Reevaluation of the Molecular Weights of Glycogen Phosphorylases *a* and *b* Using Sephadex Gel Filtration*

Donald L. DeVincenzi† and Jerry L. Hedrick

ABSTRACT: The molecular weights of phosphorylases *a* and *b* previously reported in the literature were in error. In the work reported here, Stokes radii for these enzymes were determined by Sephadex G-200 gel filtration. Using the Stokes–Einstein equation, the diffusion coefficients of phosphorylases *a* and *b* were calculated to be 3.39 and 4.33 $\times$ 10^{-7} cm^2 sec^{-1}. These values were verified by an independent technique. The molecular weights of phosphorylases *a* and *b*, calculated utilizing the Svedberg equation, $s_{20,w}$, $D_{20,w}$, and the partial specific volume, were 367,000 $\pm$ 10,000 and 177,000 $\pm$ 3000. Redetermination of adenosine monophosphate (AMP) binding stoichiometry gave values consistent with the above molecular weights.

In addition, the pyridoxal 5′-phosphate stoichiometry, which previously did not correlate satisfactorily with molecular weight data, is consistent with the new molecular weights. Previous molecular weights were in error primarily due to $D_{20,w}$ determinations. Oxidation of enzyme sulfhydryl groups probably occurred during these determinations and resulted in the formation of polymers, thus yielding low diffusion coefficients. Considering the AMP and pyridoxal 5′-phosphate stoichiometry and the Stokes radii, the molecular weights of phosphorylases *a* and *b* were approximately 360,000 and 180,000 under the conditions of these experiments.

Glycogen phosphorylase (EC 2.4.1.1 α-1,4-glucan: orthophosphate glucosyltransferase) from rabbit skeletal muscle was shown to exist in two basic forms, a form inactive in the absence of AMP,[1] denoted as phosphorylase *b*, and a form active in the absence of AMP, denoted as phosphorylase *a*. The work of the Cori's and their collaborators had established the molecular weight of phosphorylase *b* as 242,000 and phosphorylase a as 495,000 (Keller and Cori, 1953; Keller, 1953, 1955). These investigators also demonstrated that both phosphorylases *a* and *b* could be dissociated into subunits of molecular weight approximately 125,000 (Madsen and Cori, 1956; Madsen, 1956). Thus, phosphorylase *b* was a dimer, composed of two 125,000 molecular weight subunits and phosphorylase *a* was a tetramer, composed of four such units.

During investigations on the association–dissociation characteristics of the various forms of this enzyme, utilizing the techniques of Sephadex gel filtration and disc gel electrophoresis, results were obtained which were inconsistent with the above-mentioned molecular weights. Therefore, the molecular weights were reinvestigated using the gel filtration technique. The molecular weights of phosphorylases *a* and *b* were found to be 367,000 and 177,000, respectively, under conditions of high protein concentration (milligram per milliliter), 30°, and pH 6.5. Similar results have been obtained by Seery *et al.* (1967) utilizing an independent technique

Materials and Methods

Crystalline rabbit muscle phosphorylase *b* was prepared according to Fischer *et al.* (1958) with the modification of Krebs *et al.* (1964). Phosphorylase *a* was prepared from phosphorylase *b* using purified phosphorylase *b* kinase (Krebs *et al.*, 1964). Enzyme preparations used in all experiments were recrystallized two times and were homogeneous by the criteria of disc gel electrophoresis.

Phosphorylase activity was determined by a zero-order kinetic assay, as described by Hedrick and Fischer (1965) with the exception that 0.01 M β-glycerophosphate was used in place of maleate and no buffer was added to the substrate. Protein concentration was determined spectrophotometrically using an absorbancy index of $A_{278}^{1\%}$ 11.9 for both phosphorylases *a* and *b* (Appleman *et al.*, 1963).

Stock solutions of phosphorylase *b* were freed from AMP by passage through a small charcoal-cellulose column (Fischer and Krebs, 1958) and kept at 0° in 0.05 M sodium glycerophosphate–0.05 M mercaptoethanol adjusted to pH 7.0 with HCl.

The β-glycerophosphate used as a Sephadex column buffer was converted from the sodium salt to the free acid by passage over a Dowex 50 (H^+ form) column. When the inorganic phosphate contamination was

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[1] Abbreviation used: AMP, adenosine monophosphate.

greater than 0.5%, the phosphate was precipitated as the Ba^{2+} salt and the β-glycerophosphate recrystallized from water–ethyl alcohol before passage over a Dowex 50 column.

The [^{3}H]AMP used in the AMP binding studies was obtained from Schwarz BioResearch, Inc. Ferritin was obtained from Pentex and bovine serum albumin and catalase were obtained from Sigma Chemical Co. Aldolase was prepared as per Taylor (1955). All other chemicals were obtained from commercial sources and used without further purification.

Sephadex G-200 (Pharmacia) (40–120 μ) was allowed to swell for periods up to 1 month in buffer at room temperature. Hydrated gel and buffer were routinely deaerated under vacuum prior to use, and the gel was poured into jacketed columns (Pharmacia) (1.5 × 90 or 2.5 × 40 cm) at the operating temperature. In all experiments the buffer was 0.03 M β-glycerophosphoric acid–0.001 M EDTA adjusted to pH 6.5 with a solution of 2 M Tris. The sample solutions (1.5 ml) containing 10% sucrose were carefully layered under the buffer solution above the gel. Eluate fractions (1.0 ml) were collected and the ultraviolet absorption of the column eluate was monitored at 230 mμ with a Beckman DB recording spectrophotometer. Constant flow rates were maintained between 6 and 10 ml/hr by means of a pump and the temperature of the column was maintained at 30 ± 0.1° by a thermostated bath.

The elution volume (V_e) of a given solute zone was taken in all cases as the volume of the concentration maximum in the zone. All elution volumes were corrected for the volume of tubing from the column tip to the collecting tubes. Protein concentrations refer to the maximum concentration of protein in the eluate. Blue Dextran 2000 (Pharmacia), which was completely excluded from the gel interior, was used to determine the void volume (V_0), the volume exterior to the gel phase. Tritiated water, which occupies both interior and exterior phases, was used to determine the total volume (V_t). The difference between the total and exterior volumes is equal to the imbided volume (V_i).

Results of gel filtration experiments have been interpreted according to the method of Ackers (1964). The basic equation of the molecular sieve column was predicated on the assumption that the passage of a molecule through the column was characterized as a restricted diffusion process dependent upon both the Stokes' radius of the effluent molecule and the effective pore radius of the molecular sieve. The equation relating these parameters is

$$\frac{V_e - V_0}{V_i} = \left(1 - \frac{a}{r}\right)^2\left[1 - 2.104\left(\frac{a}{r}\right) + 2.09\left(\frac{a}{r}\right)^3 - 0.95\left(\frac{a}{r}\right)^5\right] \quad (1)$$

where a is the Stokes' radius of the molecule and r is the effective pore radius of the gel.

By use of eq 1 together with the column parameters V_0, V_i, and r, the Stokes' radius of an effluent molecule can be calculated from the elution volume. The free diffusion coefficient (D) can be calculated from the Stokes' radius by use of the Stokes–Einstein equation

$$D = \frac{kT}{6\pi\eta a} \quad (2)$$

where k is the Boltzman constant, T is the absolute temperature, and η is the system viscosity. The effective pore radius (r) of the gel is calculated from the elution volume data for a series of standard proteins of known Stokes' radii.

The molecular weight can be calculated from the free diffusion coefficient and the sedimentation coefficient by use of the Svedberg equation

$$M = \frac{s}{D}\frac{RT}{(1 - \bar{v}\rho)} \quad (3)$$

where R is the gas constant, ρ the density, and $\bar{v}$ the partial specific volume.

Free diffusion coefficients of phosphorylases a and b were measured in a Spinco Model H electrophoresis–diffusion apparatus using a Tiselius cell. All experiments were performed at 20° for 60–80 hr. Boundary spreading as a function of time was measured from photographs of Rayleigh fringe patterns taken at various intervals during the experiment. The diffusion coefficient for phosphorylase b was measured at a concentration of 4.0 mg/ml in the microcell (2 ml), while that for phosphorylase a was determined in the macrocell (11 ml) at 1.0 mg/ml. Diffusion coefficients for each enzyme at various times were computed from the Rayleigh fringe patterns by the method of Longsworth (1952).

Diffusion coefficients for phosphorylase b were also determined according to the method of Ehrenberg (1957). These experiments were carried out in a valve-type synthetic boundary cell at 10,000–12,000 rpm in the Beckman Model E ultracentrifuge. Photographs were taken at various times with the schlieren optical system and peak spreading as a function of time in the absence of sedimentation was evaluated by both the "maximum ordinate–area" and "maximum ordinate" methods. The concentration of enzyme was 7–8 mg/ml.

The binding of AMP to phosphorylase was measured by the gel filtration technique introduced by Hummel and Dreyer (1962) and as applied by Kemp and Krebs (1967) to phosphofructokinase.

A column with a diameter of 1.3 cm and a volume of 53 ml of Sephadex G-25 (Pharmacia) was equilibrated with the β-glycerophosphoric acid–Tris–EDTA (pH 6.5) buffer used in the G-200 experiments and containing tritiated AMP. Phosphorylase used in the binding studies was treated with charcoal to remove nucleotides. The efficiency of this treatment was verified spectrophotometrically by determination of the 260:278 mμ ratio which was always less than 0.54 for phosphorylase b and 0.60 for phosphorylase a. Phosphorylase a (1 mg/ml) in 1.5 ml of the solution used to equilibrate

the column was applied to the top of the column and allowed to enter the gel. The column was eluted with more of the same buffer and fractions of 1.0 ml were collected at a flow rate of 25 ml/hr. All binding studies were carried out at 23°. Each fraction (0.2 ml) was counted in 10 ml of Bray's (1960) solution in a scintillation counter.

Results

Elution Patterns from Sephadex G-200 for Phosphorylases a and b. Typical elution patterns for phosphorylases *a* and *b* at the concentrations indicated are shown in Figure 1. Normalized protein and activity measurements on both enzymes were coincident on smooth curves. In all experiments, recovery of protein from the column averaged greater than 90% and both enzymes were recovered after gel filtration with the same specific activities as when they were applied.

Calibration of Gel Pore Radius for a Sephadex G-200 Column. The gel pore radius (*r*) was determined from the elution volumes and calculated Stokes' radii (*a*) of bovine serum albumin, aldolase, catalase, and ferritin (Table I). Column calibrations were routinely carried out three times and the elution volumes of any one standard protein usually agreed to within ±1.0 ml.

TABLE I: Calibration of a Sephadex G-200 Column.[a]

Compd	Elution Vol. (ml)	Stokes' Radius[b] (A)	Pore Radius[g] (A)
Blue Dextran	61.6		
Ferritin	75.6	79.0[c]	200
Catalase	95.2	52.2[d]	195
Aldolase	98.7	50.0[e]	199
Albumin	119.3	36.1[f]	206
Tritiated water	196.7		

[a] Column dimensions were 2.5 × 40 cm. Buffer, pH, temperature, and flow rate were as described in Materials and Methods. [b] Stokes' radii were calculated by means of eq 2 from diffusion coefficients reported in the literature. [c] Rothen (1944). [d] Sumner and Gralen (1938). [e] Edsall (1953). [f] Tanford (1961). [g] The gel pore radius was calculated as described in Results. The average pore radius was 200 ± 4 A.

Included in the table are elution properties of Blue Dextran and tritiated water used for the determination of the column parameters V_0, V_t, and V_i. By use of these parameters together with the elution volumes and Stokes' radii of the standard proteins, eq 1, and Table III in Ackers' (1964) publication, the gel pore radius (*r*) was calculated.

The validity of the relationship proposed by Ackers and quantitated in eq 1 was shown by the excellent agreement obtained for the value of the constant *r* using different standard proteins which spanned a wide Stokes' radius (molecular weight) range. This range included the expected values for phosphorylases *a* and *b*. On all G-200 columns used in this study, these standard proteins resulted in pore radii which agreed to within 1–5 A on any one column.

Stokes' Radii for Phosphorylases a and b by Gel Filtration. Phosphorylases *a* and *b* were individually subjected to gel filtration on calibrated Sephadex G-200 columns over effluent protein concentrations of 1.5–0.03 mg/ml for phosphorylase *b* and 1.4–0.11 mg/ml for phosphorylase *a* (Table II). Below 0.1 mg/ml, the elution volume and therefore Stokes' radius for phosphorylase *a* exhibited a marked concentration dependence indicative of dissociation with decreasing concentration.

TABLE II: Stokes' Radii for Phosphorylases *a* and *b* Determined from Sephadex G-200 Gel Filtration.[a]

Enzyme	Av Pore Radius, *r* (A)	No. of Samples[b]	Av Stokes' Radius, *a*[c] (A)
Phosphorylase *b*	192	6	
	200	3	49.3 ± 0.9
	188	2	
Phosphorylase *a*	192	8	
	200	1	63.0 ± 1.7
	188	1	

[a] Buffer, pH, temperatures and flow rates were as described in Materials and Methods. All columns had 2.5-cm diameters with varying heights. [b] A range of protein concentrations was used in those experiments having more than one sample. [c] Average Stokes' radius represents the average of all samples on all columns for each enzyme and was calculated as described in Materials and Methods. All errors reported are standard deviations.

The experiments listed were carried out with three different enzyme preparations, and on three Sephadex columns of different total volumes with the indicated average pore radii. The average Stokes' radius for each enzyme is indicated in the last column.

Each stock solution of enzyme was stored in the presence of sulfhydryl compounds prior to use. Samples of these stock solutions were then subjected to gel filtration for 10–12 hr in the absence of sulfhydryl compounds. In each experiment, only one protein peak was eluted.

The data in Table II indicated that the Stokes' radii for phosphorylases *a* and *b* were constant and reproducible irrespective of the enzyme preparation, the effluent

TABLE III: Diffusion Coefficients and Molecular Weights of Phosphorylases *a* and *b* Calculated from Sephadex G-200 Gel Filtration Data.

Enzyme	Stokes' Radius (A)	$D_{20,w} \times 10^{7}$[a] (cm^2 sec^{-1})	Mol Wt[b]
Phosphorylase *b*	49.3 ± 0.9	4.33 ± 0.08	177,000 ± 3,000
Phosphorylase *a*	63.0 ± 1.7	3.39 ± 0.09	367,000 ± 10,000

[a] Diffusion coefficients were calculated from eq 2 using the average Stokes' radii from gel filtration, a temperature of 20°, and the viscosity of water at 20°. [b] Molecular weights were calculated as described in Results.

protein concentration (except as noted above for phosphorylase *a*), the total column volume, and the particular column used.

Diffusion Coefficients and Molecular Weights of Phosphorylases a and b from Gel Filtration Data. The free diffusion coefficients ($D_{20,w}$) were calculated from the average Stokes' radii by use of the Stokes–Einstein equation. The results are shown in Table III. These values differ markedly from those reported in the literature (3.3×10^{-7} cm^2 sec^{-1} for phosphorylase *b* and 2.6×10^{-7} cm^2 sec^{-1} for phosphorylase *a*; Keller, 1953; Keller and Cori, 1953).

Molecular weights for phosphorylases *a* and *b* were calculated from the physical constants for the enzymes by means of the Svedberg equation (eq 3). Sedimentation coefficients for phosphorylases *a* and *b* were redetermined in this laboratory over a concentration range of 0.1–7.0 mg/ml. The values of 8.4 ± 0.2 S for phosphorylase *b* and 13.5 ± 0.3 S for phosphorylase *a* agreed well with the values of 8.2 and 13.2 S reported in the literature (Keller, 1953). In the molecular weight calculations, the value of 0.737 ml/g for the partial specific volume was used as determined by Seery *et al.* (1967), the temperature was 20°, and the density was that of water at 20°. The last column of Table III shows the calculated molecular weights of phosphorylases *a* and *b* based on diffusion coefficients determined by gel filtration.

Verification of Diffusion Coefficients of Phosphorylases a and b by Independent Methods. The following experiments were designed to verify the diffusion coefficients for phosphorylases *a* and *b* as determined by gel

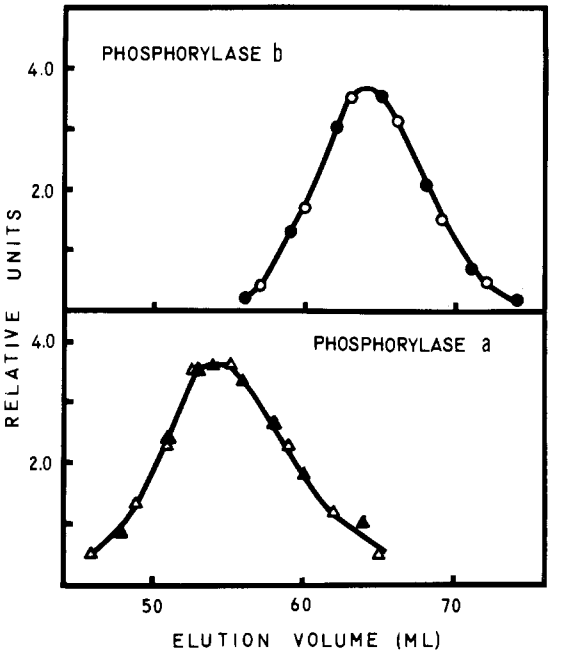

FIGURE 1: Typical elution patterns for phosphorylases *b* and *a* from Sephadex G-200. The enzymes were eluted from a 1.5 × 84 cm column with a total volume of 127 ml under experimental conditions as described in Materials and Methods. The elution volumes of phosphorylase *b* and *a* differed by 8 ml. On a 2.5 × 40 cm G-200 column with a total volume of 197 ml, phosphorylases *b* and *a* separated by 15 ml. (–●–, –▲–) Relative protein concentrations for phosphorylases *b* and *a* determined spectrophotometrically. (–○–, –△–) Relative enzymatic activities for phosphorylases *b* and *a*. Eluate concentration of phosphorylase *b* was 1.5 mg/ml and *a* was 1.4 mg/ml.

TABLE IV: Diffusion Coefficients for Phosphorylase *b* by the Method of Ehrenberg.[a]

Expt	Buffer (M)	Temp (°C)	$D_{20,w} \times 10^{7}$[b] (cm^2 sec^{-1})
1	β-Glycerophosphate (0.03)–Tris–EDTA, pH 6.5	30	4.2
2	β-Glycerophosphate (0.03)–Tris–EDTA, pH 6.5	23.5	4.5
3	β-Glycerophosphate (0.05)–Na^+–mercaptoethanol (0.05), pH 7.0	30	4.1

[a] Experimental details were as described in Materials and Methods. [b] The diffusion coefficients for each experiment represent the averages obtained by analyzing peak spreading by both methods noted in Results.

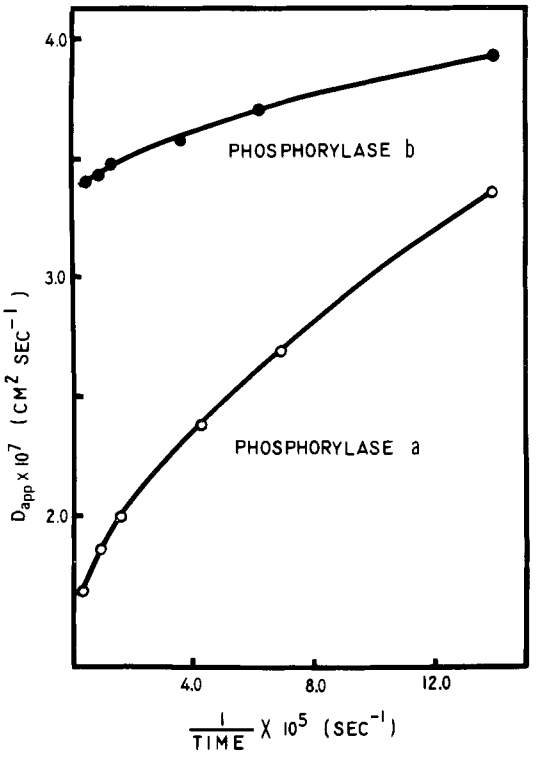

FIGURE 2: Variation of diffusion coefficients with time for phosphorylases *a* and *b*. Free diffusion coefficients were determined experimentally as indicated in Results and calculated as listed in Materials and Methods.

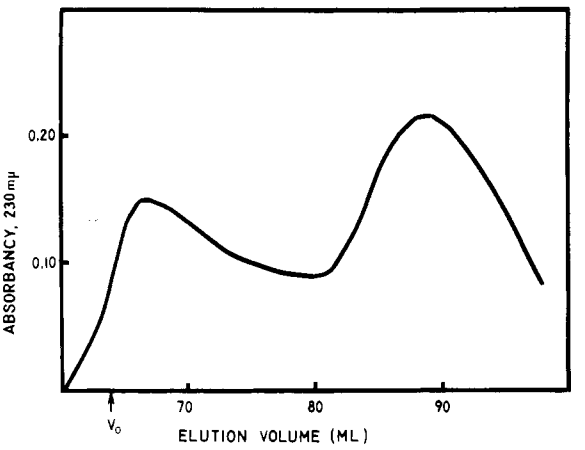

FIGURE 3: Elution pattern from Sephadex G-200 of phosphorylase *a* aged in the absence of added sulfhydryl compounds. Column conditions were as stated in Materials and Methods. The peak eluted first was aggregated material, and the later eluting peak was normal phosphorylase *a* under these experimental conditions. This curve was obtained from continuous ultraviolet monitoring of the column eluate.

filtration and to resolve the differences between these values and those reported in the literature.

Diffusion coefficients for phosphorylase *b* in the presence and absence of sulfhydryl compounds were determined according to the method of Ehrenberg (1957). According to this method, a boundary between solution and solvent was formed in the center of the cell and peak spreading as a function of time evaluated by both the maximum ordinate–area method and the maximum ordinate method.

In expt 1 and 2 of Table IV, the enzyme was subjected to the same conditions as on the Sephadex G-200 columns. An aliquot of the stock enzyme solution stored in the presence of mercaptoethanol was dialyzed for 12 hr in the glycerophosphate–Tris–EDTA buffer (pH 6.5) used for gel filtration. The 2-hr Ehrenberg run was carried out at 30° as well as at 23.5°. The calculated diffusion coefficients agreed closely with those determined by gel filtration. Aliquots of the same short-term-dialyzed samples of enzyme used in these experiments were also subjected to gel filtration. Only one peak was present and had a Stokes' radius identical with that reported above for phosphorylase *b*.

The effect of sulfhydryl compounds on the diffusion coefficient is indicated in expt 3. In this case, a sample of phosphorylase *b* was subjected to the Ehrenberg treatment in the presence of mercaptoethanol at 30°. No significant change in diffusion coefficient was detected. From these experiments, it was concluded that the diffusion coefficient as determined by gel filtration in the absence of sulfhydryl compounds was valid, could be verified by an independent technique, and was unchanged in the presence of sulfhydryl compounds.

The reason for the difference between these values and those reported in the literature remained to be established. Determination of diffusion coefficients for phosphorylases *a* and *b* by the free-diffusion method using the Tiselius electrophoresis cell were carried out under conditions similar to those employed by Keller (1953) in the original determinations. Both enzymes were dialyzed for 40 hr in the glycerophosphate–Tris–EDTA buffer (pH 6.5) and then subjected to diffusion at 20° for 60 hr for phosphorylase *b* and 80 hr for phosphorylase *a*. The calculated apparent diffusion coefficients were plotted *vs.* 1/time. The intercept of this linear plot at 1/time = 0 represented the true diffusion coefficient. As indicated in Figure 2, plots of D_{app} *vs.* 1/time for phosphorylases *a* and *b* were decidedly nonlinear and showed a progressive decrease in diffusion coefficient with increasing time. This effect, indicative of formation of higher aggregates due to prolonged absence of sulfhydryl compounds, was most pronounced with phosphorylase *a*. At the conclusion of the diffusion experiments samples were removed from the Tiselius cell and assayed in the presence of mercaptoethanol. After 40-hr dialysis and 60–80-hr diffusion at 20° both enzymes retained full activity. As will be shown below, higher polymers were present, however. Thus, the parameter of specific activity, when activity was measured in the presence of sulfhydryl groups, cannot

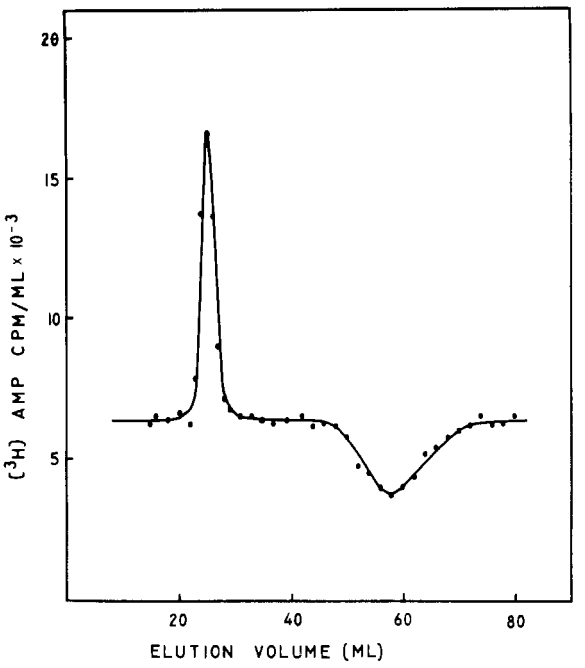

FIGURE 4: Representative elution profile for measurement of [^{3}H]AMP binding by phosphorylase *a*. A column of Sephadex G-25 was equilibrated with 2.0 × 10^{-6} M [^{3}H]AMP, and 1.5 mg of phosphorylase *a* was applied. All other conditions were as described in Materials and Methods.

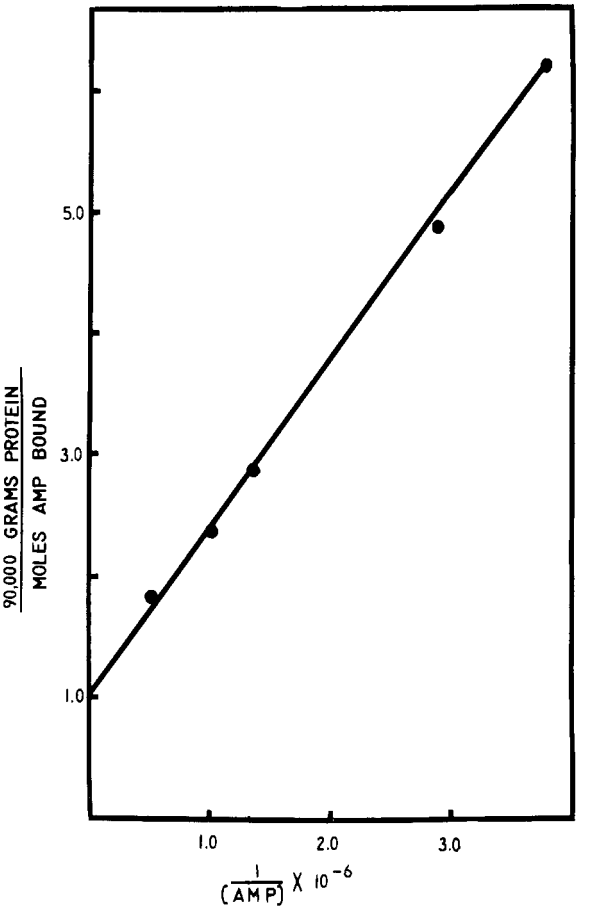

FIGURE 5. Double-reciprocal plot of [^{3}H]AMP binding by phosphorylase *a*. The amount of enzyme was held constant at 1.5 mg while the concentration of [^{3}H]AMP was varied as indicated.

be used in this instance to show that the enzyme has undergone any changes. From the range of values of D_{app} in this graph for each enzyme, it is clear that they approximated closely the values published in the literature but were decidedly lower than those obtained by gel filtration.

The sample of phosphorylase *a* used in the diffusion experiment was reassayed for activity and chromatographed on a calibrated Sephadex G-200 column. Enzymatic activity was measured in the presence and absence of mercaptoethanol. Both in the presence and absence of AMP the activity was decreased by 30% in the absence of mercaptoethanol. Figure 3 shows the elution pattern obtained from G-200 for the same sample of phosphorylase *a* after completion of the diffusion run. Whereas phosphorylase *a* preparations have consistently resulted in elution patterns similar to that of Figure 1 under normal circumstances, it is clear that the preparation used for the diffusion run in the absence of sulfhydryl contained a significant proportion of higher aggregates. The Stokes' radius of the slower eluting peak corresponded exactly to the Stokes' radius normally exhibited by phosphorylase *a* at this concentration. The major component in the faster eluting aggregated species had a calculated Stokes' radius of 107 A as compared to the normal value of 63 A for phosphorylase *a*.

Stoichiometry of Binding of AMP to Phosphorylase. The results of a typical experiment demonstrating the binding of [^{3}H]AMP to skeletal muscle phosphorylase *a* is shown in Figure 4. The return of the base-line concentration of the labeled AMP to its initial value after the emergence of the enzyme and after the appearance of the trough in the elution pattern was indicative of attainment of equilibrium during gel filtration. The amount of radioactivity bound by the enzyme was determined from the total activity under the protein peak. This value together with the specific radioactivity of the AMP were used to calculate the amount of AMP bound.

A double-reciprocal plot of AMP binding by phosphorylase *a* under experimental conditions similar to those used in the molecular weight studies is shown in Figure 5. The experimental data were evaluated by the method of least squares and were found to fit a straight line over a 130-fold range of AMP concentrations.

A dissociation constant was evaluated from the least-squares slope and was found to be 1.37 ± 0.07 × 10^{-6} M. The intercept on the ordinate indicated maximum binding of 1.04 ± 0.11 moles of AMP/90,000 g of enzyme. If the molecular weight of the subunit were

125,000 rather than 90,000, the intercept on this plot would be 0.72.

The AMP binding stoichiometry for phosphorylase *b* was complicated by the fact that it exhibited substrate dependence. Owing to technical limitations, evaluation of AMP binding to phosphorylase *b* has not, as yet, been accomplished.

These results indicated that in the same glycerophosphate–Tris–EDTA (pH 6.5) buffer used in the molecular weight determinations and at a temperature of 25°, the stoichiometry of the binding of AMP by phosphorylase *a* required that phosphorylases *a* and *b* have molecular weights of 360,000 and 180,000, respectively.

Discussion

The empirical use of Sephadex gel filtration for the physicochemical characterization of proteins has been adequately demonstrated by the work of Whitaker (1963) and Andrews (1965). Attempts to provide a more theoretical basis for the technique have been made by Squire (1964) and Ackers (1964).

The theoretical basis of Ackers' treatment of gel filtration phenomenon was verified by the close correlation between the theoretically predicted behavior of well-characterized proteins and that which was experimentally observed. The usefulness of correlating the behavior of proteins on Sephadex with their Stokes' radii, as suggested by Ackers, is amply demonstrated by the findings reported here.

Physicochemical characterization of proteins by Sephadex is subject to certain limitations, however. Anomalous behavior can be expected from at least four different types of proteins: (1) glycoproteins, (2) basic proteins, (3) those capable of undergoing association–dissociation reactions, and (4) certain carbohydrate-metabolizing enzymes which interact with the dextran gel (Whitaker, 1963; Squire, 1964; Andrews, 1965).

The Stokes' radii of glycogen phosphorylases *a* and *b* by this technique could have been subject to the above limitations; therefore it was necessary to ascertain that the Stokes' radii determined for the two forms of the enzyme were not in error due to these factors. Certain of these limitations can be categorically dismissed. No evidence has been presented which indicates that glycogen phosphorylase is a glycoprotein; in addition, neither form is a basic protein, as they have isoelectric points of less than 6 (Green, 1945). Under the conditions described here, the enzymes are not subject to association–dissociation behavior (D. L. DeVincenzi and J. L. Hedrick, unpublished observations). The possibility of interaction between glycogen phosphorylase and Sephadex under the fourth category above was tested as phosphorylase catalyzes the phosphorolysis of polysaccharides and Sephadex is a polysaccharide. However, all efforts to detect such interaction have been negative, and have included: (a) kinetic experiments utilizing Sephadex as a possible competitive inhibitor with glycogen, (b) use of Sephadex as a primer, and (c) use of Sephadex G-25 columns (1.3 × 40 cm) from which the enzyme should be totally excluded, but which would be eluted at positions greater than the void volume if interaction occurred. Thus, none of the limitations known to give anomalous results with this technique are applicable to the behavior of glycogen phosphorylase in these experiments. Therefore, the molecular weight of glycogen phosphorylase determined by this technique can be considered valid.

That this molecular weight value was correct was further verified by the stoichiometry of AMP binding and the pyridoxal phosphate content of the enzymes. The stoichiometry of the AMP sites indicated that there was approximately one binding site/90,000 g of protein. As the number of binding sites must be whole numbers, phosphorylase *b* must have a molecular weight of 180,000 for two binding sites and phosphorylase *a*, molecular weight of 360,000 for four binding sites.

The pyridoxal phosphate content of phosphorylase *b* as determined by Hedrick *et al.* (1966) also did not correlate well with a molecular weight of 250,000 for phosphorylase *b*. Phosphorylase *b* contained 2.4 to 2.7 moles of pyridoxal phosphate/250,000 g of protein. This number reduced to 1.7–1.9 moles of pyridoxal phosphate/180,000 g of protein, which was much more consistent with the idea of 1 mole of pyridoxal phosphate/mole of monomer subunit. The stoichiometry of the AMP and pyridoxal phosphate sites, and the Stokes' radii, unequivocably required that the molecular weights of phosphorylases *b* and *a* be 180,000 and 360,000, respectively.

As stated earlier, Cori and collaborators utilized the Svedberg equation to determine the molecular weights of phosphorylases *a* and *b*. There are of course three independent parameters in the Svedberg equation which can give rise to errors in the calculated molecular weight: the sedimentation coefficient, the partial specific volume, and the diffusion coefficient. Many investigators, including ourselves, have repeated the determination of the sedimentation coefficient and found it not to be substantially in error. The partial specific volume originally determined by Keller (1953) has been redetermined by Seery *et al.* (1967) and found to be slightly in error (0.737 ml/g *vs.* the original value of 0.751 ml/g). The experimentally determined partial specific volume now agrees well with the theoretical value calculated by Keller (1953). The errors in determining the partial specific volume of phosphorylase contributed in a minor way to the incorrect molecular weight calculated by the Svedberg equation. The major error occurred in the diffusion coefficient determination. From the work presented here, the erroneously low diffusion coefficient was probably caused by the formation of polymers due to sulfhydryl oxidation during the experiment. These higher polymers were clearly present (Figure 3) and would cause the calculated diffusion coefficient to be smaller than it should have been.

The Sephadex columns used to determine the diffusion coefficient in this study also did not contain added sulfhydryl compounds. However, this work was not subject to the same errors as in the previous

determinations of the diffusion constant as the time involved to perform the experiment was relatively short, and virtually no sulfhydryl oxidation occurred during the Sephadex runs. In addition, the enzyme was added to the column in a buffer which contained mercaptoethanol, so the enzyme was in the absence of a reducing medium only for the 10–12 hr required to run the column. Addition of 0.05 M mercaptoethanol to the column buffer did not significantly affect the observed Stokes' radii of phosphorylases *a* and *b*.

It is interesting to note that other indications that the diffusion coefficients and molecular weights of phosphorylases *a* and *b* were incorrect have appeared in the literature. In fact, the original determination of the molecular weight and diffusion coefficient by Oncley (1943) for phosphorylase *a* was correct ($D_{20,w}$ = 3.2–3.8 × 10^{-7} cm^2 sec^{-1}, molecular weight 340,000–400,000). Oncley's results were discounted due to experimental limitations. He used a cysteine–glycerophosphate buffer in the diffusion run which complicated the experiment due to the formation of insoluble cystine. It is apparent now, however, that although this makes for a less than perfect experiment, it did not invalidate his results. In addition, it was suggested that the high diffusion value was caused by contamination of phosphorylase *a* by phosphorylase *b* due to the lack of a good purification scheme which effectively separated phosphorylase *a* and phosphorylase phosphatase. It is also now apparent that this objection was not valid.

Appleman (1962) characterized the molecular weights of phosphorylases *a* and *b* by a sedimentation equilibrium study as 266,000 and 177,000, respectively; although the phosphorylase *a* value was incorrect, the phosphorylase *b* value was correct. This discrepancy was noted, but not further investigated. Appleman also went so far as to suggest that the molecular weight calculated by the Svedberg equation might be "erroneously high because of the difficulties encountered in measuring diffusion coefficients of muscle phosphorylase."

From the work presented here it is clear that under conditions of high protein concentration (milligrams per milliliter), neutral pH, and ambient temperatures, phosphorylase *b* had a molecular weight of approximately 180,000 and phosphorylase *a*, 360,000. However, the molecular weight of phosphorylase *a* has been observed to be highly dependent on the conditions used in its determination. Under certain conditions using Sephadex gel filtration, phosphorylase *a* has been observed to dissociate to a dimer with the same molecular weight as phosphorylase *b*. The conditions were such that it appeared that phosphorylase *a* was enzymatically active as a dimer. Kinetic evidence of Wang and Graves (1964) has also indicated that phosphorylase *a* dissociated under certain conditions. This association–dissociation phenomenon is presently being further investigated.

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Added in Proof

To further substantiate that phosphorylase was not interacting with the Sephadex polysaccharide matrix, the Stokes' radii for both enzymes were redetermined by gel filtration on Bio-Gel P-300, a polyacrylamide gel. In these experiments, all procedures were identical with those described in Methods for Sephadex. On a calibrated P-300 column, phosphorylases *a* and *b* had Stokes' radii which agreed within experimental error with those obtained using Sephadex and shown in Table III.

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Feedback Inhibition and Ionizing Radiation. Mechanism of Inactivation of Allosteric Properties of Aspartate Transcarbamylase by X-Rays*

Kjell Kleppe† and Unni Spaeren‡

ABSTRACT: The effect of X-rays on aspartate transcarbamylase from *Escherichia coli* in solution has been studied. The feedback inhibition properties of native aspartate transcarbamylase are three to four times more readily destroyed by X-rays than the catalytic activity. The catalytic subunit aspartate transcarbamylase is inactivated two to three times more easily by X-rays than the native enzyme. X-Rays cause native aspartate transcarbamylase to dissociate into subunits and this accounts for the major part of the loss of allosteric properties. Substrates, activators, and inhibitors were found to have a profound effect on the ease of destruction of various properties. L-Aspartate plus a competitive inhibitor, such as PP_i or P_i, fully protected the active site against destruction by X-rays, but not the allosteric properties.

Carbamyl phosphate and nucleotides were less effective as protectors. The destruction of allosteric properties of aspartate transcarbamylase was found to be irreversible.

The feedback inhibition concept, first proposed by Novick and Szilard (1954), has become of great importance for the understanding of cellular control mechanisms, particularly in bacteria where many biosynthetic pathways are known to possess feedback inhibition. In higher organisms metabolic control by end-product inhibition is more uncertain. A recent theory by Monod and co-workers (1963, 1965) on the nature of allosteric enzymes provides an insight into the molecular mechanisms of feedback inhibition.

In the pyrimidine metabolism in *Escherichia coli* the work of Gerhart *et al.* (1962, 1965) has demonstrated that the first enzyme in this pathway, aspartate transcarbamylase (EC 2.1.3.2), is inhibited by the end product, CTP.[1] These workers also showed that this enzyme consists of two catalytic subunits and several regulatory subunits. The regulatory subunits bind CTP but have no catalytic activity. Feedback inhibition by CTP can be abolished by treating the enzyme with such agents as *p*-mercuribenzoate, Hg^{2+}, and urea or by heating the enzyme. Desensitization leads to dissociation of the enzyme into active catalytic subunits and noncatalytic regulatory subunits.

In the present work we have examined the effect of X-rays on aspartate transcarbamylase from *E. coli* in solution. A preliminary note has appeared (Kleppe *et al.*, 1966).

Materials

Enzyme. Aspartate transcarbamylase was prepared from a mutant of *E. coli*, R-185-482, kindly provided by Dr. A. B. Pardee, Princeton University. The enzyme was purified according to a procedure slightly modified from that of Gerhart and Pardee (1962). A column of Sephadex G-200 (1.5 × 90 cm) was used to separate native aspartate transcarbamylase from subunit aspartate transcarbamylase in the final purification step. Subunit aspartate transcarbamylase accounted for approximately one-third of the protein and activity before passing it through the column. The subunit enzyme obtained from this column was kinetically identical with that obtained by heating or treating native aspartate transcarbamylase with *p*-mercuribenzoate. Both native and subunit aspartate transcarbamylase gave a single protein band on sucrose density

* From the Norsk Hydro's Institute for Cancer Research, Montebello, Oslo, Norway. *Received June 1, 1967.* This investigation was supported by the Norwegian Cancer Society.

† Present address: Department of Biochemistry, University of Bergen, Bergen, Norway.
‡ Fellow of the Norwegian Cancer Society.

[1] Abbreviations used: CTP, cytidine triphosphate; ATP, adenosine triphosphate; UTP, uridine triphosphate.