Subunit Structure of Glycogen Phosphorylase*

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ABSTRACT: High-speed sedimentation equilibrium experiments and viscosity measurements were carried out on skeletal muscle glycogen phosphorylase using a number of conditions which might dissociate the enzyme to its ultimate subunits. The solvents which were tested included 7.2 M guanidine·HCl, 0.1 M sodium bicarbonate, or 0.02 M sodium borate at pH 9–9.5, 0.1 M acetic acid, concentrated formic acid, and detergents such as sodium dodecyl sulfate and β-phenylpropionate. In all cases, the sulfhydryl groups of the enzyme were protected by alkylation with iodoacetate or iodoacetamide, high concentrations of β-mercaptoethanol, or both. For one experiment in 7.2 M guanidine·HCl, the S-alkylated enzyme was further modified by reaction of its lysyl residues with maleic anhydride.

All evidence obtained is consistent with the view that the subunit of rabbit muscle glycogen phosphorylase consists of a single polypeptide chain of molecular weight *ca.* 92.5 × 10^3 g/mole.

Rabbit muscle glycogen phosphorylase (EC 2.4.1.1) can exist in two states of aggregation: phosphorylase *b*, a dimer with a molecular weight of 185 × 10^3 g, and phosphorylase *a*, a tetramer with a molecular weight of 370 × 10^3 g. The molecular weight of the native enzyme and the results of molecular weight determinations in low molarities of guanidine·HCl indicated that the phosphorylase monomer has a molecular weight of approximately 92.5 × 10^3 g (Seery *et al.*, 1967; De Vincenzi and Hedrick, 1967; Ullmann *et al.*, 1968). Some data were observed, however, that appeared to indicate a further dissociation of the phosphorylase monomer, as the concentration of guanidine·HCl was increased to 7.2 M; a value of 70 × 10^3 g was estimated for the molecular weight of the smallest component. In addition, although preparations of the carboxymethylated derivatives of phosphorylase *b* in 8 M urea contained as much as 20% of dimeric material, a component which was smaller than the monomer also appeared to be present.

In this study an attempt was made to find additional evidence for or against dissociation of the phosphorylase monomer using a number of conditions including 7.2 M guanidine·HCl (pH 9–9.5), 0.1 M acetic acid, concentrated formic acid, and sodium dodecyl sulfate. Interpretation of the results presented in this paper indicate that the 92.5 × 10^3 g/mole species is the lowest molecular weight subunit of rabbit muscle glycogen phosphorylase.

Experimental Section

Materials. Urea (J. T. Baker), guanidine·HCl (Eastman Organic Chemicals), iodoacetate (Fischer), and iodoacetamide (Nutritional Biochemicals) were further purified as described previously (Seery *et al.*, 1967). Dialysis tubing (Union Carbide) was boiled successively in dilute EDTA, water, then ethanol, and finally washed with water. For experiments in urea and guanidine·HCl the tubing was soaked in a solution of the denaturant to remove any material which would absorb in the ultraviolet region of the spectrum.

The preparation of phosphorylase *b* from rabbit skeletal muscle and the alkylation of the enzyme with iodoacetate and iodoacetamide were carried out as previously described (Seery *et al.*, 1967).

Materials and Methods

Phosphorylase *b* which had been fully reacted with maleic anydride was obtained from Dr. Hanna Waron; the preparation which was treated with ^{35}S-labeled sodium dodecyl sulfate was obtained from Dr. John Perkins.

Aldolase and D-glygeraldehyde 3-phosphate dehydrogenase were crystalline preparations obtained from rabbit muscle and kindly supplied by Dr. Marian Kochman. Both preparations have been thoroughly examined both in terms of their purity and physical characteristics (Penhoet *et al.*, 1967; Kochman and Rutter, 1968; Hoagland and Teller, 1969).

Preparations of Samples for Physical Studies. For the ultracentrifuge experiments on unmodified and carboxymethylated phosphorylase *b* in guanidine·HCl, the protein (1.5–3 mg/ml) was dissolved in 7.2 M guanidine·HCl containing 0.05 M Tris·HCl–0.0025 M EDTA (pH 7.5) and equilibrated by dialysis against solvent for at least 48 hr. Just prior to ultracentrifugation, protein and solvent were made 0.1 M in β-mercaptoethanol. The protein was further diluted with solvent to yield a final concentration of 0.50–0.75 mg/ml.

In the presence of denaturing agents other than guanidine·HCl or urea, the sulfhydryl groups of the protein were protected by carboxymethylation or high concentrations of β-mercaptoethanol or both. The protein was dissolved in the particular solvent and either used directly or dialyzed 16–48 hr against this solvent.

Ultracentrifuge Techniques. High-speed sedimentation equi-

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librium methods and calculations were those described in a previous publication (Seery *et al.*, 1967).

In order to convert the observed concentrations into a milligram per milliliter scale, a factor of 4.0 fringes/mg per ml of protein was used (Perlman and Longsworth, 1948) in spite of the fact that in guanidine·HCl this factor might be reduced by as much as 10% (Noelkin and Timasheff, 1967). The protein concentration at the reference point close to the meniscus, C_a, was determined by computational procedures (Teller *et al.*, 1969). When the solvent contained 7.2 M guanidine·HCl, the concentration, C_a, was usually equal to or less than 0.03 fringe. In experiments using denaturing solvents of low density, C_a ranged from 0.03 to 0.08 fringe.

The cell utilized for high-speed equilibrium contained the six-channel centerpiece described by Yphantis (1964). All cell components had orientation marks so that the cell could be reproducibly assembled. Care was also taken to ensure that the cell was always aligned in the same position in the rotor. Solution columns of 2–3 mm were placed over a base formed by 0.01 ml of Fluorocarbon FC-43 (3M Co). When the solvent was anhydrous formic acid, a Kel-F centerpiece with eight circular cross-section observation channels was used (Yphantis, 1960).

It was very difficult to match menisci precisely even when extreme care was used in filling the cell. The sectors of the centerpiece do not appear to be perfectly matched and, therefore, provide a constant source of error. The overlap at the meniscus in 15 experiments in 7.2 M guanidine·HCl was estimated to range from 20 to 46 μ with the average value of about 35 μ. For the centerpiece used, 30 μ of column height corresponds to about 0.001 ml of solution.

Since the overlap at the base of the cell could not readily be determined, it was not possible to predict the redistribution of the solvent components. Consequently, a study was made of the possible effect of redistribution of guanidine·HCl on the base-line patterns. Four experiments were conducted: (a) the 3200-rpm base line taken at the start of an ultracentrifuge run on phosphorylase *b* in 7.2 M guanidine·HCl was measured; (b) the base line after 20 hr at 30,000 rpm with freshly prepared 7.2 M guanidine·HCl in all sectors was determined; (c) water–water base lines were obtained at both 3200 and 30,000 rpm; and (d) to control for material eluted from the dialysis tubing, 7.2 M guanidine·HCl was dialyzed against itself for 9 days. The solution inside the sac was placed in the solute cell sector; the dialysate in the solvent sector. A base line was measured on this sample after 20 hr at 30,000 rpm.

All base lines could be superimposed with a maximum deviation of 0.03 fringe. If the menisci had been appreciably mismatched, displacement of the fringe pattern with respect to distance would have been observed (Small and Lamm, 1966). The base line determined for a cell was fairly constant from experiment to experiment and reasonably independent of rotor speed. In fact, the base line of the cell used in most experiments did not vary systematically by more than 0.03 fringe over a period of 18 months of use.

Viscometry. All viscosity measurements were made in an Ostwald viscometer mounted in a water bath maintained at 20 ± 0.02° following the procedure of Schachman (1957). The capillary tubing of the viscometer was 0.6 mm × 90 cm with efflux time of approximately 110 sec for water. Sample flow times were measured with an electric timer that could be read to 1 msec or with a Model 5901 A Hewlett-Packard Autoviscometer timer. Density increments for the protein solutions were calculated from the partial specific volume of the protein, the concentration, and the solvent density. The intrinsic viscosity, $[\eta]$, was obtained by fitting the data using least squares to the expression: $\eta_{sp}/C = [\eta] + k[\eta]^2C$, where k is a dimensionless constant known as the Huggins constant.

Determination of Apparent Specific Volume. The apparent specific volume of the phosphorylase *b*–sodium dodecyl sulfate complex was also determined by the method of Casassa and Eisenberg (1964). The protein was initially treated by adding 0.35 mg of sodium dodecyl sulfate/mg of protein. The resulting solutions (1 ml) were then dialyzed at concentrations of approximately 6, 12, and 20 mg per ml against 1 l. of 0.005 M ^{35}S-labeled sodium dodecyl sulfate–0.05 M glycine buffer at pH 9.0 for 4 weeks at room temperature with one change of medium.

Density measurements were made in a water bath at 20° ± 0.005° using the gradient tube and a modification of the method described by Hvidt *et al.* (1954). The light and heavy density components were prepared by mixing appropriate proportions of two silicone fluids, Dow Corning 200 fluid (ρ = 0.954928 g/cm^3) and Versilube F-50 from General Electric (ρ = 1.030787 g/cm^3). The gradient was calibrated with KCl solutions of known densities and found to be essentially linear. Protein concentrations were determined using the absorbancy index at 278 nm (1%, 1 cm) of 11.9 (Appleman *et al.*, 1963). An apparent specific volume of 0.737 ml/g was used for the protein in 7.2 M guanidine·HCl (Seery *et al.*, 1967).

Results

Intrinsic Viscosity of Phosphorylase b. In order to obtain an independent measure of the relative effectiveness of urea and guanidine·HCl in denaturing the phosphorylase molecule, viscosity determinations were carried out. The results are presented in Figure 1. The intrinsic viscosity of native phosphorylase *b* was found to be 7.2 ± 0.8 ml per g, and the Huggin's constant −4.8. When the solvent contained 7.2 M guanidine·HCl, a large increase in the reduced viscosity, η_{sp}/C, was observed. The data were fit to an equation of the form: $\eta_{sp}/C = (56 \pm 1) + (1860 \pm 290)C$, from which a value of k = 0.59 is obtained. In 8 M urea the data conform to the equation: $\eta_{sp}/C = (48 \pm 1) + (1170 \pm 260)C$, with k estimated at 0.51. The lower intrinsic viscosity observed in urea suggests that the molecule is not as completely denatured in this solvent. Local protein–protein interaction would lead to a reduction in η_{sp}/C. Although there is considerable uncertainty in the value of k, the value obtained in 7.2 M guanidine·HCl is of the same order of magnitude as the values found under similar conditions for other proteins. Tanford *et al.* (1966, 1967a) reported values of k ranging from 0.16 to 0.67 for a series of globular proteins in 6 M guanidine·HCl and 0.1 M β-mercaptoethanol.

Sedimentation Equilibrium of Phosphorylase b in Guanidine·HCl. Since guanidine·HCl appeared to be a better denaturant for the phosphorylase molecule than urea, a series of experiments were carried out in solvents containing this compound. Results of three experiments in 7.2 M guanidine·HCl were essentially similar to those previously reported (Seery *et al.*, 1967). A typical experiment is presented in Figure 2: above a

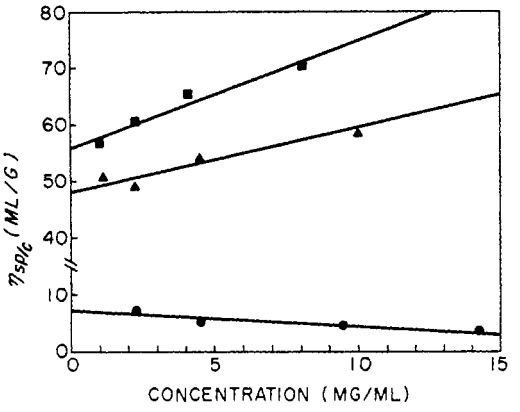

FIGURE 1: Reduced viscosity of phosphorylase *b*. Measurements were made in: (●) 0.005 M EDTA–0.03 M β-mercaptoethanol, pH 6.9 ([η] = 7.2 ± 0.8 ml per g); (▲) 8 M urea ([η] = 48 ± 1 ml per g); (■) 7.2 M guanidine·HCl ([η] = 56 ± 1 ml per g). Both denaturing solvents also contained 0.05 M Tris·HCl, 0.03 M β-mercaptoethanol, and 0.005 M EDTA adjusted to pH 7.5 before addition of either solid urea or guanidine·HCl.

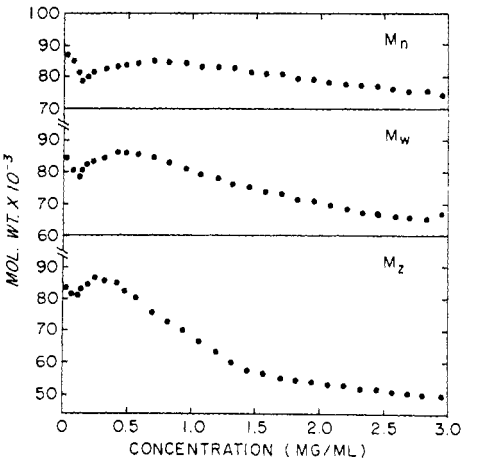

FIGURE 2: Molecular weight moments of phosphorylase *b* in 7.2 M guanidine·HCl. Experimental details are given in the Methods.

concentration of 1 mg/ml the preparation can be characterized as nonideal since the apparent molecular weight averages at each point ($M_{w,r}$, $M_{n,r}$, and $M_{Z,r}$)[1] increase with decreasing concentration and $M_{n,r} > M_{w,r} > M_{Z,r}$. Below 1 mg/ml, $M_{w,r}$, $M_{n,r}$, and $M_{Z,r}$ reach maxima and then decrease. Consequently, the function $2M_{n,r} - M_{w,r}$, which can be used to correct for thermodynamic nonideality (Yphantis, 1964), has a minimum at 0.25 mg/ml. The estimated size of the smallest component is highly dependent on the lowest concentration used in the extrapolation of $2M_{n,r} - M_{w,r}$ to $C = 0$. In a previous communication (Seery *et al.*, 1967), all data below 0.25 mg/ml were omitted.

As demonstrated below, unless the molecular weight data at concentrations less than the observed maxima in $M_{n,r}$ and $M_{w,r}$ *vs.* C plots are neglected, misleading calculations result. Consequently, the data presented in Table I are results of extrapolation to $C = 0$ from concentrations greater than the maxima in the molecular weight distributions.

Apparent molecular weight averages were determined in 7.2 M guanidine·HCl using the S-alkylated derivatives of the enzyme. In all experiments a maximum was observed in the plot of $M_{n,r}$ and $M_{w,r}$ *vs.* C occurring below 0.75 and 0.50 mg per ml for the iodoacetate and iodoacetamide derivatives, respectively. The results from experiments on the iodoacetate derivative were essentially similar to those obtained with the unmodified enzyme while, for the iodoacetamide derivative, slightly higher molecular weight averages were found (Table I). When the negative charges on the S-carboxymethylated protein were further increased by substitution of the lysyl residues with maleic anhydride, no additional evidence for dissociation was obtained. Assuming that the partial specific volume was lowered to 0.72 ml/g by the introduction of the negative charges, a value of $(2M_{n,r} - M_{w,r})_{C=0}$ of approximately 100×10^3 g/mole was calculated from two experiments in 7.2 M guanidine·HCl–0.1 M β-mercaptoethanol. The complete substitution of the 43 lysyl side chains (Sevilla and Fischer, 1969) would result in an increase in molecular weight of *ca.* 4300 g/mole.

If the decrease in $M_{n,r}$ and $M_{w,r}$ as C approaches zero were due to partial dissociation, then one may calculate the amount of light material present by constructing simple models for the dissociation process. The first model to be considered is one in which the monomer of 92.5×10^3 g/mole (M_2) dissociates into two subunits of equal size, M_1. The following expression for the concentration, C_1, of the subunits can be obtained by straightforward algebraic manipulation of the defining equations M_w and M_n: $C_1 = C_t(M_2 - M_{w,r})/M_1$ for the weight-average data and $C_1 = C_t(M_2 - M_{n,r})/M_{n,r}$ for the number-average data, where C_t is the total concentration at the point under consideration. The observed molecular weights of Figure 2 were first corrected for nonideality by using an average virial coefficient of 15.8×10^{-4} mole ml/g^2 (Table I average from row 1 B_1's). Figure 3A presents the results of these calculations. In the concentration region from 0.25 to 1 mg per ml where the function $2M_{n,r} - M_{w,r}$ decreases, the amount of dissociation is predicted to be no greater than 10%. Moreover, if the values of C_1 are summed up across the cell by trapezoidal integration, the initial concentration of C_1 is estimated to be 5% of the total initial concentration from M_w data and 4% from M_n data. For the second model, dissociation of the monomer M_3 into unequal subunits in which the molecular weight of the heavy subunit, M_2, would be twice that of the light subunit, M_1, was tested. The corresponding equations for the concentration of M_1 are: $C_1 = C_t(M_3 - M_{w,r})/6M_1$ and $C_1 = C_t(M_3 - M_{n,r})/3M_{n,r}$. The per cent C_1 estimated from $M_{n,r}$ and $M_{w,r}$ data is given in Figure 3B. The total per cent of dissociated material is three times the per cent of C_1 or less than 10% in the concentration range 0.25–1.0 mg/ml.

On the other hand, one may also ask how the data depart from the behavior expected for a nonideal system with molecular weight 92.5×10^3 g/mole and virial coefficient equal to the value of 15.8×10^{-4} mole ml/g^2 observed in 7.2 M guanidine·HCl. The per cent deviation of the observed molecular weights from the predicted values is shown in Figure 4. The agreement

[1] Abbreviations: $M_{n,r}$, $M_{w,r}$, and $M_{Z,r}$, the number-, weight-, or Z-average molecular weights, respectively, at individual radial distances, r; TDH, triose phosphate dehydrogenase (D-glyceraldehyde 3-phosphate dehydrogenase).

TABLE I: Summary of Molecular Weight Determinations in Denaturing Solvents.

Run	Enzyme	Expt	Denaturing Agent[b]	Mol Wt ($\times 10^{-3}$ (g/mole)[a] $M_{w,C=0}$	$M_{n,C=0}$	$(2M_n - M_w)_{C=0}$	Virial Coeff ($\times 10^4$), mole ml/g^2 B_1 (M_w)	B_1 (M_n)
1	Phosphorylase *b*	6	Gd·HCl (7.2 M)	89.6 (4.0)	85.4 (3.7)	100.3 (4.3)	16.7	14.9
2	Phosphorylase *b* (iodoacetate)	4	Gd·HCl (7.2 M)	86.1 (2.1)	85.0 (2.4)	106.2 (2.8)	13.2	14.6
3	Phosphorylase *b* (iodoacetamide)	4	Gd·HCl (7.2 M)	102.4 (3.5)	100.6 (4.4)	101.5 (5.8)	20.7	21.5
4	Phosphorylase *b* (iodoacetamide)[c]	4	HCOOH (anhydrous)	92.5	85.0		44.9	39.4
5	Phosphorylase *b*[c]	2	HOAc (0.1 M)	59.9 (1.0)	74.7 (3.8)	65.2 (1.2)	167.0	210.0
6	Phosphorylase *b* (iodoacetate)	1	SDS (0.005 M)	97.2 (1.5)	90.7 (0.6)		36.9	33.2
			Av[d]	93.6 (5.7)	89.6 (6.0)			

[a] Numbers in parentheses are root-mean-square errors; data at concentrations below the molecular weight maxima were neglected in the extrapolation to $C = 0$. [b] All solutions except that of line 4 also contained 0.1 M β-mercaptoethanol. Abbreviations used: Gd·HCl, guanidine·HCl; SDS, sodium dodecyl sulfate; HOAc, acetic acid. [c] $\bar{v} = 0.72$ ml/g. [d] HOAc data not included in this average. Experimental details are given in the text.

is good above 0.5 and 0.75 mg per ml for M_w and M_n, respectively. The M_Z data conform reasonably well to the predicted behavior from 0.25 to 1.25 mg per ml but diverge somewhat at higher concentrations. Calculations on simulated data containing random numbers with standard deviation corresponding to 5 μ have shown that the point-by-point $M_{z,r}$ values are only precise to $\pm 5\%$ and the most unreliable section of the cell is toward the cell bottom. Between 0.25 and 0.50 mg per ml the per cent deviation in any of the molecular weight moments is never greater than 10%. Such deviation might be caused by experimental error or by contamination of the phosphorylase preparations with low molecular weight material. These calculations and the estimates of the per cent light material serve to illustrate that the decreases in $M_{n,r}$ and $M_{w,r}$ observed at low concentrations are small relative to the values predicted for a homogeneous nonideal solute with molecular weight equal to that of the monomer.

Sedimentation Equilibrium Studies on Other Proteins of Known Molecular Weight in Guanidine·HCl. In order to obtain additional information supporting the results obtained

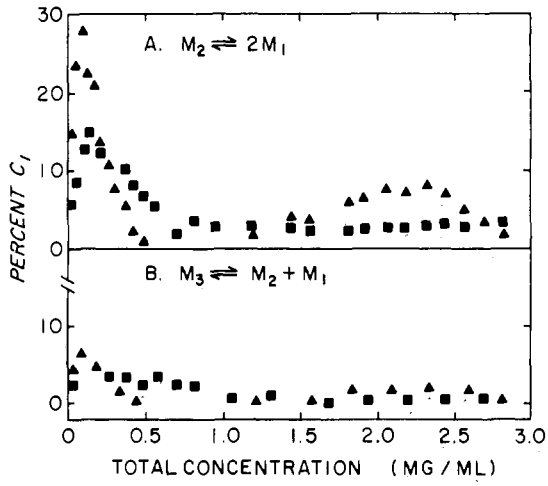

FIGURE 3: Per cent of species having a molecular weight smaller than that of phosphorylase monomer as calculated from the data of a typical experiment in 7.2 M guanidine·HCl. Data have been taken from the experiment described in Figure 2, calculated from $M_{n,r}$ [■] and $M_{w,r}$ (▲).

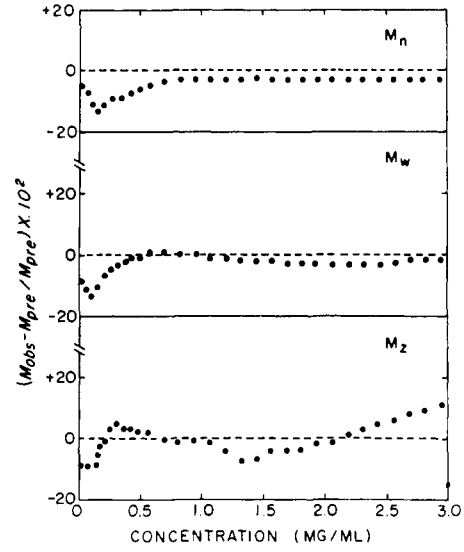

FIGURE 4: Per cent deviation of the observed molecular weight moments from those predicted for a homogeneous nonideal solute with a molecular weight of 92.5 $\times 10^3$ g per mole and a virial coefficient equal to 15.8 $\times 10^{-4}$ mole ml per g^2. M_{obsd} represents the observed molecular weight and M_{pre}, the predicted molecular weight at the specified concentration.

TABLE II: Molecular Weight Determinations of Aldolase and TDH in 7.2 M Gd·HCl and 0.1 M β-Mercaptoethanol.[a]

Enzyme	H	Expt	$(C_b + C_m)/2$[b] (mg/ml)	Mol Wt ($\times 10^{-3}$) (g/mole)[b] M_n	M_w	M_Z	M_{Z+1}	$(2M_n - M_w)_{C=0}$	$M_{w,C=0}$	$M_{n,C=0}$	Virial coeff ($\times 10^4$), mole ml/g^2 B_1 (M_w)	B_1 (M_n)
Aldolase	4.3	3	1.03	37.8 (1.1)	35.1 (1.2)	31.9 (1.2)	27.1 (2.7)	39.4 (1.8)	42.6 (1.9)	41.4 (1.7)	46.0	39.5
TDH	4.3	1	1.19	28.5	28.5	26.0	21.7	24.0 (0)	30.8 (0.9)	27.7 (0.4)	18.9	18.3
TDH	3.0	1	0.69	34.0	33.9	32.0	28.9	36.5 (0)	36.2 (0.2)	36.5 (0)	28.4	32.4

[a] The solvent also contained 0.05 M Tris·HCl and 0.0025 M EDTA (pH 7.5). [b] Numbers in parentheses are root-mean-square errors. [c] $(C_b + C_m)/2$ represents the concentration at which the apparent weight average molecular weights apply (van Holde and Baldwin, 1958).

for phosphorylase in guanidine·HCl, experiments were carried out in 7.2 M guanidine·HCl on aldolase (Kawahara and Tanford, 1966; Penhoet *et al.*, 1967) and D-glyceraldehyde 3-phosphate dehydrogenase (TDH, Harrington and Karr, 1965), two proteins whose molecular weight and subunit structure had been well investigated. An apparent specific volume of 0.729 ml/g (Harrington and Karr, 1965) for TDH in guanidine·HCl was used, while for aldolase the value of 0.742 ml/g reported for the native molecule (Taylor and Lowry, 1956) was assumed to be lowered to 0.72 ml/g (Hade and Tanford, 1967; Reisler and Eisenberg, 1969). The results are presented in Table II. TDH was examined at two different centrifugal force fields corresponding to H values (van Holde and Baldwin, 1958; Teller *et al.*, 1969) of 3.0 and 4.3, the conditions used for phosphorylase. By definition $H = M(1 - \bar{v}\rho)\cdot\omega^2(x_b^2 - x_m^2)/4RT$.

Increasing the equilibrium speed of the rotor results in an increase in the value of H when other conditions are held constant. At $H = 3$, the only indication of heterogeneity in the sample of TDH occurred below 0.25 mg/ml (Figure 5A). The values of $2M_{n,r} - M_{w,r}M_{n,r}$ and $M_{w,r}$ extrapolated to $C = 0$ are in good agreement with each other and with the subunit molecular weight reported for the enzyme from rabbit muscle (Harrington and Karr, 1965), pig muscle (Perham and Harris, 1965), and lobster muscle (Davidson *et al.*, 1967). When TDH was centrifuged under conditions comparable with those used for phosphorylase *b* ($H = 4.3$), the preparation appeared to be heterogeneous as demonstrated in Figure 5B. At infinite dilution the molecular weights were estimated to be smaller than those ascribed to the TDH subunit. In three experiments on aldolase at $H = 4.3$, a decrease in $2M_{n,r} - M_{w,r}$ was observed. When all of the data at concentrations greater than 0.25 mg/ml are combined, $(2M_{n,r} - M_{w,r})_{C=0}$ is found to be 39.4 ± 1.8 × 10^3 g/mole which is consistent with the proposal that the aldolase molecule with a molecular weight of approximately 160 × 10^3 g/mole contains four polypeptide chains (Kawahara and Tanford, 1966; Penhoet *et al.*, 1967). Thus, when the results of experiments in which $H = 4.3$ are treated in the same manner as the phosphorylase data, the correct value is obtained for the subunit of aldolase but not for TDH. Low estimates for the molecular weight of the subunit of aldolase are obtained only when data at concentrations below 0.25 mg/ml are included in the extrapolation to $C = 0$, a value of 29.5 ± 1.6 × 10^3 g per mole being calculated from all of the data above 0.08 mg/ml. It will be noted that the whole cell average molecular weight of 35 ± 1 × 10^3 g per mole (Table II) determined for aldolase agrees fairly well with the value of 37 × 10^3 g per mole reported by Kawahara and Tanford (1966) from the results of similar experiments. The two sets of data differ in that the preparation examined by Kawahara and Tanford was characterized by

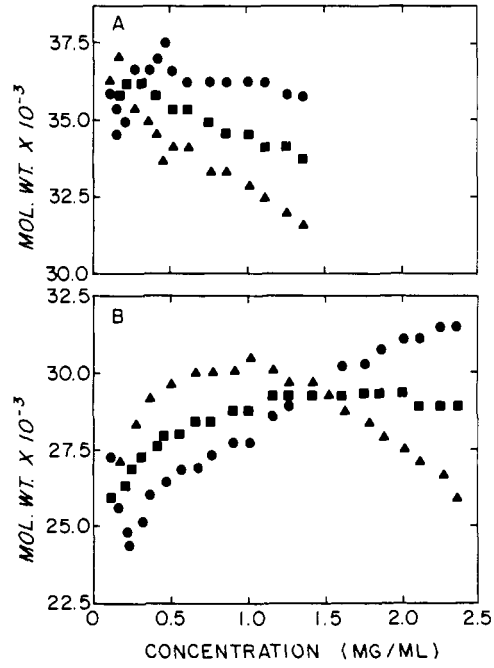

FIGURE 5: Molecular weight distributions for TDH in 7.2 M guanidine·HCl plus 0.1 M β-mercaptoethanol at 20°. The solvent also contained 0.05 M Tris·HCl and 0.003 M EDTA adjusted to pH 7.5. (A) 36,000 rpm at sedimentation equilibrium. (B) 44,000 rpm at sedimentation equilibrium. ▲ represents $M_{w,r}$; ■, $M_{n,r}$; and ●, $2M_{n,r} - M_{w,r}$.

heterogeneity, *i.e.*, $M_n < M_w < M_Z$, while the preparation used in this study exhibited nonideality, *i.e.*, $M_n > M_w > M_Z$.

Sedimentation Equilibrium Studies on Phosphorylase b in Denaturing Solvents Other than Urea or Guanidine·HCl. Phosphorylase *b* was also examined in a number of other solvents that might dissociate the enzyme. In all cases, the sulfhydryl groups were protected by either high concentrations of β-mercaptoethanol or carboxymethylation or both.

In 0.1 M acetic acid and concentrated formic acid the molecular weight distributions were similar to those obtained in 7.2 M guanidine·HCl but with greater nonideality. The values of the apparent molecular weight averages depend on the choice of the apparent specific volume. A maximum value for electrostriction can be calculated from the amino acid composition (Sevilla and Fischer, 1969). For 150 charged pairs with -17 cm³/mole per charged pair (Linderstrøm-Lang and Jacobsen, 1941) $\bar{v}$ will be decreased by approximately 0.02 ml/g. The results obtained in acetic and formic acid (Table I) were calculated by using apparent specific volume of 0.72 ml/g. Correcting the observed weight-average molecular weight in acetic acid by using the average virial coefficient obtained from $M_{n,r}$ and $M_{w,r}$ of 189×10^{-4} mole ml/g² yielded an estimate of the true M_w of approximately 90×10^3 g/mole at $(C_b + C_m)/2$. The extrapolation of $2M_{n,r} - M_{w,r}$ to $C = 0$ is somewhat uncertain for both the acetic and formic acid data due to the maxima (at 0.5 mg/ml) displayed by both $M_{w,r}$ and $M_{n,r}$ when plotted *vs.* C. At pH 9–9.5 both native phosphorylase *b* and phosphorylase *b* fully substituted with maleic anhydride were found to behave as self-associating systems with molecular weights of the smallest components varying from 75 to 100×10^3 g per mole.

In sedimentation equilibrium experiments, the iodoacetate derivative of phosphorylase *b* in sodium dodecyl sulfate exhibited nonideal behavior (Table I). The value of $2M_{n,r} - M_{w,r}$ extrapolated from all data obtained for concentrations above 0.25 mg/ml was comparable to that obtained in 7.2 M guanidine·HCl. In the presence of sodium dodecyl sulfate, the apparent specific volume, ϕ', determined by the method of Casassa and Eisenberg (1964) was found to be 0.644 ± 0.005 ml per g indicating that 1 g of protein binds approximately 0.6 g of sodium dodecyl sulfate. Radioactivity measurements of these samples indicated 0.44 g of [^{35}S]-sodium dodecyl sulfate was bound per g of protein. Considering the errors in both determinations, these are in reasonable agreement. This binding of sodium dodecyl sulfate has led to occasional reports of dissociation of phosphorylase to species of molecular weight less than 90×10^3 g per mole, as determined by sedimentation velocity experiments. However, the resulting decrease in sedimentation coefficients is due to the binding of sodium dodecyl sulfate rather than to further dissociation of the protein (Schachman, 1960).

β-Phenylpropionate caused association of phosphorylase *b*, giving relatively homogeneous species with molecular weights approximately equal to that of phosphorylase *a*. This effect was antagonized by addition of either 0.1 M acetic acid or sodium borate at pH 9.5 with molecular weights decreasing to those of the phosphorylase dimer and monomer, respectively. Finally, an attempt was made to use a 9:1 mixture of dimethyl sulfoxide and 1 M acetic acid as a solvent. However, in this solvent, the protein aggregated.

Discussion

The possibility that the phosphorylase monomer contains structural elements which are resistant to the action of guanidine·HCl cannot be excluded but appears unlikely. Tanford and coworkers have presented evidence that a series of proteins ranging in size from insulin to myosin are highly disorganized in a solvent containing 6 M guanidine·HCl and 0.1 M β-mercaptoethanol as judged by such techniques as sedimentation velocity and viscosity (Tanford *et al.*, 1967a), titration (Nozaki and Tanford, 1967), osmotic pressure (Lapanje and Tanford, 1967), and optical rotatory dispersion (Tanford *et al.*, 1967b). Within the limitations of both theory and experiments, all of the proteins which were studied could be reasonably well described as random coils in this solvent. On the other hand the calculations of Miller and Goebel (1968) suggest that segments of the polypeptide chain may not be completely dissociated in 6 M guanidine·HCl and 0.1 M β-mercaptoethanol. Nevertheless, using the equations presented by Tanford *et al.* (1967a), the viscosity behavior of phosphorylase *b* in 7.2 M guanidine·HCl corresponds to a molecular weight of approximately 91×10^3 g per mole. If the monomer were merely extended and not dissociated, the presence of one or two bonds between nonterminal portions of individual chains should decrease the intrinsic viscosity. In the case of β-lactoglobulin, two disulfide bridges lower the intrinsic viscosity by about 20% (Tanford *et al.*, 1967a). By recalculating the data for phosphorylase assuming both a 10 and a 20% decrease in $[\eta]$, molecular weights of 76 and 67 $\times 10^3$ g per mole, respectively, were obtained. The good agreement between the monomer molecular weight determined by other methods and that obtained from viscosity in guanidine·HCl suggests that intrachain bonds are absent. Experiments presented here and data in the literature indicate that typical globular proteins such as aldolase (Kawahara and Tanford, 1966) and TDH (Harrington and Karr, 1965) are dissociated to their fundamental subunits in concentrated guanidine·HCl. In addition, Small and Lamm (1966) have fractionated carboxymethylated rabbit γG-immunoglobulin into individual polypeptide chains on Sephadex G-200 in the presence of 5 M guanidine·HCl. Thus in order to postulate a subunit structure for the monomer, one must assume that the individual polypeptide chains are held together by forces which are stronger than those which have been observed in other multichain proteins. Another possibility, unlikely as it may be, could be considered, namely, that the species of molecular weight 92.5×10^3 are made up of two identical peptide chains covalently linked by their terminal groups (either as a straight chain or a cyclic peptide). Indeed, no amino or carboxyl end group has ever been detected in rabbit muscle phosphorylase (Appleman *et al.*, 1966).

In addition to the chemical evidence against this alternative (Fischer *et al.*, 1958, 1959; Nolan *et al.*, 1964; Zarkadas *et al.*, 1968), the viscosity results yield a root-mean-square end-to-end distance of 291 Å, quite consistent with the data of Tanford *et al.* (1966) for proteins in 6 M guanidine·HCl and 0.1 M β-mercaptoethanol.

The virial coefficients observed in this study are larger than those found by Lapanje and Tanford (1967). The reason for the discrepancy is probably due to the use of buffered solutions in this study, so that the subunits have a

net electrostatic charge. Indeed, the virial coefficient for the iodoacetate derivative is larger than that observed for the iodoacetamide derivative. Based on the data of Lapanje and Tanford (1966), the expected virial coefficient for phosphorylase is 6.06×10^{-4} ml/mole g^2. If we ascribe the excess virial coefficient to charges distributed on a rigid molecule (Tanford, 1961), the net charge is 71, while from the amino acid composition (C. B. Sevilla and E. H. Fischer, unpublished data) the net charge should be 12 at pH 7.5. The discrepancy in the data is almost certainly due to the flexibility of phosphorylase in 7.2 M guanidine·HCl.

The cause of the decrease in $(2M_{n,r} - M_{w,r})_{C=0}$ to values below that of the monomer is not certain. Assuming simple models for the subunit structure of the monomer, as pointed out earlier, the results in 7.2 M guanidine·HCl cannot be interpreted as representing more than 5% of dissociation. Even if a minimum value of 0.72 ml/g is used for the apparent specific volume in guanidine·HCl, the concentration of dissociated material calculated for two chains of equal size is found to be less than 25% of the original concentration of monomer. The effects of small errors in the base-line corrections result in changes in the point-by-point molecular weights below 0.5 mg/ml of approximately 5% (Teller *et al.*, 1969). In order to bring about a decrease in $M_{n,r}$ and $M_{w,r}$ in such a reproducible manner, these errors would have to be systematic rather than random. While systematic shifts in base lines can and do occur, the results appear anomalous in ways other than those observed in this study (T. A. Horbett and D. C. Teller, in preparation). The assignment of an absolute value for the protein concentration at the meniscus is another potential source of error. However, in order to raise both $M_{n,r}$ and $M_{w,r}$ at concentrations below 0.5 mg/ml to the values predicted for a homogeneous, nonideal solute of 92.5×10^3 g per mole, a negative value must be assigned to the protein concentration at the meniscus. Since negative concentrations have no physical meaning, the computational procedures could not be expected to give rise to the low values of $M_{n,r}$ and $M_{w,r}$. Moreover, when the computer program was tested using perfect data for a nonideal solute with the properties of the phosphorylase monomer, the extrapolated value of $2M_{n,r} - M_{w,r}$ was slightly greater than 92.5×10^3 g per mole and neither $M_{n,r}$ nor $M_{w,r}$ exhibited a maximum when plotted as a function of concentration. The decrease in $M_{n,r}$ and $M_{w,r}$ at low concentrations is most simply interpreted as due to the presence of low molecular weight material still contaminating the enzyme preparation after several recrystallizations. Such material does not appear to be formed in the guanidine·HCl solvent as, for example, by the polymerization of guanidine molecules, for two reasons. First, no change was observed in the guanidine·HCl–guanidine base-line patterns before or after prolonged dialysis indicating that no nondialyzable material was formed in the solvent or extracted from the dialysis bag. Secondly, $M_{n,r}$ and $M_{w,r}$ also decreased as the protein concentration approached zero in acetic acid and formic acid. Although peptide bonds might be split in acetic acid and formic acid, there is no evidence for their rupture in guanidine·HCl. In the present experiments, the equilibrium pattern for a given preparation was not significantly altered when the initial 3 days of treatment with guanidine·HCl was extended to 17 days. On the other hand, a small amount of contamination of the preparations with low molecular weight proteins which remain bound to phosphorylase during purification could easily account for the heterogeneity. When the meniscus region of the equilibrium cell is depleted of the major component by the use of high centrifugal force fields, the presence of small molecules could decrease the molecular weight in that region. At lower force fields, such molecules might not be detected. It is not unreasonable to speculate that all proteins contain bound peptides at concentrations which are generally below the sensitivity of most physical and chemical techniques. The experiments on the other proteins that served as controls for these studies were too limited to provide convincing support for this proposal. If the data obtained at $H = 4.3$ for aldolase and TDH are treated exactly as the phosphorylase data, the right value is obtained for the subunit of aldolase but not for TDH.

The fact that the aldolase preparation exhibited heterogeneity only at concentrations below 0.25 mg/ml might be interpreted as indicating that the aldolase preparations contained a smaller amount of low molecular weight material than either the phosphorylase or TDH preparations. In summary, even though the true explanation for the decrease in $(2M_{n,r} - M_{w,r})_{C=0}$ observed for phosphorylase in 7.2 M guanidine·HCl is unknown, dissociation of the 92.5×10^3 g per mole subunit is not indicated.

The deviations from the predictions observed in Figure 4 could potentially arise from nonidentical subunits. The possibility that the monomers of phosphorylase are nonidentical has been suggested by Valentine and Chignell (1968). Although this possibility cannot be excluded, no chemical evidence presently available supports this hypothesis (Nolan *et al.*, 1964; Zarkadas *et al.*, 1968). Similarly, since errors in $M_{n,r}$ are propagated throughout the solution columns in sedimentation equilibrium experiments (Teller *et al.*, 1969) we believe that deviations of $M_{n,r}$ in Figure 4 are due to experimental error and contamination by light material rather than to the existence of nonidentical subunits.

Phosphorylase exists in the monomer form in guanidine·HCl, sodium dodecyl sulfate, and formic acid. Although the molecular weight moments found in acetic acid are lower than 92.5×10^3 g per mole, the large virial coefficient makes the extrapolation to infinite dilution in protein rather hazardous, and probably the third as well as the fourth virial terms are nonnegligible in this case. Taken together, the data of Table I provide no reliable evidence for dissociation beyond that of the phosphorylase monomer.

Thus, phosphorylase may contain a fundamental subunit which is larger than those found in most other proteins. Recently, another pyridoxal 5′-phosphate containing enzyme, arginine decarboxylase, has been found to have a molecular weight of the order of 80×10^3 g per mole in both 5 M guanidine·HCl–0.1 M β-mercaptoethanol and 70% formic acid (Boeker and Snell, 1968). β-Galactosidase with a molecular weight of 134×10^3 in 6 M guanidine·HCl (Ullmann *et al.*, 1968) and the major component of about 200×10^3 g per mole observed for myosin preparations in 5 M guanidine·HCl (Dreizen *et al.*, 1966) may, among other examples, also fall into this category (see also Klotz and Darnall, 1969, and Weber and Osborn, 1969).

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