Characterization of the Aggregated States of Glycogen Phosphorylases by Gel Electrophoresis*

Jerry L. Hedrick, Alan J. Smith, and George E. Bruening

ABSTRACT: The disc gel electrophoretic method developed previously for the characterization of size and/or charge differences in proteins has been applied to glycogen phosphorylase. We have concluded that phosphorylases *a* and *b* have the same molecular size but different net charges under the conditions of electrophoresis employed. The enzymes are both dimers with molecular weights of 170,000–180,000 g/mole at pH 8.5, 35°, and a protein concentration of 0.5 mg/ml. Phosphorylase dimer can be dissociated to monomers using sodium dodecyl sulfate or 2 M urea at 4°. The binding of various ligands (adenosine monophosphate, glucose 6-phosphate, and dextrins) changes the relative mobility of phosphorylase. Plots of the logarithm of the relative mobility *vs.* gel concentration in the presence of ligands yields lines parallel to the line obtained with phosphorylase alone. This behavior is interpreted as an alteration in the net charge of the migrating macromolecule.

In the case of dextrin binding, however, an alternative explanation is proposed. A mathematical model is derived which indicates that parallel lines can arise with no change in charge if an equilibrium exists between a slowly migrating protein–ligand complex and free protein. The size–charge relations of chemically modified forms of phosphorylase ($NaBH_4$-reduced phosphorylase *b* and apophosphorylase *b*, phosphorylase *b'*) have also been determined and are compared with the native enzyme. In addition to characterizing the various aggregated forms of glycogen phosphorylase, this study extends the use of the gel electrophoretic method for analyzing the size–charge relations of proteins to situations involving ligand binding.

During an investigation of the molecular sizes of phosphorylases *a* and *b* by disc gel electrophoresis, the relative mobilities of the two enyzme forms could not be explained utilizing the 2:1 molecular weight ratio previously established (Davis *et al.*, 1967); phosphorylase *a*, 360,000 g/mole, migrated faster than phosphorylase *b*, 180,000 g/mole (DeVincenzi and Hedrick, 1967; Seery *et al.*, 1967). Phosphorylase *a* was expected to have a smaller R_m than phosphorylase *b* as the larger molecule would experience a greater "sieving effect" due to the polyacrylamide gel.[1,2] However, as both size and charge contribute to the mobility of proteins on disc gel electrophoresis, it was possible that charge differences could account for these results. A method, therefore, was devised to evaluate the relative contribution of a protein's size and charge to its mobility (Hedrick and Smith, 1968).

In this paper the application of the disc gel electrophoretic method to the characterization of phosphorylase *a*, phosphorylase *b*, and various chemically modified forms of the enzyme is reported. A description of the size and/or charge alterations induced in phosphorylase by interaction with different ligands (*e.g.*, AMP, glucose 6-phosphate, and dextrin) is presented. In addition, a theoretical treatment of ligand binding during gel electrophoresis has been derived. Alteration in a protein's electrophoretic mobility due to ligand binding has been evaluated as a function of (a) the extent of protein–ligand association at equilibrium and (b) the difference in size between the protein and the protein–ligand complex.

Materials and Methods

Crystalline rabbit muscle phosphorylase *b* was prepared according to Fischer *et al.* (1958a) with the modification of Krebs *et al.* (1964). Phosphorylase *a* was prepared from phosphorylase *b* using purified phosphorylase *b* kinase (Krebs *et al.*, 1964). Stock solutions of phosphorylase *b* were freed from AMP by passage through a charcoal-cellulose column (Fischer and Krebs, 1958) and stored at 0° in 0.05 M sodium glycerophosphate–0.05 M mercaptoethanol adjusted to pH 7.0 with HCl. $NaBH_4$-reduced phosphorylase *b* was prepared essentially by the method Fischer *et al.* (1958b) as more recently described by Strausbauch *et al.* (1967); apophosphorylase *b* was made by the method of Shaltiel *et al.* (1966); phosphorylase *b'* was prepared by the method of Keller (1955) as modified by Nolan *et al.* (1964).

All materials other than acrylamide were obtained commercially and used without further purification. An impurity in commercially obtained acrylamide, presumably acrylic acid, gave rise to artifacts when the histochemical stain for phosphorylase activity was used. This impurity was greatly reduced by recrystallization of the acrylamide from ethyl acetate.

Phosphorylase activity was determined by a zero-order kinetic assay as previously described (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. Phosphorylase concentration was determined

* From the Department of Biochemistry and Biophysics, University of California, Davis, California 95616. *Received May 26, 1969.*

[1] Abbreviations used are relative mobility, R_m; *p*-hydroxymercuribenzoate, PMB; sodium dodecyl sulfate, SDS.

[2] The phrase "sieving effect" is used here to describe the phenomenalogical relationship between protein mobility and gel concentration. It is not intended that this term be identified with the physical process(es) responsible for the phenomenon.

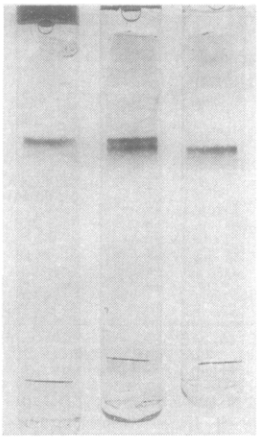

FIGURE 1: Resolution of phosphorylases *a* and *b* by disc gel electrophoresis. Electrophoresis was performed as described in the text using a 6% polyacrylamide gel. Migration is from the top of the figure with the copper pin indicating the position of the dye front. The gels are, from left to right, phosphorylase *b*, a mixture of phosphorylases *a* and *b*, and phosphorylase *a*.

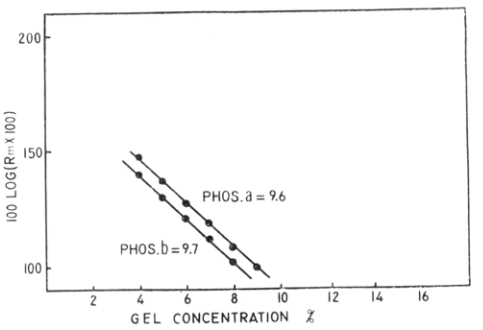

FIGURE 2: The effect of different gel concentrations on the relative mobilities of phosphorylases *a* and *b*. The negative slopes of the lines are noted on the figure.

spectrophotometrically using an absorbancy index of $A_{278}^{1\%}$ 11.9 (Appleman *et al.*, 1963).

Disc gel electrophoresis was performed using an asparagine buffer system (Davis *et al.*, 1967). Incorporation of charged compounds into the electrophoresis system was made by adding them to all components including the small and large pore gels, the sample solution, and the upper buffer reservoir. Addition of noncharged compounds was made only to the small and large pore gels and to the sample solution.

The staining procedure for phosphorylase activity was essentially that of Davis *et al.* (1967) with the following exceptions: (1) the gels were incubated with 0.075 M glucose-1-P, 5% bacteriological dextrin (pH 6.5), in the presence or absence of 1×10^{-3} M AMP at 37° for 10 min, and (2) the gels after rinsing several times with water were placed in the Tris-maleate–$PbNO_3$ solution for 20 min with occasional inversion, then washed for 40 min with several changes of 0.1 M Tris-maleate (pH 7.0) and finally treated with a 5% $(NH_4)_2S$ solution.

An additional staining technique for phosphorylase which was dependent upon the binding of oligosaccharides rather than enzymatic activity was the periodate–Schiff base stain. This method can be applied to any enzyme or protein which exhibits polysaccharide binding. The gels were incubated with 5% bacteriological dextrin for 10 min at 37°. The unbound dextrin was removed by washing the gels with water. The bound dextrin was then visualized by the method described in Pearse (1961).

In some instances, phosphorylase and other proteins which possessed free sulfhydryl groups were detected using the dihydroxydinaphthyl disulfide method of Barrnett and Seligman (1952).

Results

Phosphorylases a and b. It should be recalled from our earlier publication (Hedrick and Smith, 1968) that "charge isomer" proteins (proteins having the same size but different net charges) were characterized by parallel lines when 100 log ($R_m \times 100$) was plotted *vs.* gel concentration. "Size isomer" proteins (proteins having different sizes, but the same charge/mass ratios) were characterized by nonparallel lines intersecting at or about 0% gel concentration in the same type of plot. Proteins differing in both charge and size gave rise to nonparallel lines intersecting at points other than 0% gel concentration. In addition, the slopes of such plots were directly related to molecular weights.

The resolution of phosphorylases *a* and *b* on disc gel electrophoresis is illustrated in Figure 1. In a 6% gel, phosphorylase *a* had an R_m of 0.230 ± 0.010 (standard deviation) while phosphorylase *b* had an R_m of 0.204 ± 0.010. The two proteins were resolved on the basis of differences in net charge rather than differences in molecular size (Figure 2) and are therefore, "charge isomer" proteins (Hedrick and Smith, 1968). Furthermore, the slopes of both lines corresponded to a molecular weight of 164,000 g/mole, a value in approximate agreement with values previously published for phosphorylase *b* (DeVincenzi and Hedrick, 1967; Seery *et al.*, 1967). Both enzymes were catalytically active at the conclusion of the run as shown by activity stains; thus, the enzymes had not been irreversibly denatured during electrophoresis.

Phosphorylases *a* and *b* have the same molecular size on disc gel electrophoresis due to the pH (8.5) and temperature (35°) of the electrophoresis medium. Under these conditions, the aggregated state of both enzymes has been shown to be dimeric by ultracentrifugal studies (Table I). Furthermore, phosphorylase *b* was relatively unaffected by limited variations of temperature or pH while phosphorylase *a* tended to dissociate with increasing temperature or pH. These findings are consistent with those obtained using Sephadex G-200 to characterize the aggregated state of phosphorylase[3] (D. L. DeVincenzi and J. L. Hedrick, 1969, unpublished data) and with predictions based on kinetic evidence (Wang and Graves, 1964).

It should be pointed out that the relative mobility of phosphorylase *a* was altered when thioglycolate was used in its preparation. Modification of the physical properties of phosphorylase *a* was also noted by Seery *et al.* when thioglycolate

[3] Several factors are important in determining the aggregated state of the enzyme; protein concentration is one crucial parameter. The approximate protein concentration in the bands on disc gel electrophoresis can be calculated from the amount of protein applied and the volume of the stained band. In these experiments, the protein concentration of the stained bands ranged from 0.2 to 0.5 mg per ml.

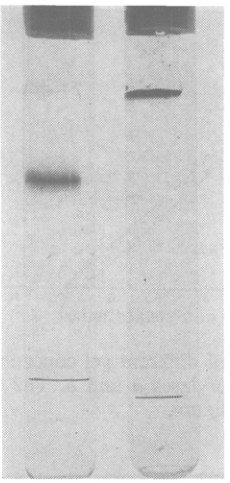

FIGURE 3: The mobility of phosphorylase *b* in the presence of 2 M urea. Electrophoresis performed as described in the text using a 6% polyacrylamide gel. Migration is from the top of the figure. The gel on the left was run at 4° (2 mA/tube) and the gel on the right at room temperature (4 mA/tube). After electrophoresis at room temperature, the gel was approximately 35° with the temperature increase due to ohmic heating.

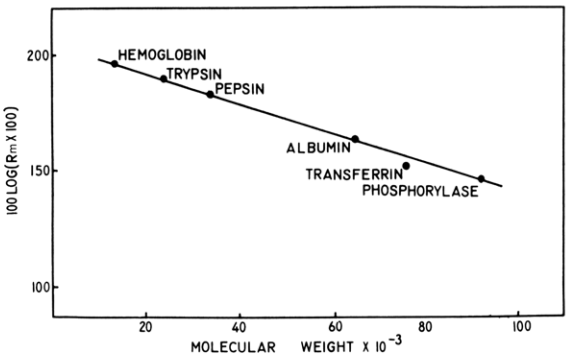

FIGURE 4: The relative mobilities of proteins (in the presence of SDS) as a function of their molecular weights. Samples were dialyzed overnight against 0.1% SDS, 0.05 M mercaptoethanol, and 0.01 M sodium phosphate buffer (pH 7.1) and then electrophoresed in a 6% gel containing 0.1% SDS. The proteins were stained using the dihydroxydinaphthyl disulfide method (Barrnett and Seligman, 1952) and/or with sulfosalicylic acid–coomassie blue (Shapiro *et al.*, 1967). The molecular weights of the various proteins used were hemoglobin, 17,000; trypsin, 24,000; pepsin, 34,000; bovine serum albumin, 65,000; and transferrin, 74,000 (Hedrick and Smith, 1968; Braunitzer *et al.*, 1964).

was used (Seery *et al.*, 1967). The R_m of phosphorylase *a* prepared utilizing thioglycolate was 0.280, while the R_m with an L-cysteine preparation was 0.230. The increase in R_m in the presence of thioglycolate is probably due to a change in the charge of the enzyme. Thiolation of the protein amino groups by the thioglycolide impurity in the thioglycolate could account for this increased mobility (Schoeberl, 1948; Benesch and Benesch, 1956).

The monomeric state of phosphorylase has also been characterized. Ultracentrifugal studies have led to the conclusion that phosphorylase *b* will dissociate to monomers (90,000 g/mole) in 2 M urea at 4° (Hedrick *et al.*, 1969). Disc gel electrophoresis under comparable conditions gave results subject to the same interpretation. When urea was incorporated into the gels and sample solution, the disc electrophoretic method gave a molecular weight of 90,000 g/mole for phosphorylase *b*. Temperature was a critical factor in this experiment, however. Electrophoresis performed at room temperature resulted in a highly aggregated form of the enzyme which did not migrate into the gel (Figure 3). Apparently the monomer form of the enzyme was unstable at the ambient temperature and tended to aggregate, as shown by its relative mobility at different gel concentrations. At both temperatures, however, the urea-treated and electrophoretically resolved enzymes were devoid of enzymatic activity.

TABLE 1: Sedimentation Velocity of Phosphorylases *a* and *b* As a Function of Temperature and pH.[a]

	s_{20} (S)			
	Phosphorylase *a*			Phosphorylase *b*
pH	4°	22°	33°	33°
6.5	14.7	13.9	10.6	8.2
7.5	14.8	14.3	9.8	8.4
8.5	17.1	11.4	8.5	8.8

[a] Experiments at pH 7.5 and 8.5 were carried out in 0.12 M Tris-HCl while those at pH 6.5 were in 0.09 M 2-(*N*-morpholino)ethanesulfonic acid–NaOH at an enzyme concentration of 1 mg/ml.

Similar results, in terms of monomer instability, were observed when the enzyme was treated with 1×10^{-4} M PMB. It was first observed by Madsen and Cori that PMB treatment of phosphorylase resulted in a species sedimenting in the ultracentrifuge as a monomer (Madsen and Cori, 1956). However, we have been unable to demonstrate a PMB monomer by either disc gel electrophoresis or in a continuous pH system, at ambient temperatures or in the cold (4°) (PMB present in the gels and reservoir buffers). At ambient temperatures the protein was highly aggregated as shown by its relative mobility at different gel concentrations. That PMB treatment does result in a monomer species has been verified by many investigators using a variety of physical techniques other than electrophoresis. The reason the electrophoretic method failed to demonstrate the presence of monomer was probably due to monomer instability under the conditions employed. However, others have reported that PMB treatment of the enzyme altered its mobility and electrophoretic pattern but unequivocable evidence that the enzyme was monomeric in these cases was lacking (Huang and Madsen, 1966; Chignell *et al.*, 1968).

Treatment of phosphorylase *b* with SDS dissociated the enzyme into monomers as previously shown by sedimentation equilibrium, sedimentation velocity and diffusion, and Sephadex gel filtration experiments (J. Perkins, University of Colorado, personal communication). Characterization by gel electrophoresis of the dissociated state of phosphorylase *b* produced by SDS is shown in Figure 4. Advantage has been taken of the observation of Shapiro *et al.* (1967) that in

SDS all proteins have the same net charge due to SDS binding. Thus, differences in protein mobilities are due only to size inequalities. As can be seen in Figure 4, there is a good correlation between the electrophoretic mobilities observed for the standard proteins and their molecular weights. The molecular weight of phosphorylase under these conditions was 92,000 g/mole, corresponding to the monomer.

We have not been able to unequivocally demonstrate the existence of a phosphorylase tetramer using either discontinuous (disc gel electrophoresis) or continuous gel electrophoresis. In disc gel electrophoresis the running pH has always been such that the dimer form predominates. In continuous gel electrophoresis, performed at lower pH values (pH 6.5 and 7.5), the dimer and tetramer forms were insufficiently resolved to permit identification of the different aggregated states.

Chemically Modified Forms of Phosphorylase. PHOSPHORYLASE *b'*. Phosphorylase *b'* and a positively charged phosphohexapeptide are produced from phosphorylase *a* by brief treatment with trypsin (Fischer *et al.*, 1959). Phosphorylase *b'* has the same enzymatic and molecular weight properties as phosphorylase *b* (Keller, 1955).

However, from the basic nature of the hexapeptide released, it might be anticipated that phosphorylase *b'* would have a larger net negative charge at pH 8.5 than phosphorylase *b* and, therefore, a larger R_m. As trypsin does produce proteolysis in other regions of the protein as well as at the serine phosphate site it was expected that "charge isomer" forms of phosphorylase *b'* might be produced. The first prediction was verified, but the second was not (Figure 5). The action of trypsin on phosphorylase *a* as a function of time was followed both enzymatically and by gel electrophoresis. The R_m values changed from 0.250 (phosphorylase *a*) to 0.273 (phosphorylase *b'*). Activity stains for phosphorylase *b'* showed it to be enzymatically active and AMP dependent. In comparison, the R_m of phosphorylase *b* under these conditions was 0.215. However, since only a single band was observed at all times during the experiment, "charge isomers" of phosphorylase *b'* were not present. These results agree in general with those recently published by Wang *et al.* (1968). Comparison of our results with those of Graves *et al.* (1968) is very difficult due to the diffuse electrophoretic patterns published by the latter authors.

$NaBH_4$-Reduced Phosphorylase b and Apophosphorylase b. Reduced phosphorylase *b* has the same physicochemical properties as phosphorylase *b* (Strausbauch *et al.*, 1967; D. L. DeVincenzi and J. L. Hedrick, 1969, unpublished data) with two exceptions: (a) pyridoxal phosphate is linked to the protein *via* a secondary amine bond to the ε-amino group of a lysine residue and, (b) the enzyme has different association–dissociation characteristics indicative of a change in protein structure. Neither of these differences was detectable by disc gel electrophoresis, since reduced phosphorylase *b* had the same R_m in a 6% gel as the native enzyme (R_m = 0.205). Again, the enzyme was active after electrophoresis.

Apophosphorylase *b* exhibits aggregation properties which are highly sensitive to temperature variations. At 35°, the enzyme is monomeric while at lower temperatures it can be dimeric and/or tetrameric (Hedrick *et al.*, 1966). However, when characterized by disc gel electrophoresis, an aggregate was produced at 35° which did not migrate much beyond the spacer-small pore gel junction (6% gel). Thus, as noted previously, under conditions of gel electrophoresis the monomeric form of the enzyme appeared to be unstable and to aggregate. At 4°, an enzymatically inactive dimer and a higher aggregate of the enzyme were present. An adequate characterization of the association–dissociation behavior of apophosphorylase *b* could not be carried out due to the limited stability of the enzyme under these conditions.

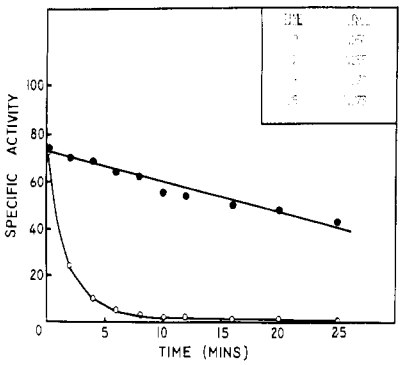

FIGURE 5: The conversion of phosphorylase *a* into phosphorylase *b'* by trypsin. A 2:1 molar ratio of phosphorylase *a* to trypsin was used. Initially, phosphorylase *a* was present at a concentration of 4.5 mg/ml. At various times 0.2-ml aliquots were removed and diluted 1:4 in the sample buffer for electrophoresis. The buffer also contained 6 mg/ml of soybean trypsin inhibitor. Electrophoresis was performed on the diluted samples and enzymatic assays on aliquots further diluted 1:50. The insert refers to the times at which samples were removed and subjected to electrophoresis. Only one broad band was observed after the reaction was initiated. (●) Activity in the presence of AMP and (○) activity in the absence of AMP.

Effect of Substrates and Other Ligands. It has previously been shown that substrates and allosteric effectors can influence the state of phosphorylase aggregation (Wang and Graves, 1964; Kent *et al.*, 1958; Metzger *et al.*, 1967, 1968). Glycogen, for instance, promotes the dissociation of phosphorylase *a* tetramer to dimer. The effect of 1% glycogen on phosphorylase structure was difficult to determine by gel electrophoresis as the R_m values were so low that precise measurements were difficult. The R_m of phosphorylase *a* was always greater than that of phosphorylase *b*, but both were <0.1 (6% gel). Lowering the glycogen concentration to 0.1% did not significantly increase the R_m of phosphorylase *b*. This drastic reduction in mobility was undoubtedly due to the strong binding of the enzyme to the immobile glycogen. An additional factor, however, that may play a role is the "sieving effect" produced by glycogen. Glycogen is a sufficiently large macromolecule that its presence may cause a retardation of proteins in a way analogous to that of polyacrylamide. This is illustrated by the effect of glycogen addition on mobility of bovine serum albumin, a protein which does not bind glycogen. In the presence of 0.1% glycogen the R_m of albumin is 0.73 (7% gel) while the absence of glycogen, the R_m is 0.84 (7% gel).

The effect of polysaccharide binding on the state of aggregation of phosphorylase could be examined when smaller molecular weight oligosaccharides were used. As indicated in Figure 6, the mobility of phosphorylase *b* was less in the presence of maltodextrin than in its absence (*e.g.*, 6% gel, R_m phosphorylase *b* = 0.204 *vs.* R_m = 0.173 when dextrin was present). However, the slope of a log R_m *vs.* gel concentration plot was essentially the same whether maltodextrin

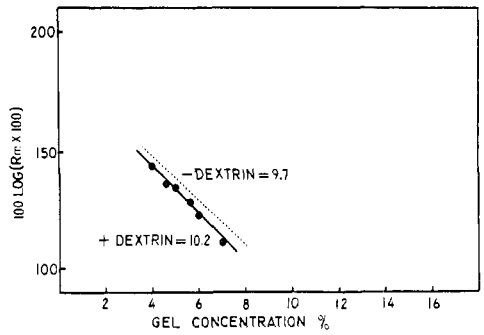

FIGURE 6: The effect of different gel concentrations on the relative mobility of phosphorylase *b* in the presence of maltodextrin. Maltodextrin (chain length of 5–12 glucose units) was present at a concentration of 1%. The dotted line was derived from Figure 2. The negative slopes of the lines are noted on the figure.

was present or absent. This slope corresponded to the dimer form of the enzyme. Under the same conditions, bovine serum albumin showed no change from the slope obtained in the absence of dextrin, indicating that the "sieving effect" of the medium was unaffected by the presence of 1% dextrin. In addition, glucose by the same criterion did not affect the aggregated state of the enzyme or its R_m.

Since the lines in Figure 6 are parallel, it would appear that the binding of dextrin causes a change in the charge of the protein. This is unlikely as the bound dextrin is uncharged. It is possible, however, that binding of the dextrin could cause a conformational change in the protein thus altering the pK values of a few ionizable groups thereby changing the net charge of the protein–ligand complex. We prefer to interpret the data as indicating that the addition of dextrin superimposes a binding phenomenon on the protein which results in a decrease of the observed mobility. If it is assumed that the dextrin–enzyme complex has a low mobility relative to that of the free enzyme, then the observed R_m is a function of the equilibrium between the enzyme–dextrin complex and the free enzyme. Thus the observed migration results from the electrophoretic transport of free enzyme with only a small contribution from the transport of the complex. The apparent molecular size of the enzyme determined by gel electrophoresis, then, would be the same in the presence as in the absence of dextrin, although the mobility at any one gel concentration would be less when dextrin is present. That this is a potentially valid interpretation of the data is shown by the following theoretical treatment of the binding phenomenon.

The relationship between gel concentration and protein mobility observed in a plot of gel concentration *vs.* log R_m can be expressed by

$$\log R_m^{P} = A - SG \qquad (1)$$

where R_m^{P} is the mobility of the protein relative to the dye, A is the intercept, S the slope, and G the gel concentration. If a neutral ligand, L, were to bind to the protein thereby changing the size of the protein but not the net charge, then a change in slope would be observed as defined by

$$\log R_m^{PL} = A - \alpha SG \qquad (2)$$

where α is the coefficient or multiplier of S giving rise to the different slope of the line. Expressing the above in an exponential form, $R_m^{P} = 10^{(A-SG)}$ for the protein and $R_m^{PL} = 10^{(A-\alpha SG)}$ for the protein–ligand complex.

In the rapidly attained equilibrium

$$P + L \rightleftharpoons PL$$

the fraction of the protein, P, in the unbound form can be designated as F and the fraction in the protein–ligand complex, PL, as $1 - F$. Thus the observed R_m of a protein in the presence of a ligand is

$$R_m = F \times 10^{(A-SG)} + (1 - F)10^{(A-\alpha SG)} = (10^{A-SG})[F + (1 - F)10^{-SG(\alpha-1)}] \qquad (3)$$

and

$$\log R_m = A - SG + \log [F + (1 - F)10^{-SG(\alpha-1)}] \qquad (4)$$

If the fraction of the protein in the protein–ligand complex approaches zero, the above equation reduces to eq 1. If the fraction of the protein in the protein–ligand complex approaches one, then eq 4 reduces to eq 2.

The slope of a log R_m *vs.* gel concentration is given by the differential form of eq 4

$$\text{slope} = \frac{d \log R_m}{dG} = -S - \frac{(1 - F)(\alpha - 1)(S)10^{-SG(\alpha-1)}}{F + (1 - F)10^{-SG(\alpha-1)}} \qquad (5)$$

The limits of the slope can be seen to be $-S$ when $G \rightarrow \infty$ and $-S[1 + (1 - F)(\alpha - 1)]$ when $G \rightarrow 0$. In the same manner, as $F \rightarrow 0$, slope $\rightarrow -S$, and when $F \rightarrow 1$, slope $\rightarrow \alpha S$. Thus nonlinear curves should be obtained which become parallel with a slope of $-S$ at high gel concentrations.

In order to evaluate the nature of the curves at values intermediate to the above limits, a computer program was prepared and R_m values obtained as a function of varying F and α values. Figures 7 and 8 depict the results of two such computations.

For protein–ligand complexes where the slope of the line differs by less than a factor of two ($\alpha < 2$), a series of nonparallel lines are obtained at different values of F which have a common point of intersection on the ordinate (Figure 7). At values of $\alpha > 3$, nonlinear curves are obtained which intersect at a common point when the gel concentration approaches zero, and become parallel when the gel concentration is relatively high (Figure 8). The degree of curvature is a function of F.

It is immediately apparent that as the mobility of the protein–ligand complex approaches zero ($\alpha \rightarrow \infty$), a series of parallel lines would be obtained at gel concentrations between 4 and 12% as F varies between one and a number approaching zero. This theoretically derived relation generates the same type of data as was experimentally observed for the binding of phosphorylase *b* to dextrin (Figure 6).

Thus, ligand binding which causes a reduction in the R_m of a protein, due to a large increase in the size of the complex relative to the protein, can also give rise to parallel lines when plotted in the above manner. The previous explanation

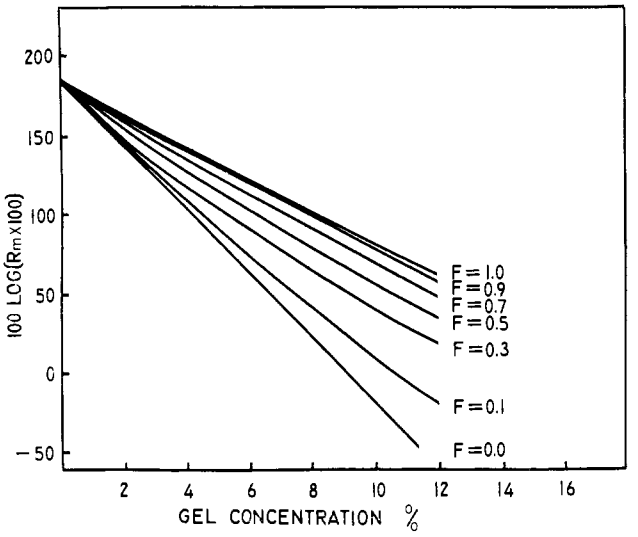

FIGURE 7: Computer-generated curves of the protein–ligand mobility. The curves were generated by substitution of the following values in eq 7: A = 185, S = 10.2, α = 2, G = 4–12%, and F = 0–1.

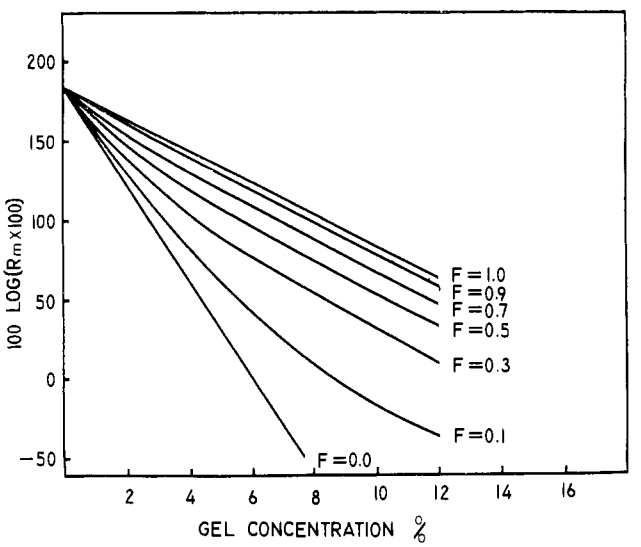

FIGURE 8: Computer-generated curves of the protein–ligand mobility. The curves were generated as in Figure 7 with the exception of α = 3.

for such an observation was that the protein species involved had different charges but the same size. The exception noted here must now be taken into consideration when interpreting data from binding experiments where ligand binding can considerably change the size of the molecules being studied. It should also be possible to obtain protein–ligand binding constants from this type of analysis by varying the concentration of ligand present.

It is fortuitous that the "sieving effects" noted for proteins on gel electrophoresis can be directly related to their molecular weights. This is due to the relative uniformity of protein density and shape (predominantly globular). As shown by Ackers, the behavior of proteins should be more properly related to Stokes radii than molecular weights when utilizing techniques which involve "molecular sieving" or processes analogous to restricted diffusion (Ackers, 1964). Binding of dextrin to phosphorylase *b* could cause a relatively large change in the size of the molecule when one considers the Stokes radii of the molecules. As 2 moles of dextrin bind to 1 mole of phosphorylase *b*, the Stokes radius of the complex can be considered to increase by the diameter of the dextrin molecule. The Stokes radius of phosphorylase *b* has been measured and is 49 Å (DeVincenzi and Hedrick, 1967). Estimates for the Stokes radii of dextrins are more difficult to make, however, as they have not been experimentally determined and the dextrin preparation used here was heterogeneous with regard to chain length (5–12 glucose units). However, values for the Stokes radius or diameter of γ-Schardinger dextrin, a cyclic octadextrin, can be calculated from diffusion and X-ray crystallographic data reported in the literature (Weber, 1956; French, 1957). The diameter of this cyclic compound is 16 Å. However, the dextrin chains with which we are working are linear, and therefore, more likely correspond to the extended form of the γ-Schardinger dextrin which is approximately 50 Å (the circumference of the 16-Å cyclic octadextrin). Thus, the diameter of a linear octadextrin can be considered to lie in the range of 16–50 Å. The geometry of dextrin binding to the surface of the enzyme is likely to be approximately perpendicular as phosphorylase works on the nonreducing end of a polyglucose chain. In addition, the spacing of branch points in a glycogen molecule would sterically prevent the binding of anything but the outermost glucose residues of an α-(1–4)-glycosidic chain (Brown and Cori, 1961). The binding of a linear octadextrin would increase the Stokes radius of phosphorylase *b* from 49 to 65–99 Å, a change of 1.3–2.0-fold. Since the volume of a sphere is related to the cube of its radius, this would increase the apparent volume of the complex 2.4–8.3-fold. From a previous publication (Hedrick and Smith, 1968) a 2-fold change in molecular weight (equivalent to a volume change here) changes the slope of a log R_m *vs.* gel concentration plot 1.5-fold. This slope change corresponds to the symbol α, the size factor of eq 2. Thus, volume changes of 2.4–8.3-fold correspond to α changes of 1.8–6.2. Since α values of approximately three and more give rise to a series of parallel lines as shown in Figure 8, and the larger α value is probably the better estimate for the size of a linear octadextrin, it is not unreasonable to accept this model of the phosphorylase–dextrin complex as fitting the experimental data.

AMP has been shown to promote the aggregation of phosphorylase *b* and the dissociation of phosphorylase *a* (D. L. DeVincenzi and J. L. Hedrick, 1969, unpublished data; Kent *et al.*, 1958). Its effect on both enzymes under the conditions of disc gel electrophoresis was only one of changing the charge as indicated in Figure 9. After electrophoresis both phosphorylases *a* and *b* were enzymatically active and had higher R_m values when AMP was present than when it was absent. The lines in the figure had equivalent slopes, indicating that the enzymes had the same molecular size, but different net charges. The slope in this instance corresponded to a molecular weight of 172,000 g/mole, evidence that the aggregated state was dimeric.

Two other proteins were characterized under the above conditions in order to ascertain that incorporation of AMP into the electrophoresis medium had not changed the polyacrylamide gel structure and also to verify that the AMP

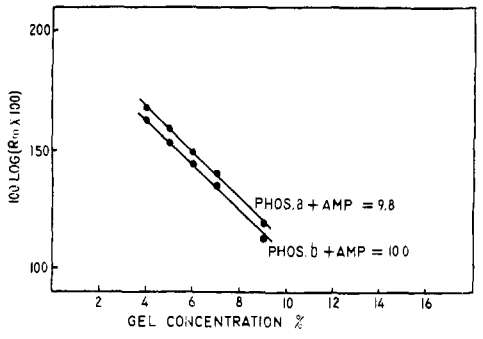

FIGURE 9: The effect of different gel concentrations on the relative mobility of phosphorylases *a* and *b* in the presence of AMP (1×10^{-3} M). The negative slopes of the lines are noted on the figure.

effect was specific for phosphorylase. Bovine serum albumin and ferritin, both of which should not bind AMP, had the same mobilities in the presence as in the absence of AMP. Thus, the gel structure was not changed by inclusion of AMP and the AMP effect was specific for phosphorylase.

Glucose-6-P promotes dissociation of phosphorylase *a* as well as the hybrid form of the enzyme composed of phosphorylated and nonphosphorylated subunits (Hurd *et al.*, 1966). By the same electrophoretic criterion used for the AMP effect, glucose-6-P bound to both phosphorylases *a* and *b* changed the net charge of the enzymes but not their molecular sizes. At 1×10^{-3} M glucose-6-P, the R_m in a 6% gel of phosphorylase *b*, increased more (from 0.206 to 0.258) than the R_m of phosphorylase *a* (from 0.238 to 0.276). These data lead to the conclusion that phosphorylase *b* had a higher affinity for G-6-P than phosphorylase *a*. However, no glucose-6-P binding constants for the two enzyme forms have been reported in the literature to verify this conclusion.

Discussion

Using the previously developed analysis technique we have demonstrated that phosphorylases *a* and *b* have the same size but different charges under the conditions of disc gel electrophoresis. Thus, by this criterion, they are "charge isomers." The fact that many substrates and ligands (dextrin, AMP, and glucose-6-P) shown by other physical techniques to dissociate phosphorylase *a* tetramer to dimer do not affect the apparent size of phosphorylases *a* and *b* under the conditions of electrophoresis is further evidence that the enzymes are present as dimers.

We have also demonstrated that even though ligands are bound to a protein and alter its relative mobility, the protein molecular size can still be determined by the disc gel electrophoretic method. Thus, phosphorylase *b* exhibited increased mobility when AMP or glucose-6-P were bound due to a change in the charge of the protein; its relative mobility was decreased when dextrin was bound; but again, it was most probably not due to a change in the molecular size of the enzyme.

Characterization of a monomer form of the enzyme (90,000 g/mole) by gel electrophoresis was possible using SDS or 2 M urea at 4°. This value agrees with those obtained using ultracentrifugation and guanidine hydrochloride to dissociate the enzyme (Seery *et al.*, 1967; Ullman *et al.*, 1968). Treatment of the enzyme with PMB, urea at 35°, or resolution of the enzyme appeared to produce a highly aggregated state of the enzyme. Failure to show the monomer form of the enzyme in these cases was probably due to the instability of the modified enzyme under the electrophoretic conditions employed. Even when AMP was added to the electrophoretic medium, no stabilization of the monomer or change in the electrophoretic pattern was observed. As stated earlier, we have been unable thus far to demonstrate tetrameric forms of either phosphorylase *a* or *b* on gel electrophoresis.

The results reported here on the characterization of the aggregated state of phosphorylase *a* by gel electrophoresis differ markedly from those reported by Chignell *et al.* (1968). Using a continuous gel electrophoresis system, Chignell *et al.* reported that phosphorylases *a* and *b* had the same mobilities at pH 8.75. They also stated that a second phosphorylase *a* band was present under these conditions which they interpreted as a tetrameric form of the enzyme. In addition, in their hands AMP caused formation of the tetrameric species of both phosphorylases *a* and *b*, and PMB resulted in monomers plus a number of aggregated forms. We have repeated this work using the methods reported by Chignell *et al.* and cannot verify their results. Instead, using their methods, we obtained results consistent with the data reported here. It should be pointed out that Chignell *et al.* had no unequivocal way of assigning the state of enzyme aggregation to the various bands observed in their gels. Confirmation of our observation that phosphorylase *a* has a higher mobility on gel electrophoresis than phosphorylase *b* have appeared (Davis *et al.*, 1967; Huang and Madsen, 1966; Wang *et al.*, 1968).

The theoretical equations involving ligand binding derived in this report place certain limitations on the interpretation of size–charge relations of proteins. Binding of uncharged ligands can give rise to results which appear to reflect a change in the charge of the protein. These observations are clearly predictable by mathematical relations and permit the interpretation that no change in charge is occurring. These findings should extend the usefulness of the disc gel electrophoresis method to the investigation of protein–ligand interactions on polyacrylamide gels.

Acknowledgements

The authors are indebted to Dr. William J. Whelan for the sample of maltodextrin and Dr. Dexter French for a very enlightening and helpful discussion on the molecular size of γ-Schardinger dextrin.

References

Ackers, G. K. (1964), *Biochemistry 3*, 723.

Appleman, M. M., Yunis, A. A., Krebs, E. G., and Fischer, E. H. (1963), *J. Biol. Chem. 238*, 1358.

Barrnett, R. I., and Seligman, A. M. (1952), *Science 116*, 322.

Benesch, R., and Benesch, R. E. (1956), *J. Am. Chem. Soc. 78*, 1597.

Braunitzer, G., Hilse, K., Rudloff, V., and Hilschmann, N. (1964), *Advan. Protein Chem. 19*, 1.

Brown, D. H., and Cori, C. F. (1961), *Enzymes 5*, 207.

Chignell, D. A., Gratzer, W. B., and Valentine, R. C. (1968), *Biochemistry 7*, 1082.

Davis, C. H., Schlieselfeld, L. H., Wolf, D. P., Leavitt, C. A., and Krebs, E. G. (1967), *J. Biol. Chem. 242*, 4824.
DeVincenzi, D. L., and Hedrick, J. L. (1967), *Biochemistry 6*, 3489.
Fischer, E. H., Graves, D. J., Crittenden, E. R. S., and Krebs, E. G. (1959), *J. Biol. Chem. 231*, 65.
Fischer, E. H., Kent, A. B., Synder, E. R., and Krebs, E. G. (1958b), *J. Am. Chem. Soc. 80*, 2906.
Fischer, E. H., and Krebs, E. G. (1958), *J. Biol. Chem. 231*, 65.
Fischer, E. H., Krebs, E. G., and Kent, A. D. (1958a), *Biochem. Prepn. 6*, 68.
French, D. (1957), *Advan. Carbohydrate Chem. 12*, 189.
Graves, D. J., Mann, S. A. S., Philip, G., and Oliveira, R. J. (1968), *J. Biol. Chem. 243*, 6090.
Hedrick, J. L., and Fischer, E. H. (1965), *Biochemistry 4*, 1337.
Hedrick, J. L., Shaltiel, S., and Fischer, E. H. (1966), *Biochemistry 5*, 2117.
Hedrick, J. L., Shaltiel, S. S., and Fischer, E. H. (1969), *Biochemistry 8*, 2422.
Hedrick, J. L., and Smith, A. J. (1968), *Arch. Biochem. Biophys. 126*, 155.
Huang, C., and Madsen, N. B. (1966), *Biochemistry 5*, 116.
Hurd, S. S., Teller, D., and Fischer, E. H. (1966), *Biochem. Biophys. Res. Commun. 24*, 79.
Keller, P. J. (1955), *J. Biol. Chem. 214*, 135.
Kent, A. B., Krebs, E. G., and Fischer, E. H. (1958), *J. Biol. Chem. 232*, 549.
Krebs, E. G., Love, D. S., Bratvold, G. E., Trayser, K. A., Meyer, W. L., and Fischer, E. H. (1964), *Biochemistry 3*, 1022.
Madsen, N. B., and Cori, C. F. (1956), *J. Biol. Chem. 223*, 1055.
Metzger, B. E., Glaser, L., and Helmreich, E. (1968), *Biochemistry 7*, 2021.
Metzger, B., Helmreich, E., Glaser, L. (1967), *Proc. Natl. Acad. Sci. U. S. 57*, 994.
Nolan, C., Novoa, W. B., Krebs, E. G., and Fischer, E. H. (1964), *Biochemistry 3*, 542.
Pearse, A. G. E. (1961), Histochemistry, Boston, Mass., Little, Brown, p 831.
Schoeberl, A. (1948), *Angew. Chem. 60*, 7.
Seery, V. L., Fischer, E. H., and Teller, D. C. (1967), *Biochemistry 6*, 3315.
Shaltiel, S., Hedrick, J. L., and Fischer, E. H. (1966), *Biochemistry 5*, 2108.
Shapiro, A. L., Vinuella, E., and Maizel, J. V. (1967), *Biochem. Biophys. Res. Commun. 28*, 815.
Strausbauch, P. H., Kent, A. B., Hedrick, J. L., and Fischer, E. H. (1967), *Methods Enzymol. 11*, 671.
Ullman, A., Goldberg, M. E., Perrin, D., and Monod, J. (1968), *Biochemistry 7*, 261.
Wang, J. H., and Graves, D. J. (1964), *Biochemistry 3*, 1437.
Wang, J. H., Humniski, P. M., and Black, W. J. (1968), *Biochemistry 7*, 2037.
Weber, R. V. (1956), *J. Am. Chem. Soc. 78*, 536.