A Reinvestigation of the Molecular Weight of Glycogen Phosphorylase*

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ABSTRACT: The molecular weights of skeletal muscle phosphorylases *a* and *b* have been investigated by high-speed sedimentation equilibrium, low-speed sedimentation equilibrium, and sedimentation and diffusion in the analytical ultracentrifuge. The molecular weight of phosphorylase *b* has been determined to be 185 $\times 10^3$ g/mole by high-speed sedimentation equilibrium. The value obtained by sedimentation and diffusion was 188 $\times 10^3$ g/mole. By low-speed sedimentation equilibrium experiments the molecule has been shown to be slightly associated in relatively dilute solution. Phosphorylase *a*, on the other hand, exhibits slight dissociation in solution as demonstrated by high-speed sedimentation equilibrium. From experiments in a variety of buffers, temperatures, and ionic strength, the molecular weight has been estimated to be 370 $\times$ 10^3 g/mole. Higher values of the molecular weight were obtained for phosphorylase *a* when it was crystallized in the absence of reducing thiol. However, addition of β-mercaptoethanol to such preparations decreased the molecular weight to that obtained when thiol was present throughout the purification procedure. In spite of the doubling of molecular weight the frictional ratio remains the same in the conversion of phosphorylase *b* to *a*. Phosphorylase has also been studied in urea and guanidine hydrochloride. Alkylated phosphorylase *b* in 8 M urea yielded molecular weight estimates in the range 70–80 $\times 10^3$ g/mole but exhibited heterogeneity of molecular weight. Both 3.6 and 5.4 M guanidine hydrochloride with 0.1 M β-mercaptoethanol produced homogeneous molecular weight behavior with molecular weights of 100 and 95 $\times 10^3$, respectively.

In 7.2 M guanidine hydrochloride containing 0.1 M β-mercaptoethanol some heterogeneity of molecular weights was observed with values comparable to those in urea but with greater nonideality. This heterogeneity has been interpreted as due primarily to molecular weight heterogeneity and not preferential interactions with the two-component solvent.

Rabbit muscle glycogen phosphorylase (EC 2.4.1.1) exists in two states of aggregation: phosphorylase *b*, a dimer inactive in the absence of AMP[1] and phosphorylase *a*, a tetramer active in the absence of this nucleotide (Brown and Cori, 1961). Conversion of phosphorylase *b* to *a* is effected by means of Mg^{2+}, ATP, and phosphorylase kinase (EC 2.7.1.38). Phosphorylase *a* is converted back to *b* by phosphorylase phosphatase (EC 3.1.3.17). These two enzymes are part of an elaborate system of control involving other enzymes, nucleotides, metal ions, hormones, etc. (Krebs and Fischer, 1962; Krebs *et al.*, 1966; Fischer and Krebs, 1966). Both phosphorylases *a* and *b* dissociate when treated with an excess of *p*-mercuribenzoate; nonphosphorylated units in the case of *b* and phosphorylated monomers in the case of *a* (Madsen and Cori, 1956).

Two sets of data, difficult to reconcile with the accepted values for the molecular weight of phosphorylases *a* and *b* (495 and 248 $\times 10^3$ g/mole, respectively), necessitated reassessment of these figures. First, analysis of pyridoxal 5′-phosphate (PLP) in freshly crystallized preparations of phosphorylase *b* or *a* consistently gave too high stoichiometric ratios, averaging 2.5 $\pm$ 0.2 moles of PLP/250 $\times 10^3$ g (Kent *et al.*, 1958). Furthermore, titrations of crystalline apophosphorylase *b* with PLP gave typical saturation curves with maximum activity attained after addition of 1 mole of PLP to 92 $\pm$ 2 $\times 10^3$ g of protein (C. B. Sevilla and E. H. Fischer, unpublished data).

Secondly, attempts to fully disaggregate phosphorylase in 8 M urea or in low molarities of guanidine hydrochloride yielded material in the molecular weight range of 70–100 $\times 10^3$ g/mole, much lower than the expected 125 $\times 10^3$ g/mole which would have been observed if phosphorylase were fully dissociated and the *p*-mercuribenzoate subunit (Madsen and Cori, 1956) were the fundamental monomer. At the same time the data were not consistent with a model for a fundamental subunit of 62 $\times 10^3$ g/mole in these solvents.

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[1] Abbreviations: AMP, adenosine 5′-monophosphate; ATP, adenosine triphosphate; glucose-1-P, glucose 1-phosphate; glucose-6-P, glucose 6-phosphate; PLP, pyridoxal 5′-phosphate.

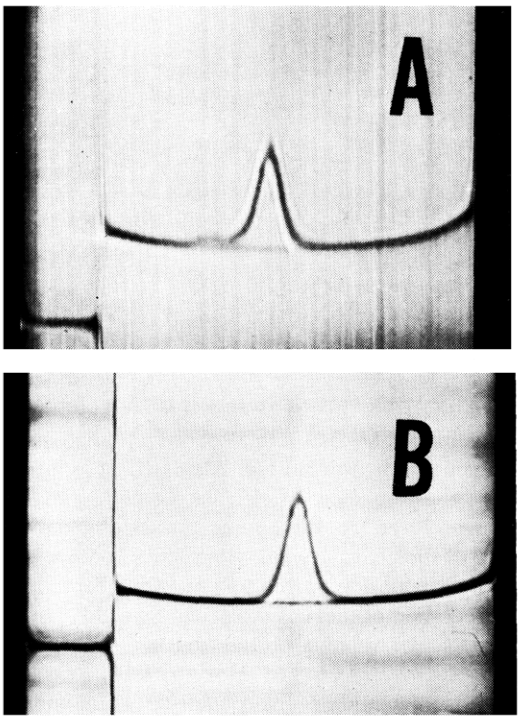

FIGURE 1: Sedimentation velocity and schlieren diagram of three-times-crystallized phosphorylase *a*. (A) Sedimentation velocity of three-times-crystallized phosphorylase *a* prepared by method I in the presence of thioglycolate. Sedimentation 22 min at 59,000 rpm. (B) Schlieren diagram of three-times-crystallized phosphorylase *a* prepared by method II in β-mercaptoethanol.

A major difficulty in the determination of molecular weights of an associating–dissociating system of the complexity of phosphorylase is that one is rarely certain which condition might favor one state of aggregation rather than another. Consequently, the experiments which are reported here have been performed under a variety of conditions. The results in all cases appear to favor the hypothesis that phosphorylase *a* is always slightly dissociated, whereas phosphorylase *b* is always slightly associated in relatively dilute solutions.

Materials and Methods

Urea was the product of J. T. Baker Chemical Co. and recrystallized from ethanol in the presence of 0.005 M 8-hydroxyquinoline. Guanidine hydrochloride was purchased from Eastman Organic Chemicals and purified by recrystallization from methanol after charcoal treatments. The optical density of 7.2 M solutions was always less than 0.05 at 280 mμ in the 1-cm cell. Sodium glycerophosphate and glucose 1-phosphate (Nutritional Biochemical Co.) were recrystallized from ethanol prior to use. Iodoacetate (Fischer Scientific) was recrystallized from petroleum ether, iodoacetamide (Nutritional Biochemical Co.) from ethyl acetate. AMP (Pabst Laboratories) and glucose 6-phosphate (Calbiochem) were used without further treatment.

Samples of phosphorylase *b* were prepared according to the method of Fischer *et al.* (1958) and recrystallized two to three times. The AMP which was used for crystallization was removed on a Norit cellulose column of 2 × 1 cm; the $A_{260}:A_{280}$ ratio of all preparations was less than 0.54. Protein concentration was determined spectrophotometrically using the absorbancy index at 278 mμ (1%, 1 cm) of 11.9 (Appleman *et al.*, 1963). The only time that this coefficient was of any importance in these studies was in the partial specific volume determinations; for all other experiments fringe numbers suffice to define the concentration scale. Enzymatic assays were carried out following the procedure of Hedrick and Fischer (1965) except that 0.05 M sodium glycerophosphate buffer was used instead of the maleate buffer.

Two methods were used for the preparation of phosphorylase *a*. For preliminary experiments, phosphorylase *a* was prepared according to the method of Fischer *et al.* (1958) with 0.03 M thioglycolate in place of the cysteine used in the original purification. Hereafter we shall denote the phosphorylase *a* obtained by this procedure as method I phosphorylase *a*. In order to have enzyme of the highest purity for sedimentation equilibrium experiments, the twice-recrystallized method I protein was further treated by chromatography on TEAE-cellulose and concentrated by ultrafiltration. Since the recovered protein was too dilute to form crystals, it was equilibrated with buffer by dialysis prior to ultracentrifugation.

As judged by the schlieren pattern in sedimentation velocity, preparations made by this method were found to contain a considerable amount of light material (Figure 1A) which could not be removed by recrystallization. The percentage of the observed light material was variable and increased with time. In contrast, the phosphorylase *a* which has been obtained by a modified procedure (denoted here as method II) using β-mercaptoethanol as the sulfhydryl compound was essentially homogeneous in sedimentation velocity as shown in Figure 1B. The details of this modified preparation for phosphorylase *a* are given below (J. M. Vidgoff and E. H. Fischer, unpublished data).

Method II phosphorylase *a* was prepared from the phosphorylase *b* which emerges as a first fraction from a TEAE-cellulose column during the purification of glycogen synthetase (Hickenbottom, 1967) or phosphorylase–phosphatase (Hurd, 1966). In these preparations the glycogen–phosphorylase *b* complex obtained by sedimentation at 80,000*g* for 1 hr (Krebs *et al.*, 1964) was digested with approximately 0.03 mg/ml of crystalline human salivary amylase for 1 hr at 30° in the presence of 0.01 M β-mercaptoethanol. The digest was clarified by centrifugation at 40,000*g* for 20 min and applied to a TEAE-cellulose column. The fractions collected during the wash of the column with equilibration buffer (0.05 M Tris-HCl–0.05 M NaCl–0.01 M β-mercaptoethanol–0.001 M EDTA,

pH 7.5) contained phosphorylase *b*. The pH of the pooled fractions was raised to 8.5 with 2 M Tris (free base), then phosphorylase *b* kinase, 10^{-3} ATP, and 10^{-2} M Mg^{2+} were added. After approximately 1 hr at 30°, the pH was lowered to 7 by the addition of acetic acid. Crystals of phosphorylase *a* which formed when the solution was placed at 4° overnight were collected by centrifugation. For recrystallization, the pellet was dissolved in 0.02 M Tris-HCl–0.005 M EDTA buffer at pH 7.2 containing 0.2 M KCl and dialyzed against the buffer without KCl. Two to three recrystallizations were made on all preparations. One sample of method II phosphorylase *a* was further purified by chromatography on TEAE-cellulose. The phosphorylase *a* fraction was concentrated by ultrafiltration and crystallized. This sample behaved identically in terms of ultracentrifuge properties to the three-times-crystallized starting material, so that this extra step was routinely omitted.

The recrystallization process proceeded well either with or without the addition of β-mercaptoethanol. For two preparations of the enzyme by this method β-mercaptoethanol was added to the crystallization buffer. For one sample of enzyme the effect of absence of thiol was to be tested; this sample also crystallized readily. The physical properties of the enzyme were different from that prepared with β-mercaptoethanol, as discussed in Results, but upon addition of β-mercaptoethanol to the dissolved crystals, the sample was identical with that crystallized in the presence of β-mercaptoethanol.

The specific activity of a typical sample of method II phosphorylase *a* was found to be 76 units/mg when assayed in the presence of AMP comparable to 80 units/mg reported by Hedrick and Fischer (1965). A single band was observed when the material was examined by disc gel electrophoresis according to the method of Davis *et al.* (1967). The physical homogeneity and reproducibility of preparation of this material lead to its choice as the molecule to study as a contrast to phosphorylase *b*. We attribute this stability to the use of β-mercaptoethanol rather than thioglycolate in the preparation of the enzyme. A sample of method II phosphorylase *a* stored in thioglycolate for 3 months was observed to exhibit significantly more light material than the control stored in β-mercaptoethanol (Figure 2). More recent samples of the enzyme prepared by method I in the presence of β-mercaptoethanol rather than thioglycolate exhibit homogeneity comparable to that of the method II enzyme. Thus we conclude that the thioglycolate is primarily responsible for the observed physical heterogeneity of phosphorylase *a* under our conditions.

For the ultracentrifuge experiments in guanidine hydrochloride phosphorylase *b* (1.5–3 mg/ml) was dissolved in 7.2 M guanidine hydrochloride containing 0.05 M Tris-HCl–0.0025 M EDTA (pH 7.5) and equilibrated by dialysis against solvent for 2 weeks. Just prior to ultracentrifugation, protein and solvent were made 0.1 M in β-mercaptoethanol. For experiments in 7.2 M guanidine hydrochloride the protein was diluted 1:1 with the solvent to yield a final concentration of 0.75 mg of protein/ml. For lower concentrations of guanidine hydrochloride, gravimetric dilutions of stock protein and solvent were made. In all cases the final concentrations of β-mercaptoethanol was 0.1 M.

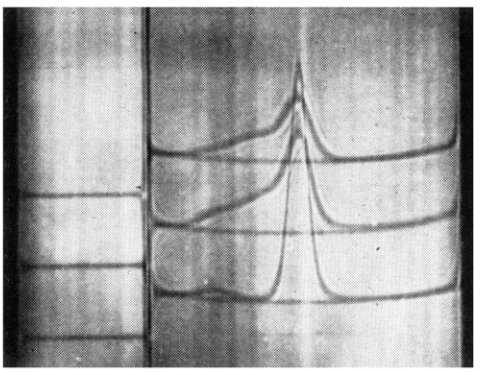

FIGURE 2: Effect of storage for 3 months on phosphorylase *a* in thioglycolate (upper patterns) and β-mercaptoethanol (lowest schlieren pattern). Sedimentation at 52,000 rpm for 28 min.

Phosphorylase *b* used for experiments in 8 M urea was treated with 0.1 M β-mercaptoethanol (a 1000-fold molar excess of β-mercaptoethanol over the concentration of the protein's sulfhydryl groups) and alkylated with a fivefold excess of iodoacetate or iodoacetamide over total SH according to the method of Craven *et al.* (1965) except with the substitution of Tris-HCl for the bicarbonate buffer. Amino acid analyses performed on similarly alkylated samples did not detect any free cysteine residues within the limit of experimental error. Prior to ultracentrifugation dry protein was dissolved in 8 M urea containing 0.2 M NaCl, 0.005 M EDTA, and 0.05 M Tris-HCl. The buffer was preadjusted to pH 7.5 before addition of solid urea. Owing to the high viscosity and density of the medium, sedimentation velocity experiments were carried out for about 10 hr at 60,000 rpm at 20°.

Ultracentrifugation was performed in a Spinco Model E analytical ultracentrifuge equipped with electronic speed control and focused at the two-thirds plane of the cell (Svensson, 1954, 1956; Yphantis, 1964).

For most of the molecular weight determinations, high-speed equilibrium (Yphantis, 1964) was used with the modification that higher initial concentrations and lower speeds were used. Low-speed sedimentation equilibrium was performed according to the method of Richards and Schachman (1959). In order to reduce the time to sedimentation equilibrium, the overspeeding–underspeeding technique of Richards (1960) was applied for low-speed experiments while a modified overspeeding–underspeeding procedure was used for the high-speed experiments (D. C. Teller, E. G. Richards, and H. K. Schachman, unpublished data). The cell utilized for high-speed equilibrium consisted of the six-channel Kel-F centerpiece described by Yphantis

TABLE I: Molecular Weight of Phosphorylase *b* in High-Speed Equilibrium.

Expt	Treatment[a]	Temp (°C)	Init Concn (mg/ml)	Molecular Weights ($\times 10^{-3}$) (g/mole) M_n	M_w	M_z	M_{z+1}	$(2M_n - M_w)_{c=0}$
A-1	None	5	0.25	165	175	188	198	152
A-2	None	5	0.50	184	189	199	202	176
A-3	None	5	0.75	182	189	198	207	166
B-1	None	20	0.2	190	191	198	211	187
B-2	None	20	0.4	195	195	195	196	196
B-3	None	20	0.6	183	188	196	198	174
C-1	Buffer + 0.01 M G-6-P[b]	20	0.2	194	194	197	199	193
C-2	Buffer + 0.01 M G-6-P[b]	20	0.4	193	188	176	138	194
C-3	Buffer + 0.01 M G-6-P[b]	20	0.6	192	191	187	180	193
D-2	Buffer + 0.01 M G-6-P	20	0.6	193	196	197	199	186
D-3	Buffer + 0.01 M G-6-P	20	0.6	191	194	207	262	184
E	None	20	0.2	204	199	194	185	201
F	None	5	0.2	198	199	200	200	196
Av ± RMS[c]				190 ± 9	191 ● 6	195 ± 7	198 ± 25	185 ± 14

[a] Buffer is 0.02 M sodium glycerophosphate–0.03 M β-mercaptoethanol–0.06 M NaCl, adjusted to pH 6.8 with HCl at 20°. [b] Glucose 6-phosphate. [c] Root mean square deviation.

(1964). For low-speed work multiple cells with appropriate masks were used in the AN F rotor (Teller, 1965).

Photographic plates of both the equilibrium and water–water base lines were read on a modified Nikon microcomparator (Teller, 1967) up to the concentration point at which the fringes could no longer be resolved. Extrapolation to the base of the cell, when performed, did not change the molecular weights significantly. Data reduction was performed on the IBM 7090–7094 IBSYS system using computer programs developed in this laboratory.

For all sedimentation velocity experiments, centrifugation was carried out at 20° in double-sector cells and continued until the plateau region was lost. The position of the boundary with time was measured from the schlieren diagrams using a Nikon microcomparator. For concentration-dependence studies, sedimentation coefficients were evaluated at the initial concentration of solute.

Diffusion coefficients were determined in the ultracentrifuge at 20° using a synthetic boundary cell. The layering of solvent which occurred at about 3000 rpm was carefully observed to detect convective disturbance. When convection was observed, the experiment was repeated. The spread of the boundary with time was photographed using the schlieren optical system at various bar angles and the photographs were traced on graph paper from the screen of the Nikon microcomparator with 20 times enlargement. Diffusion coefficients were calculated by plotting the square of the inflection point *vs.* time, which is proportional to 2 D_{app} (Svedberg and Pedersen, 1940). At the speeds used, the correction for sedimentation was negligible (Schachman, 1957). Concentration dependence was evaluated at one-half the initial solute concentration.

The partial specific volume calculated from amino acid analysis by the method of McMeekin *et al.* (1949) was 0.737 ml/g (Appleman *et al.*, 1963). The apparent specific volume of phosphorylase *b* was also determined from density measurements at 20° using a Cahn electrobalance. The protein was dialyzed at a concentration of *ca.* 70 mg/ml at least 24 hr at 4° against 0.02 M sodium glycerophosphate–0.06 M NaCl–0.002 M EDTA (pH 6.8). The value determined as the average of these measurements was 0.739 ± 0.004 ml/g; nearly equal to that calculated from the amino acid composition but slightly lower than that previously reported (Keller and Cori, 1953). The apparent specific volume of the phosphorylase *b*–guanidine hydrochloride system was determined according to the method of Casassa and Eisenberg (1964). For this measurement, phosphorylase *b* was dialyzed at a concentration of *ca.* 35 mg/ml for 5 weeks at room temperature against 7.2 M guanidine hydrochloride with the same buffer system as used in the ultracentrifugation. The long

period of dialysis was used to ensure osmotic equilibrium. The protein solution at the end of this time formed a highly viscous gel which was solubilized by adding 10 μl of the 15 M β-mercaptoethanol to a weighed amount (*ca.* 2 ml) of gel sample. The same amount of β-mercaptoethanol was added to the solvent before density measurements were performed. An absorbancy index of 12.2 (1%, 1 cm) at 277 mμ was determined by weighing a protein solution into 7.2 M guanidine hydrochloride and comparing the spectrum with that obtained by a known dilution of the protein in a low salt buffer. The value of the apparent specific volume determined in this way was 0.736 ml/g, identical within experimental error to that of the native enzyme. The apparent specific volume of phosphorylase *a* was assumed to be 0.737 ml/g, since the amino acid composition is identical with that of phosphorylase *b*. For experiments at low temperature the correction factor of $d\bar{v}/dT = 0.0005$ ml/g per deg (Svedberg and Pedersen, 1940) was applied to the apparent specific volumes.

Results

Sedimentation Equilibrium and Velocity Studies on Phosphorylase b. Since skeletal muscle phosphorylase exists either in the form of a tetramer (phosphorylase *a*) or a dimer (phosphorylase *b*), it could not be predicted whether a particular experimental condition might favor one form or the other. Preliminary high-speed sedimentation equilibrium experiments on phosphorylase *b* yielded weight-average molecular weights of the order of 190×10^3 considerably lower than the expected value of 250×10^3. One possible explanation for these results was that under our conditions, the molecule dissociated during ultracentrifugation. Consequently, attempts were made to establish optimum conditions which would prevent dissociation and favor the dimeric form of the enzyme: (a) the temperature was varied; (b) the time required to reach equilibrium was decreased by the use of shorter columns and an overspeeding–underspeeding procedure; (c) the concentration was increased and experiments performed by low-speed sedimentation equilibrium; and (d) experiments were performed in the presence of glucose-6-P. This substrate analog has been shown to affect the structure of the enzyme since it prevents removal of PLP from the enzyme in the presence of imidazole–citrate buffer (Shaltiel *et al.*, 1966) and blocks hybridization of smooth and skeletal muscle phosphorylases (Davis *et al.*, 1967), both reactions are assumed to proceed through monomerization of the enzyme.

Table I presents a summary of the results from high-speed equilibrium experiments. Under all of the conditions employed, no significant change could be observed in the various molecular weight averages. In Figure 3 is presented a typical distribution of molecular weight with concentration within a single cell. These data exhibit slight inhomogeneity. By assuming that rather than dissociation, some association of the phosphorylase *b* dimer occurred and using the approximation that the smallest major component (M_1) in a dimerizing system is given by $2M_n - M_w$ (Yphantis, 1964), we obtained a molecular weight of phosphorylase *b* of $185 \pm 14 \times 10^3$ g/mole when the data from each cell were extrapolated to infinite dilution. It is to be noted that the last two entries of Table I were experiments performed in 30-mm centerpieces so that the concentration at which the first reliable data may be obtained (0.03 mg/ml) is 2.5-fold less than for the usual high-speed experiments with 12-mm centerpieces (0.075 mg/ml). These experiments did not show any evidence of dissociation of phosphorylase *b* either at 5 or 20°. Thus, we have concluded that the apparent heterogeneity of phosphorylase *b* is due to slight association of the 185×10^3 mol wt molecules.

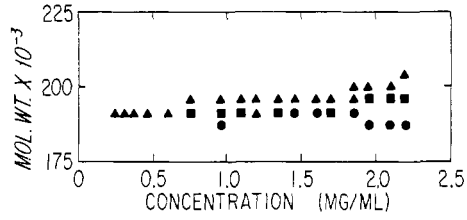

FIGURE 3: Representative distribution of molecular weight moments for phosphorylase *b* obtained in expt D-2 (Table I). ▲ represents $M_{w,z}$; ■ represents $M_{n,z}$; and ● represents $2M_{n,z} - M_{w,z}$; 12,000 rpm at sedimentation equilibrium.

In order to establish that the heterogeneity of molecular weight can be characterized by an association reaction, low-speed sedimentation equilibrium experiments were performed. It is clear from the results of these experiments (Table II) that both M_w and M_z increase with increasing concentration, consistent with the conclusion that there are weak interactions between phosphorylase *b* dimers causing a slight amount of tetramer to be formed.

In sedimentation velocity experiments, phosphorylase exhibited only slight concentration dependence as shown in Figure 4A. The least-squares equation of the form $s = s^0(1 - kc)$ which gives the best fit to these data is $s^0_{20,w} = 8.42 \pm 0.03$ S and $k = 0.005 \pm 0.003$ ml/mg. The value of 8.42 S for $s^0_{20,w}$ is in close agreement with the value of 8.2 S reported by Keller and Cori (1953). However, the diffusion coefficient of 4.2×10^{-7} cm^2/sec (Figure 4B) obtained at infinite dilution differs significantly from the value of 3.3×10^{-7} cm^2/sec reported by Green (1944). Combination of $s^0_{20,w}$ and $D^0_{20,w}$ yields a molecular weight of 188×10^3 g/mole in excellent agreement with the results of high-speed sedimentation equilibrium.

Sedimentation Equilibrium and Velocity Studies of Phosphorylase a. As mentioned in the Methods section, preparations of phosphorylase *a* made by the procedure outlined as method I were quite variable in physical

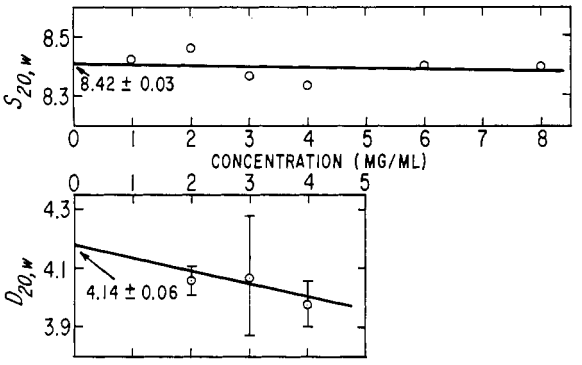

FIGURE 4: Concentration dependence studies. (A) (top) Concentration dependence of the sedimentation coefficient for phosphorylase *b*. (B) (bottom) Concentration dependence of the diffusion coefficient of phosphorylase *b*. In both cases, experiments were carried out at 20° in a buffer containing 0.02 M sodium glycerophosphate–0.002 M EDTA–0.03 M β-mercaptoethanol–0.1 M NaCl (pH 6.8). Error bars in this figure are standard errors in the x_i^2 *vs.* *t* plots. The negative slope, although not statistically significant, is characteristic of associating systems.

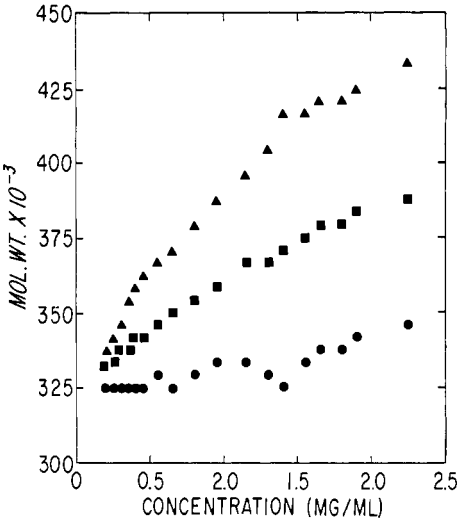

FIGURE 5: Phosphorylase *a* prepared by method I in 0.03 M thioglycolate, 5°. The buffer also contained 0.04 M Tris–HCl–0.001 M EDTA–0.06 M NaCl (pH 7.2). ▲ represents $M_{w,z}$; ■ represents $M_{n,z}$; and ● represents $2M_{n,z} - M_{w,z}$ for 7000 rpm for 1354 min.

properties, although relatively constant in enzymatic properties. A summary of four sedimentation equilibrium experiments on this material is presented in Table III, line 1, and a representative distribution of molecular weights in a single cell is given in Figure 5.

TABLE II: Summary of Molecular Weights for Phosphorylase *b* Low-Speed Equilibrium.[a]

Init Concn (mg/ml)	Method of Fringe Labeling[c]	Molecular Weights ($\times 10^{-3}$) (g/mole)[b]			
		M_w	M_m	M_b	M_z
2.08	CM	194	187	217	230
2.67	WL	193	198	203	205
	CM	192	207	200	197
6.45	WL	205	196	214	226
	CM	203	196	219	234

[a] All experiments were performed at 5° in a solvent containing 0.02 M sodium glycerophosphate, 0.03 M β-mercaptoethanol, and 0.06 M NaCl adjusted to pH 6.8 with HCl at 20°. [b] M_w = weight-average molecular weight. M_m = weight-average molecular weight at the meniscus. M_b = weight-average molecular weight at the cell bottom. M_z = *z*-average molecular weight. [c] WL is the white light method of fringe number assignment; CM is the mass conservation method (Richards and Schachman, 1959).

It is evident from these data that such variability would make analysis of this system very difficult.

In contrast to the variability of enzyme prepared by method I, method II gave relatively reproducible results. Table III (line 2) presents the molecular weight moment distribution for many runs at ionic strength less than 0.3 M. A representative sample is also depicted in Figure 6. Fifteen experiments on three separate preparations in three different buffers are included in the data of line 2 of Table III. Adsorption of the phosphorylase *a* to TEAE followed by elution with high salt did not change the distribution of molecular weights, and these experiments are included in the data of Table III (line 2).

In addition to sedimentation equilibrium experiments, sedimentation velocity and diffusion experiments yielded the parameters in the equation $s = s^0(1 - kc)$ as $s^0_{20,w} = 13.52 \pm 0.10$ S, $k = 0.007 \pm 0.002$ ml/mg. A single diffusion experiment with an initial concentration of 5.50 mg/ml yielded $D_{20,w} = 3.31 \times 10^{-7}$ cm²/sec. At 2.75 mg/ml, $s_{20,w} = 13.26$ S, to that $M_{s/D,\,app} = 370 \times 10^3$ g/mole. This figure is in very good agreement with the high-speed equilibrium results presented above. The sedimentation coefficient is comparable to the value reported by Keller and Cori (1953). However, as in the case of phosphorylase *b*, the diffusion coefficient is significantly different. A similar buffer to that of Keller and Cori (1953) was used in this experiment, except that β-mercaptoethanol was included in our buffer.

Taken together, the data in line 2 of Table III, the

TABLE III: Molecular Weight of Phosphorylase *a*.

	Prepn Method	Expt	Buffer[a]	Molecular Weight ($\times 10^{-3}$) (g/mole) $(2M_n - M_w)_{c=0}$ ± RMS	M_n ± RMS	M_w ± RMS	M_z ± RMS
1	I	4	a	335 ± 30	364 ± 14	394 ± 25	444 ± 22
2	II	3	b				
		8	c	355 ± 16	361 ± 10	364 ± 10	370 ± 8
		2	d				
		2	e				
3	II	2	f	320 ± 4	352 ± 3	365 ± 0	375 ± 5
4	II + 0.001 M AMP	3	c	335 ± 9	350 ± 5	365 ± 2	381 ± 16
5	II, no β-mercapto-ethanol	1	g	385	434	470	533
6	II, no β-mercapto-ethanol	1	h	364	395	425	496

[a] Buffer: a = 0.04 M Tris-HCl–0.03 M thioglycolic acid–0.06 M NaCl (pH 7.2), 5°; b = 0.05 M Tris-HCl–0.002 M EDTA–0.03 M β-mercaptoethanol–0.25 M NaCl (pH 7.1), 20°; c = 0.02 M Tris-HCl–0.005 M EDTA–0.05 M β-mercaptoethanol–0.18 M KCl (pH 7.1), 20°; d = same as c but TEAE-treated enzyme, 20°; e = 0.03 M sodium glycerophosphate–0.002 M EDTA–0.02 M β-mercaptoethanol–1% KCl (pH 6.8), 20°; f = same as c but 0.30 M KCl, 20°; g = same as e but without β-mercaptoethanol; and h = same as c, but without β-mercaptoethanol.

value obtained from sedimentation and diffusion, and the molecular weight established for phosphorylase *b* strongly indicate that the molecular weight of phosphorylase *a* is 370 × 10^3 g/mole. The values of Table III which are slightly less than this figure may be ascribed to slight dissociation of the tetrameric species under the conditions of these experiments.

Line 3 of Table III presents the result of an experiment performed in 0.3 M KCl. Phosphorylase *a* appeared somewhat more dissociated than under the conditions shown in line 2 of this table as reflected by $(2M_n - M_w)_{c=0}$. A similar amount of dissociation was observed (line 4, Table III) in the experiment carried out in the presence of 0.001 M AMP. However, the amount of dissociation observed under these two conditions is not great.

In order to determine whether the presence of thiol was responsible for the difference in diffusion coefficients reported previously and that obtained here, a preparation of phosphorylase *a* was crystallized in the absence of thiol. Under these conditions a second protein peak with $s_{20,w}$ of 19.4 S was observed in addition to the main peak of $s_{20,w}$ of approximately 14.5 S. The diffusion coefficient evaluated at this same concentration was 2.95 × 10^{-7} cm^2/sec, somewhat lower than that obtained in the same preparation in the same buffer with β-mercaptoethanol (reported above). $M_{s/D,app}$ from these values was calculated to be 454 × 10^3 g/mole. The value of the diffusion coefficient obtained here is somewhat greater than that reported previously (Keller and Cori, 1953). Sedimentation equilibrium experiments in the absence of thiol yield quite a disperse mixture of components as seen in Figure 7. The data from two experiments performed in the absence of thiol are presented in Table III (lines 5 and 6). Line 5 corresponds to the buffer in which $M_{s/D}$ was determined. Clearly, the values obtained by sedimentation diffusion and by sedimentation equilibrium are in reasonable agreement for such a disperse system.

Sedimentation Equilibrium and Velocity in Urea and Guanidine Hydrochloride. As mentioned in the introduction, one of the reasons for the reinvestigation of the molecular weight of phosphorylases *b* and *a* was the observation of low molecular weight in urea and guanidine hydrochloride. The data for the sedimentation coefficient of the iodoacetate derivative of the phosphorylase *b* in 8 M urea at pH 7.5 are presented in Figure 8. In this case it was necessary to evaluate the concentration dependence as $s = s°/(1 + kc)$

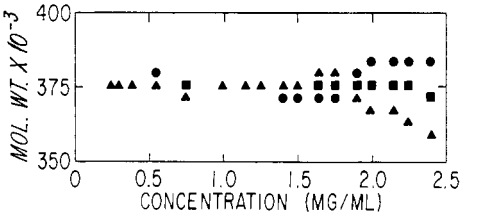

FIGURE 6: Phosphorylase *a* prepared by method II in the presence of 0.05 M β-mercaptoethanol, 20°. The solvent is buffer c of Table III. ▲ represents $M_{w,z}$; ■ represents $M_{n,z}$; and ● represents $2M_{n,z} - M_{w,z}$; 9000 rpm for 1408 min.

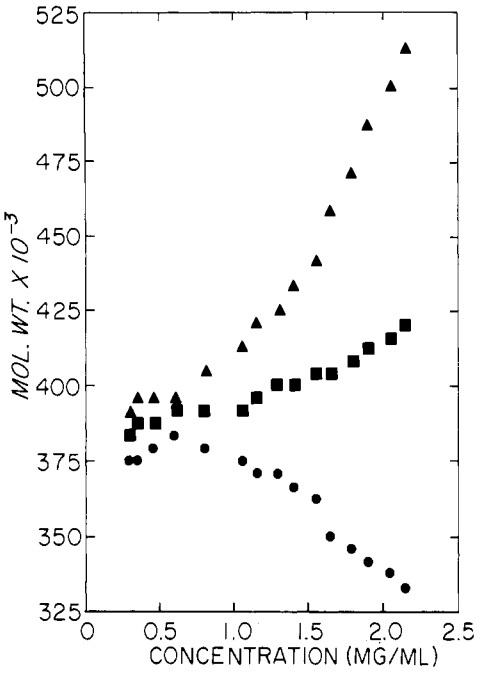

FIGURE 7: Effect of omission of thiol in the crystallization of phosphorylase *a* on molecular weight. The conditions here for sedimentation equilibrium were those used by Keller and Cori (1953) for a diffusion experiment: 0.03 M sodium glycerophosphate–0.002 M EDTA–1% KCl (pH 6.8). ▲ represents $M_{w,x}$; ■ represents $M_{n,x}$; and ● represents $2M_{n,x} - M_{w,x}$.

(Schachman, 1959). In this equation we have $s^0_{20,w}$ = 2.34 ± 0.03 S and k = 0.064 ± 0.002 ml/mg. The concentration dependence of the diffusion coefficient which we have determined is rather small as shown in Table IV. Combination of $s_{20,w}$ with the corresponding $D_{20,w}$ together with the partial specific volume of the native enzyme yields an average M_{app} of 76 ± 7 × 10^3 g/mole. This figure is somewhat unreliable because of the uncertainty in the diffusion coefficients. High-speed sedimentation equilibrium experiments on both iodoacetate and iodoacetamide derivatives in 8 M urea were similar enough to be combined together. In all cases the distributions indicated heterogeneity.

TABLE IV: Diffusion Coefficient of Phosphorylase *b* in 8 M Urea[a] at 20°.

Concn (mg/ml)[b]	$D_{20,w}$ (× 10^7) (cm²/sec)
1.13	2.4
2.30	2.6
2.98	2.5

[a] Experimental details are given in the text. [b] The protein concentrations presented in this table are values of one-half the initial concentration placed into the cell.

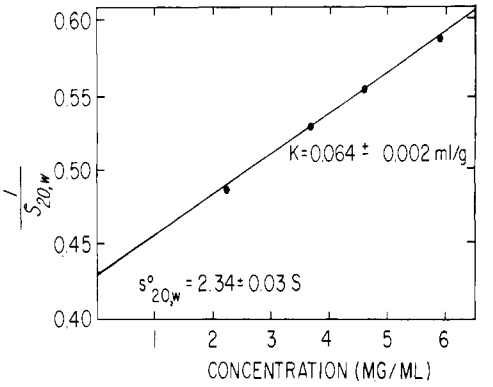

FIGURE 8: Concentration dependence of the sedimentation coefficient in 8 M urea at 20°. The experimental details are given in the text. The line is from the best-fitting least-squares equation.

Analysis of the data from seven experiments gave the results summarized in Table V. The determination of dimer and per cent of heavy material was performed as previously described (Teller *et al.*, 1965). The results showed that the preparation could be characterized either by 18 ± 10% of dimeric material or 21 ± 12% of heavy material of M_n = 108 × 10^3 and M_w = 128 × 10^3 g/mole (Table V). However, because of the danger of incomplete alkylation of all sulfhydryl groups in these preparations and since guanidine hydrochloride appears to be a better denaturant than urea (Tanford *et al.*, 1967) we have performed a series of experiments at a variety of concentrations of guanidine hydrochloride in the presence of 0.1 M β-mercaptoethanol.

While guanidine hydrochloride is a good denaturing agent, several problems arise in its use: specifically, preferential solvation (Schachman and Edelstein, 1966; Hade and Tanford, 1967), thermodynamic nonideality (Kielley and Harrington, 1960; Tanford *et al.*, 1967), and, in some cases, macromolecular heterogeneity.

There are presently available two methods of eliminating preferential solvation. The measurement of apparent

TABLE V: Molecular Weight Moments from Alkylated Phosphorylase in 8 M Urea (pH 7.5).[a]

Mol Wt Moment	Mol Wt (× 10^{-3}) (g/mole)
$(2M_n - M_w)_{c=0}$	78 ± 6
M_n	82 ± 3
M_w	86 ± 3
M_z	94 ± 7
M_{z+1}	103 ± 14
M_n of heavy material	108 ± 11
M_w of heavy material	128 ± 20

[a] Experimental details are given in the text.

TABLE VI: Molecular Weight Determinations in Guanidine Hydrochloride Plus β-Mercaptoethanol.

Guanidine Hydrochloride Concn (M)	No. of Samples	$1 - \bar{v}\rho$	ρ (g/ml)	$(C_b + C_m)/2$ (mg/ml)[b]	Molecular Weight ($\times 10^{-5}$) (g/mole)[a]					Virial Coefficient ($\times 10^4$) mole ml/g^2	
					M_n	M_w	M_z	M_{z+1}	$(2M_n - M_w)_{c=0}$	$M_{w,c=0}$	$M_{n,c=0}$	$B_1(M_w)$	$B_1(M_n)$
3.6	3	0.1981	1.0880	2.0	96.2 (0.2)	91.3 (0.8)	83.7 (1.9)	74.1 (2.8)	100.4 (1.5)	102.4 (2.7)	100.5 (1.1)	5.5	4.0
5.4	5	0.1661	1.1314	1.88	89.4 (0.8)	82.5 (1.7)	73.3 (3.4)	65.5 (6.7)	96.1 (2.4)	99.9 (3.4)	97.8 (1.8)	11.6	10.0
7.2	4	0.1345	1.1743	1.63	78.5 (0.4)	73.3 (0.5)	64.9 (2.9)	56.8 (3.4)	69.8 (2.2)	88.9 (2.3)	83.2 (2.0)	14.9	8.5
At $\rho = 1$		0.2643	1.00		105.0	99.8	92.5	82.4	117	110	110		

[a] Numbers in parentheses are root-mean-square errors. [b] $(C_b + C_m)/2$ represents the concentration at which the apparent weight-average molecular weights apply (Van Holde and Baldwin, 1958).

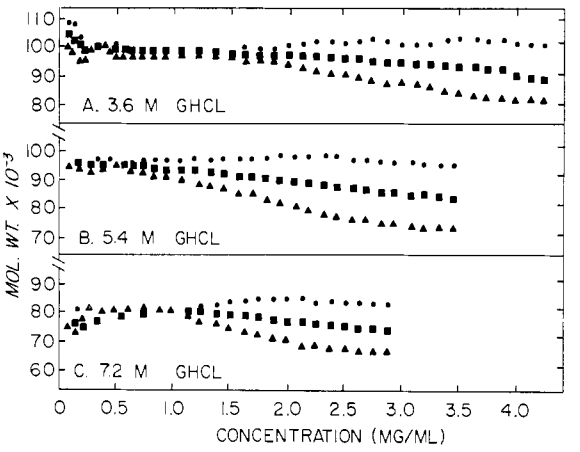

FIGURE 9: Molecular weight distributions of representative individual samples in 0.05 M Tris-HCl, 0.1 M β-mercaptoethanol, and various concentrations of guanidine hydrochloride. ▲ represents $M_{w,x}$; ■ represents $M_{n,x}$; and ● represents $2M_{n,x} - M_{w,x}$.

specific volumes by the method of Casassa and Eisenberg (1964) and the treatment of reduced molecular weight moments by the method of Schachman and Edelstein (1966). Also, two methods are available for the elimination of thermodynamic nonideality. The first is the elimination of virial terms at finite concentration by the functions $2M_n - M_w$ (Yphantis, 1964) and M_w^2/M_z (D. A. Yphantis, personal communication). The second method is extrapolation of M_n and M_w data to infinite dilution. All four methods of eliminating the two factors have been used. In principle, the results of the treatments should yield identical answers. However, it must be emphasized that if the molecule aggregates upon dilution of the guanidine hydrochloride, then the results of the calculations by methods dependent on comparisons of data at different guanidine hydrochloride concentrations become difficult to interpret.

Representative distributions of molecular weights in 3.6, 5.4, and 7.2 M guanidine hydrochloride are presented in Figure 9A–C, respectively. The quantity $2M_{n,app} - M_{w,app}$ at each point cancels virial terms to a large extent (Yphantis, 1964). Upward curvature in the distribution of $2M_n - M_w$ indicates the lack of complete cancellation of virial terms or heterogeneity due to molecules smaller than a dimer of the smallest component; downward curvature is indicative of heterogeneity due to large molecules relative to the smallest component. The data from these experiments have been treated by three methods with the results as summarized in Table VI; the fourth method consists of using the apparent specific volume determined by the method of Casassa and Eisenberg (1964).

The first treatment of the guanidine hydrochloride data is the elimination of preferential interactions by an extension of the method of Schachman and Edelstein (1966). Figure 10 presents the reduced apparent molec-

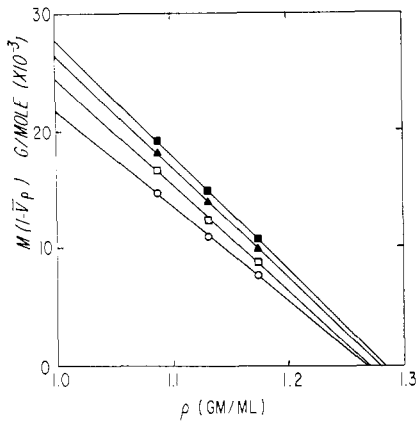

FIGURE 10: Reduced molecular weight moments in guanidine hydrochloride–0.1 M β-mercaptoethanol as a function of density. The molecular weights at $\rho = 1$ are given in Table VI. The values of ρ at $(1 - \bar{v}\rho) = 0$ are 1.282, 1.277, 1.269, and 1.267 g/ml for the number, weight, z, and $z + 1$ averages, respectively. ■ represents number average, ▲ represents weight average, □ represents z average, and ○ represents the $z + 1$ average data.

ular weight moments, $M_{n,\mathrm{app}}(1 - \bar{v}\rho_0)$, $M_{w,\mathrm{app}}(1 - \bar{v}\rho_0)$, $M_{z,\mathrm{app}}(1 - \bar{v}\rho_0)$, and $M_{z+1,\mathrm{app}}(1 - \bar{v}\rho_0)$ as a function of solvent density (ρ_0). At $\rho_0 = 1$ g/ml, where preferential interactions should disappear, we obtain the apparent molecular weights shown in the last line of Table VI. Correcting these values for virial coefficients by $2M_n - M_w$ (Yphantis, 1964) yields 110×10^3 g/mole when the partial specific volume of 0.737 ml/g was employed in the calculation. The quantity M_w^2/M_z, which also may be used to eliminate virial terms (D. A. Yphantis, personal communication), gives 108×10^3 g/mole at $\rho = 1$ g/ml. The buoyant densities calculated from extrapolation of the reduced apparent molecular weight moments to zero on the ordinate yield the values listed in the legend of Figure 10. The reciprocals of these values (0.78–0.79 ml/g) are much larger than the observed apparent specific volumes implying that guanidine hydrochloride is preferentially excluded from the domain of the protein. Similar results have been obtained for aldolase (Schachman and Edelstein, 1966), even though the molecule has been reported to bind guanidine hydrochloride preferentially as determined by isopiestic measurements (Hade and Tanford, 1967). The value of 0.78 ml/g obtained as the reciprocal buoyant density is different from the value of the apparent specific volume (0.736 ml/g) measured by the method of Casassa and Eisenberg (1964); but, in the absence of complicating factors, the two measurements should be identical. In addition, the molecular weights of 110×10^3 g/mole obtained at $\rho = 1$ are incompatible with the observed molecular weights of the dimeric and tetrameric states of the enzyme.

A second treatment of these data is to assume homogeneity of the protein at all guanidine hydrochloride concentrations and eliminate thermodynamic nonideality by extrapolation to infinite dilution. In this case, all of the data at each guanidine hydrochloride concentration can be combined in the reciprocal plots of $1/M_{n,\mathrm{app}}$ and $1/M_{w,\mathrm{app}}$ *vs.* protein concentration. These graphs should give straight lines with slope proportional to the virial coefficients and intercept given by the reciprocal molecular weight of the homogeneous material. Defining the virial coefficient after Fujita (1962) gives the slope equal to B_1 in the case of the weight-average data and 0.5 B_1 for the number-average data. These data are summarized in the last four columns of Table VI. The plotted data for 5.4 M guanidine hydrochloride show reasonable linearity as given in Figure 11. Figure 11A depicts the reciprocal M_w function while the reciprocal M_n function is given in Figure 11B. At this concentration of guanidine hydrochloride these functions had the greatest linearity. This is reflected by the agreement of B_1 calculated by the two methods. However, the disagreement of the values of B_1 shown in Table VI indicates that the material was not completely homogeneous in any of the guanidine concentrations. By assuming that the observed heterogeneity is apparent rather than real, the values of M_n and M_w at $c = 0$ may be used, in turn, in the treatment of Schachman and Edelstein (1966) to obtain the values of M_n and M_w in the absence of preferential interactions. When this calculation is performed the values in the bottom row of Table VI are obtained. The numbers agree with each other and with the value of 110×10^3 g/mole determined on the apparent molecular weight moments, above. The principle difficulty with this treatment is that the wrong answer may be obtained if heterogeneity or nonlinear preferential interactions increase with increasing guanidine hydrochloride in these solutions.

A third treatment of the data is to combine all data at each guanidine concentration in the function $2M_n - M_w$ and extrapolate this to $c = 0$. If no preferential interactions are present, but heterogeneity is present then the molecular weight of the smallest major component will be obtained at $c = 0$. Such data are shown in Figure 12 for four determinations in 7.2 M guanidine hydrochloride. It may be seen from this figure that superposition of the data is relatively good, and that the data always appear heterogeneous. Extrapolation to $c = 0$ for these data yielded 69.8×10^3 g/mole as the molecular weight of the smallest major component. In 3.6 and 5.4 M guanidine hydrochloride the data are in approximate agreement with each other when treated in this fashion, in spite of a doubling of the apparent virial coefficients. Further, the results of 7.2 M guanidine hydrochloride when corrected for the virial terms by the quantity $2M_n - M_w$ resemble strongly the results obtained in 8 M urea.

The three treatments of the data of Table VI taken together are interpreted as indicating that phosphorylase dissociates in low molarities of guanidine hydrochloride to a unit of molecular weight in the range of 100×10^3 g/mole but upon increasing the concentration of guanidine hydrochloride, further dissociation occurs,

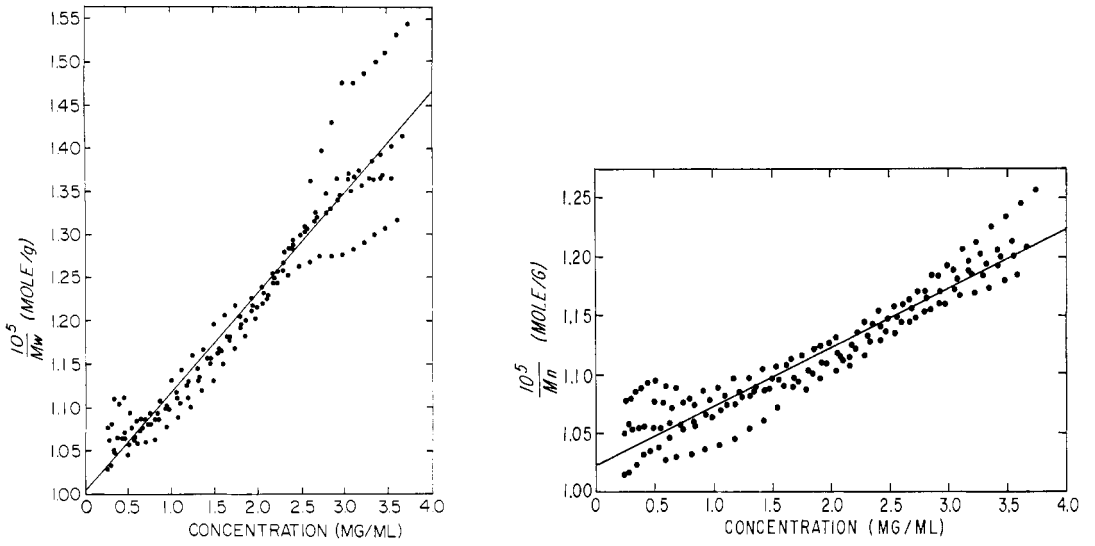

FIGURE 11: Reciprocal molecular weights as a function of concentration for phosphorylase *b* in 0.05 M Tris-HCl–5.4 M guanidine hydrochloride–0.1 M β-mercaptoethanol. The lines are those determined by least squares. (A) Reciprocal weight-average data. (B) Reciprocal number-average data. Curvature in such a plot indicates heterogeneity.

possibly with the acquisition of preferential interactions.

However, in spite of the acquisition of preferential interactions, the correct molecular weights should have been obtained in the 7.2 M guanidine hydrochloride solution. The use of the apparent specific volume determined by the method of Casassa and Eisenberg (1964) should account for any preferential solvation unless the assumptions incorporated into the theory are violated. These assumptions are (Casassa and Eisenberg, 1961): (1) the numbers of diffusible ions in the complex macroion are relatively small, and (2) the binding of ions is pressure independent. In this report the additional assumption is made that dilute β-mercaptoethanol added during the volume determinations does not seriously disrupt the osmotic equilibrium.

Discussion

The physical parameters for phosphorylases *b* and *a* which we have determined in this study are summarized in Table VII. The sedimentation and diffusion coefficients are almost identical with those presented by De Vincenzi and Hedrick (1967). The striking finding of Keller and Cori (1953) that the frictional ratio does not change upon conversion of phosphorylase *b* to *a* has been found in these experiments as well. The results of Keller and Cori (1953) indicate that phosphorylase *a* has twice the molecular weight of phosphorylase *b* and that result has been confirmed here in spite of the difference in absolute values of the molecular weights obtained in the two studies. The molecular weights presented in Table VII are our best present estimates. They are probably accurate to within 3%, although the determinations of the values for phosphorylases *b* and *a* are less precise than this figure. The existence of the dimer-tetramer relation between phosphorylases *b* and *a* places an additional constraint on the values which reduces the probable error.

All of the results which have been obtained to date on phosphorylase *b* indicate that this molecule has a molecular weight of 185 × 10^3 g/mole and tends to self-associate slightly in solution as the concentration is increased. If phosphorylase *b* were also slightly dissociated, then the addition of glucose-6-P, which is presumed to prevent monomerization of the molecule, should lead to higher molecular weights than that obtained for the enzyme alone. The accuracy of our data in the presence of glucose-6-P is not sufficiently good to distinguish any dissociation, although the results of Madsen and Cori (1956) can be interpreted as implying such is the case.

The difficulty presented by association–dissociation

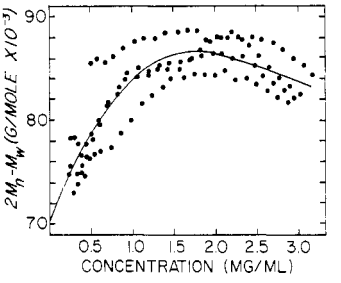

FIGURE 12: Combined data of $2M_{n,z} - M_{w,z}$ for four samples in 0.05 M Tris-HCl–7.2 guanidine hydrochloride–0.1 M β-mercaptoethanol. The line is from the best-fitting least-squares equation. The observed curvature is discussed in the text.

TABLE VII: Physical Parameters of Glycogen Phosphorylase.

Constant	Phosphorylase	
	a	*b*
$s_{20,w}$ (S)	13.5 ± 0.1	8.42 ± 0.03
$D_{20,w}$ (× 10^7) (cm²/sec)	3.3	4.14 ± 0.06
$\bar{v}_{20}$	0.737	0.737
Molecular weight (× 10^3)	185	370
f/f_0	1.35	1.35

equilibria is encountered also with phosphorylase *a*. Under almost all of the conditions in which we have examined phosphorylase *a* in the presence of β-mercaptoethanol, it appears as a heterogeneous mixture arising from slight dissociation of the 370 × 10^5 g/mole particle. Further, in the presence of thioglycolate it dissociates as well as polymerizes as indicated by Figure 5. In the absence of thiol it polymerizes, presumably by oxidation of cysteine to cystine, since the polymerization process is reversed by the addition of β-mercaptoethanol.

In connection with the native molecules of phosphorylases *a* and *b* from skeletal muscle, it is of interest to compare their molecular weights with those obtained on the enzyme found in heart muscle (Davis *et al.*, 1967). Heart muscle phosphorylase *b* type I yielded the parameters M_w = 218 × 10^3 and M_n = 190 × 10^3. However, heart muscle type I phosphorylase *a* does not tetramerize as is the case of the skeletal muscle enzyme. The data obtained for the heart muscle phosphorylase *a* were M_w = 207 × 10^3 and M_n = 196 × 10^3. All of these values are consistent with those reported here for skeletal muscle phosphorylase *b*.

With respect to the subunit structure of skeletal muscle phosphorylase, we are presently unable to give an exact model of the protein. The results in 7.2 M guanidine hydrochloride and on carboxymethylated derivatives in urea suggest, but do not prove, that the fundamental subunit is less than 90 × 10^3 g/mole. In both of these solvents, preferential solvation could lead to the observed results, in spite of a value for the true molecular weight of about 90 × 10^3 g/mole.

The agreement between the values of 110 × 10^3 g/mole by the first two procedures outlined under Results may be misleading. If no preferential interactions occur but increasing degrees of dissociation do occur, as the guanidine hydrochloride concentration is increased, then a high value of molecular weight of the fundamental subunit will be obtained at $\rho = 1$. The observed distributions in 3.6 and 7.2 M guanidine hydrochloride indicate heterogeneity in these solvents, more extreme in the case of 7.2 M guanidine hydrochloride. The value of the smallest major component evaluated as 70 × 10^3 g/mole in this case could be misleading either because of incomplete dissociation, lack of the presence of integral multiples of molecular weight for the fundamental subunits, or preferential interactions.

The approximate virial coefficients obtained at the various guanidine hydrochloride concentrations exhibit a striking dependence on guanidine hydrochloride concentration, but at 5.4 M compare well with literature values of 9.9 × 10^{-4} ml moles/g² obtained by Kielley and Harrington (1960) for the myosin subunits and 7.8 × 10^{-3} ml moles/g² obtained for bovine serum albumin in 5 M guanidine hydrochloride–0.1 M β-mercaptoethanol (Teller, 1965). Because of the observed molecular weight heterogeneity, the value of B_1 obtained in 7.2 M guanidine hydrochloride is an underestimate. But the increase of B_1 observed from M_w data between 5.4 M and 7.2 M indicates a greater extension of the polypeptide chains in the latter solvent (Tanford, 1961) consistent with the interpretation that preferential interactions are not the major difficulty but the heterogeneity is due to further dissociation of phosphorylase in 7.2 M guanidine hydrochloride. Three further points support this conclusion. First, the use of the apparent specific volume measured by the method of Casassa and Eisenberg in the calculations should account for any preferential solvation and yield the correct molecular weight unless the assumptions on which the theory is based are violated. Second, the plots of reduced molecular weight (Figure 10) do not all have the same intercept at $M(1 - \bar{v}\rho) = 0$ for the various moments, indicating heterogeneity either in $\bar{v}$ or M. Third, the downward curvature of $2M_n - M_w$ in 7.2 M guanidine hydrochloride (Figure 12) is most simply interpreted as due to heterogeneity of $\bar{v}$ or M. Experiments are presently in progress in this laboratory to attempt to resolve the difficulties associated with guanidine hydrochloride and urea and obtain the molecular weight of the phosphorylase subunits.

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