Molecular Weight and Subunit Structure of Bovine Adrenal Glucose-6-phosphate Dehydrogenase†

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ABSTRACT: The bovine adrenal glucose-6-phosphate dehydrogenase used in these studies was highly purified and crystallized three times. The mean value and standard deviation of six measurements of the sedimentation coefficient within the concentration range of 2–6 mg/ml was 9.45 ± 0.065 S. The diffusion coefficient obtained from boundary spreading experiments in the synthetic boundary cell was 3.30×10^{-7} cm^2 sec^{-1}. The partial specific volume calculated from a comparison of low-speed sedimentation equilibrium experiments in H_2O and D_2O was 0.737. This value is in good agreement with the value 0.736 calculated from the amino acid composition. Several methods of dissociating the enzyme into its subunits were tested, but only reduction and carboxymethylation in 0.1% sodium dodecyl sulfate produced essentially complete dissociation. The following parameters were obtained for the subunit: $s_{20,w} = 3.70$ S and $D_{20,w} = 5.33 \times 10^{-7}$ cm^2 sec^{-1}. The molecular weight calculated from these values is 65,200. The value calculated from sedimentation equilibrium measurement is 63,900, and from polyacrylamide gel electrophoresis measurements in sodium dodecyl sulfate is 64,500. The best estimate of the molecular weight of the subunit therefore is 64,600. We conclude from these and other data that the enzyme is composed of four subunits that are indistinguishable by sedimentation and electrophoretic criteria, and that each subunit has a molecular weight of 64,600, and consists of a single polypeptide chain.

Since the discovery of glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate:NADP$^+$-oxidoreductase, EC 1.1.1.49) by Warburg and Christian (1931a,b), the enzyme has been isolated from brewer's yeast (Kornberg, 1950; Noltmann *et al.*, 1961), human erythrocytes (Kirkman, 1962; Chung and Langdon, 1963), mammary glands of cows (Julian *et al.*, 1961) and of guinea pigs (Andrews, 1962), *Escherichia coli* (Sanwal, 1970), rat liver (Matsuda and Yugari, 1967), bovine ovary (Nielson and Warren, 1965), adrenal glands (McKerns, 1964; Squire and Sykes, 1970), and many other biological systems. As the first enzyme of the hexose monophosphate pathway, this enzyme plays a key role in maintaining the level of the reduced coenzyme NADPH in the cell by catalyzing the following reaction

$$\text{Glc-6-P} + \text{NADP}^+ \rightleftharpoons \text{6-phosphogluconolactone} + \text{NADPH} + \text{H}^+$$

Control of this enzyme is of special importance in the bovine adrenal cortex since NADPH provided by glucose oxidation through the hexose monophosphate shunt is required for the synthesis of steroid hormones. Control by a variety of metabolites has in fact been reported (McKerns, 1963, 1964; Criss and McKerns, 1968b; McKerns and Kaleita, 1960; Schachet and Squire, 1971). Preliminary estimates of the molecular weight based on the sedimentation coefficient have been reported by Criss and McKerns (1968a), and by Squire and Sykes (1970), but no systematic determination of the parameters required for the precise calculation of the molecular weight of this polysubunit enzyme has been reported.

The initial studies conducted in this laboratory were directed toward the determination of the molecular weight of the native enzyme by sedimentation measurements. Analysis of these experiments showed that even though the dependence of the sedimentation coefficient and the weight average molecular weight on concentration was small, plots of log c *vs.* X^2 from the sedimentation equilibrium measurements were nonlinear, and the deviation from linearity increased with protein concentration. We concluded that the enzyme at this protein concentration, 2–6 mg/ml, was reversibly associating, and that the predominant association constant was small, but not negligible. These experiments provided valuable information about the state of aggregation of the native enzyme, but did not permit characterization of the mass of the enzyme with the precision we wanted. The alternative was to completely dissociate the enzyme, and determine the number and molecular weight of the subunits under conditions where association equilibria were absent or negligible.

Materials and Methods

Nicotinamide adenine dinucleotide diphosphate (NADP$^+$), maleic anhydride, and sodium dodecyl sulfate were obtained from Sigma Chemical Co. Urea and D_2O (99.8%) were purchased from Mallinckrodt Chemical Works and Stohler Isotope Chemicals, respectively. Eastman Organic Chemicals was the source of the chemicals used in polyacrylamide gel electrophoresis. Gdn·HCl[1] Ultra Pure grade, used in some of our studies, was purchased from Mann Research Laboratories Inc. Fast Green FCF and dithioerythritol were the products of Fischer Scientific Co. and P-L Biochemicals Inc., respectively.

Protein standards used for the determination of the molecular weight of bovine adrenal glucose-6-phosphate dehydrogenase by sodium dodecyl sulfate gel electrophoresis were:

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[1] Abbreviation used is: Gdn·HCl, guanidine hydrochloride.

bovine serum albumin from Armour Pharmaceutical Co., lot no. F 71703; ovalbumin, pepsin, and hemoglobin from Pentex Inc.; γ-globulin from Mann Research Laboratories Inc.

Dimethyl suberimidate used in the cross-linking experiment was synthesized from 1,6-dicyanohexane according to the procedure described by Davies and Stark (1970). Urea was recrystallized from 70% ethanol by the method of Steinhardt (1938). All other chemicals were of reagent grade and used without further purification.

Glucose-6-phosphate dehydrogenase was isolated from bovine adrenal glands according to the procedure of Squire and Sykes (1970). The enzyme was crystallized three times before using it in our studies. The concentrations of samples used in sedimentation studies were determined refractometrically by means of the synthetic boundary cell. The activity of the enzyme was determined as previously described by Squire and Sykes (1970).

Reduction and carboxymethylation of Glc-6-P-dehydrogenase were conducted essentially as described by Crestfield *et al.* (1963), except dithioerythritol was substituted for 2-mercaptoethanol. The alkylated protein was examined for free sulfhydryl groups with a Beckman amino acid analyzer. When necessary, a second cycle of reduction and carboxymethylation was carried out to assure complete alkylation of the protein. The reduced and carboxymethylated enzyme was stored at $-30°$. In preparing samples for sodium dodecyl sulfate gel electrophoresis, the reduction and carboxymethylation were carried out in the presence of 0.1% sodium dodecyl sulfate rather than 8 M urea as recommended by Crestfield *et al.* (1963).

Dissociation of the enzyme with maleic anhydride was carried out essentially as described by Cohen and Rosemeyer (1969). Prior to treatment with maleic anhydride, the enzyme was dialyzed for at least 24 hr against 0.1 M potassium phosphate buffer, containing 10^{-3} M EDTA and 0.15 M KCl at pH 8.0, to remove ammonium ions remaining from the crystallization step. After dialysis a small volume of 2.0 M dibasic potassium phosphate was added to the protein solution in order to neutalize the maleic acid produced during the course of the reaction. The protein solution was then pipetted onto solid maleic anhydride, amounting to 30 equiv of the lysine residues present. The reaction mixture was incubated at 37° with constant stirring of the solution. No enzyme activity was observed after incubation for 30 min. The solution was then prepared for sedimentation and electrophoresis studies by dialysis against 0.1 M potassium phosphate (pH 7.0), containing 10^{-3} M EDTA, 0.15 M KCl, and 0.1% 2-mercaptoethanol.

The procedure used for the determination of the molecular weight of the subunit, as well as the reduced and carboxymethylated Glc-6-P-dehydrogenase, combines features of the methods of Weber and Osborn (1969) and of Hedrick and Smith (1968). Gel concentration varied from 5 to 10%, but the Bis:acrylamide ratio was maintained at 1:30. Operating procedures and relative mobility calculations were done according to Weber and Osborn (1969), but molecular weights were calculated from the slope of log of relative mobility *vs.* acrylamide concentration according to Hedrick and Smith (1968). Gels were stained with 0.1% solution of Fast Green FCF dissolved in 10% acetic acid. After destaining they were scanned at 620 nm by the Gilford uv recorder, Model 2000, connected to a Beckman DU recording spectrophotometer.

The procedure of Davies and Stark (1970) was used for cross-linking the subunits with dimethyl suberimidate. The best results as judged by the appearance of a maximum number of bands were obtained with an enzyme to reagent ratio of 1:2.5. The enzyme treated with the bifunctional reagent was subjected to sodium dodecyl sulfate gel electrophoresis (7.5% gel) as described earlier.

All samples analyzed by sedimentation analysis were first dialyzed for at least 24 hr against an appropriate buffer, and the dialysate was used as the reference solvent. All transfers of the solution and the dialysate were performed with care to prevent differential evaporation. The sedimentation velocity and the equilibrium experiments of the native and dissociated glucose-6-phosphate dehydrogenase were carried out at 20° in a Beckman Spinco Model E analytical ultracentrifuge equipped with a schlieren diaphragm, a Raleigh interference optical system, and a rotor-temperature control (RTIC) unit. A double-sector cell was used in all experiments. Fluorochemical FC-43 was used to provide a visible lower meniscus in the sedimentation equilibrium experiments. All measurements on the photographic plate were made with a Nikon Model 6C optical micro comparator equipped with a rotational stage. The optical constants of the centrifuge were checked with the solution of a known refractive index by a fringe count as well as with a calibration cell furnished by the manufacturer. A capillary-type synthetic boundary cell was used for the determination of the initial protein concentration, c_0, and plots of fringe positions *vs.* time were used to determine the hinge point, *i.e.*, the radial position at which the concentration is independent of time during the approach to equilibrium. Sedimentation coefficients were determined and corrected to $s_{20,w}$ as described by Schachman (1957).

The diffusion coefficient, D, of the enzyme was determined by two different methods. One approach was the measurement of boundary spreading in the double-sector synthetic boundary cell by means of the Rayleigh interference optical system. The diffusion coefficient was calculated according to the procedure outlined by Schachman (1957) from the slope of the plot of $(\Delta H/\Delta h)^2(4Dt/F^2)$ with respect to the corresponding time, t. Both sequential- and symmetrical-fringe pairing methods were utilized in order to determine the value of the diffusion coefficient. Unpublished experiments we have performed by this method on standard proteins have yielded results agreeing within a few tenths of a percent with published values.

The second method was based on measurements taken during the approach to the sedimentation equilibrium, and utilized the equation derived by Van Holde and Baldwin (1958)

$$\log \epsilon = \log \left\{\frac{4[1 + \cosh (^1/_2\alpha)]}{\pi^2 U^2(\alpha)}\right\} - \left\{\frac{D\pi^2 U(\alpha)t}{2.303(r_b - I_a)^2}\right\} \quad (1)$$

Here, $\epsilon = (\Delta c_{eq} - \Delta c_t)/\Delta c_{eq}$ represents the departure of the system from sedimentation equilibrium. Δc_t is the concentration change between the inner and outer limits of the solution, r_a and r_b, at the time, t. Δc_{eq} refers to the system at equilibrium

$$\alpha \equiv (c_0/\Delta c_{eq}) = RT/M(1 - \bar{v}\rho)\omega^2\bar{r}(r_b - r_a) \quad (2)$$

where c_0 is the initial protein concentration determined by an independent experiment with the synthetic boundary cell, and

$$U(\alpha) \equiv (1 + {}^1/_4\pi^2\alpha^2) \quad (3)$$

The apparent molecular weight of the protein, M, was calculated from the slope of the plots of log c with respect to r^2 by

$$M = \frac{(2.303)(2RT)(\mathrm{d}\ \log\ c/\mathrm{d}r^2)}{(1 - \bar{v}\rho)\omega^2} \quad (4)$$

TABLE I: Physical Properties of Bovine Adrenal Glucose-6-phosphate Dehydrogenase in the Native State.

A. The sedimentation coefficient		
Concn (mg/ml)	Buffer[a]	$s_{20,w} \times 10^{13}$ sec
4.37		9.44
5.50	0.3 M $(NH_4)_2SO_4$	9.44
2–5.0	0.15 M KCl	9.46[b]
6.00	0.10 M NaCl	9.42
	$s_{20,w} = 9.45 \pm 0.065$ S	
B. The diffusion coefficient ($D_{20,w}$)		
By boundary spreading (concn 3.57 mg/ml)		3.30×10^{-7} cm^2 sec^{-1}
C. Partial specific volume, $\bar{v}$		
Sedimentation equilibrium in D_2O and H_2O buffers		0.737 ml/g
From amino acid composition		0.736 ml/g
D. The molecular weight		
Sedimentation equilibrium	283,000	
Sedimentation and diffusion	285,000	
Best value	284,000	

[a] Buffer used in these studies was 0.1 M sodium acetate (pH 5.5), containing 2×10^{-5} M $NADP^+$, 10^{-3} M EDTA, and 10^{-2} M 2-mercaptoethanol with additions as indicated. [b] Average of four experiments.

Results

Characterization of the Native Enzyme. The partial specific volume, $\bar{v}$, was determined by the method of Edelstein and Schachman (1967). Two sedimentation equilibrium experiments were carried out with temperature, angular velocity, and the protein concentration kept constant. The density, ρ, was varied by substituting D_2O in one of the experiments for H_2O.

The partial specific volume was calculated by applying the following relationship

$$\bar{v} = \frac{k - [(\mathrm{d} \ln c/\mathrm{d}r^2)_{D_2O}/(\mathrm{d} \ln c/\mathrm{d}r^2)_{H_2O}]}{\rho_{D_2O} - \rho_{H_2O}[(\mathrm{d} \ln c/\mathrm{d}r^2)_{D_2O}/(\mathrm{d} \ln c/\mathrm{d}r^2)_{H_2O}]} \quad (5)$$

where k is a measure of exchangeability of hydrogen atoms of the protein with deuterium of D_2O. Experiments to determine the relative weight increase due to deuterium exchange have been carried out with a number of proteins, and gave an average value of k equal to 1.0155 (Hvidt and Nielsen, 1966). The partial specific volume of the native bovine adrenal Glc-6-P-dehydrogenase, calculated from the slopes of log c *vs.* X^2 plots (Figure 1), was found to be 0.737 ml/g.

Preparations of freshly purified Glc-6-P-dehydrogenase were characterized by sedimentation velocity experiments performed at 20°, and at a speed of 59780 rpm. The buffer used in four of these experiments was 0.10 M sodium acetate (pH 5.50), containing 2×10^{-5} M $NADP^+$, 10^{-2} M 2-mercaptoethanol, 10^{-3} M EDTA, and 0.15 M KCl. This buffer was chosen because it approximates the conditions of maximal stability of the native enzyme. The results obtained from sedimentation velocity experiments in other buffers also are shown in Table I. Since no concentration dependence was observed in the concentration range we studied, the mean value of 9.45 ± 0.065 S of six experiments ($s_{20,w}$) was taken as the best value of this parameter.

Measurements of boundary spreading in a double-sector synthetic boundary cell gave a value of 3.30×10^{-7} cm^2 sec^{-1} for the diffusion coefficient ($D_{20,w}$) of this enzyme.

The molecular weight calculated from s and D by the Svedberg equation was 285,000. This is in good agreement with the average value of 283,000 calculated from the sedimentation equilibrium experiments for the native complex (eq 4). The plots of log c *vs.* X^2 for the native enzyme were not strictly linear. Since the linearity improved as the concentration decreased, the non-linearity was attributed to self-association. It was clear that it would be difficult to determine a precise value of molecular weight by this approach; nevertheless, an approximate value proved to be useful in determining the state of aggregation of the native enzyme. The molecular weight determined from the linear region of the plot corresponding to the lower values of concentration was 283,000.

Dissociation of the Enzyme into Subunits and Its Physical Properties. Attempts were made to dissociate the enzyme by treatment with maleic anhydride, with sodium dodecyl sulfate, by lowering the pH to 1.3 in 0.20 M HCl–KCl buffer, and by reduction followed by carboxymethylation in 8 M urea, in 8 M Gdn·HCl, and in 0.1% sodium dodecyl sulfate. All of these treatments resulted in substantial, but incomplete, dis-

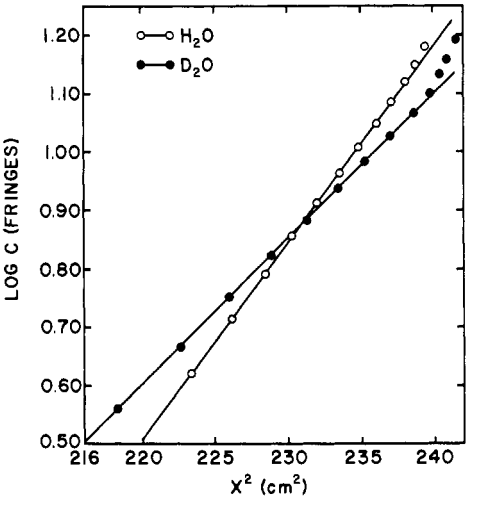

FIGURE 1: Determination of $\bar{v}$ from plots of logarithm of the concentration in fringes, c, *vs.* X^2, at sedimentation equilibrium. Here $X = Fr$, where F is the magnification factor and r the radial distance. The experiments were performed in H_2O and D_2O solutions containing 0.10 M sodium acetate (pH 5.50), 0.30 M ammonium sulfate, and 10^{-5} M $NADP^+$. Sedimentation equilibrium measurements were performed on the native enzyme at 4908 rpm and 20°. The protein concentration was 2.381 mg/ml.

TABLE II: Physical Properties of Bovine Adrenal Glucose-6-phosphate Dehydrogenase Subunit.

A. The sedimentation coefficient		
Treatment	$s_{20,w}$ (S)	$(s_n/s_d)^{3/2}$
Maleic anhydride	3.67	4.13
Reduced and alkylated in sodium dodecyl sulfate	3.73	4.03
HCl–KCl (pH 1.3)	3.57[a]	
Best value[b]	3.70	4.08
B. The diffusion coefficient ($D_{20,w} \times 10^7$ cm^2 sec^{-1})		
Boundary spreading	5.35	Average = 5.33
Approach to sedimentation equilibrium	5.30	
C. The molecular weight	M_1	
Sedimentation equilibrium	63,900	Average = 64,600
Sedimentation and diffusion	65,200	
Acrylamide gel electrophoresis	64,500	
D. Other parameters		
Frictional coefficient (f)	7.48×10^{-8} g sec^{-1}	
Frictional ratio (f/f_0)	1.48	
Stokes' radius, R_e	39.5×10^{-8} cm	

[a] Not corrected for charge effects. [b] Values obtained in HCl–KCl buffer were not included in the best value for $s_{20,w}$.

sociation of the native enzyme. While the polydispersity of these preparations precluded accurate determination of the molecular weight of the subunit by sedimentation equilibrium, reliable measurements of the sedimentation coefficients could be made. These are recorded in Table II. The values of 3.73 and 3.67 S for the enzyme dissociated by reduction and carboxymethylation, and by maleylation, respectively, are in good agreement. The low value of 3.57 S obtained in the HCl–KCl buffer at pH 1.30 may be attributed to charge effects. The ratios of $(s_n/s_d)^{3/2}$ recorded in Table II are all approximately equal to four. This provides strong evidence that the native enzyme is a tetramer. Essentially complete dissociation of the enzyme was achieved when the reduced and carboxymethylated sample was dialyzed against 0.10 M sodium phosphate buffer (pH 7.0), containing 0.1% sodium dodecyl sulfate, and incubated at 37°. The plot of log c *vs.* X^2, unlike that obtained for the native enzyme, was linear throughout the radial distance. In a more sensitive test for linearity, plots of fringe deviations from the regression line showed only random scatter. The linearity of the plot provides evidence for the monodisperse nature of the subunit and the purity of original sample as well. The sedimentation equilibrium experiment yields a value for buoyant molecular weight, $M(1 - \bar{v}\rho)$, for the sodium dodecyl sulfate–protein complex. The extent of sodium dodecyl sulfate binding, expressed as grams of sodium dodecyl sulfate per gram of protein, was determined from the difference in refractive index between the undiluted dialyzed solution and the equilibrium dialysate as described by Olins and Warner (1967). McBain *et al.* (1939) report that micelle formation is negligible at the low concentrations of sodium dodecyl sulfate (0.1%, 3.47 mM) used in this study. The synthetic boundary cell experiment gives a fringe number, J_0, proportional to the refractive index contribution of the sodium dodecyl sulfate–protein complex. The fraction of these fringes due to the enzyme was calculated from its known concentration, its specific refractive increment, $(dn/dc)_{Enz}$, and the optical constants of the ultracentrifuge. This fraction is proportional to the contribution of the protein to the refractive index increment, $(\Delta n)_{Enz}$. From this value, the extent of sodium dodecyl sulfate binding, δ_2 (grams of sodium dodecyl sulfate (SDS) per gram of protein), was determined by the following relationship

$$\delta_2 = \frac{(\Delta n)_{SDS}(dn/dc)_{Enz}}{(\Delta n)_{Enz}(dn/dc)_{SDS}} \quad (6)$$

where $(dn/dc)_{Enz}$, the specific refractive increment of the protein, was taken as 1.86×10^{-4} ml/mg; $(dn/dc)_{SDS}$, the specific refractive increment of sodium dodecyl sulfate, is 1.209×10^{-4} ml/mg (Hersh and Schachman, 1958), and $(\Delta n)_{SDS}$, the refractive index increment due to the sodium dodecyl sulfate bound to the protein, was obtained by subtracting $(\Delta n)_{Enz}$ from the $(\Delta n)_{complex}$. The amount of sodium dodecyl sulfate bound per gram of Glc-6-P-dehydrogenase was found to be 0.423 g. With the use of this value of δ_2, and $\bar{v}_2 = 0.863$ ml/g (Anacker *et al.*, 1964), the molecular weight of Glc-6-P-dehydrogenase in the complex, M_p, was computed from the following expression derived by Hersh and Schachman (1958)

$$M_p = \frac{\{M(1 - \bar{v}\rho)\}_{complex}}{(1 - \bar{v}_p\rho) + \delta_2(1 - \bar{v}_2\rho)} \quad (7)$$

where the numerator is the buoyant molecular weight term calculated experimentally. Here $\bar{v}_p$ and $\bar{v}_2$ are the partial specific volumes of protein and sodium dodecyl sulfate, respectively. The molecular weight of the subunit calculated in this manner (eq 7) was also corrected for the amount of iodoacetamide covalently linked to the cysteine residues during carboxymethylation of the enzyme. The calculated value of the molecular weight of the subunit, after these corrections were made, was 63,900 (Table II).

The data used in calculating the diffusion coefficient from boundary spreading in a double sector synthetic boundary cell are plotted in Figure 2. The diffusion coefficient ($D_{20,w}$) calculated from the slope was 5.35×10^{-7} cm^2 sec^{-1}. Figure 3 illustrates the results of the measurements taken during the approach to sedimentation equilibrium. The diffusion coefficient, 5.30×10^{-7} cm^2 sec^{-1}, estimated by this method, is in good agreement with the value obtained from boundary spreading (Table II). The linearity of these plots for the determination of the diffusion coefficient (Figures 2 and 3) provides an additional evidence of homogeneity of the sample.

The molecular weight of the subunit was also estimated by polyacrylamide gel electrophoresis in 0.1% sodium dodecyl sulfate. The denatured protein moved as a single band, again indicating that these subunits have similar or identical molec-

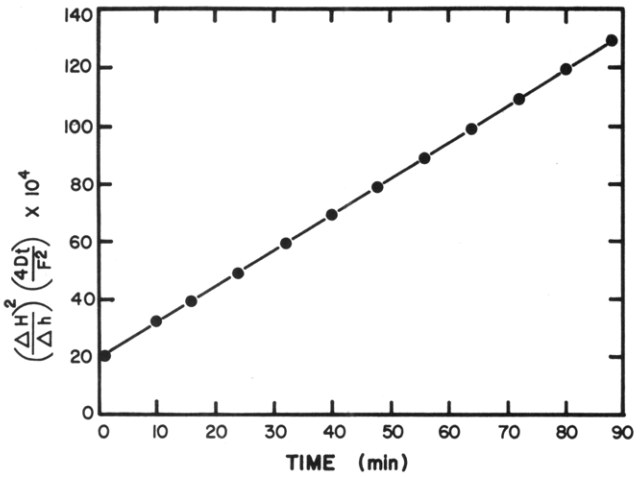

FIGURE 2: Determination of the diffusion coefficient of the dissociated enzyme from the measurement of boundary spreading in a double-sector synthetic boundary cell by means of a Raleigh interference optical system. The slope of this graph yields the value of the diffusion coefficient.

ular weights. The log of relative mobility was plotted against the corresponding gel concentrations as recommended by Hedrick and Smith (1968), and a linear relationship was obtained for all the proteins tested. The slopes of these lines were plotted as a function of their molecular weights and by interpolation the molecular weight of bovine adrenal Glc-6-P-dehydrogenase was found to be 64,500 (Table II), in excellent agreement with the values determined by the sedimentation studies.

Since the subunits had a tendency to aggregate below pH 6.0, higher pH buffers (pH 6.3, 7.3, and 8.6) were used in order to examine the electrophoretic homogeneity of the subunits. In all cases only one band was observed suggesting that all the subunits have essentially the same mobility in this range of pH.

Davies and Stark (1970) have shown how dimethyl suberimidate may be used to determine the number and the type of subunits contained in an oligoprotein. If the native bovine adrenal glcuose-6-P-dehydrogenase consists of four identical subunits, four bands should be obtained on sodium dodecyl sulfate gel electrophoresis with the dimethyl suberimidate treated enzyme. As shown in Figure 4, this was the result obtained.

Discussion

The sedimentation coefficient of the native enzyme under conditions approximating those of the adrenal, but measured at a protein concentration in the range of 2–6 mg/ml, is 9.45 ± 0.065 S. This value is in good agreement with the value 9.47 S previously reported from this laboratory (Squire and Sykes, 1970), but is somewhat lower than the value 9.80 S reported by Criss and McKerns (1968a) for the enzyme from the same source.

Measurement of the diffusion coefficient of the native enzyme by the boundary-spreading technique yielded the value, $D_{20,w} = 3.30 \times 10^{-7}$ cm^2 sec^{-1}. The molecular weight calculated from $s_{20,w}$ and $D_{20,w}$ is 285,000. This is in good agreement with the value 283,000 obtained from measurements at sedimentation equilibrium. Since we had evidence of mild association equilibria under the conditions of these experiments, we were uncertain as to the significance of these values. The other values in the literature, $M = 190{,}000$ (Squire and Sykes, 1970), and 239,000 (Criss and McKerns, 1968a), were subject to the same uncertainty, but in addition, these values were calculated from sedimentation coefficients alone by means of approximate equations involving assumptions which, while useful, cannot be expected to yield results of high accuracy.

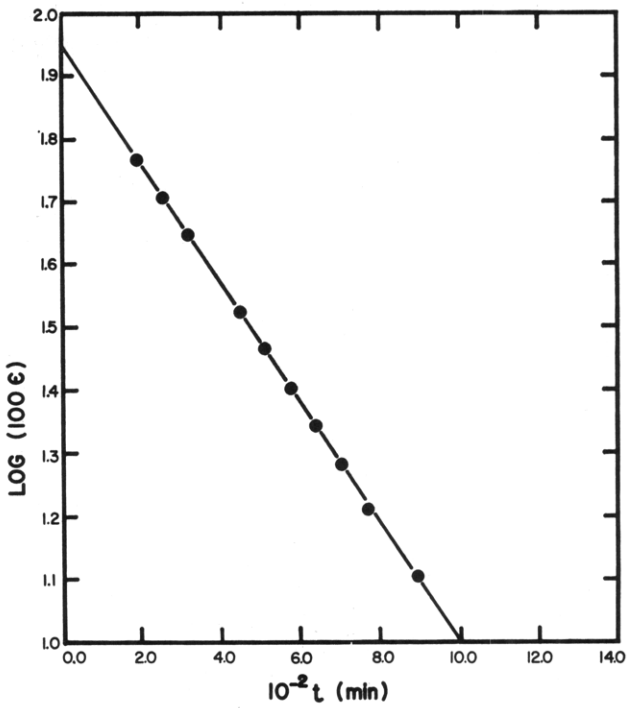

FIGURE 3: Determination of the diffusion coefficient of the enzyme subunit from the measurements taken during the approach to the sedimentation equilibrium. The diffusion coefficient of the subunits was calculated from the slope according to eq 1, derived by Van Holde and Baldwin (1958).

Because of the complications mentioned above, we have chosen to determine the molecular weight of the native enzyme complex by accurately determining the molecular weight and number of subunits under conditions where dissociation is

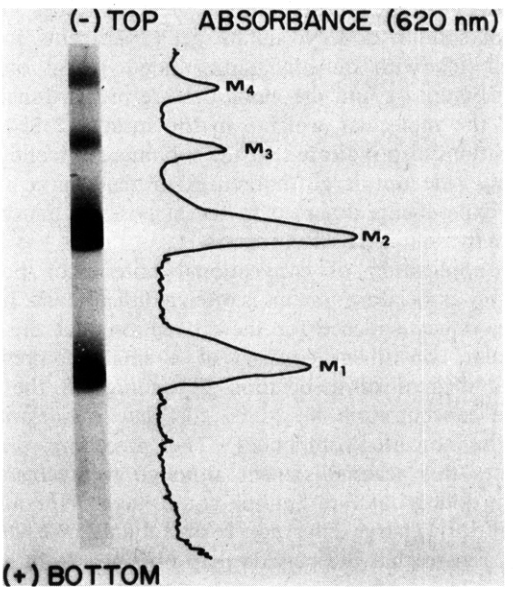

FIGURE 4: The cross-linking of the bovine adrenal glucose-6-phosphate dehydrogenase. Electrophoresis was performed in 7.5% polyacrylamide gel containing sodium dodecyl sulfate as described under the Methods section. Here M_1, M_2, M_3, and M_4 represent monomer, dimer, trimer, and tetramer species, respectively.

complete. We accomplished this by complete reduction and carboxymethylation in 8 M urea followed by dialysis against a buffer containing 0.1% sodium dodecyl sulfate at room temperature for 24 hr. Attempts to dissociate the enzyme by other agents and even our early attempts by this method were discouraging in that dissociation, while substantial, appeared to be incomplete, and the resulting preparation was polydisperse. We surmise that the denatured protein aggregates during removal of the urea by dialysis, and that subsequent complete dissociation requires incubation in 0.1% sodium dodecyl sulfate at room temperature for several hours.

The subunit molecular weight was determined by sedimentation equilibrium, and by measurements of the sedimentation and diffusion coefficients. Our best value, 64,600, is the mean of the values obtained by these methods (Table II). The good agreement of this value with the value 64,500 obtained by acrylamide gel electrophoresis is remarkable.

All these data lead to the conclusion that the native enzyme consists of four subunits. The ratio of the molecular weight of the native enzyme to that of the subunit is 284,000/64,600 = 4.4. The ratio of the sedimentation coefficients raised to the $^3/_2$ power is 4.08. (For a discussion of this formulation, see Squire *et al.*, 1968.) Acrylamide gel electrophoresis of the partially cross-linked enzyme gives four bands as expected for a tetrameric structure. The fact that the ratio of molecular weights exceeds 4.0 may be attributed to a tetramer–octamer equilibrium which is significant in this concentration range. We, therefore, take as the best value for the molecular weight of the native tetramer four times the molecular weight of the monomer, or 258,000.

All the data concerning the subunits are consistent with the hypothesis that the four subunits are identical. The linearity of the plot of log c *vs.* X^2 of data at sedimentation equilibrium of the dissociated subunits rules out the possibility of two populations of subunits having substantially different molecular weights. Analysis of the photographs of the sedimentation velocity studies on the same preparation leads to the same conclusion. Acrylamide gel electrophoresis of the subunit at pH values 6.3, 7.3, and 8.6, after treatment with maleic anhydride, showed only one band indicating that all subunit derivatives had the same mobility at these pH values. Observations of sodium dodecyl sulfate gel experiments following cross-linking with dimethyl suberimidate reveal only four bands (Figure 4), and the mobilities are proportional to the log of the molecular weights in the ratio 1:2:3:4. These observations also indicate that the subunits are identical, and certainly rule out large differences in the charge to mass ratio. Experiments designed to reveal possible minor differences in the subunits are in progress.

The application of conventional criteria of purity to reversibly associating systems is often a difficult task. In such a system, direct evidence for the contention that the macromolecular constituent consists of a single component in various degrees of association depending on the overall protein concentration would be complete dissociative to a single monomeric component. The preceding discussion describes this accomplishment although we recognize that sodium dodecyl sulfate binding could have obliterated possible intrinsic charge differences in the subunits. We would add in this regard that the peptide map obtained from a tryptic digest of this preparation is also consistent with the amino acid analysis based on a single monomeric compound of mol wt 64,500.

The fact that subunits were prepared by complete reduction and carboxymethylation of the enzyme suggests that the subunits consist of a single polypeptide chain. Even though our attempts to dissociate the enzyme with maleic anhydride, urea, Gdn·HCl, and low pH did not result in complete dissociation, well over 80% of the protein had a sedimentation coefficient in fair agreement with our best value, 3.70 S. These observations lend additional support to this contention.

The translational frictional coefficient, f, and the frictional ratio, (f/f_0), were calculated by means of the equations

$$f = \frac{RT}{ND} = \frac{M(1 - v_p\rho)}{Ns} \tag{8}$$

and

$$f_0 = 6\pi\eta(3M\bar{v}_p/4\pi N)^{1/3} \tag{9}$$

These are true molecular constants calculated from experimental data, and are free of questionable assumptions. Their interpretation in terms of shape and hydration, however, would either require additional experimental data from other physicochemical methods, or would require the use of assumed values for shape or hydration. This problem is discussed in detail by several authors. For a recent review, see Squire (1974).

The frictional ratio, f/f_0, calculated from eq 8 and 9 is 1.48 for the monomer–sodium dodecyl sulfate complex. While this frictional ratio could correspond to a rather wide range of models, the assignment of reasonable values to some of the undetermined parameters permits calculation of molecular dimensions that are also reasonable and consistent with other physicochemical data on protein–sodium dodecyl sulfate complexes. Thus if we assume a hydration value of 0.2 g of H_2O/g of protein (Edsall, 1953) and utilize our experimentally determined value of 0.423 g of bound sodium dodecyl sulfate per g of protein, we calculate the values a = 73.6 Å and b = 16.4 Å for the major and minor axes of the equivalent prolate ellipsoid of revolution.

In a systematic study of the viscosity of solutions of protein–sodium dodecyl sulfate complexes, Reynolds and Tanford (1970) concluded that the proteins studied all appeared to have minor axes ranging from 13.4 to 15.1 Å. Our value is 16.4 Å, a somewhat higher value. In view of the fact that we have used a different hydrodynamic method to characterize the protein–sodium dodecyl sulfate complex, and have studied a protein not included in the work by Reynolds and Tanford (1970), we consider the agreement with the conclusion of these authors as satisfactory, and favor the prolate model of axial ratio 4.5 as best representing the Glc-6-P-dehydrogenase–sodium dodecyl sulfate complex.

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Selenium as a Component of Glutathione Peroxidase Isolated from Ovine Erythrocytes†

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ABSTRACT: By a nine-step procedure, glutathione peroxidase (glutathione:H_2O_2 oxidoreductase, EC 1.11.1.9) was purified 4000-fold from ovine erythrocytes to homogeneity as determined by polyacrylamide gel electrophoresis and gel filtration chromatography. During purification the selenium concentration per unit of protein increased 3000-fold indicating that this enzyme accounted for about 75% of the total ovine erythrocyte selenium. The purified enzyme contained 0.34% selenium and the approximate molecular weight as determined by gel filtration and polyacrylamide gel electrophoresis was 88,000, giving a stoichiometry of 3.8 g-atoms of selenium/mol of glutathione peroxidase. This is considered to represent 4 g-atoms of selenium/mol of enzyme, presumably one selenium per enzyme subunit since sodium dodecyl sulfate gel electrophoresis suggested the presence of 4 subunits/mol of enzyme. Glutathione peroxidase is the first positively identified selenoenzyme of animal tissues.

Previous studies in our laboratory (Rotruck *et al.*, 1971) demonstrated that dietary selenite exerted a marked protective effect against oxidant damage to the membrane and hemoglobin of rat erythrocytes incubated *in vitro* in the presence of ascorbate or H_2O_2. This effect depended upon the presence of glucose in the incubation medium and was clearly distinguishable from the protective effects of vitamin E. The Se–glucose relationship was shown to involve the need for glucose metabolism in the continued regeneration of reduced glutathione (GSH) in the erythrocyte and a role for Se in the utilization of this GSH in protecting the cell against oxidant damage (Rotruck *et al.*, 1971, 1972). Because GSH protects the cell, at least in large part, by serving as the hydrogen donor in the glutathione peroxidase catalyzed reduction of H_2O_2 and various hydroperoxides (Mills and Randall, 1958; Clive and Obrien, 1968), we attempted to determine if Se is a necessary component of this enzyme. It was found that most of the ^{75}Se of rat erythrocytes labeled *in vivo* by injecting rats 2–4 weeks earlier with $Na^{75}SeO_3$ followed glutathione peroxidase activity through two highly effective purification steps, but we did not purify the rat enzyme to homogeneity (Rotruck *et al.*, 1973). In order to obtain a larger amount of glutathione peroxidase than was easily provided by rat blood, we selected ovine blood as a source material in our further attempts at

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