activity of the back fractions increased threefold to 51.

The 450-mμ fluorescence of the pyridine nucleotide cofactor of the epimerase was measured by excitation at 350 mμ. The specific fluorescence of the epimerase in the front and middle fractions was significantly higher than that of the back fractions. Enhancement of both the front and back fractions was brought about by increasing the Tris concentration to 0.1 M and reducing the bound NAD to NADH by the addition of UMP and L-arabinose (see Methods). This produced an increase of 15-fold in the front fractions to a final specific fluorescence of 990 while the fluorescence of the back fractions increased 33-fold to a specific fluorescence of 500.

Removal of the Cofactor in Low Ionic Strength Buffers. The cofactor could easily be removed from the epimerase by heating in low ionic strength buffer. The enzyme was equilibrated by gel filtration or dialysis with 0.001 M Tris-HCl buffer. The enzyme was heated to 60° for 3 min, cooled in ice, and passed through a Sephadex G-50 column (1 × 16 cm) equilibrated with 0.001 M Tris-HCl buffer, pH 7.5 at 25°. The apoenzyme prepared by heating was essentially 100% resolved as measured by enzymatic activity or by measurement of NAD and NADH by the alkaline fluorescence method (Lowry *et al.*, 1957). No cofactor was removed by heating in buffers of higher ionic strength.

Dissociation of the Epimerase in Guanidine Hydrochloride and Urea. In order to determine whether the 60,000 molecular weight subunit was the smallest subunit, the molecular weight of the epimerase was determined after exposure to the denaturing agents, 6 M guanidine hydrochloride and 8 M urea. In 6 M Gdn·HCl–0.1 M Tris-HCl buffer (pH 7.4)–10^{-3} M EDTA–1.4 × 10^{-2} M ME, the epimerase had an $s_{20,w}$ value of 2.06 S (4.4 mg/ml) and a diffusion coefficient of 2.68 cm^2/sec (4.4 mg/ml) which yields a value of 72,300 for the molecular weight. The molecular weights in 6 M guanidine hydrochloride and 8 M urea were also determined by high-speed sedimentation equilibrium. This method, in 6 M guanidine hydrochloride and at a speed of 35,600 rpm, yielded molecular weights ranging from 63,000 to 79,000 (Table V) when the value of 0.74 cm^3/g, the partial specific volume calculated from the amino acid composition (Darrow and Rodstrom, 1968), was used in the calculations. It has been found, however, that the value of the effective specific volume in 6 M Gdn·HCl is on the order of 0.01 cm^3/g lower than the partial specific volume for several proteins (Tanford *et al.*, 1967). When the value of 0.73 cm^3/g was used for the evaluation of the epimerase subunit molecular weight, the range of values dropped to 59,300–73,700 (Table V), which would be more consistent with the expected value of 60,000. The plots of ln j *vs.* x were linear, suggesting that the subunits of the enzyme are of equal or nearly equal molecular weight. The molecular weight of the enzyme in 6 M Gdn·HCl was independent of Tris concentration, cofactor binding, mercaptoethanol concentration, and duration of the exposure to 6 M Gdn·HCl. Two determinations of the molecular weight in 8 M urea–0.01 M ME gave an average value of 66,900 for the apoepimerase.

TABLE V: Sedimentation Equilibrium Molecular Weight of Yeast UDP-galactose 4-Epimerase in 6 M Guanidine Hydrochloride at 20° and 35,600 rpm.

Buffer	Co-enzyme	Time in 6 M Gdn·HCl	Mol Wt $\bar{v}$ = 0.74	Mol Wt ϕ = 0.73
0.1 M Tris-HCl (pH 7.4)–1 × 10^{-3} M EDTA–0.014 M ME	Holo	1 hr	75,800[a]	70,100
0.1 M Tris-HCl (pH 7.4)–1 × 10^{-3} M EDTA–0.014 M ME	Holo	1 hr	68,700	64,500
0.001 M Tris-HCl–0.001 M ME	Apo	48 hr	66,000	61,000
0.01 M Tris-HCl (pH 7.4)–1 × 10^{-4} M EDTA–0.001 M ME	Apo	1 hr	78,500	73,700
0.01 M Tris-HCl (pH 7.4)–1 × 10^{-4} M EDTA–0.001 M ME	Apo	5 days	72,500	68,700
0.01 M Tris-HCl (pH 7.4)–1 × 10^{-4} M EDTA–0.1 M ME	Apo	1 hr	63,200	59,300
0.01 M Tris-HCl (pH 7.4)–1 × 10^{-4} M EDTA–0.1 M ME	Apo	48 hr	76,800	72,200
0.01 M Tris-HCl (pH 7.4)–1 × 10^{-4} M EDTA–0.1 M ME	Apo	22 days	76,300	71,800

[a] The speed was 25,900 instead of 35,600.

The epimerase was chromatographed on Bio-Gel A-5M in 6 M Gdn·HCl–0.01 M Tris-HCl buffer (pH 7.5)–10^{-4} M EDTA–0.1 M ME according to the method of Fish *et al.* (1969). The column dimensions were 1 × 53 cm. The standards used were chymotrypsinogen (mol wt 25,700), aldolase (mol wt 40,000), and bovine serum albumin (mol wt 69,000). Blue Dextran (Pharmacia) was used to determine the void volume, and [^{14}C]UMP was used to determine the total volume. Protein was determined by measurement of turbidity at 450 mμ after precipitation with 4% trichloroacetic acid.

By this method, the molecular weight of the epimerase monomer was determined to be 59,130 (Figure 4). No heterogeneity was apparent in the elution pattern of the epimerase.

Discussion

When the ions are removed from the epimerase by equilibration with the very dilute buffers such as 0.001 M Tris-HCl, a decrease in the sedimentation coefficient is observed. This

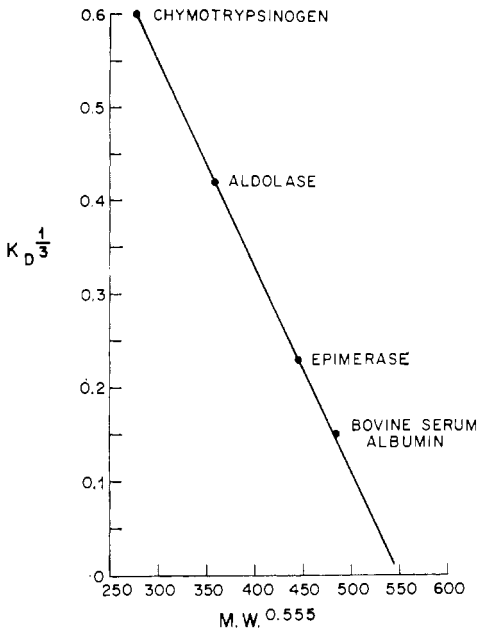

FIGURE 4: Chromatography of yeast UDP-Gal-4-epimerase on Bio-Gel A-5M in 6 M guanidine hydrochloride–0.01 M Tris-HCl buffer (pH 7.5)–10^{-4} M EDTA–0.1 M ME. The column dimensions were 1 × 53 cm.

decrease could result from dissociation, conformational change, or from charge effects due to sedimentation of the macro molecule at low concentrations of counterions (Pedersen, 1958). In the latter case, a strong negative dependence of s value on concentration would be expected which was not observed in the present situation. A small decrease in the sedimentation coefficient of the 60,000 molecular weight apoenzyme, from 4.4 in 0.01 M Tris-HCl buffer (pH 7.4) to 3.7 in 0.001 M Tris-HCl buffer (pH 7.4), was observed. This change is not of the magnitude of the decrease of the sedimentation coefficient of the native enzyme, and is of the same order as the s values observed for the native enzyme in 0.001 M Tris-HCl (pH 7.4). High-speed sedimentation-equilibrium ultracentrifugation consistently gave molecular weights of 55,000–65,000 for the native enzyme in the very dilute buffer; the apoenzyme also gave the expected value of 60,000 in this buffer, indicating that the molecular weights obtained by the equilibrium method are not affected by charge. The decrease in the molecular weight measured by sedimentation equilibrium coupled with the decrease in the sedimentation coefficient are interpreted, therefore, to represent true dissociation rather than conformational changes resulting from the removal of the counterions.

Sedimentation-equilibrium centrifugation in both 0.001 M Tris buffer and in guanidine hydrochloride indicated that the subunits of the enzyme were the same size. However, both sedimentation-velocity centrifugation and gel filtration chromatography in 0.001 M Tris buffer separated the native enzyme into two components. We attribute the presence of these two components to two conformations of identical subunits. Since the apoenzyme sedimented as a single protein and since the enzyme has 1 mole of cofactor/2 subunits, it was possible to speculate that the two components were due to cofactor-free and cofactor-bound subunits. If it can be assumed that the elution pattern of the column correlates with the sedimentation pattern, then the difference between the two fractions is not one of cofactor binding.

A previous study (Darrow and Rodstrom, 1966) found that the apoenzyme subunits prepared in low ionic strength buffers did not require NAD for reassociation to dimers although it did reactivate the dimers; and that the reactivated dimers would not reassociate to form tetramers at high ionic strengths. We have, on the contrary, found that apoenzyme subunits prepared in 0.01 M Tris require the addition of a pyridine nucleotide cofactor for dimer formation, and in the absence of this cofactor, will remain as a 4S subunit even in 0.1 M Tris. Once the cofactor is bound, reactivation occurs and partial aggregation to tetramers occurs at high ionic strength. Darrow and Rodstrom carried out their experiments in glycine and spermine, and it is possible that the disparity in the results is due to a different type of interaction of these compounds with the epimerase.

The dissociation of the epimerase with decreasing ionic strength may be attributed to electrostatic repulsions resulting from exposure of charged groups when their masking counterions are removed. It may be speculated that a certain conformation of the subunits is necessary for total reaggregation to the tetramer, and that this conformation is maintained in a cooperative manner by both the cofactor and counterions. In 0.1 M Tris, the apoenzyme subunits immediately form an inactive dimer which can neither be reactivated nor reassociated to tetramers. The high Tris concentration apparently overcomes any electrostatic repulsion between the subunits which form a dimer with an active site which is inaccessible to NAD. In very dilute buffers, unfolding of the subunits may occur in such manner that the conformation required for reassociation to tetramers is lost even though NAD is still bound. In 0.01 M Tris, the counterion concentration maintains the conformation of the subunits, enabling NAD to be bound in the proper manner for restoration of enzymatic activity and reaggregation. Similarly, yeast glyceraldehyde 3-phosphate dehydrogenase has been found to require both NAD and high ionic strength for reassociation (Deal, 1969).

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Proportion of Keto and Aldehydo Forms in Solutions of Sugars and Sugar Phosphates*

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ABSTRACT: A number of aldoses and ketoses have been examined in deuterium oxide and aqueous solution by infrared, ultraviolet, and circular dichroic spectroscopy. Only infrared spectroscopy in deuterium oxide is a reliable index of the proportion of the free carbonyl tautomer present in solution. Ultraviolet spectra are due principally to impurities and circular dichroism spectra cannot be interpreted quantitatively. The percentages of keto or aldehydo forms in the sugars tested are D-fructose, <0.4; D-fructose 1-phosphate, <1.0; D-fructose 6-phosphate, 2.5; D-glucose 6-phosphate, <0.4; D-glyceraldehyde, 4.4; DL-glyceraldehyde 3-phosphate, 4.4.

Reducing sugars, *i.e.*, carbohydrates having a potentially free aldehydo or keto function, are capable of existing in aqueous solution in several tautomeric forms and as a hydrate. A stable enol form has been suggested also for D-fructose 1,6-diphosphate (McGilvery, 1965). The proportions of these forms are important to considerations of the mode of action of enzymes that use the reducing sugars as substrates and to the chemistry of the substances in water. The phosphorylated monosaccharides are of particular importance. Gray and Barker (1970) have presented nuclear magnetic resonance and infrared evidence that D-fructose 1,6-diphosphate exists in deuterium oxide solution in a furanose form, probably β, and that less than 2% is in the free keto form. Similarly, D-*glycero*-D-*altro*-octulose 1,8-diphosphate exists entirely in ring form(s). Several acyclic ketose phosphates were examined also and found to exist predominantly in the keto rather than the hydrated (gem diol) form, thus making improbable the presence of acyclic structures in the case of D-fructose 1,6-diphosphate.

Avigad *et al.* (1970) examined a number of ketoses, ketose phosphates, and substituted ketoses using ultraviolet and circular dichroic spectroscopy. These authors concluded that aqueous solutions of D-fructose 1-phosphate, D-fructose 6-phosphate, and D-fructose 1,6-diphosphate may contain up to 20% of the acyclic forms; presumably these are the keto form. They did not consider the possibility of hydrated forms which would not be detected by these methods. This difference between the two studies is sufficiently large to warrant further examination of the ketoses in solution.

The experimental difficulty in these studies lies in finding an analytical method that allows the determination of relatively small amounts of free keto forms. Chemical methods can all be suspected of perturbing the system and the methods of choice are physical methods. In the nuclear magnetic resonance spectra of the ketoses the signals due to the keto forms can only be identified in the simplest cases, *e.g.*, 1,3-dihydroxy-2-propanone phosphate (Gray and Barker, 1970). Ultraviolet absorptions in the 260- to 280-nm region cannot be assigned unequivocally to be carbonyl function and can be enhanced enormously by small amounts of impurities, *e.g.*, D-fructose 1,6-diphosphate (Gray and Barker, 1970). Circular dichroic spectra may be more reliable because they depend on a chromophore associated with an asymmetric center but impurities that might be present in reducing sugar preparations, such as osazones, could contribute to ellipticities. Infrared spectroscopy in deuterium oxide solution allows observation of the carbonyl absorption and only impurities with a carbonyl group interfere with the analysis. Large molar extinction coefficients for this region as compared to the ultraviolet ($n-\pi^*$) lead to greater sensitivity for the detection of carbonyl groups in the infrared. If the molar extinction coefficient of a suitable model of known per cent keto form can be obtained the per cent keto form for similar compounds can be estimated readily as shown by Gray and Barker (1970) in their study of several acyclic ketose phosphates. The infrared method was shown to agree well with the nuclear magnetic resonance analyses when discrimination between keto and hydrated keto forms is possible by this latter technique.

Proton magnetic resonance spectroscopy can be used to advantage in examining aldoses for the presence of aldehyde forms; *e.g.*, Trentham *et al.* (1969) have examined D-glyceraldehyde 3-phosphate and Horton and Wander (1971) have studied a number of acetylated *aldehydo*-aldoses.

Materials and Methods

Infrared spectra were obtained on a Perkin-Elmer Model 521 grating spectrometer at 25 ± 2°. Samples dissolved in deuterium oxide solution were placed in a 0.05-mm calcium

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