Physical properties and catalytic function of glycogen phosphorylase

Charles Yung Huang
Iowa State University

Follow this and additional works at: https://lib.dr.iastate.edu/rtd

Part of the Biochemistry Commons

Recommended Citation
Huang, Charles Yung, "Physical properties and catalytic function of glycogen phosphorylase " (1968). Retrospective Theses and Dissertations. 3475.
https://lib.dr.iastate.edu/rtd/3475

This Dissertation is brought to you for free and open access by the Iowa State University Capstones, Theses and Dissertations at Iowa State University Digital Repository. It has been accepted for inclusion in Retrospective Theses and Dissertations by an authorized administrator of Iowa State University Digital Repository. For more information, please contact digirep@iastate.edu.
This dissertation has been microfilmed exactly as received 69-4242

HUANG, Charles Yung, 1935-
PHYSICAL PROPERTIES AND CATALYTIC FUNCTION OF GLYCOGEN PHOSPHORYLASE.

Iowa State University, Ph.D., 1968
Biochemistry

University Microfilms, Inc., Ann Arbor, Michigan
PHYSICAL PROPERTIES AND CATALYTIC FUNCTION
OF GLYCOGEN PHOSPHORYLASE

by

Charles Yung Huang

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Major Subject: Biochemistry

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

Head of Major Department

Signature was redacted for privacy.

Dean of Graduate College

Iowa State University
Ames, Iowa

1968
TABLE OF CONTENTS

DEDICATION iii

GENERAL INTRODUCTION 1

EXPERIMENTAL PROCEDURE 4

PART I. EXTINCTION COEFFICIENTS AND MOLECULAR WEIGHTS 9a

INTRODUCTION 9b

RESULTS 11

DISCUSSION 29

PART II. CORRELATION BETWEEN SUBUNIT INTERACTION AND ENZYMATIC ACTIVITY OF PHOSPHORYLASE a 34a

INTRODUCTION 34b

THEORY: A METHOD FOR DETERMINING EQUILIBRIUM CONSTANT FROM RATE MEASUREMENTS 36

RESULTS 51

DISCUSSION 78

SUMMARY 85

APPENDIX 88

BIBLIOGRAPHY 94

ACKNOWLEDGMENTS 97
DEDICATION

To My Parents
GENERAL INTRODUCTION

Glycogen phosphorylase (EC 2.4.1.1) catalyzes the first reaction in the utilization of glycogen:

\[
glycogen (n \text{ glucosyl units}) + \text{inorganic phosphate} \rightleftharpoons \text{glucose-1-phosphate} + \text{glycogen (n-1 glucosyl units)}
\]

Although the equilibrium of the reaction favors the reverse process, this enzyme has been shown to function in vivo only in the breakdown of glycogen. It is part of a subtle control system involving several enzymes, substrates, nucleotides, divalent metal ions, and hormones.

Phosphorylase from rabbit skeletal muscle has been isolated in two forms, designated as \text{a} and \text{b}. These two species are interconvertible through specific enzymic phosphorylation and dephosphorylation according to the following stoichiometry:

\[
\text{2 phosphorylase } b \xrightarrow{\text{Mg}^{++} \text{Phosphorylase } b \text{ kinase}} \text{phosphorylase } a \xrightarrow{\text{Phosphorylase phosphatase}} 2 \text{phosphorylase } b
\]

\[
\begin{align*}
4\text{ATP} & \quad \text{4ADP} \\
\text{Mg}^{++} & \\
\text{Phosphorylase } b \text{ kinase} & \quad \text{phosphorylase } a \\
\text{Phosphorylase phosphatase} & \quad 4\text{P}_1 \\
& \quad 4\text{H}_2\text{O}
\end{align*}
\]

Phosphorylase \text{b} is a dimer (consisting of two identical subunits) of molecular weight 177,000-195,000 and is active only
in the presence of adenosine-5'-monophosphate (AMP). Phosphorylase a differs from b in that it does not require AMP for activity, has a molecular weight twice that of b (hence a tetramer), and contains four phosphoseryl residues (cf. Brown and Cori, 1961; Krebs and Fischer, 1962). The finding by Wang and Graves (1964, 1965a, 1965b) of a catalytically more active dimeric form of phosphorylase a indicates that the catalysis of glycogen breakdown in the absence of AMP by this enzyme is more directly related to phosphorylation of the seryl residues rather than the doubling of molecular weight. Kinetic studies by the same authors (Wang and Graves, 1964) also showed that there is a dynamic equilibrium between tetrameric and dimeric species. Since most enzymes involved in the regulation of metabolism are composed of subunits and often undergo reversible dissociation-association reactions, it is widely thought that such a general phenomenon may be a means by which metabolism is controlled in vivo. Monod et al. (1965) have proposed a model, based on the transition between two conformational states of different affinities for ligands, to interpret the cooperative type kinetics displayed by many multi-site enzymes. Such apparent cooperativity may arise in reversibly dissociating systems where molecular aggregates of varying degrees dif-

1 Abbreviations: AMP, adenosine-5'-monophosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; DTT, dithiothreitol; glycerol-P, glycerophosphate; G·HCL, guanidine hydrochloride.
fer in their intrinsic activities and/or ligand binding affinities (Frieden, 1967). The subunit aggregation and deaggregation of phosphorylase a seems to be a good example of such a system especially in view of the fact that the formation of higher activity dimeric form is greatly enhanced by the presence of the substrate glycogen and several other carbohydrates (Wang et al., 1965a, 1965b; Metzger et al., 1967).

To further characterize the dissociation-association reaction, the investigations reported here are keyed to the following themes: (1) to provide direct evidence for the dissociation of phosphorylase a by molecular weight measurements; (2) to establish the relationship of molecular weight to enzymatic activity; (3) to examine the model of dynamic equilibrium between a more active dimer and a less active tetramer. Several physical constants for phosphorylases a and b have been determined or reexamined; a method for evaluating equilibrium constant from activity measurements has been developed; and a list for calculating specific refractive increment for a protein from its amino acid composition has been compiled.
EXPERIMENTAL PROCEDURE

Materials

Crystalline phosphorylase b from rabbit skeletal muscle was prepared according to the procedure of Fischer and Krebs (1958) except that dithiothreitol (DTT) was used in place of cysteine throughout the preparation. Phosphorylase a was prepared from phosphorylase b using phosphorylase b kinase as described by Fischer and Krebs (1962). Both enzymes were recrystallized at least four times and made AMP-free by treatment with acid-washed Norit A. Before separating Norit A from protein by centrifugation, the mixture was incubated at 35° for 10-15 min, which in general favored removal of AMP as judged by the ratio of absorbancies at 260 μm and 280 μm. Enzyme stock solutions were dialyzed against the buffer to be used for at least twenty-four hours at 5°. Enzymatic activity was assayed before each experiment in order to ensure that the enzyme was fully active.

Shellfish glycogen and glucose-1-phosphate were purchased from Sigma Chemical Co. Glycogen was made AMP-free according to the procedure of Sutherland and Wosilait (1956). Frozen rabbit muscle was purchased from Pel-Freeze Biologicals. Glycero-P was obtained from either Calbiochem or Sigma Chemical Co.; DTT from Calbiochem; G·HCl from Kodak Organic Chemical; and AMP from Pabst Laboratories. Standard bovine albumin in an ampul was purchased from Armour Pharmaceutical Co. Ludox was
the product of E. I. DuPont. All other chemicals were reagent grade.

Methods

Specific refractive increments were measured by means of a Brice-Phoenix differential refractometer, model BP-2000-V, equipped with temperature control. The instrument was calibrated, as suggested by the manufacturer, with KCl solutions prepared from samples dried in vacuum oven at 110° over Drierite. The data of Kruis (1936) were used to calculate the correction factor. In measuring refractive index increment, at least 20 minutes were allowed for the samples and the reference buffer to attain the same temperature. Duplicate measurements were carried out for each sample. Refractive index of water was calculated from International Critical Tables. Protein concentrations were determined by absorbance at 280 μm, using $A_{280}^{1mg/ml} = 1.27$ for phosphorylase a and $A_{280}^{1mg/ml} = 1.18$ for b determined as described in Part I. Absorption measurements at single wavelengths were made with a Beckman DU with Gilford absorbance indicator. Spectra of phosphorylases a and b were measured with a Cary 15 double beam spectrophotometer. All cuvettes used were calibrated with appropriate blank solutions prior to each measurement.

In dry weight determinations, duplicate measurements of absorbance of stock protein solution were made at all dilutions. Varying portions of protein and buffer solutions were
transferred into weighing bottles of known constant dry weights to make up a final total volume of 5 ml. Samples were first allowed to evaporate at $60^\circ-70^\circ$ and then vacuum dried at $110^\circ$ over Drierite. After at least 24 hours, samples were allowed to cool to room temperature under vacuum, quickly transferred to a desiccator, and immediately weighed on an Ainsworth Type 12 electrical balance. The balance was checked with standard weight before each series of weighing and the cabinet was desiccated. The drying and weighing process was repeated over and over until attainment of constant weights.

Phosphorylases a and b used in biuret reactions and in G-HCl experiments were dialyzed against the same 40 mM glycero-P buffer, PH 6.8. Values of $\% N$ used for protein concentration calculations were 16.05 for bovine serum albumin (Perlmann and Longsworth, 1948) and 16.9 for phosphorylase (Appleman et al., 1963).

Molecular weight measurements were performed with a Sofica light scattering photometer (Wippler and Scheibling, 1954) with thermostated benzene vat. The instrument was calibrated with Ludox twice, once according to the method of Maron and Lou (1954) and once according to the modified method of Goring et al. (1957). It was found that, when pure benzene was used as reference, a correction factor of $(\text{refractive index of solvent/refractive index of benzene})^2$ for excess scattering volume as derived by Hermans and Levinson (1951) was essentially correct. Rayleigh ratios for benzene at 546 m$\mu$ used were
15.7 \times 10^{-6} \text{ cm}^{-1} \text{ at } 20^\circ \text{ (cf. Kratohvil et al. 1962)}, 16.3 \times 10^{-6} \text{ cm}^{-1} \text{ at } 25^\circ, \text{ and } 16.9 \times 10^{-6} \text{ cm}^{-1} \text{ at } 30^\circ \text{ (Carr and Zimm, 1950). Molecular weights were calculated as described in Part I. Protein samples were dedusted by filtration through either 0.45 \mu \text{ pore size HA type or 0.22 \mu GS type Millipore filters directly into 2 cm-diameter cylindrical cells and recyled through the same filter three times. The average dissymmetry ratio, } \frac{I_{450}}{I_{1350}}, \text{ was 1.16. All samples were incubated at the desired temperature for at least 30 min and allowed another 15 min in the benzene bath prior to taking readings. Protein concentrations were determined spectrophotometrically after the light scattering measurements. Specific refractive increments used in the calculation of molecular weights are listed in Table 1 in Part I.}

Enzymatic activity was measured in the reverse direction (inorganic phosphate formation) according to the procedure of Illingworth and Cori (1953). This method was chosen because it permits rate measurements to be carried out in a wide range of enzyme concentration. Assay time varies with protein concentration and temperature as stated in figure legends. In preincubation with glycogen experiments, enzyme samples were incubated with 2\% glycogen for at least two hours at room temperature and then at least 30 minutes at the desired temperature before assay. Arsenolysis was carried out according to the method described by Kent (1959). In kinetic studies, prog-
ress curves of phosphate release were run in duplicates for each sample and a tangentimeter was employed to estimate initial velocities.
PART I. EXTINCTION COEFFICIENTS AND MOLECULAR WEIGHTS
INTRODUCTION

The concentration of rabbit skeletal muscle glycogen phosphorylases \(a\) and \(b\) is ordinarily determined by their absorption in the ultraviolet region. An absorbance index, \(A^{1\text{mg/ml}}_{280}\), of 1.17 at 280 \(\mu\)m was first obtained by Velick and Wicks (1951) for phosphorylase \(a\). Appleman et al. (1963) reported a value of 1.19 at 278 \(\mu\)m for phosphorylase \(b\). These two values agree quite well and have been used to determine the concentration of phosphorylase in a wide variety of investigations. In the course of studying subunit dissociation-association of phosphorylase \(a\) by light scattering, a specific refractive increment, \((\text{dn/dc})_{T, P, \mu}^{1}\), of 0.160-0.163 at 546 \(\mu\)m, 25\(^{\circ}\), was consistently obtained when computed on the basis of \(A^{1\text{mg/ml}}_{280} = 1.18\). This value is unusually low for a protein like phosphorylase that is not composed predominantly of amino acid residues of low refraction. The low \(\text{dn/dc}\) is therefore suggestive of an inaccurate extinction coefficient; it also appears to support the higher figures of 1.35 for phosphorylase \(b\) reported by Buc and Buc (1967), and more recently 1.32 reported by Metzger et al. (1968) and 1.31 reported by

\(^{1}\text{All absorbance indices mentioned in this report are for 1 cm light path.}\)
Gold (1968). Since a correct absorbance index is important not only to the determination of molecular weights by light scattering (dn/dc and protein concentration) but also to the results of partial specific volume (hence molecular weights measured by ultracentrifugation and gel filtration), ligand binding studies, etc., it seems necessary to redetermine the extinction coefficient of phosphorylase by dry weight—the ultimate method on which all the values reported so far were directly or indirectly based.
RESULTS

Specific Refractive Increments of Phosphorylase a

The specific refractive increment of a protein solution at constant temperature, constant pressure, and constant chemical potentials of diffusible solutes is independent of its concentration over a wide range of this variable and is defined as \(\frac{n-n_0}{c}\), where \(n\) and \(n_0\) are the refractive indices of the solution and the solvent, and \(c\), the protein concentration in g/ml. Alternatively it is defined as \(dn/dc\) since in general the refractive index of a solution can be expressed as

\[ n = n_0 + c \frac{dn}{dc} . \]

Thus all the specific refractive increments listed in Table 1 were computed from the least square slopes of \((n-n_0)\) vs. \(c\) plots. In all cases, the lines passed through, or very close to, the zero point. When the concentration of phosphorylase a was calculated by taking \(A_{280}^{1\text{mg/ml}}\) to be 1.18, a value of 0.163 was obtained in 40 mM glycero-P-1 mM EDTA buffer containing 1% KCl, pH 6.8, at 546 m\(\nu\), 25\(^\circ\). This value is substantially lower than the average value of 0.185 for proteins in water at this wavelength and temperature. Although refractive index increment for protein in salt solution has a lower value, the difference is usually small. To assure that the calibration factor for the differential refractometer was not in error the instrument was recalibrated twice and its optical system was
Table 1. Specific refractive increments of phosphorylases a and b at 546 mp, 25°

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Specific refractive increments (dn/dc) in ml/g</th>
<th>Phosphorylase a</th>
<th>Phosphorylase b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of samples</td>
<td>$A_{280}^{1 \text{mg/ml}}$</td>
<td>$A_{280}^{1 \text{mg/ml}}$</td>
</tr>
<tr>
<td>40 mM glycero-P-1 mM EDTA, 1% KCl, pH 6.8</td>
<td>11</td>
<td>0.163</td>
<td>0.176</td>
</tr>
<tr>
<td>40 mM glycero-P-1 mM EDTA, 1% NaCl, pH 6.8</td>
<td>8</td>
<td>0.160</td>
<td>0.172</td>
</tr>
<tr>
<td>40 mM glycero-P-1 mM DTT, 0.5% NaCl, pH 6.8</td>
<td>7</td>
<td>0.161</td>
<td>0.173</td>
</tr>
<tr>
<td>40 mM glycero-P-1 mM DTT, pH 6.8</td>
<td>7</td>
<td>0.167</td>
<td>0.180</td>
</tr>
<tr>
<td>Water a</td>
<td>---</td>
<td>---</td>
<td>0.182</td>
</tr>
<tr>
<td>Water b</td>
<td>---</td>
<td>---</td>
<td>0.185</td>
</tr>
</tbody>
</table>

*Calculated from the values obtained in 40 mM glycero-P-1 mM DTT, at pH 6.8, using the equation of Casassa and Eisenberg (1964).*

*Calculated from the amino acid composition determined by Appleman et al. (1963) and the refraction data of McMeekin et al. (1964).*
realigned once. Again, the values of 0.160 and 0.161 were observed (Table 1). The dn/dc obtained in buffer containing no NaCl, 0.168, though higher, was still low for a protein of no seemingly abnormal amino acid composition. And, calculation from amino acid composition (Appleman et al., 1963) using the refraction data of McMeekin et al. (1964) yielded a theoretical value of 0.185 ± 0.007 in water (cf. Appendix). All these observations and calculations seemed to imply that the extinction coefficient for phosphorylase should have a higher value than 1.18.

**Dry Weight Determinations of the Absorbance Indices of Phosphorylases a and b**

The extinction coefficient of phosphorylase b was investigated first for two reasons: (1) Buc and Buc (1967) reported an A\textsubscript{280}\textsuperscript{1} mg/ml of 1.35 determined by biuret and amino acid analyses, which is considerably higher than the 1.19 reported by Appleman et al. for b. Since the phosphorylase b sample used in the experiments of Appleman et al. was exhaustively dialyzed against distilled water, possible change in protein conformation might have led to low absorption. (2) AMP-free phosphorylase b has high solubility and does not crystallize, making it a better choice than a for absorbance and dry weight measurements. To ensure that the absorbance of the native enzyme was measured, stock phosphorylase b solution was dialyzed, instead of distilled water, against 40 mM glycero-P-1 mM DTT buffer, pH 6.8 and its enzymatic activity assayed prior to
each series of experiments. A series of samples containing 0, 20, 40, 60, 80, and 100% stock enzyme solution in a final total volume of 5 ml was prepared. This method has two advantages: (1) It permits the extinction coefficient to be determined in the presence of buffer, thus avoiding any undesirable changes that may be introduced due to the absence of thiol reagent or change in pH and ionic strength. (2) When all the samples reach constant weights and all the points in a dry weight vs. % stock protein solution plot fall on a straight line, it is a good indication that the samples are void of moisture. The same stock solution was also prepared in at least three dilutions in duplicates for absorbance measurements to ascertain that Beer-Lambert's Law was obeyed. The results of two determinations using different enzyme preparations are shown in Figure 1. It was found that phosphorylase b has an absorbance index of $A_{280}^{1 \text{mg/ml}} = 1.18$, confirming that the $A_{278}^{1 \text{mg/ml}} = 1.19$ reported by Appleman et al. (1963) was correct.

When measurements of specific refractive increments of phosphorylase b were carried out in the same buffer under the same conditions where the dn/dc's for a were measured, values of 0.173 and 0.177 were observed (Table 1). This observation seems to support that $A_{280}^{1 \text{mg/ml}} = 1.18$ for b is correct and strongly suggests that phosphorylase a has an extinction coefficient higher than that of b by about 6-8%.

Consequently, similar dry weight experiments were carried
Figure 1. Dry weight determination of extinction coefficient for phosphorylase b

A. Absorbance measurements of stock protein solution at different dilutions at 280 mp (light path = 1 cm). Absorbances of stock solutions are indicated on graph.

B. Dry weight of different % stock protein solution (final total volume of protein solution + buffer = 5 ml). Concentrations of stock solutions are indicated on graph.

Extinction coefficients were obtained by dividing absorbance of stock solution by its concentration, in 40 mM glycero-P-1 mM DTT, at pH 6.8. •, in 40 mM glycero-P-1 mM DTT -1 mM EDTA, at pH 6.8.
PHOSPHORYLASE b SOLUTION

% BUFFER

DRIY WEIGHT (mg/5 ml SAMPLE)

27.42 mg/ml

7.06 mg/ml

% PHOSPHORYLASE b SOLUTION

% BUFFER
out for phosphorylase a, and a reproducible higher value of $A_{280}^{1 \text{ mg/ml}} = 1.27$ was obtained (Figure 2). Using this new constant for $a$, the specific refractive increments for this enzyme corrected to 0.173, 0.176 in buffers containing NaCl or KCl and 0.130 in buffer without these salts. These values now agree quite well with the 0.173 and 0.177 for $b$. Furthermore, the value 0.180 can be corrected to 0.182 in water using the equation derived by Casassa and Eisenberg (1964), which is now within the range of the theoretical value of 0.185 $\pm$ 0.007.

Determination of Extinction Coefficients by Biuret Reaction

Since phosphorylases $a$ and $b$ were shown to have different extinction coefficients by dry weight, the difference should also be detectable, then, when a common reference was used for comparison. Phosphorylases $a$ and $b$ prepared from the same source and dialyzed against the same buffer in the same container were used for biuret experiments. The experiments were carried out in a similar manner as the dry weight experiments except that biuret reaction replaced direct dry weight measurement. A higher value of $A_{280}^{1 \text{ mg/ml}} = 1.38$ was obtained for $a$, and a lower value of 1.31 for $b$. But surprisingly, the extinction coefficients were considerably higher than those obtained from dry weight. In addition, the value 1.31 for phosphorylase $b$ is in good agreement with the 1.31-1.35 reported by Buc and Buc (1967), Metzger et al. (1968), and Gold (1968) for the same enzyme.
Figure 2. Dry weight determination of extinction coefficient for phosphorylase a

A. Absorbance measurements of stock protein solution at different dilutions at 280 nm (1 cm light path). Absorbances of stock solutions are indicated on graph.

B. Dry weight of different % stock protein solution (final total volume of protein + buffer = 5 ml). Concentration of stock solutions are indicated on graph.

Extinction coefficients were calculated as described in Figure 1. ● in 40 mM glycero-P-1 mM DTT, at pH 6.8. ○ in 40 mM glycero-P-1 mM DTT - 1 mM EDTA, at pH 6.8.
70

LU

4.71 mg/ml

6.11 mg/ml

50

PHOSPHORYLASE α SOLUTION

BUFFER

100 80 60 40 20 0

% PHOSPHORYLASE α SOLUTION

% BUFFER

0 20 40 60 80 100

40 50 60 70

DRY WEIGHT (mg/5ml SAMPLE)
Guanidine·HCl Experiments

To further demonstrate that phosphorylases a and b have different absorbance indices, experiments utilizing G·HCl as denaturant were carried out. If the observed difference in absorption is due to variation in the immediate environment of the chromophores, tryptophan and/or tyrosine, it would be expected that completely unfolded a and b would have identical absorbance indices. The following relationships may be written:

\[ A_a = E_a \cdot C_a \]

\[ A_a \text{ (denatured)} = E(\text{denatured}) \cdot C_a \]

\[ A_b = E_b \cdot C_b \]

\[ A_b \text{ (denatured)} = E(\text{denatured}) \cdot C_b \]

where \( A \) = absorbance

\( E \) = extinction coefficient

\( C \) = protein concentration

subscripts \( a \) and \( b \) denote phosphorylases \( a \) and \( b \) respectively.

Consequently,

\[ \frac{A_a / A_a \text{ (denatured)}}{A_b / A_b \text{ (denatured)}} = \frac{E_a}{E_b} \cdot \]

Thus the ratio of the two extinction coefficients can be determined by measuring absorbancies of native and denatured \( a \) and \( b \). Two experiments employing 8.2 M G·HCl and 7.1 M G·HCl were carried out. Samples were carefully diluted in
40 mM glycero-P buffer, and G·HCl, both at pH 6.8, and then their absorptions read at 280 mp against blanks containing the same amounts of buffer and G·HCl. With 8.2 M G·HCl, the $E_a/E_b$ ratio was 1.06; with 7.1 M G·HCl, 1.05. Again, the results showed that the extinction coefficient of phosphorylase a is higher than that of b.

The $E_a/E_b$ ratios obtained from different experiments are summarized in Table 2.

Table 2. Ratio of the extinction coefficients for phosphorylases a and b (at 280 mp)

<table>
<thead>
<tr>
<th>Experiment</th>
<th>$E_a/E_b$ ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry weight</td>
<td>1.08</td>
</tr>
<tr>
<td>Specific refractive increment</td>
<td></td>
</tr>
<tr>
<td>at 546 mp, 25°</td>
<td>1.07 (buffer + NaCl)</td>
</tr>
<tr>
<td>(using $A_{280}^1$ mg/ml = 1.18 for</td>
<td>1.05 (buffer - NaCl)</td>
</tr>
<tr>
<td>both enzymes)</td>
<td></td>
</tr>
<tr>
<td>Biuret</td>
<td>1.05</td>
</tr>
<tr>
<td>G·HCl denaturation</td>
<td>1.06 (8.2 M G·HCl)</td>
</tr>
<tr>
<td></td>
<td>1.05 (7.1 M G·HCl)</td>
</tr>
</tbody>
</table>

Spectral Studies

The spectra of phosphorylases a and b from 245-315 mp are very similar as shown in Figures 3A and 3B. Albeit Michaelis constant studies showed that phosphorylase a binds AMP more tightly than does b (cf. Brown and Cori, 1961), the observed
Figure 3. Spectra of phosphorylases a and b

A. Spectra of phosphorylase a in the absence and presence of AMP. Buffer used was 40 mM glycero-P, pH 6.8. ——, no AMP, $\frac{A_{260}}{A_{280}} = 0.534$; ———, + 5.89 x 10^{-6} M AMP, $\frac{A_{260}}{A_{280}} = 0.631$; ————, + 1.16 x 10^{-5} M AMP, $\frac{A_{260}}{A_{280}} = 0.752$; —————, + 2.26 x 10^{-5} M AMP, $\frac{A_{260}}{A_{280}} = 0.985$.

B. Spectrum of phosphorylase b in the absence of AMP. Buffer used was 40 mM glycero-P, pH 6.8.
The difference in absorbance indices does not seem to be due to the presence of unremoved AMP in a. When \(5.89 \times 10^{-6}\) M AMP was added to 0.48 mg/ml phosphorylase a solution, a shift of the absorption peak towards shorter wavelength and an increase in absorption took place (Figure 3A). The absorbance at 280 nm increased 3%, while the absorbance at 260 nm increased 22%; in the meantime, the \(A_{260}/A_{280}\) ratio changed from 0.534 to 0.630. Additional AMP caused further blue shift and increase of absorbance, in line with the spectral change reported by Madsen and Cori (1957). Since phosphorylases a and b used in these experiments have very similar spectra and since a large increase in the \(A_{260}/A_{280}\) ratio should be observed to account for 5-8% increase of absorbance at 280 nm, it seems unlikely that bound AMP is responsible for the higher extinction coefficient for a. This difference does not seem to be due to the effect of buffer salts either in view of the fact that dilution of phosphorylase a in 40 mM glycerol-\(\cdot\)1 mM DTT + 0.5% NaCl and in water yielded absorbancies at 280 nm which differed by less than 1%, well within the experimental error introduced by the uncertainties in dilution and cuvette calibration.

**Molecular Weights of Phosphorylases a and b Measured by Light Scattering**

Molecular weights determined by light scattering were calculated from the equation given by Townend and Timasheff (1960) for systems undergoing one-step dissociation-association.
\[ \frac{Kc}{R_{90^\circ}} = \frac{1}{M_w} + \frac{2Bc}{M_D} \]

where \( K = \frac{2\pi^2 n_o^2 (dn/dc)^2}{N\lambda^4} \)

- \( n_o \) = refractive index of solvent
- \( N \) = Avogadro's number
- \( \lambda \) = wavelength of incident beam in vacuum
- \( c \) = protein concentration
- \( R_{90^\circ} \) = Rayleigh ratio measured at an angle of 90° to the incident beam

\[ = (I_{\text{solution}} - I_{\text{solvent}}) \times \frac{R_B}{I_B} \times \left( \frac{n_o}{n_B} \right)^2 \]

- \( I \) = intensity of scattered light
- \( I_B \) = intensity of scattered light by pure benzene
- \( R_B \) = Rayleigh ratio for benzene
- \( n_B \) = refractive index for benzene

\( \left( \frac{n_o}{n_B} \right)^2 \) = correction factor for excess scattering volume (cf. Methods)

- \( M_w \) = weight average molecular weight
- \( M_D \) = molecular weight of the non-aggregated dimer
- \( 2Bc/M_D \) = second virial coefficient of the non-aggregated dimer.

In the case of phosphorylase a, by plotting \( Kc/R_{90^\circ} \) vs. \( c \), the intercept on the ordinate will yield a molecular weight slightly lower than the tetrameric form due to dissociation (see
Part II, also cf. Wang and Graves, 1964). In the case of phosphorylase b, due to slight aggregation of this enzyme (see Figure 4B; also cf. Seery et al., 1967; and Chignell et al., 1968), the intercept on the ordinate will yield a value somewhat higher than the true molecular weight.

As shown in Figure 4A, the apparent molecular weight for phosphorylase a extrapolated to zero protein concentration is 380,000. As shown in Figure 4B, the apparent molecular weight for b similarly obtained is 215,000. Using the equilibrium constant at 25° reported in Part II, $4.7 \times 10^{-3}$ g/l, and utilizing the equation

$$K_{eq} = \frac{c(M_T - \bar{M}_w)^2}{M_D(\bar{M}_w - M_D)}$$

where $M_T = \text{molecular weight of tetramer} = 2 M_D$, the molecular weight of phosphorylase a is estimated to be 390,000 ± 20,000. The molecular weight of b is accordingly estimated to be 195,000 ± 20,000.
Figure 4. Molecular weights of phosphorylases a and b measured by light scattering

A. Molecular weight of phosphorylase a extrapolated to zero protein concentration. All measurements were carried out at 546 μm. ○, in 40 mM glycerol- P-1 mM EDTA, 1% KCl, pH 6.8, 25°. Δ, in 40 mM glycerol- P-1 mM DTT, 0.5% NaCl, pH 6.8, 30°.

B. Molecular weight of phosphorylase b extrapolated to zero protein concentration. Measurements were carried out at 546 μm, 25°. ●, in 40 mM glycerol- P, 1 mM DTT, 0.5% NaCl, pH 6.8.
DISCUSSION

It has long been accepted that phosphorylases a and b have identical extinction coefficients mainly because of the similarity in their amino acid compositions and because of the close agreement between the values $A_{280}^1$ mg/ml 1.17 for a reported by Velick and Wicks (determined by nitrogen content analysis) and $A_{278}^1$ mg/ml 1.19 for b reported by Appleman et al. (determined by dry weight). The dry weight determinations of absorbance indices presented in Results showed that, while the value 1.19 for b is correct, the 1.17 for a is low by about 8%.

That phosphorylase a has a higher extinction coefficient than b is supported by the following evidence: (1) $dn/dc$ for phosphorylase a is unusually low (0.161-0.163) when $A_{280}^1$ mg/ml 1.18 is used for protein concentration calculations. From Table 4 (Appendix) it is evident that such low value is possible only when the protein consists overwhelmingly of amino acid residues of low refractive increments. The theoretical $dn/dc$ calculated from the amino acid composition determined by Appleman et al., 0.185 ± 0.007 in water, is substantially higher, and the discrepancy cannot be accounted for by variation in solvents. Furthermore, when $A_{280}^1$ mg/ml = 1.27 was employed, the $dn/dc$'s for phosphorylase a corrected to 0.173 and 0.180 which agreed quite well with the 0.173 and 0.177 for b measured under identical conditions. While it is possible for a and b to have different refractive increments, the 5-8%
difference is too large for two proteins of virtually identical amino acid constituents (with the exception of four phosphate groups on a). Since denaturation of ovalbumin by urea (Kauzmann, 1959) and of β-lactoglobulin by heat (McMeekin et al., 1964) resulted in small changes in the refractive index, it seems unlikely that the conformational change brought about by phosphorylation and dimerization in the case of phosphorylases b and a should produce a greater difference in refraction. Rather, in view of the insensitivity of dn/dc to detailed molecular structure (cf. Tanford, 1961), the difference may be better explained by changes in absorbance indices.

(2) When compared with a common standard, bovine serum albumin, by means of biuret reaction, phosphorylase a gave a value that is 5% higher than that for b. (3) By assuming that G'HCl-denatured a and b have identical extinction coefficients and using the relationships described in Results, the ratio of the absorbivities of native a to native b was shown to be 1.05-1.06. All the experiments done by various methods consistently show that the extinction coefficient of phosphorylase a has a significantly higher value than that of b.

Since addition of AMP to phosphorylase a solution showed that a 5-8% increase at 280 mp would be accompanied by a large increase in absorption at 260 mp, the observed difference, therefore, is most likely due to changes in polarizability in the immediate environment of certain tryptophanyl and tyrosyl residues. Whether the difference is related to dimerization
or not cannot be determined owing to the difficulty that one cannot prepare a solution containing only the dimeric phosphorylase \( \text{a} \) without at the same time perturbing the spectrum, e.g., utilizing glucose to dissociate the enzyme. One is tempted to think, however, that dimerization may create high polarizability regions around certain chromophores. It may also be due to the structural change accompanying phosphorylation of this enzyme or due to the combined effect of dimerization and phosphorylation.

The higher extinction coefficients of 1.31-1.35 for phosphorylase \( \text{b} \) obtained by biuret reaction, % nitrogen and amino acid analyses present a puzzling problem. All these higher figures were based on the % nitrogen and amino acid composition reported by Appleman et al. Since Appleman et al. used the result of the same protein dry weight determinations to calculate absorbance index, % \( N \), and amino acid composition, theoretically, extinction coefficients computed by using their data should agree with the 1.19 reported by them. The higher values seem unlikely, however, by the following reasons:

1. If 1.31-1.35 is correct, \( \frac{dn}{dc} \) for phosphorylase \( \text{b} \) at 546 \( \mu \)m, \( 25^\circ \), would assume a value of 0.199-0.205 in water, which is unusually high for a protein at this wavelength and is beyond the range of the value 0.185 \( \pm \) 0.007 calculated from amino acid composition.

2. The higher extinction coefficient implies that Appleman et al.'s and our vacuum dried protein samples contained 10-14% moisture. But we have continually
dried and weighed protein samples over a period of two weeks and were unable to detect any significant change in weight. One criticism of dry weight method is that the protein may form a film inside the weighing bottle, preventing further evaporation of water. Even a film does form, however, the protein film per se is hygroscopic and will absorb the moisture trapped beneath it. After repeated drying at 110° in vacuum oven, the moisture should eventually be driven out through the film.

Thus it may be concluded that the $A_{280}^{1}$mg/ml 1.18 for phosphorylase b and $A_{280}^{1}$mg/ml 1.27 for phosphorylase a are more reliable because the results were reproducible and because dry weight determination is the methods of choice considering that all other methods, biuret, % N, and amino acid analyses, etc., depend ultimately on dry weight determination of protein samples.

The molecular weight of phosphorylase a was first estimated by Oncley (1943) to be between 340,000 and 400,000. Later Keller and Cori (1953) established the molecular weight of phosphorylase a as 495,000 and that of phosphorylase b as 242,000. Recently, numerous reports on redetermination of these molecular weights have appeared: Seery et al. (1967) reported 370,000 and 185,000 for phosphorylases a and b; Buc and Buc (1967) reported 182,000-195,000 for b; DeVincenzi and Hedrick (1967) reported 367,000 and 177,000 for a and b; and
Metzger et al. reported 185,000 for \( b \). The molecular weights of phosphorylases \( a \) and \( b \) reported here, 390,000 and 195,000, are therefore in good agreement with recent literature values. Since all but one of these reported values were determined by ultracentrifugation or gel filtration in which the knowledge of partial specific volume is vital, the molecular weights reported here by light scattering are a good support for the validity of the new values. Buc and Buc (1967) have reported a molecular weight of 188,000 \( \pm \) 8,000 for phosphorylase \( b \) also by light scattering by assuming \( \text{dn/dc} \) to be 0.185 and using the higher extinction coefficient of 1.32-1.35. If the values \( \text{dn/dc} = 0.173 \) and \( A_{280}^{\text{mg/ml}} = 1.18 \) for \( b \) reported here are employed, the molecular weight becomes 189,000 \( \pm \) 10,000, virtually unchanged. This shows that our light scattering data in essence agree with those of Buc and Buc; it also suggests that absorbance index and specific refractive increments determined by us are valid.
PART II. CORRELATION BETWEEN SUBUNIT
INTERACTION AND ENZYMATIC ACTIVITY OF PHOSPHORYLASE α
INTRODUCTION

Rabbit skeletal muscle glycogen phosphorylase a, molecular weight 367,000-390,000, is generally designated as a tetramer since treatment of this enzyme with p-hydroxymercuribenzoate resulted in four subunits (Madsen and Cori, 1956). In addition, AMP binding studies revealed four equivalent sites per enzyme molecule (Madsen and Cori, 1957; DeVincenzi and Hedrick, 1967; Helmreich et al., 1967).

For some time it was thought that the active form of phosphorylase a is the tetramer. Wang and Graves found that, in fact, phosphorylase a exists in two states of aggregation: a catalytically more active dimer and a less active tetramer. In 2.8 M NaCl, pH 7.0, phosphorylase a was transformed into a new species with a molecular weight of ca. 258,000 (Wang and Graves, 1963). Rapid assay following dilution of this sample yielded high initial enzymic activity which decayed to the activity of samples not incubated in NaCl (Wang and Graves, 1964). The decay followed second order kinetics, suggesting that this is a dimerization reaction and that the dimeric form is catalytically more active. Further support for the existence of a more active dimer was obtained from studies of activation of phosphorylase a by α-1,4-glucosyl compounds.
Incubation of the enzyme with glucose resulted in a species of high enzymic activity, which sediments as a dimer (Wang et al., 1965a). Preincubation with glycogen and hydrolyzed amylose also generated the more active species (Wang et al., 1965b). It was also shown (Wang and Graves, 1964) that the two subunit forms are in equilibrium with each other since decreasing protein concentration is accompanied by increasing specific activity; by consideration of the law of mass action, dilution would favor the dissociated component. More recently, Metzger et al. (1967) also found that the dimeric species is more active when assayed in the forward direction by way of arslenolysis and that in the presence of amyloheptaose the enzyme sediments as a dimer. And, from centrifugal experiments with phytoglycogen at 15°, they concluded that the tetrameric form of phosphorylase a cannot bind to glycogen and is therefore catalytically inactive with this substrate.

The following report is concerned with several aspects of the dissociation reaction of phosphorylase a: (1) to obtain physical evidence for dissociation of this enzyme upon dilution and to correlate enzymic activity with subunit structure; (2) to evaluate the thermodynamic parameters of dissociation; (3) to examine whether the tetrameric form is capable of catalysis.
THEORY: A METHOD FOR DETERMINING EQUILIBRIUM
CONSTANT FROM RATE MEASUREMENTS

In an effort to correlate molecular weight change and enzymic activity of phosphorylase a as a function of protein concentration, it was found that such relationship can be quantitatively defined. It is felt that the determination of subunit equilibrium by activity measurements may find wider application to other enzyme systems. This method permits the study of dissociation at low protein concentrations which are inaccessible to ordinary means of molecular weight measurements. It is also easier to carry out the experiments as far as time, technique, material, and equipment are concerned. Thus a rather general treatment is presented here, including situations which are not encountered in the case of phosphorylase a.

For a polymeric enzyme, $E_p$, which undergoes one-step dissociation-association reactions, we can write

$$E_p \leftrightarrow p E_m$$

and

$$K_d = \frac{[E_m]^p}{[E_p]} = \text{dissociation constant} \quad (1)$$

where $E_m$ = dissociated enzyme species.

If the following conditions are satisfied, $K_d$ (and a variety of kinetic parameters) can be estimated: (1) the equilibrium is essentially between $E_p$ and $E_m$, no significant
amount of intermediate aggregates; (2) The two enzyme species differ in their intrinsic activities and/or ligand binding affinities.

For such enzyme systems, the observed enzymatic activity is the sum of the activities contributed by the aggregated and the dissociated species.

\[ v = v_m + v_p \]  \hspace{1cm} (2)

where \( v \) = observed initial velocity

\( v_m, v_p \) = activities contributed by the dissociated and polymeric forms, respectively.

From Equation 2, it can be shown that the observed specific activity (defined as activity per unit weight of protein\(^1\)), \( \bar{\varphi} \), is the weight-average value of the specific activities of the polymer, \( \varphi_p \), and the monomer, \( \varphi_m \):

\[ \bar{\varphi} = \frac{v}{E_o} = \frac{v_p + v_m}{E_o} = \frac{v_p}{E_p} + \frac{v_m}{E_m} = \frac{E_p \varphi_p + E_m \varphi_m}{E_o} \]  \hspace{1cm} (3)

where \( E_o \) = total weight concentration of the enzyme = \( E_p + E_m \)

\( E_p, E_m \) = weight concentration of the polymeric and monomeric species

\( \varphi_p, \varphi_m \) = specific activity of the polymeric and monomeric species

Substituting \( E_p = E_o - E_m \) and \( E_m = E_o - E_p \) into Equation 3, we

\(^1\)\( \mu \) moles per min per mg' is the basis of the definition of specific activity now adopted by international agreement (Report of the Commission on Enzymes of the International Union of Biochemistry, 1961).
Let us first consider the case that the equilibrium between the two molecular species is slow compared with the overall enzymatic reaction, that is, the relative amounts of polymeric and monomeric forms, $E_p$ and $E_m$, remain essentially unchanged during initial rate measurements. If this condition is met, we can substitute Equations 4a and 5a into Equation 1 to express $K_d$ in terms of specific activities:

\[
K_d = \frac{E_o^{p-1} (\overline{\phi} - \phi_p)^p}{(\phi_m - \overline{\phi})(\phi_m - \phi_p)^{p-1} (g/1)^{p-1}} \quad (6)
\]

or,

\[
K_d = \frac{pE_o^{p-1}(\overline{\phi} - \phi_p)^p}{M_m^{p-1} (\phi_m - \overline{\phi})(\phi_m - \phi_p)^{p-1} (\text{moles}/1)^{p-1}} \quad (6a)
\]
where $M_m =$ molecular weight of the dissociated form. Upon rearrangement, Equation 6 becomes

$$
\frac{1}{\bar{\theta} - \theta_p} = \frac{1}{K_d (\theta_m - \theta_p)^p} \cdot \left[ E_o (\bar{\theta} - \theta_p) \right]^{p-1} + \frac{1}{\theta_m - \theta_p} \tag{6b}
$$

Thus a plot of $1/(\bar{\theta} - \theta_p)$ vs. $\left[ E_o (\bar{\theta} - \theta_p) \right]^{p-1}$ will yield $1/(\theta_m - \theta_p)$ as intercept on the ordinate and $-K_d (\theta_m - \theta_p)^{p-1}$ as intercept on the abscissa. If $\theta_p$ and $p$ are known, $K_d$ and $\theta_m$ can be evaluated. From Equation 4, we have

$$
\lim_{\frac{1}{E_o} \to 0} \bar{\theta} = \theta_p .
$$

By plotting $\bar{\theta}$ vs. $1/E_o$ and extrapolating to $1/E_o = 0$, the value of $\theta_p$ can be obtained. The value of $p$, which is the number of monomeric units in a polymer, usually can be determined by physical or chemical methods; theoretically $p$ can also be determined from activity measurements because the $1/(\bar{\theta} - \theta_p)$ vs. $\left[ E_o (\bar{\theta} - \theta_p) \right]^{p-1}$ plot will yield a straight line when the correct $p$ is chosen.

**Relationship Between Specific Activity and Molecular Weight**

Since the observed specific activity is a weight-average quantity, if we simultaneously solve Equation 3 and the expression for weight-average molecular weight, $\bar{M}_w$, of the same
enzyme system

$$\overline{M}_w = \frac{E_p M_p + E_m M_m}{E_o}$$

where $M_p$ = molecular weight of the polymeric form = $pM_m$

we can express $\overline{M}_w$ in terms of specific activities:

$$\overline{M}_w = \frac{M_m \left( p\phi_m - \phi_p - (p-1)\overline{\phi} \right)}{\phi_m - \phi_p}.$$ (7)

The weight average molecular weight at any given protein concentration, $E_o$, then, can be calculated from the specific activity at that concentration, provided $M_m$ is known. Alternatively, Equation 7 can be written as

$$\overline{\phi} = \frac{\phi_m + (p-2)\phi_p}{p-1} - \frac{\phi_m - \phi_p}{p-1} \left( \frac{\overline{M}_w}{M_m} - (p-1) \right).$$ (7a)

If both molecular weight and specific activity data are available, a plot of $\overline{\phi}$ vs. $[\overline{M}_w/M_m - (p-1)]$ will yield $[\phi_m + (p-2)\phi_p]/(p-1)$ as y-intercept and $\phi_p$ at $[\overline{M}_w/M_m - (p-1)] = 1$. This allows checking of the values of $\phi_p$ and $\phi_m$ determined from rate measurements alone.

Validity of the Equations under Various Conditions

1. One or both forms of the enzyme exist in more than one conformational state, of different catalytic activities, e.g., the R state $\rightarrow$ T state transition popularized
by Monod et al. (1965).

Suppose we have three conformational states, \( M_1 \), \( M_2 \) and \( M_3 \), of the same molecular weight in equilibrium with one another:

\[
\begin{array}{c}
M_1 \xrightarrow{K_{12}} M_2 \xrightarrow{K_{23}} M_3
\end{array}
\]

Let \( E_0 = E_1 + E_2 + E_3 \)

\( E_1, E_2, E_3 = \) weight concentrations for \( M_1, M_2, \) and \( M_3 \) and \( K_{12}, K_{23} = \) equilibrium constants for the transitions \( M_1 \rightleftharpoons M_2 \) and \( M_2 \rightleftharpoons M_3 \) respectively,

then,

\[
K_{12} = \frac{E_2}{E_1}
\]

\[
K_{23} = \frac{E_3}{E_2}
\]

and

\[
E_2 = K_{12} E_1
\]

\[
E_3 = K_{12} K_{23} E_1
\]

The specific activity for such a system is therefore

\[
\phi = \frac{E_1 \phi_1 + E_2 \phi_2 + E_3 \phi_3}{E_0}
\]

\[
= \frac{\phi_1 + K_{12} \phi_2 + K_{12} K_{23} \phi_3}{1 + K_{12} + K_{12} K_{23}} = \text{constant (8)}
\]
where \( \phi_1, \phi_2, \phi_3 \) = specific activities for \( M_1, M_2, M_3 \).

This simple derivation shows two things: (1) As long as there is no molecular weight change, the specific activity of the system can be represented by a single constant. Thus the existence of R and T states, for instance, does not alter the appearance of Equations 1-7. (2) Specific activity is independent of protein concentration if the system does not involve subunit dissociation-association. Therefore, concentration dependence of specific activity is indicative of molecular weight change, barring non-specific surface denaturation or the presence of small amounts (concentration high enough to affect activity but not high enough to saturate the enzyme) of activator or inhibitor in the assay mixture. However, while concentration-dependence of specific activity is suggestive of subunit interactions, the lack of it does not necessarily mean the absence of dissociation-association since the two molecular species may have identical specific activity.

2. Rapid equilibrium (compared with overall enzymatic reaction) between the molecular species.

If the polymeric and monomeric species have different affinities for the substrate and if there are \( n \) equivalent and independent active sites (i.e., the binding of substrate on one site does not influence the binding on another site) on the monomer, by assuming rapid equilibration of all enzyme species, the following kinetic equations can be written:
where $S = \text{substrate}$

$P = \text{product}$

$K_m, K_p = \text{substrate dissociation constants for } E_m, E_p$

$k_m, k_p = \text{rates of breakdown for } E_m, E_p$.

Taking into account the probability factor for dissociation of substrate (Klotz, 1953):

\[
\text{number of sites available} = \frac{n - 1 + 1}{1} \quad \text{or} \quad \frac{pn - 1 - 1}{1}
\]

where $i = 1, 2, \ldots n, \ldots pn$, we can write

\[
E_m S = n E_m \frac{S}{K_m} \quad \quad E_p S = pnE_p \frac{S}{K_p}
\]
\[
E_m s_2 = \frac{n-1}{2} \left( E_m s \right) \frac{S}{K_m} \quad \quad E_p s_2 = \frac{p_n-1}{2} \left( E_p s \right) \frac{S}{K_p}
\]

\[
= \frac{n(n-1)}{2} E_m (S/K_m)^2 \quad \quad = \frac{p_n(p_n-1)}{2} E_p (S/K_p)^2
\]

\[
E_m s_n = \frac{1}{n} \left( E_m s_{n-1} \right) \frac{S}{K_m} \quad \quad E_p s_{p_n} = \frac{1}{p_n} \left( E_p s_{p_n-1} \right) \frac{S}{K_p}
\]

\[
= E_m (S/K_m)^n \quad \quad = E_p (S/K_p)^{p_n}
\]

\[
v = k_m \sum_{i=1}^{n} (E_m s_i) + k_p \sum_{i=1}^{p_n} (E_p s_i)
\]

\[
n E_o = n (E_m + \sum_{i=1}^{n} E_m s_i) + p_n (E_p + \sum_{i=1}^{p_n} E_p s_i) = n E_{m_o} + p_n E_{p_o}
\]

Consequently, \( \frac{v}{n E_{m_o} + p_n E_{p_o}} = \)

\[
n k_m E_m (S/K_m) \left( 1 + (n-1)(S/K_m) + \ldots + (S/K_m)^{n-1} \right) + p_n k_p E_p (S/K_p) \left( 1 + (p_n-1)(S/K_p) + \ldots + (S/K_p)^{p_n-1} \right) \]

\[
n E_m \left( 1 + n(S/K_m) + \ldots + (S/K_m)^n \right) + p_n E_p \left( 1 + p_n(S/K_p) + \ldots + (S/K_p)^{p_n} \right)
\]

\[
= \frac{k_m E_m (S/K_m) (1 + S/K_m)^{n-1} + p_n k_p E_p (S/K_p) (1 + S/K_p)^{p_n-1}}{E_m (1 + S/K_m)^n + p E_p (1 + S/K_p)^{p_n}}
\]
Converting the expression for enzyme concentration from 'moles of sites' to molar or weight scale, we have

\[
\frac{\varnothing}{E_0} = \frac{k_m E_m(S/K_m)(1 + S/K_m)^{n-1} + k_p E_p(S/K_p)(1 + S/K_p)^{p_n-1}}{E_m(1 + S/K_m)^n + E_p(1 + S/K_p)^{p_n}}
\]

Equation 9 is analogous to Equation 3 except that \( E_m(1 + S/K_m)^n \) now replaces \( E_m \); \( E_p(1 + S/K_p)^{p_n} \) replaces \( E_p \); \( \varnothing_m = k_m S/(K_m + S) \); and \( \varnothing_p = k_p S/(K_p + S) \). Accordingly, the expression for the dissociation constant for the rapid equilibrium case, \( K_d(s) \), is

\[
K_d(s) = \frac{\left[E_m(1 + S/K_m)^n\right]^p}{E_p(1 + S/K_p)^{p_n}}
\]

\[
= K_d \left(\frac{1 + S/K_m}{1 + S/K_p}\right)^{p_n} \left(s/1\right)^{p-1}.
\]

It is evident, then, the dissociation constant obtained by use of the plot utilizing Equation 6b, in the case of rapid equilibrium between the two molecular species, is the dissociation constant of the system under the influence of substrate. To obtain the intrinsic dissociation constant in the absence of substrate, the substrate binding constants, \( K_m \) and \( K_p \), must be known. These two constants can be estimated by the following method.
From Equation 9, one can write

$$\frac{v}{S(V_m - v)} = \frac{(k_m/K_m)E_m(1+S/K_m)^{n-1} + (k_p/K_p)E_p(1+S/K_p)^{n-1}}{k_mE_m(1+S/K_m)^{n-1} + k_mE_p(1+S/K_p)^{n-1}}$$  \hspace{1cm} (10)$$

where $V_m$ = maximum velocity = $k_mE_{m0} + k_mE_{p0}$.

At infinitely low protein concentration, only the monomeric species is present such that

$$\lim_{E_0 \to 0} \frac{v}{S(V_m - v)} = \frac{1}{k_m}.$$  

Similarly, at infinitely high protein concentration, only the polymeric form is present and

$$\lim_{E_0 \to 0} \frac{v}{S(V_m - v)} = \frac{1}{k_p}.$$

The Case of Interacting Sites

The above equations are derived on the basis of equivalent and independent active sites. If there is interaction among sites on the same enzyme molecule, i.e., the binding of a ligand on one site alters the binding constants and breakdown rates on the other sites, then we have
\[ v = \sum_{i=1}^{n} k_i E_m S_i + \sum_{i=1}^{pn} k'_i E_p S_i. \]

Consequently, it can be shown that \( v/V_m = \)

\[ \frac{(E_m/n)(S/K_1)}{k_n E_m [1 + (S/K_1) + \ldots + (S^n/K_i \ldots K_n)]} \]

\[ + \frac{(E_p/pn)(S/K'_1)}{k'_n E_p [1 + (S/K'_1) + \ldots + (S^{pn}/K'_1 \ldots K'_{pn})]}, \]

\[ + \frac{nk_n (S^{n-1}/K_2 \ldots K_n)}{2k_2 + \ldots + nk_n (S^{n-1}/K_2 \ldots K_n)}, \]

\[ + \frac{nk'_n (S^{pn-1}/K'_2 \ldots K'_{pn})}{2k'_2 + \ldots + nk'_n (S^{pn-1}/K'_2 \ldots K'_{pn})}. \]
\[ S(V_m - v) = \frac{(E_m/nK_1)(k_1 + \ldots + nk_n(S^{n-1}/K_2 \ldots K_n))}{E_m k_n + (k_n - k_1/n)(S/K_1) + \ldots + k_n - \frac{(n-1)k_n-1}{n}\frac{S^{n-1}}{K_1 \ldots K_n}} \]

\[ + E_p\left[k_{pn} + (k_{pn} - k_1/pn)(S/K_1) + \ldots + k_{pn} - \frac{(pn-1)k_{pn}-1}{pn}\frac{S^{pn-1}}{K_1 \ldots K_{pn}}\right] \]

If we define
\[ \lim_{S \to 0} S(V_m - v) = \frac{k_1E_m + k_1E_p}{nK_1 + \frac{pnK_1}{k_1E_m + k_1E_p}} = k^\neq(E_o) \quad (11) \]

it is clear that
\[ \lim_{E_o \to 0} k^\neq(E_o) = k_1/nk_n K_1 \quad (12) \]

and
\[ \lim_{\frac{1}{E_o} \to 0} k^\neq(E_o) = \frac{k_1}{pnk_1} K_1 \cdot (13) \]

Also,
\[ \lim_{E_o \to 0} \frac{V_m}{E_o} = k_n \quad (14) \]

and
\[ \lim_{\frac{1}{E_o} \to 0} \frac{V_m}{E_o} = k_{pn} \cdot (15) \]
Thus from Equations 11-15 and the conservation equation
\[ E_o = E_{m_0} + E_{p_0} \]

\( E_m \) and \( E_p \) in the absence of substrate can be determined, and
\( K_d \) can be calculated from Equation 1.

It should be noted that the above treatment can be extended to two substrate systems and to steady-state kinetics if random mechanism of binding is assumed.

Equations for the Case of Phosphorylase a

The dissociation of tetrameric phosphorylase a into dimer is a simple case where \( p = 2 \) and \( n = 2 \). The following equations describing the phosphorylase a system, basing on slow equilibrium between dimer and tetramer, can be easily obtained:

\[ \bar{\theta} = \theta_T + \frac{(\theta_D - \theta_T)E_D}{E_o} \] (16)

\[ \frac{1}{\bar{\theta} - \theta_T} = \frac{1}{K_d(\theta_D - \theta_T)^2} \cdot E_o(\bar{\theta} - \theta_T) + \frac{1}{\theta_D - \theta_T} \] (17)

\[ \frac{1}{K_w} = \frac{\theta_D - \theta_T}{M_D(2\theta_D - \theta_T - \bar{\theta})} \] (18)

where the subscripts D and T denote dimer and tetramer.

If one of the subunit forms is inactive, for instance, the inactive tetrameric phosphorylase a proposed by Metzger et al. (1967), then \( \theta_T = 0 \), and the above equations reduce to
\[ \frac{1}{\bar{\theta}} = \frac{1}{K_a \theta_D^2} \cdot E_0 \bar{\theta} + \frac{1}{\bar{\theta}_D} \]  

(17a)

and

\[ \frac{1}{\bar{M}_W} = \frac{\theta_D}{M_D (2\theta_D - \bar{\theta})} \]  

(18a)

Therefore, theoretical lines of \( \bar{M}_W \) as a function of protein concentration can be calculated by assuming active or inactive tetrameric phosphorylase \( a \) and compared with experimentally determined molecular weights to test the validity of either model.
RESULTS

Concentration-Dependent Dissociation of Phosphorylase a

Figures 5A and 6A show plots of reciprocal apparent weight-average molecular weight of phosphorylase a obtained from light scattering measurements at 546 m at 25° and 30°, respectively, as a function of protein concentration. These plots are typical of dissociation-association systems and demonstrate clearly that molecular weight decreases directly with the decrease in concentration. At 25°, the extent of dissociation is moderate. The change is, however, quite significant, considering the fact that in weight-average molecular weight, the contribution from large molecule is more influential. The lowest molecular weights obtained at this temperature were 306,000-317,000, representing the presence of 37-43% dimer. As temperature was elevated to 30°, the change in molecular weight was much more pronounced (Figure 6A). At low protein concentrations, the molecular weight seems to rapidly approach that of the dimer. From either plot it can be seen that at higher protein concentrations, molecular weight reaches a finite value of 380,000 ± 10,000, indicating that the aggregation is not a random phenomenon, but a rather specific reaction.
Figure 5. Molecular weight and specific activity of phosphorylase a as a function of protein concentration at 25°. All experiments carried out in 40 mM glycero-P-1 mM EDTA, 1% KCl buffer, pH 6.8.

A. Reciprocal apparent molecular weight of phosphorylase a as a function of protein concentration. Molecular weights measured by light scattering at 546 m\(\mu\) as described in Methods. Different enzyme preparations were used. — — — —, theoretical line of molecular weight as a function of protein concentration calculated from specific activity curve shown in B. Values used for the calculation: specific activity of tetramer = 1.86, specific activity of dimer = 1.17. All values obtained by least square method as described in the text. — — — —, theoretical line of molecular weight as a function of protein concentration calculated from specific activity curve shown in B by assuming inactive tetramer. Specific activity of dimer used for the calculation = 9.48, obtained from least square method as described in the text.

B. Specific activity of phosphorylase a as a function of protein concentration. Assay carried out in AMP-free substrate. Assay time varied from 20 sec to 10 min as protein concentration decreased from 1.71 mg/ml (data at high protein concentrations not shown) to 0.0024 mg/ml. Specific activity is expressed as pmoles p\(\_\) released per min per mg protein. Data were obtained from same enzyme preparation.
Figure 6. Molecular weight and specific activity of phosphorylase a as a function of protein concentration at 30°. All experiments carried out in 40 mM glycero-P-1 mM DTT, 0.5% NaCl buffer, pH 6.8.

A. Reciprocal apparent molecular weight of phosphorylase a as a function of protein concentration. Molecular weight measured by light scattering at 546 m\(\mu\) as described in Methods. Different enzyme preparations were used. - - - - , theoretical line of molecular weight as a function of protein concentration calculated from specific activity curve shown in B. Values used for the calculation: specific activity of tetramer = 3.30, specific activity of dimer = 15.8; all values obtained by least square method as shown in Figure 7A. - - - - , theoretical line of molecular weight as a function of protein concentration calculated from specific activity curve shown in B by assuming inactive tetramer. Specific activity of dimer used for the calculation = 13.5, obtained from least square method as shown in Figure 7B.

B. Specific activity of phosphorylase a as a function of protein concentration. Assay carried out in AMP-free substrate. Assay time varied from 10 sec to 5 min as protein concentration varied from 1.42 mg/ml (data at high protein concentration shown in insert of Figure 7A) to 0.0056 mg/ml. Specific activity is expressed as \(\mu\)moles P\(\_1\) released per min per mg protein. Data were obtained from same enzyme preparation.
Correlation Between Molecular Weight and Enzymatic Activity

Wang and Graves (1964) have shown that specific activity of phosphorylase a increases directly with the decrease in protein concentration. This can be best explained as the formation of smaller, catalytically more active species in view of Le Chatelier's Principle. Theoretical basis for concentration dependence of activity as indication of subunit dissociation has been given in Theory. Physical evidence of dissociation has been presented in the previous section. If the dimeric and tetrameric forms of phosphorylase a have distinctly different intrinsic activities, the change in enzymatic activity should closely parallel the change in molecular weight. Thus activities of phosphorylase a were measured under conditions identical to those of the light scattering measurements except for the presence of substrates, in order to make the correlation more relevant. The specific activity profiles so obtained at 25° and 30° are shown in Figures 5B and 6B. Although the enzymic assays were carried out in the reverse direction, assays done in the forward direction by means of arsenolysis yielded similar results. It was assumed that activation by glycogen was negligible during assay because progress curves of phosphate release and of glucose formation by arsenolysis always appeared linear.

If one compares the light scattering data with the specific activity curves, one can see the first sign of correlation
between them since they both level off at approximately the same enzyme concentration, around 0.4 mg/ml at 25° and around 0.8 mg/ml at 30°, and both seem to asymptotically approach finite values. To obtain the specific activity for tetramer, \( \varphi_T \), Equation 16 was utilized. By plotting specific activity, \( \varphi \), observed at high protein concentrations against reciprocal enzyme concentration, \( \varphi_T \) was obtained by extrapolating to \( 1/\varphi_0 = 0 \). An example of such plot is shown in the insert of Figure 7A. The \( \varphi_T \)'s resulting from such extrapolations were 1.86 and 3.30 at 25° and 30°, respectively. Having determined \( \varphi_T \), the values of specific activity for dimer, \( \varphi_D \), and dissociation constant, \( K_d \), were obtained by a \( 1/(\varphi - \varphi_T) \) vs. \( E_o (\varphi - \varphi_T) \) plot using Equation 17. The values of \( \varphi_D \)'s so determined were 11.7 at 25° and 15.8 at 30°. From values of these specific activities, theoretical lines of molecular weight as a function of protein concentration were calculated according to Equation 18 and compared with actual molecular weights obtained by light scattering (solid lines, Figures 5A and 6A). In both cases, the theoretical lines fit the experimental points quite well, demonstrating that activity change can be quantitatively accounted for by a change in molecular weight. The correlation between molecular weight and enzymic activity strongly suggests that the increase in activity is

---

\(^1\)All specific activities are expressed as \( \mu \) moles P\(_i\) released per minute per mg protein.
Indeed the consequence of the formation of a catalytically more active species. It also supports the concept that the dissociated form is a dimer since Equation 18, which is derived on the assumption of dimer-tetramer equilibrium, seems to describe the system quite adequately.

The hypothesis of inactive tetramer was tested by using Equations 17a and 18a. To be objective, the specific activity data obtained at 25° and 30° were plotted according to Equation 17 or Equation 17a (for $\varphi_T \neq 0$ and $\varphi_T = 0$, respectively) and least square methods were employed to obtain specific activities and dissociation constants. As an example, graphical analyses of data at 30° are shown in Figures 7A and 7B. In the case of $\varphi_T = 0$, the $\varphi_D$'s calculated were 9.48 at 25° and 13.5 at 30°. Theoretical lines of molecular weight as a function of protein concentration were then calculated from Equation 18a using these values. From Figures 5A and 6A (dashed lines), it can be seen that the lines calculated by assuming $\varphi_T = 0$ do not fit the experimental points. It is evident that the proposed inactive tetramer is unlikely since the enzyme would have to undergo more dissociation than seen from molecular weight measurements to account for the observed specific activities; this is manifested by the fact that $K_d$'s obtained by assuming $\varphi_T = 0$, $1.43 \times 10^{-2}$ g/l at 25° and $1.53 \times 10^{-1}$ g/l at 30°, are considerably larger than their corresponding values $3.59 \times 10^{-3}$ g/l and $3.53 \times 10^{-2}$ g/l obtained by assuming active tetramer.
Figure 7. Graphical analyses of specific activity data at 30° (shown in Figure 6B)

A. Determination of specific activities and dissociation constant by least square method, assuming active phosphorylase a tetramer. Insert. Determination of specific activity for tetramer, $\phi_T$, by extrapolation according to Equation 16. The $\phi_T$ so obtained, 3.30, was used for calculation of $1/(\bar{\phi} - \phi_T)$ and $E_0(\bar{\phi} - \phi_T)$ as shown in the main plot (cf. Equation 17). Specific activity of dimer, $\phi_D = 15.8$, was obtained from y-intercept. Dissociation constant, $K_d = 3.53 \times 10^{-2}$ g/l, was obtained from x-intercept.

B. Determination of specific activity of dimer and dissociation constant by least square method, assuming inactive phosphorylase a tetramer (cf. Equation 17a). Specific activity of dimer, $\phi_D = 13.5$, and dissociation constant, $K_d = 1.53 \times 10^{-1}$ were obtained from y- and x-intercepts, respectively.
Activation Energies and Thermodynamic Parameters of Dissociation

Having shown that the equilibrium between tetrameric and dimeric phosphorylase \( a \) can be studied by activity measurements, it is feasible, then, to determine activation energies of the two forms and thermodynamic parameters of dissociation from specific activity curves. Since specific activity varies somewhat with different enzyme preparations and with age, it is necessary to carry out assays at different temperatures in a short period using the same batch of enzyme. Figures 8A-8D show specific activity profiles for the same enzyme preparation at 35°, 30°, 25°, and 20°. Specific activities of enzyme pre-incubated in glycogen in the same temperature range were also measured. The specific activities of dimer calculated from the graphic methods were in good agreement with those obtained from preincubation with glycogen experiments (Figure 9). Theoretical specific activity curves calculated from dissociation constants, obtained also from graphic methods, in general fit the experimental points quite well, indicating that the dissociation reactions obey the law of mass action and that the \( K_d \)'s are of the right magnitude. The results also suggest that the species generated by incubation with glycogen is the dimeric form, confirming the data of Wang et al. (1965b).

Figure 9 shows Arrhenius plots for the two species of phosphorylase \( a \). The activation energies for dimer and tetramer are 11.3 kcal and 22.6 kcal, respectively. This again
Figure 8. Specific activities of phosphorylase a with and without preincubation with glycogen at different temperatures. All data obtained in 40 mM glycero-P, 1 mM DTT, 0.5% NaCl, pH 6.8 buffer, using same enzyme preparation. o, preincubated in 2% glycogen before assay; ●, without preincubation; — — — —, theoretical lines calculated from dissociation constants obtained by graphical method as described in the text. Data obtained at protein concentration > 0.8 mg/ml are not shown.

A. 35°C. Assay time varied from 10-30 sec as enzyme concentration decreased from 0.366-0.055 mg/ml in preincubation with glycogen experiments; without preincubation, assay time varied from 5-40 sec as enzyme concentration decreased from 2.19 mg/ml to 0.050 mg/ml. \( K_d = 1.56 \times 10^{-1} \) g/l.

B. 30°C. Assay time varied from 12-40 sec as enzyme concentration decreased from 0.453 mg/ml to 0.054 mg/ml in preincubation with glycogen experiments; without preincubation, assay time varied from 12 sec to 2 min as enzyme concentration decreased from 1.05 mg/ml to 0.0087 mg/ml. \( K_d = 4.50 \times 10^{-2} \) g/l.

C. 25°C. Assay time varied from 12 sec-2 min as enzyme concentration decreased from 0.685 mg/ml to 0.018 mg/ml, in preincubation in glycogen experiments; without preincubation, assay time varied from 15 sec to 5 min as enzyme concentration decreased from 1.37 mg/ml to 0.0091 mg/ml. \( K_d = 6.69 \times 10^{-3} \) g/l.

D. 20°C. Assay time varied from 15-80 sec as enzyme concentration decreased from 0.446 mg/ml to 0.0045 mg/ml in preincubation with glycogen experiments; without preincubation, assay time varied from 1-10 min as enzyme concentration decreased from 0.892 mg/ml to 0.0036 mg/ml. \( K_d = 1.17 \times 10^{-3} \) g/l.
Figure 9. Activation energies of dimeric and tetrameric phosphorylase a. All specific activities were obtained from data shown in Figure 8. •, specific activities of dimer calculated according to Equation 17; o, specific activities of dimer obtained from preincubation with glycogen experiments. Activation energy for dimer = 11.3 Kcal. ▲, specific activities of tetramer obtained from extrapolation using Equation 16. Activation energy for tetramer = 22.6 Kcal.
is in line with the model of dimer being more active and tetramer being less active.

As shown in Figures 10A and 10B, the standard enthalpy change, $\Delta H^\circ_d$, and entropy change, $\Delta S^\circ_d$, of dissociation for phosphorylase a at pH 6.8, in 40 mM glycerol-P-1 mM DTT, and 0.5% NaCl, calculated from activity measurements are 60.0 kcal/mole and 170 entropy units, respectively. The thermodynamic parameters of dissociation are summarized in Table 3. The findings that an elevation in temperature favors dissociation (cf. Wang and Graves, 1964) and that $\Delta H^\circ_d$ is quite large and positive seem to imply that the forces involved in dimer-dimer bonding are more electrostatic (as opposed to hydrophobic) in nature.

Table 3. Thermodynamic parameters of dissociation for phosphorylase a at pH 6.8

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>$\Delta F^\circ_d$ (kcal/mole)</th>
<th>$\Delta H^\circ_d$ (kcal/mole)</th>
<th>$\Delta S^\circ_d$ (e.u.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20°</td>
<td>10.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25°</td>
<td>9.18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30°</td>
<td>8.21</td>
<td>60.0</td>
<td>170</td>
</tr>
<tr>
<td>35°</td>
<td>7.56</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$Buffer used: 40 mM glycerol-P, 1 mM DTT, and 0.5% NaCl.

$^b$Calculated from the $K_d$ obtained from Figure 7A using a different enzyme preparation.
Figure 10. Standard ethalpy (A) and entropy (B) changes of dissociation of phosphorylase a at pH 6.8. All experiments were carried out in 40 mM glycerol-P-1 mM DTT, 0.5% NaCl buffer, , data obtained from specific activity curves shown in Figure 8, $\Delta H^0 = 60.0$ kcal/mole, $\Delta S^0 = 170$ e.u.; $\Delta$, data obtained from molecular weight measurements by light scattering, enzyme concentration = 0.095 mg/ml, $\Delta H^0 = 58.5$ kcal/mole, $\Delta S^0 = 165$ e.u.; o, data obtained from molecular weight measurements by light scattering, enzyme concentration = 0.262 mg/ml, $\Delta H^0 = 60.0$ kcal/mole, $\Delta S^0 = 172$ e.u.
The magnitude of $\Delta H^\circ_d$ and $\Delta S^\circ_d$ were checked by measuring molecular weight change by means of light scattering in the temperature range of 15°-35°. Two enzyme samples at 0.095 mg/ml and 0.262 mg/ml were subject to cooling, warming, and recooling. The dissociation was found to be completely reversible, indicating a truly equilibrating system. Although the dissociation constants calculated from the two samples show deviations, the $\Delta H^\circ_d$'s, 58.5 kcal and 60.0 kcal, and the $\Delta S^\circ_d$'s, 165 e.u. and 172 e.u., agree very well with those determined from rate measurements (Figures 10A and 10B). The results further support the validity of the equations derived for the phosphorylase a system, which is based on the model of a more active dimer and a less active tetramer.

Concentration Dependence of Apparent Michaelis Constant for Glycogen for Phosphorylase a

To further demonstrate that both subunit species of phosphorylase a have affinity for glycogen, apparent Michaelis constants were determined at four different enzyme concentrations, ranging from 0.0060 mg/ml to 0.302 mg/ml, at 25°. On the basis of slow equilibrium between dimer and tetramer, the kinetic equation for the phosphorylase a system is

$$v = \frac{k_D E_D S}{K_D + S} + \frac{k_T E_T S}{K_T + S}$$

where D and T denote dimer and tetramer. This equation is analogous to the case of two enzymes acting on the same sub-
_booksstrate_ (Dixon and Webb, 1964) except that the relative amounts of dimer and tetramer (E_D and E_T) will be dependent on the total protein concentration E_o. As has been shown by Dixon and Webb, when the two enzyme forms differ significantly in their maximum velocities and substrate affinities, Lineweaver-Burk plots will yield lines indistinguishable from ordinary Michaelis-Menten kinetics. If only dimeric a is capable of binding glycogen, one would expect the apparent Michaelis constant, K_app, to show no protein concentration dependence since the kinetic equation will contain only the K_D term. If the two forms can both bind glycogen but differ in their affinities, then at high enzyme concentrations, K_app would be expected to approach K_T; at very low enzyme concentrations, K_app would approach K_D. At protein concentrations intermediate to the two extremes, extrapolation of the curve would yield a value somewhere between K_D and K_T. In Figure 11, Lineweaver-Burk plots show that the K_app's vary from 1.39 mM to 0.52 mM as enzyme concentration decreases, which is consistent with the concept that both subunit species are catalytically active, with dimer having higher affinity and tetramer having lower affinity for glycogen.

To estimate the value of Michaelis constants for the two forms, K_app's were plotted against enzyme concentration (Figure 12A) and against reciprocal enzyme concentration (Figure 12B). Extrapolations show that K_D is ca. 0.20 mM and K_T is ca. 1.55 mM. The approximately eight-fold difference in Michaelis
Figure 11. Protein concentration dependence of apparent Michaelis constant for glycogen for phosphorylase a. Assay was carried out in AMP-free substrate, in 40 mM glycerol-P-1 mM DTT, pH 6.8 buffer, at 25°. Enzyme concentrations are as follows: □, 0.302 mg/ml; ○, 0.0302 mg/ml; ▲, 0.0071 mg/ml; Δ, 0.0060 mg/ml.
\[ \frac{1}{\nu} = \frac{\text{min}}{\mu \text{ moles}} \]
GLYCOGEN (mM GLUCOSYL UNITS)
Figure 12. Estimation of Michaelis constants for phosphorylase a dimer and tetramer, \(K_{\text{app}}^*\) taken from Figure 11.

A. Extrapolated Michaelis constant for glycogen for phosphorylase a dimer = 0.20 mM

B. Extrapolated Michaelis constant for glycogen for phosphorylase a tetramer = 1.55 mM
PROTEIN CONCENTRATION (mg/ml)
constants well explains the fact that preincubation of this enzyme in glycogen yields the dimeric species. From Equation 6c, it is evident that at saturating substrate level, the expression for dissociation constant for phosphorylase a becomes

$$K_d(\text{in glycogen}) = K_d \left( \frac{K_T}{K_D} \right)^4.$$ 

Thus the $K_d$ in the presence of sufficiently high glycogen concentration is greater by $\sim (8)^4 \approx 4,000$ fold, greatly favoring the formation of dimer.

To show that the change in $K_{\text{app}}$ is due to the presence of different molecular forms, the Michaelis constant for phosphorylase b, a dimer, was determined at five different protein concentrations. As shown in Figure 13, a value in the vicinity of 0.50 mM was obtained at all five concentrations, showing virtually no protein concentration dependence of Michaelis constant for this enzyme.
Figure 13. Michaelis constant for glycogen for phosphorylase b at different protein concentrations at 25°.
Assay was carried out in substrate containing $10^{-3}$ M AMP, in 40 mM glycero-P-1 mM DTT, pH 6.8 buffer. Enzyme concentrations are as follows: ●, 0.250 mg/ml; ○, 0.100 mg/ml; ▲, 0.0250 mg/ml; △, 0.0156 mg/ml; ■, 0.0082 mg/ml.
DISCUSSION

Application and Limitation of Initial Rate Measurement as a Tool for Studying Enzyme Equilibria

The derivations presented in Theory deal with several situations of one-step polymerization-depolymerization reactions of enzyme systems. In the case of phosphorylase a—a relatively slow equilibrium between a more active dimer and a less active tetramer—the equations have been shown to describe the system quite adequately. Thus initial rate measurement seems to be a valid tool for studying equilibria of biologically active macromolecules. Although ultraviolet scanning device promises to overcome some of the problems which confront molecular weight determination at low protein concentration, activity measurement is probably the only tool for studying dissociation-association phenomena for very dilute solutions. The relatively short time needed in enzymic assay may also make studies of dissociation possible at higher temperature without causing denaturation. Furthermore, many enzymes dissociate or associate into inactive subunit species, thereby greatly simplifying the analysis of data.

One limitation of this method is that a suitable way of assay which is effective at both high and low protein concentrations may not be available. For instance, in coupled assays, at high enzyme concentration the required high levels of auxiliary enzymes may interfere with the result or render it
impracticable. Also, when the degree of polymerization is great, i.e., the number \( p \) is large, or when the dissociated and aggregated states differ only slightly in their specific activities, the accuracy and sensitivity of this method greatly declines.

In using Equation 4, it is obvious that extrapolation to \( 1/E_o = 0 \) is meaningful only when the data are taken from where specific activity tends toward a finite value. It should also be noted that, when making plots according to Equation 6b, only the part of data where \( \bar{\phi}_p \) is reasonably large should be used since as \( \bar{\phi}_p \) decreases, \( 1/\bar{\phi}_p \) rapidly increases and the plot will no longer be linear.

Many of the problems discussed here are, of course, also present in other methods. For example, the expression for equilibrium constant in terms of molecular weights (Rao and Kegeles, 1958) is analogous to Equation 6:

\[
K_d = \frac{E_o^{p-1}(M_p - \bar{M}_w)^P}{(\bar{M}_w - M_m)(p-1)K_m^{p-1}} (g/1)^{P-1}
\]

If the monomeric and polymeric forms have similar activities but differ significantly in their affinities for substrate, i.e., \( k_m/(K_m + S) = k_p/(K_p + S) \) at certain substrate levels, yet \( K_m \neq K_p, k_m \neq k_p \), even for the slow equilibrium case, Equation 6b is useless and a method similar to that used for the analysis of the case of interacting sites (Equations 11-15) should be employed. In using this method, however,
another problem may be encountered in the determination of maximum velocity since the $\frac{1}{v}$ vs. $\frac{1}{S}$ plot would be non-linear; it may be further complicated by the occurrence of substrate inhibition.

The kinetic equations and methods for treatment of data presented in Theory are similar to those for ligand binding but are more complex due to the presence of rate constants. The thermodynamic basis for multiple equilibria has been established for some time (Wyman, 1948, 1964; Klotz, 1953). The fundamental principle of evaluating binding constants was developed by Scatchard and several methods have been discussed by Hareon and Gilbert (1955). Applications to ligand binding for reversibly associating protein systems have recently appeared (Klapper and Klotz, 1968). Monod et al. (1965) have proposed a model, based on transition between two conformational states of different ligand binding affinities, to account for the cooperative type kinetics shown by regulatory enzymes. Frieden (1967) has pointed out that such apparent cooperativity may occur in dissociating-associating enzyme systems and has converted the binding equations of Monod et al. into kinetic ones, including an equation for the dimerization case. Equations similar to Equations 17 and 17a for the case of slow equilibrium between monomer and dimer have been independently developed by Kurganov (1967) at about the same time the equations presented in Theory were derived (Huang and Graves, 1967; Graves et al., 1967). The procedure described in this paper
should facilitate the study of the catalytic properties of enzymes undergoing one-step dissociation-association reactions.

Correlation Between Molecular Weight and Catalytic Function of Phosphorylase a

It has been shown that theoretical lines of molecular weight as a function of protein concentration calculated from specific activity fitted the light scattering data quite well at 25° and 30°. The fact that correlations were demonstrated at two temperatures seems to rule out coincidence and strongly suggests the following: (1) Each state of aggregation of this enzyme is associated with a well-defined catalytic activity under a given set of conditions (pH, temperature, ionic strength, buffer salts, etc.). (2) The model of a relatively slow equilibrium between a catalytically more active dimer and a less active tetramer is correct.

Evidence for a slow equilibrium between the two species has also been given by Wang and Graves (1964), Wang et al. (1965b), and more recently by Chignell et al. (1968). The concept of a more active dimer and a less active tetramer is further supported by the observations that the dimeric form has a lower activation energy of 11.3 kcal and a higher affinity for glycogen (Michaelis constant = 0.20 mM), and the tetrameric form has a higher activation energy of 22.6 kcal and a lower affinity for glycogen (Michaelis constant = 1.55 mM). The activation energies reported here are lower than the 21.0 kcal and 32.6 kcal reported by Wang and Graves (1964).
The difference can be easily explained. In Wang and Graves' experiments the enzymic activity was assayed in the presence of AMP. Also, in their Arrhenius plot, the logarithm of the observed activity, which is the sum of the activities contributed by the two molecular species, was plotted against reciprocal absolute temperature; hence the activity is nearer that of the dimer at higher temperature and nearer that of the tetramer at lower temperature, resulting in a steeper slope.

On the Thermodynamic Parameters

Although $\Delta H_d^o$ and $\Delta S_d^o$ at pH 6.8 have been reported, they do not provide enough information to justify the interpretation of the forces involved in the dissociation-association process. These values merely represent the gross change of various types of interactions. One is tempted to think, however, that the interaction is more electrostatic in nature in view of the fact that elevation in temperature and high ionic strength (Wang and Graves, 1963) enhanced the dissociation and that both $\Delta H_d^o$ and $\Delta S_d^o$ are positive—contrary to the evolution of heat and loss of entropy predicted by hydrophobic interaction. The above point of view on hydrophobic bonding is based on studies on the exposure of non-polar molecules to the solvent and emphasizes the ordering of water around the solute. A different concept has been proposed by Sinanoglu and Abdulnur (1965). They held that the free energy required to form a cavity to accommodate the dissociated
molecule in the case of depolymerization will result in a large positive enthalpy change even though the interaction between the molecules is hydrophobic in nature. Thus no unambiguous interpretation of these thermodynamic parameters can be made in the absence of studies done in different solvents.

Examination of the Hypothesis of Inactive Tetramer

Metzger et al. (1967) have proposed that tetrameric phosphorylase a is inactive with glycogen as substrate. Judging from existing evidence, the proposed inactive tetramer seems unlikely: (1) The specific activities observed at high protein concentrations at four temperatures all seem to approach finite values. The specific activity vs. $1/E_0$ plots could not be extrapolated to zero activity. (2) When tetramer was assumed to be inactive, the change in specific activity did not correlate with the change in molecular weight. The dissociation constants obtained by assuming inactive tetramer were too high. (3) The $K_{app}$'s for glycogen for phosphorylase a showed protein concentration dependence. If dimer is the sole active form, only one Michaelis constant should be obtained at all concentrations. Since Helmreich et al. (1967) have shown that both dimer and tetramer may exist in R and T states, it seems that the variation in $K_{app}$ may be ascribed to the presence of two conformations of the dimeric species. However, Helmreich et al. also reported that even in the absence of reactive ligands the enzyme exists to a considerable extent in the
active R form. This is supported by the fact that phosphorylase a ordinarily yields Michaelis-Menten type kinetics. Thus the possibility that the concentration dependence of $K_{app}$ is the result of R and T states of the dimeric form appears remote. (4) Wang and Graves (1964) have shown that the decay of activity of phosphorylase a preincubated in 3.0 M NaCl followed second order kinetics, i.e., $1/(\theta - \theta_T)$ vs. time plot yielded a straight line, which is consistent with a dimerization process. When $\theta_T$ was assumed to be zero, the decay did not fit either second order or first order kinetics.

On the other hand, when active tetramer was assumed and $\theta_T$'s determined according to Equation 16, the specific activities of dimer calculated from Equation 17 agreed very well with those obtained from preincubation with glycogen experiments at four temperatures. All these observations are consistent with the concept that tetrameric phosphorylase a is capable of catalysis.

Whether or not the dissociation-association phenomenon is involved in the control of glycogen metabolism cannot be deduced from existing data. It is tempting to think, however, that aggregation of phosphorylase a may serve as a faster means of reducing the rate of glycogen breakdown than the dephosphorylation reaction catalyzed by phosphorylase phosphatase.

---

1Theoretically conformation transitions are independent of protein concentration. In practice, the way the data are presented in $1/v$ vs. $1/S$ plots may result in such apparent concentration dependence.
SUMMARY

It has long been accepted that phosphorylases α and β have identical extinction coefficients. In studying subunit dissociation of phosphorylase α by means of light scattering, an unusually low specific refractive increment at 546 μμ, 0.160-0.163, was consistently observed when protein concentration was calculated on the basis of $A_{280}^1$ mg/ml, 1 cm = 1.18. Under the same conditions, a higher specific refractive increment of 0.173 was obtained for phosphorylase β. These observations prompted reinvestigation of the absorbance indices of the two enzymes. Repeated dry weight determinations showed that the value 1.18 at 280 μμ for phosphorylase β is correct, but phosphorylase α has a higher absorptivity of 1.27. The higher absorbance index of phosphorylase α was supported by biuret method and by comparing the absorbancies of native phosphorylases α and β with guanidine hydrochloride-denatured samples. When $A_{280}^1$ mg/ml, 1 cm = 1.27 was employed, the specific refractive increment of phosphorylase α corrected to 0.173-0.176 which now agrees with the 0.173 for β. Using these constants, the molecular weights of phosphorylases α and β were estimated to be 390,000 and 195,000 respectively.

A method for determining equilibrium constant and kinetic parameters from initial rate measurements for enzymes undergoing one-step dissociation-association reaction has been developed. It permits the study of dissociation at low protein
concentrations which are inaccessible to ordinary means of molecular weight measurements, provided the two states of enzyme aggregation differ in their catalytic activity and/or substrate binding affinity. Equations and methods for treatment of data have been given for the cases of slow or rapid equilibrium relative to overall enzymatic reaction and for independent or interacting sites. The equations derived for phosphorylase α were shown to describe the system quite adequately.

Light scattering measurements of molecular weight at different protein concentrations at 25° and 30° provided direct evidence for the concentration-dependent dissociation of phosphorylase α. The increase in specific activity can be quantitatively accounted for by a decrease in molecular weight, based on a dimer-tetramer equilibrium. Standard enthalpy and entropy changes of the dissociation reaction at pH 6.8 were evaluated to be 60.0 kcal/mole and 170 e.u., respectively.

The concept of a catalytically more active dimer and a less active tetramer was reinforced by the findings that the dimeric form has a lower activation energy of 11.3 kcal and a greater affinity for glycogen, whereas the tetrameric form has a higher activation energy of 22.6 kcal and a lower affinity for glycogen. The hypothesis of inactive tetramer was also examined. When tetramer was assumed to be inactive the change in specific activity no longer correlates with the change in molecular weight. The protein concentration-dependence of
apparent Michaelis constant for glycogen also indicated that tetrameric phosphorylase a is capable of catalysis.

A list of specific refractive increments for amino acid residues has been compiled from the refraction data of McMeekin et al. to facilitate the calculation of theoretical specific refractive increment for a protein from its amino acid composition.
APPENDIX

McMeekin et al. (1964) have shown that the refractive indices of proteins can be calculated from their amino acid compositions and the values for the refraction of the amino acid residues. The calculated refractive indices were shown to agree quite well with those experimentally determined. From the data of McMeekin et al., it is possible to calculate specific refractive increments. However, this involves a tedious procedure: (1) the refraction per 100 g protein, \([R]\), is calculated by the method given by McMeekin et al.; (2) the partial specific volume is calculated by the method of Cohn and Edsall (1943); (3) the two values so obtained are then introduced into the expanded Lorenz-Lorentz equation as given by Doty and Geiduschek (1953) to solve for the refractive index of the protein solution, \(n\):

\[
\frac{n^2 - 1}{n^2 + 2} = \bar{c_v} \left(\frac{n_p^2 - 1}{n_p^2 + 2}\right) + (1 - \bar{c_v}) \left(\frac{n_o^2 - 1}{n_o^2 + 2}\right)
\]

where \(c\) = protein concentration, \(n_o\) = refractive index of solvent

\[
\bar{c_v} \left(\frac{n_p^2 - 1}{n_p^2 + 2}\right) = \frac{[R]}{100}
\]

(4) specific refractive increment is then obtained from \((n - n_o)/c\). Besides, since the expanded Lorenz-Lorentz equation is valid only at low \(c\), if one chooses \(c = 1\) (as one is
likely to do), one will get a high value for \( \frac{dn}{dc} \).

Since theoretically calculated \( \frac{dn}{dc} \) may find its use in checking the experimentally determined value and in providing reasonable values for interpreting light scattering and electrophoresis results (if experimentally determined values are not available), it seems worthwhile to compile a list of specific refractive increments of amino acid residues based on the data of McMeekin et al., to facilitate the calculation of \( \frac{dn}{dc} \) for proteins of known amino acid composition.

The specific refractive increment for a protein is calculated as the weight-average value of the refractive increments of the amino acid residues:

\[
\frac{dn}{dc} = \frac{\sum W_i (dn/dc)_i}{\sum W_i}
\]

where \( W_i \) and \( (dn/dc)_i \) are the weight per 100 g protein and refractive increment of amino acid residue \( i \), respectively. The procedure is as follows:

1. Multiply the 'g amino acid residue per 100 g protein' \(^1\) by the \( \frac{dn}{dc} \) for the residue listed in Table 4.

\(^1\) 'g amino acid residue per 100 g protein' is obtained by multiplying 'g amino acid per 100 g protein' by the ratio of the formula weights of amino acid residue (i.e., molecular weight of amino acid minus 18) and its corresponding amino acid. In practice, if the values of 'g amino acid residue per 100 g protein' are not available, 'g amino acid per 100 g protein' may be used as long as the weight-average value is calculated.
Table 4. Refractive index increments at 589 μm and specific volumes of amino acid residues in water at 25°

<table>
<thead>
<tr>
<th>Amino acid residue</th>
<th>Specific volume ($\bar{v}$)(^a)</th>
<th>Specific refractive increment ($dn/dc$)(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>glycine</td>
<td>0.64</td>
<td>0.168</td>
</tr>
<tr>
<td>alanine</td>
<td>0.74</td>
<td>0.161</td>
</tr>
<tr>
<td>valine</td>
<td>0.86</td>
<td>0.167</td>
</tr>
<tr>
<td>leucine</td>
<td>0.90</td>
<td>0.169</td>
</tr>
<tr>
<td>isoleucine</td>
<td>0.90</td>
<td>0.174</td>
</tr>
<tr>
<td>serine</td>
<td>0.63</td>
<td>0.162</td>
</tr>
<tr>
<td>threonine</td>
<td>0.70</td>
<td>0.165</td>
</tr>
<tr>
<td>hydroxyproline</td>
<td>0.68</td>
<td>0.160</td>
</tr>
<tr>
<td>proline</td>
<td>0.76</td>
<td>0.159</td>
</tr>
<tr>
<td>methionine</td>
<td>0.75</td>
<td>0.195</td>
</tr>
<tr>
<td>cysteine</td>
<td>0.61</td>
<td>0.202</td>
</tr>
<tr>
<td>phenylalanine</td>
<td>0.77</td>
<td>0.231</td>
</tr>
<tr>
<td>tryptophan</td>
<td>0.71</td>
<td>0.227</td>
</tr>
<tr>
<td>histidine</td>
<td>0.67</td>
<td>0.207</td>
</tr>
<tr>
<td>arginine</td>
<td>0.70</td>
<td>0.196</td>
</tr>
<tr>
<td>lysine</td>
<td>0.82</td>
<td>0.175</td>
</tr>
<tr>
<td>aspartic acid</td>
<td>0.60</td>
<td>0.166</td>
</tr>
<tr>
<td>glutamic acid</td>
<td>0.66</td>
<td>0.175</td>
</tr>
<tr>
<td>asparagine</td>
<td>0.62</td>
<td>0.182</td>
</tr>
<tr>
<td>glutamine</td>
<td>0.67</td>
<td>0.178</td>
</tr>
</tbody>
</table>

\(^a\)Taken from Cohn and Edsall (1943).

\(^b\)Calculated from data of McMeekin et al. (1964); $n_0 = 1.3325$ for water at 589 μm, 25°.
2. Divide the sum of the products obtained in step 1 by the sum of g residues (because total g residues seldom add up to exactly 100 g) to get $dn/dc$ for the protein.

Since the values of $dn/dc$ listed in Table 4 are measured at 589 μm, the calculated $dn/dc$ is valid only at this wavelength. To convert the calculated value into a value at any given wavelength, $\lambda$, in the visible range, the following equation may be used, which is calculated from the empirical equation given by Perlmann and Longsworth (1948):

$$\frac{dn}{dc}\lambda = \frac{dn}{dc}589 \mu m \times \left(0.9422 + \frac{2.005 \times 10^4}{\lambda^2}\right)$$

where $\lambda = \text{desired wavelength in } \mu \text{m}$. Usually light scattering experiments are conducted at 546 μm or 436 μm. For these two wavelengths, the correction factors are:

$$\frac{dn}{dc}546 = \frac{dn}{dc}589 \times 1.01$$

and

$$\frac{dn}{dc}436 = \frac{dn}{dc}589 \times 1.05$$

To correct for the effect of solvent, the equation derived by Casassa and Eisenberg (1964) may be used:

$$\frac{dn}{dc}b = \frac{dn}{dc}a + \bar{v}(n_oa - n_ob)$$
where \( \frac{dn}{dc}_a \), \( \frac{dn}{dc}_b \) = specific refractive indices in solvent a and solvent b, respectively

\[ \bar{v} = \text{specific volume of protein} \]

\( n_{o_a}, n_{o_b} \) = refractive indices of solvent a and solvent b, respectively.

Thus from the knowledge of \( \frac{dn}{dc} \) in one solvent (e.g. water), if \( \bar{v} \) and the refractive index of the second solvent are known, \( \frac{dn}{dc} \) in the second solvent can be estimated. Since the refractive increments for amino acid residues listed in Table 4 are computed by using the \( \bar{v} \)'s given by Cohn and Edsall, to be consistent, theoretical \( \bar{v} \) for protein should be used; but it is not critical in view of the rather small correction. If experimentally determined \( \bar{v} \) is not available, theoretical value can be calculated from amino acid composition in a like fashion as the calculation of theoretical \( \frac{dn}{dc} \) (Cohn and Edsall, 1943):

\[
\bar{v} = \frac{\sum W_i \bar{v}_i}{\sum W_i}
\]

where \( \bar{v}_i \) = specific volume for amino acid residue i.

The data of Cohn and Edsall are included in Table 4.

Theoretical \( \frac{dn}{dc} \) for phosphorylase has been computed by three different ways: (1) from \([R]/100 \text{ g protein} \) and expanded Lorenz-Lorentz equation, (2) weight-average \( \frac{dn}{dc} \) using \((g \text{ residue})/(100 \text{ g protein})\), and (3) weight-average \( \frac{dn}{dc} \) using \((g \text{ amino acid})/(100 \text{ g protein})\). In all cases the same value,
0.185 ± 0.007 in water at 546 μι at 25° was obtained, which agrees with experimentally determined values (corrected to the values in water) 0.179 and 0.182 quite well.
BIBLIOGRAPHY


Gold, A. M.; (1968), Biochemistry 7, 2106.


Helmreich, E., Michaelides, M. C., and Cori, C. F. (1967), Biochemistry 6, 3695.


Wang, J. H. and Graves, D. J. (1964), Biochemistry 3, 1437.


ACKNOWLEDGMENTS

The author wishes to express his sincere thanks to Dr. Donald J. Graves, his major professor, for constant guidance, encouragement, and material aid throughout the course of these investigations.

The author is indebted to Dr. Malcolm A. Rougvie for valuable discussions and technical advice.

Thanks are also due to Dr. Jerry H. Wang, Mr. George Johnson, Mr. Michael Burke, Mr. Neal Busch, and Mrs. Shirley Mann for various kinds of suggestions and help, and to Sister Burcharda for performing part of the specific activity measurements.

The author especially wishes to thank his wife, Eileen Wei-lee, for everything she has done for him in all these years.