Kinetics of Purified Liver Phosphorylase*

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SUMMARY

Glycogen phosphorylase has been purified from rabbit liver by a simplified procedure which depends on the initial separation of the enzyme from a crude extract by centrifuging it as a complex with particulate glycogen.

Initial rates have been measured with varied concentrations of both substrates in each reaction direction. The data were analyzed with double reciprocal plots and secondaryary plots of intercepts and slopes to yield the eight Dalziel kinetic coefficients.

Inhibition by uridine diphosphoglucose is competitive with glucose-1-phosphate and inorganic orthophosphate. With the latter substrate the \( K_i \) at high glycogen concentration is 0.4 mM and at low glycogen concentration it is 0.2 mM.

Consideration of the relationships among the Dalziel kinetic coefficients, various related kinetic constants, the Halldane relationship, and the patterns of inhibition by UDP-glucose has led to a tentative assignment of a kinetic mechanism which involves random order of substrate addition to enzyme, with rapid equilibria compared to a rate-limiting interconversion of ternary complexes.

On the basis of this tentative kinetic mechanism, the kinetically derived dissociation constants for glycogen, \( P_i \), and glucose-1-P from the free enzyme are 14, 11, and 1.3 mM, respectively. Dissociation constants were also deduced for glycogen and the enzyme-\( P_i \) complex (0.93 mM); glycogen and the enzyme-glucose-1-P complex (2.9 mM); \( P_i \) and the enzyme-glycogen complex (0.82 mM); and glucose-1-P and the enzyme-glycogen complex (0.28 mM). Some of these differences were rationalized by suggesting a glucose-binding site adjacent to a phosphate-binding site, both of which are occupied by glucose-1-P in one case or by the terminal glucose of glycogen and by \( P_i \).

Increasing the concentration of \( P_i \) or glucose-1-P decreases the apparent \( K_m \) for glycogen, while increasing the concentration of glycogen decreases the apparent \( K_m \) values for either of the other two substrates.

Adenosine 5'-phosphate decreases the apparent \( K_m \) values for all three substrates. The apparent \( K_m \) for AMP is \( 6 \times 10^{-5} \) M at 24 mM glucose-1-P and \( 10 \times 10^{-5} \) M at 8 mM glucose-1-P.

The apparent \( K_m \) values and dissociation constants for glycogen and liver phosphorylase are of the same order of magnitude as those commonly found for the muscle phosphorylases. The apparent \( K_m \) values of glucose-1-P and \( P_i \) are less than those found for the muscle enzymes, and this is paralleled by smaller \( K_i \) values for inhibition by a variety of naturally occurring organic phosphates and pyrophosphates. Alteration of the groups on the glucose moiety of various phosphate compounds decreases binding to the active center with an order of importance of C-4 > C-6 > C-2.

The pioneering researches on liver glycogen phosphorylase (\( \alpha-1,4\)-glucan:orthophosphate glucosyltransferase, EC 2.4.1.1) by Sutherland, Wosilait, and Rall (2-5) served to delineate some of the similarities and differences between this enzyme system and that of muscle. The mechanisms involved in the interconversion between active and inactive forms of the enzyme, and the role of hormones in this process, were defined especially well. This group also showed that, in contrast to the glycogen phosphorylase from muscle, the dephosphorylated form of liver phosphorylase could not be rendered catalytically active by the addition of adenosine 5'-phosphate (3), a fact which has been confirmed by Appleman (6). No detailed study of the kinetics of this important enzyme has yet been published, however. Kinetic studies on the muscle phosphorylase are dispersed in numerous papers, some of which will be alluded to below, but a comprehensive approach, similar to that contained herein, will be found in the work of Lowry, Schuls, and Passonneau (7).

In a previous communication, we have shown that about 60% of the glycogen phosphorylase in normal "resting" liver is present in the active form (1). These results indicated that regulatory mechanisms complementing those through which hormones mediate their effects may be operating to control the activity of liver phosphorylase in vivo. Inhibition by uridine diphosphoglucose, the substrate for the glycogen-synthesizing enzyme, as well as by other metabolites, was considered as a possible factor in such control mechanisms at the cellular level, in addition to such factors as the binding of phosphorylase to particulate glycogen. It seemed obvious that detailed kinetic studies of the interactions of the purified enzyme and its substrates, activators, and inhibitors were necessary to provide in-
formation to supplement more physiological investigations on the biological control of glycogen metabolism in the liver.

In this paper we report a somewhat simplified method for the purification of phosphorylase from rabbit livers. A detailed kinetic analysis has been performed, primarily by the method of Dalziel (8), but with some treatments suggested by Frieden (9). The analysis of data and theoretical considerations also owes much to the writings of Alberty (10, 11) and Cleland (12). The inhibition of liver phosphorylase by a series of naturally occurring organic phosphates, as well as by UDP-glucose, has been investigated.

**EXPERIMENTAL PROCEDURE**

Chemicals and auxiliary enzymes were purchased from Sigma or Calbiochem, and were the highest grade obtainable. The rabbit liver glycogen was routinely passed through a Dowex-1 chloride column. It was assayed against a glucose standard by the method of Dische as described by Ashwell (13), and its concentration is expressed as the molar equivalent of its glucose residues.

The protein concentration of crude preparations was measured by the method of Lowry et al. (14) and by the absorbance at 280 mµ for purified preparations, assuming that 1 mg per ml yields an absorbance of 1.18 (15). The two methods agreed well for the purified enzyme.

**Phosphorylase Assays**

Phosphorylase activities are expressed as micromoles of orthophosphate (P_i) released from glucose-1-P per min per mg of protein. Activities in crude homogenates were assayed at 30° by the method of Wosilait and Sutherland (3). Preparations purer than that obtained in Step 2 of the purification procedure were assayed essentially by the method of Cori, Cori, and Green (10), at 30° and pH 6.6 ± 0.1. The reaction mixture contained 55 mM imidazole, 4 mM mercaptoethanol, 0.4 mM EDTA, and, except as noted, 1 mM AMP. Glycogen and glucose-1-P concentrations were assayed below or were 28 mM and 15 mM, respectively. The enzyme was incubated with glycogen and AMP, and the reaction was started by the addition of the glucose-1-P. Initial reaction rates were calculated from the pseudo-first order reaction constants. In the kinetic experiments, 12 µg of liver phosphorylase were present per ml of reaction mixture.

The phosphorylisis of glycogen was coupled to a large excess of phosphoglucomutase and glucose-6-P dehydrogenase so that the rate-limiting step in the reduction of NADP was the phosphorylase activity. Phosphorylase was again incubated with glycogen and AMP at 30° before being added to the measuring system, also at 30°, in a thermostated cuvette. The increase in the absorbance at 340 mµ was plotted on a Sargent recorder coupled to a Zeiss PMQ II spectrophotometer equipped with a Gilford automatic cuvette changer. After a lag period of 5 to 10 sec the reaction rate was linear and proportional to the phosphorylase concentration. The reaction mixture of 1.5 ml at pH 6.6 ± 0.1 contained 3 µg of phosphorylase, 3 units (20 µµ) of glucose-6-P dehydrogenase, 50 µg of phosphoglucomutase, 35 mM imidazole, 10 mM magnesium acetate, 1 mM NADP, 4 mM mercaptoethanol, 0.4 mM EDTA, 1 mM AMP (except as noted), and 1 mM glucose 1,6-diphosphate. The glycogen and P_i concentrations are given for each experiment.

**Preparation of Liver Phosphorylase**

The purification of dog liver phosphorylase by Sutherland and Wosilait (2) required precautions and procedures to block and remove the inactivating enzyme (phosphophorylase phosphatase), as well as to remove the glycogen which accompanied the phosphorylase. Appleman (9) purified the active liver phosphorylase of pig and rabbit by taking advantage of the binding between the enzyme and glycogen and collecting the enzyme-glycogen complex by centrifugation. The binding of various phosphorylases to polynucleotides has also been studied or employed in purification procedures by other authors (17, 18). We found it possible to modify the procedure of Appleman so that the active phosphorylase can be purified. The chief modification was the use of sodium fluoride to inhibit the inactivating enzyme until it could be removed from the phosphorylase.

**Step 1: Homogenization**—Fed, adult rabbits were killed by a blow on the neck. The livers were removed, weighed, and homogenized in a Waring Blender in 3 volumes of cold 0.1 M NaF for 1.5 min. Further steps were carried out at 2–4°C.

**Step 2: Centrifugation and Straining**—The homogenates were centrifuged for 30 min at 2,000 × g, and the supernatant solution was strained through cheesecloth.

**Step 3: Collection of Glycogen and Amylase Digestion**—The extract was centrifuged for 2 hours at 35,000 × g. The glycogen pellet was collected with the aid of 0.1 M NaF and was dispersed in that medium. Ten micrograms of crystalline human salivary α-amylase (prepared by the method of Bernfeld (19)) were added to each milliliter, and the turbid suspension was dialed for 96 hours at 2°C against two 4 liter changes of 0.1 M NaF, 20 mM NaCl, and 2 mM Ca**2+**-EDTA, pH 7.

**Step 4: Ammonium Sulfate Fractionation**—To the clear brown solution from Step 3 was added one-half volume of neutral saturated ammonium sulfate, and the resultant precipitate was removed and discarded. A further 0.5 volume of ammonium sulfate was added to the supernatant solution, and the resultant precipitate was collected by centrifugation and dissolved in a minimal volume of 0.1 M NaF, 1 mM EDTA, 5 mM glycerophosphate, and 2 mM mercaptoethanol, pH 6.7.

**Step 5: Chromatography on Sephadex G-200**—The ammonium sulfate precipitate was placed on a column, 4 × 30 cm, of Sephadex G-200, previously equilibrated with the buffer solution described in Step 4. The column was eluted with the same buffer solution. Fractions, 8.0 ml, were collected, and those showing a specific activity greater than 4 amoles per min per mg were pooled.

**Step 6: Ammonium Sulfate Precipitation**—The solution from Step 5 was brought to 60% of saturation by the addition of 1.5 volumes of saturated ammonium sulfate. The precipitate was collected by centrifugation, dissolved in 15 ml of 4 mM Tris, 2 mM mercaptoethanol, and 1 mM EDTA, pH 7.2, and passed through a column, 2 × 26 cm, of Sephadex G-25 that had been equilibrated with the same buffer solution.

**Step 7: Chromatography on DEAE-cellulose**—The solution from Step 6 was placed on a column, 2 × 36 cm, of DEAE-cellulose which had been previously equilibrated with the Tris buffer solution described in Step 6. The column was washed with 150 ml of this buffer. A linear gradient of NaCl was then applied to the column, with 1 liter of the initial buffer in the mixing chamber and 1 liter of the same buffer containing 0.5 M NaCl in the reservoir. The column was eluted under pressure of gravity of 125 cm of
TABLE I
Purification of rabbit liver phosphorylase

In this preparation, 1,410 g of liver, from 12 rabbits, were used.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Protein</th>
<th>Phosphorylase</th>
<th>Recovery</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ml</td>
<td>Per ml</td>
<td>Total</td>
<td>µmoles/min</td>
<td>µmoles/min/mg</td>
</tr>
<tr>
<td>1. Homogenate</td>
<td>5,140</td>
<td>27.6</td>
<td>142,000</td>
<td>3.0</td>
<td>15,400</td>
</tr>
<tr>
<td>2. Supernatant solution</td>
<td>4,290</td>
<td>23.5</td>
<td>101,000</td>
<td>3.4</td>
<td>14,000</td>
</tr>
<tr>
<td>3. Amylase digest</td>
<td>500</td>
<td>9.5</td>
<td>4,750</td>
<td>9.9</td>
<td>5,000</td>
</tr>
<tr>
<td>4. Ammonium sulfate, 0.33-0.50% saturation</td>
<td>13</td>
<td>55.2</td>
<td>720</td>
<td>308</td>
<td>4,000</td>
</tr>
<tr>
<td>5. Sephadex G-200</td>
<td>172</td>
<td>2.5</td>
<td>422</td>
<td>21</td>
<td>3,500</td>
</tr>
<tr>
<td>6. Ammonium sulfate, 60% saturation, + Sephadex G-25</td>
<td>23</td>
<td>15.4</td>
<td>354</td>
<td>146</td>
<td>3,400</td>
</tr>
<tr>
<td>7. DEAE-cellulose</td>
<td>131</td>
<td>0.25</td>
<td>32</td>
<td>11.5</td>
<td>1,540</td>
</tr>
</tbody>
</table>

* Mean specific activity of 12 fractions, ±S.E.M.

In this preparation, 1,410 g of liver, from 12 rabbits, were used. A protein peak which began to appear at 0.1 m NaCl was found to contain the phosphorylase activity. The 12 fractions which showed a specific activity greater than 42 µmoles per min per mg were pooled, concentrated to 32 ml by evaporation from a dialysis sac in the cold room, and dialyzed against a buffer solution of 20 mM sodium glycerophosphate, 5 mM mercaptoethanol, and 1.5 mM EDTA, pH 6.7. The clear solution was stored under toluene vapor at 4°C.

RESULTS
Purified Liver Phosphorylase—Table I summarizes the preparation of liver phosphorylase used in this work. The enzyme was quite stable and retained 77% of its original activity after 9 months. The preparation was homogeneous as judged by the criterion that it produced a single band when subjected to electrophoresis on acrylamide gel (see Fig. 9 of Reference 20). The specific activity corresponds to 1800 Cori (16) units per mg and therefore compares favorably with that reported for various other glycogen phosphorylases (21), with the exception of the dog liver phosphorylase prepared by Sutherland and Wosilait (2), which was considerably more active. The latter authors have, however, obtained homogeneous preparations of dog liver phosphorylase with specific activities more comparable with the enzymes from other sources (22). Appleman reported a specific activity of 1760 Cori units per mg for the active phosphorylase which he prepared from his purified inactive rabbit liver phosphorylase (6).

Kinetic Studies—Initial rate data for the phosphorolysis of glycogen at varying concentrations of both glycogen and inorganic phosphate are shown in the primary plots of Figs. 1 and 2. In Fig. 1 the point of intersection in the upper left-hand quadrant yields the dissociation constant for glycogen and free enzyme and is labeled according to the scheme in Fig. 7. Although the application of this scheme (random addition of substrates and rapid equilibrium of all complexes, with interconversion of ternary complexes being rate-limiting) to liver phosphorylase may at present be somewhat arbitrary, the discussion of Frieden (9) makes it clear that a kinetic derivation of the dissociation constant for glycogen and free enzyme is sound because glycogen can bind to the free enzyme and would therefore be the first substrate bound were a compulsory order of binding to obtain. Similarly, in Fig. 2, the point of intersection in the upper left-hand quadrant yields the dissociation constant for P_i and free enzyme, provided...
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It is necessary to take into account the fact that the rate of the reaction slows markedly as the outer chains of the glycogen are lengthened, a phenomenon which is accentuated at low concentrations of the glycogen (16, 17). Normally, this technical difficulty is surmounted by determining the course of the reaction at various times and extrapolating to zero. In the experiment reported here, the reaction volume was increased and the time decreased to 2 min, so that for any datum reported in Figs. 3 and 4 the average increase of chain length did not exceed 2 glucose units, an amount too small to decrease the rate. The $K_m$ of glycogen for liver phosphorylase was also determined by the method of Cori et al. (16), and at 15 mM glucose-1-P was found to be 2.9 mM, in good agreement with that reported here.

In Figs. 5 and 6 the intercepts and slopes from the primary plots are replotted according to the method of Dalziel (8). Internal consistencies of the data are shown in Fig. 5 by common intercepts for the respective values of $\phi_1$ and $\phi_2$, and in Fig. 6 by common slopes yielding the values of $\phi_{12}$ and $\phi'_{12}$. Similarly, the values of $\phi_1$, $\phi_2$, $\phi_{12}$, $\phi'_{12}$, $\phi_2$, and $\phi'_{2}$ derived from the intercepts of Fig. 6 that the mechanism shown in Fig. 7 is a reasonable approximation of the real situation.

In these experiments, it is seen that the maximal velocities and apparent $K_m$ values obtained with one substrate are a function of the concentration of the other substrate.

The dissociation constant for glycogen and free enzyme derived from Fig. 3 is the same as that derived from Fig. 1, so that $K_1 = K_7$, as would be expected for this enzyme.

In determining the $K_m$ of glycogen for phosphorylase, it is
agree reasonably well with those derived from the slopes of Fig. 5 (the latter are reported in Table II).

Table II summarizes the various kinetic coefficients derived from Figs. 5 and 6. These are generalized entities which would be applicable to any kinetic mechanism. The corresponding (specific) kinetic constants, assuming that the kinetic mechanism shown in Fig. 7 applies to liver phosphorylase, are also given in Table II.

Table III summarizes certain relationships between the kinetic coefficients or the kinetic constants or both. The kinetically derived equilibrium constant (Haldane relationship) is considered to be in reasonable agreement with the equilibrium constant obtained by direct measurement by Trevelyan, Mann, and Harrison (23). Comparison of the data in Table III with Table I of Dalziel (8) indicates that several of the more commonly proposed kinetic mechanisms can be eliminated from consideration for liver phosphorylase. The data and analysis presented here indicate that only two kinetic mechanisms remain as reasonable candidates. The first, Type I(a) of Dalziel, depicted in Fig. 7, involves random, rapid equilibria between substrates and enzyme forms, with the rate-limiting step being the intramolecular conversion of the ternary complexes. The alternative is that only one of the substrates in each direction forms a binary complex (Type II(i) or II(iia)) so that there is a compulsory order of addition, and there may be either one or two ternary complexes. The initial rate equation (Equation 2, Fig. 7) would be derived by steady state treatment. It should be noted that the data presented in this section of the paper are not sufficient to distinguish between Mechanisms I and II.

Inhibition by UDP-glucose—Figs. 8 and 9 illustrate the pattern of the inhibition of liver phosphorylase by UDP-glucose as a function of the concentration of P_i. It may be seen that the in-
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FIG. 9. Velocity of liver phosphorylase activity as a function of Pi concentration at 2.8 mM glycogen and the following levels of UDP-glucose: •, 0 (apparent $K_m$ for Pi = 4.7 mM); X, 0.45 mM; ○, 1.0 mM; △, 2.0 mM; ▲, 3.3 mM.

Inhibition is strictly competitive with respect to Pi, at both high and low concentrations of glycogen. In Fig. 10, the slopes from Figs. 8 and 9 have been plotted against the concentration of UDP-glucose and the results are again consistent with the idea that UDP-glucose and Pi compete for the same site on the enzyme. It may be noted, however, that the $K_i$ for UDP-glucose is different for the two concentrations of glycogen studied. This suggests that UDP-glucose can combine either with the free enzyme or with the enzyme saturated with glycogen, but that there are different dissociation constants for the two cases.

Fig. 11 shows the effect of various concentrations of UDP-glucose at various levels of glycogen for a constant high level of Pi. The inhibition with respect to glycogen is not of the competitive type but rather follows the pattern to be expected from the addition of a competitive inhibitor for a second substrate to various concentrations of the first substrate. Furthermore, the intersection of the lines in the upper left-hand quadrant of Fig. 11 should be at a point equivalent to the negative reciprocal of the dissociation constant for glycogen and the free enzyme. The value of 15 mM glycogen agrees well with the values of 13 and 14 mM derived from the data in Figs. 1 and 3. The theoretical considerations in this paragraph stem directly from manipulations of Equation 8 of Dalziel (8) when applied to an inhibitor which competes with one substrate. Thus, with $I$ representing inhibitor concentration, and $K_i$, the dissociation of inhibitor from enzyme, the general rate equation (Equation 2) from Fig. 7 becomes

$$\frac{E}{V} = \frac{\phi_0 + \phi_1}{G} + \left(1 + \frac{I}{K_i}\right) \frac{\phi_2}{[P]} + \left(1 + \frac{I}{K_i}\right) \frac{\phi_3}{[G][P]}$$

(3)

Fig. 12 shows the results of repeating the experiment of Fig. 11 at a constant low level of Pi. The results are very similar to those of Fig. 11 except that the decrease in the $V_{max}$ values with increasing concentration of UDP-glucose is more pronounced. Again, the value of $K_i$ for the dissociation of glycogen from the free enzyme, 15 mM, agrees with the values determined earlier.

The inhibition of the synthesis of glycogen from glucose-1-P by UDP-glucose was competitive with respect to glucose-1-P, an effect reported earlier for the glycogen phosphorylases from muscle and bacteria (24). When determined under the same conditions, the $K_i$ for UDP-glucose measured in this direction was similar to the $K_i$ determined for the phosphorylase (Table V).

The intersection of the lines in the upper left-hand quadrant is equivalent to 15 mM glycogen.
Effect of AMP—As has been previously reported (2), it was found that the addition of AMP to liver phosphorylase increased the rate of reaction from 20 to 40% when the substrate was glucose-1-P. The activation in the direction of phosphorolysis was negligible except at low concentrations of Pi. The addition of AMP had a significant effect on the apparent $K_m$ values for all three substrates, however, causing a marked lowering in every case. These relationships are summarized in Table IV.

The apparent $K_m$ for AMP is also affected by the concentration of glycogen. The intersection of the lines in the upper left-hand quadrant is equivalent to 15 mM glycogen.

**Table IV**

<table>
<thead>
<tr>
<th>Effect of AMP on liver phosphorylase</th>
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<tbody>
<tr>
<td>Substrate</td>
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<tr>
<td>-------------</td>
</tr>
<tr>
<td>Glucose-1-P</td>
</tr>
<tr>
<td>+</td>
</tr>
<tr>
<td>P$_i$</td>
</tr>
<tr>
<td>+</td>
</tr>
<tr>
<td>Glycogen</td>
</tr>
<tr>
<td>+</td>
</tr>
</tbody>
</table>

* At 28 mM glycogen.
+ At 29 mM glycogen.
$^a$ At 27 mM P$_i$.

The velocity of liver phosphorylase activity as a function of glycogen concentration at 1.2 mM P$_i$ is shown in Fig. 12.

**Table V**

<table>
<thead>
<tr>
<th>Inhibition of liver phosphorylase by organic phosphates</th>
</tr>
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<tbody>
<tr>
<td>Compound tested</td>
</tr>
<tr>
<td>-----------------</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>1. ADP-glucose</td>
</tr>
<tr>
<td>2. CDP-glucose</td>
</tr>
<tr>
<td>3. GDP-glucose</td>
</tr>
<tr>
<td>4. TDP-glucose</td>
</tr>
<tr>
<td>5. UDP-glucose</td>
</tr>
<tr>
<td>6. UDP-N-acetylglucosamine</td>
</tr>
<tr>
<td>7. UDP-glucuronic acid</td>
</tr>
<tr>
<td>8. UDP-galactose</td>
</tr>
<tr>
<td>9. ADP, CDP, GDP, TDP, UDP</td>
</tr>
<tr>
<td>10. GMP, TMP, UMP</td>
</tr>
<tr>
<td>11. ATP</td>
</tr>
<tr>
<td>12. CTP</td>
</tr>
<tr>
<td>13. GTP</td>
</tr>
<tr>
<td>14. TTP</td>
</tr>
<tr>
<td>15. UTP</td>
</tr>
<tr>
<td>16. Glucose-6-P</td>
</tr>
<tr>
<td>17. Fructose-1-P</td>
</tr>
<tr>
<td>18. Galactose-1-P</td>
</tr>
<tr>
<td>19. Fructose-1,6-di-P</td>
</tr>
<tr>
<td>20. α-Glycerophosphate</td>
</tr>
<tr>
<td>21. α-Glycerophosphate</td>
</tr>
<tr>
<td>22. Pyrophosphate</td>
</tr>
</tbody>
</table>

* In the absence of AMP; no inhibition was observed in the presence of 1 mM AMP. All other compounds were tested in the presence of 1 mM AMP.

**Fig. 13** Velocity of liver phosphorylase activity as a function of glycogen concentration at 1.2 mM P$_i$. The apparent $K_m$ values for all three substrates were determined at various concentrations of glycogen. The intersection of the lines in the upper left-hand quadrant is equivalent to 15 mM glycogen.
Inhibition by Organic Phosphates — Table V lists a series of organic phosphates of biological importance which were tested for their ability to inhibit liver phosphorylase. All the compounds listed, with the exception of UDP-galactose, caused an inhibition which was strictly competitive with the substrate (P_i, or glucose-1-P) which was varied. The reciprocal plots were all straight, including those obtained with compounds such as ATP, ADP, or glucose-6-P, which cause a curved type of reciprocal plot in the case of phosphorylase b (25).

Comparison of Compounds 1 to 5 (Table V) indicates that the type of base in the nucleoside diphosphate glucose compounds may not be of great importance in determining the binding of these compounds to the active site of phosphorylase. The differences observed may be indicative of configurational differences between these compounds. On the other hand, minor alterations in the glucose moiety result in a great decrease in the tightness of binding. It is apparent that organic phosphates which have the configuration of α-β-glucose-1-P are bound more tightly than those which do not, just as the earlier kinetic experiments indicated that α-β-glucose-1-P itself is bound more tightly to the enzyme than is P_i. It is probable that those organic phosphates which do not have the configuration of α-β-glucose-1-P are unable to inhibit because they resemble P_i and can presumably bind to the binding site for P_i.

Glucose alone caused slight inhibition of the binding. It is apparent that organic phosphates which have the configuration of α-β-glucose-1-P are bound more tightly to the enzyme saturated with P_i than it does to the enzyme saturated with glucose-1-P (K_i < K_d). This would be the expected result if the glycosgen had available to it in the former case an extra binding site (for its terminal glucose moiety), which in the latter case is occupied by the glucose moiety of glucose-1-P.

In a manner similar to that discussed above, increasing the glycosgen concentration decreases the apparent K_m values for either P_i or glucose-1-P, an effect similar to that reported for muscle phosphorylase a (7), but not, in the case of P_n, for phosphorylase b (20). The apparent K_m values again form a progression between the dissociation constant for the free enzyme and that for the enzyme saturated with glycosgen. The effects of AMP on the apparent K_m values for the various substrates and vice versa are again similar to those reported for the muscle enzymes, with certain obvious differences with regard to the magnitude of the effects, and also of the effects on velocities of reaction (7, 25, 28).

Certain of the data reported here have a bearing on the nature of the binding sites at the catalytic center of liver phosphorylase. Glucose-1-P is found to have a lower dissociation constant than does P_i for either free enzyme or glycosgen-enzyme complex, suggesting that a specific binding site exists for its glucose moiety; this extra binding thus augments that provided by the binding of the phosphate group to a specific phosphate site. As suggested above, the glucose site would be occupied by a terminal glucose moiety of glycosgen during the phosphorylization reaction. Glycosgen phosphorylases appear to have absolute specificity with regard to hexose-1-P, but some idea of the relative importance of various parts of the glucose moiety with regard to the binding to the glycosgen may be gained from a comparison of the different compounds tested for their inhibitory properties, as reported in Table V. Thus, modifications of the groups on carbon 2 of glucose are less effective in reducing binding than modifications of the groups on carbon 4 or 6. The rather general, nonspecific inhibition by organic phosphates and pyrophosphate indicates that these compounds act at a specific phosphate-binding site.

Liver glycosgen phosphorylase appears to be much more sensitive to inhibition by organic phosphates in general than do the muscle phosphorylases, but the latter do exhibit the phenomenon.

\[ \text{Ki values on the order of 50 mM.} \]

\[ \text{which tended to be noncompetitive with all three substrates, with the binding site for P_i. Glucose alone caused slight inhibition because they resemble P_i and can presumably bind to which do not have the configuration of α-β-glucose-1-P are able to inhibited enzyme on which glycosgen was bound, and that therefore the same \( K_i \) should result at both high and low concentrations of glycosgen. In fact, the data show that UDP-glucose can combine with either free enzyme or enzyme bound to glycosgen, and one would therefore predict that the same would be true for P_i and glucose-1-P. These results have led us to make a tentative assignment of the Type II mechanism to liver phosphorylase, and subsequent discussion is based on the assumption that this assignment will eventually be proved substantially correct. Such proof will involve independent measurement of the various dissociation constants suggested in this paper, or demonstrations that P_i and glucose-1-P can bind to the free enzyme, or both. It is interesting that Lowry et al. (7) fitted their kinetic data on phosphorylase a into the framework of Mechanism I. It has been noted that increasing the concentration of either P_i or glucose-1-P results in a pronounced decrease in the dissociation constant for glycosgen, with the apparent \( K_m \) values forming a progression from the \( K_1 \) and \( K_2 \) for binding to the free enzyme, to \( K_3 \) and \( K_4 \) for binding to the enzyme saturated with the other substrate. A similar effect of the P_i concentration on the apparent \( K_m \) of glycosgen has been reported before for phosphorylase a of muscle (7), but this phenomenon was not observed with phosphorylase b (26). It is noteworthy that the glycosgen binds more tightly to the enzyme saturated with P_i than it does to the enzyme saturated with glucose-1-P (\( K_i < K_d \)). This would be the expected result if the glycosgen had available to it in the former case an extra binding site (for its terminal glucose moiety), which in the latter case is occupied by the glucose moiety of glucose-1-P.

\[ \text{Comparison of Compounds 1 to 5 (Table V) indicates that the type of base in the nucleoside diphosphate glucose compounds may not be of great importance in determining the binding of these compounds to the active site of phosphorylase. The differences observed may be indicative of configurational differences between these compounds. On the other hand, minor alterations in the glucose moiety result in a great decrease in the tightness of binding. It is apparent that organic phosphates which have the configuration of α-β-glucose-1-P are bound more tightly than those which do not, just as the earlier kinetic experiments indicated that α-β-glucose-1-P itself is bound more tightly to the enzyme than is P_i. It is probable that those organic phosphates which do not have the configuration of α-β-glucose-1-P are unable to inhibit because they resemble P_i and can presumably bind to the binding site for P_i. Glucose alone caused slight inhibition which tended to be noncompetitive with all three substrates, with \( K_i \) values on the order of 50 mM.} \]

\[ \text{DISCUSSION} \]

\[ \text{As is pointed out above, the analysis of the kinetic data by the method of Dalziel has permitted the elimination of all the more common kinetic mechanisms except those which he has designated as Type I and Type II. Normally, further differentiation of these two types by kinetic means would involve the use of product inhibition, but this approach is, for the present at least, precluded for glycosgen phosphorylase by technical limitations. On the other hand, UDP-glucose undoubtedly mimics the product, glucose-1-P, when it inhibits the phosphorolysis of glycosgen, and it may also mimic the product, P_i, when it inhibits the reverse reaction. Thus it may be possible to consider the inhibition by this compound as a substitute for product inhibition. Even if this should not be strictly correct, the analysis of the inhibition by UDP-glucose shows that the kinetics is that which would be predicted for the addition of an inhibitor which is competitive for one of the two substrates in either direction of the scheme outlined in Fig. 7 (kinetic mechanism Type I). The kinetics is also correct for UDP-glucose competing with the second substrate to be bound to the enzyme in the compulsory order of addition typical of kinetic mechanisms of Type II, except for the fact that the \( K_i \) differs for different concentrations of the first substrate (glycosgen). Were the Type II mechanism to be correct, one would expect that UDP-glucose could combine only with enzyme on which glycosgen was bound, and that therefore the same \( K_i \) should result at both high and low concentrations of glycosgen. In fact, the data show that UDP-glucose can combine with either free enzyme or enzyme bound to glycosgen, and one would therefore predict that the same would be true for P_i and glucose-1-P. These results have led us to make a tentative assignment of the Type I mechanism to liver phosphorylase, and subsequent discussion is based on the assumption that this assignment will eventually be proved substantially correct. Such proof will involve independent measurement of the various dissociation constants suggested in this paper, or demonstrations that P_i and glucose-1-P can bind to the free enzyme, or both. It is interesting that Lowry et al. (7) fitted their kinetic data on phosphorylase a into the framework of Mechanism I. It has been noted that increasing the concentration of either P_i or glucose-1-P results in a pronounced decrease in the dissociation constant for glycosgen, with the apparent \( K_m \) values forming a progression from the \( K_1 \) and \( K_2 \) for binding to the free enzyme, to \( K_3 \) and \( K_4 \) for binding to the enzyme saturated with the other substrate. A similar effect of the P_i concentration on the apparent \( K_m \) of glycosgen has been reported before for phosphorylase a of muscle (7), but this phenomenon was not observed with phosphorylase b (26). It is noteworthy that the glycosgen binds more tightly to the enzyme saturated with P_i than it does to the enzyme saturated with glucose-1-P (\( K_i < K_d \)). This would be the expected result if the glycosgen had available to it in the former case an extra binding site (for its terminal glucose moiety), which in the latter case is occupied by the glucose moiety of glucose-1-P.} \]

\[ \text{In a manner similar to that discussed above, increasing the glycosgen concentration decreases the apparent \( K_m \) values for either P_i or glucose-1-P, an effect similar to that reported for muscle phosphorylase a (7), but not, in the case of P_n, for phosphorylase b (20). The apparent \( K_m \) values again form a progression between the dissociation constant for the free enzyme and that for the enzyme saturated with glycosgen. The effects of AMP on the apparent \( K_m \) values for the various substrates and vice versa are again similar to those reported for the muscle enzymes, with certain obvious differences with regard to the magnitude of the effects, and also of the effects on velocities of reaction (7, 25, 28).} \]

\[ \text{Certain of the data reported here have a bearing on the nature of the binding sites at the catalytic center of liver phosphorylase. Glucose-1-P is found to have a lower dissociation constant than does P_i for either free enzyme or glycosgen-enzyme complex, suggesting that a specific binding site exists for its glucose moiety; this extra binding thus augments that provided by the binding of the phosphate group to a specific phosphate site. As suggested above, the glycosgen site would be occupied by a terminal glucose moiety of glycosgen during the phosphorolysis reaction. Glycosgen phosphorylases appear to have absolute specificity with regard to hexose-1-P, but some idea of the relative importance of various parts of the glucose moiety with regard to the binding to the glycosgen site may be gained from a comparison of the different compounds tested for their inhibitory properties, as reported in Table V. Thus, modifications of the groups on carbon 2 of glucose are less effective in reducing binding than modifications of the groups on carbon 4 or 6. The rather general, nonspecific inhibition by organic phosphates and pyrophosphate indicates that these compounds act at a specific phosphate-binding site. Liver glycosgen phosphorylase appears to be much more sensitive to inhibition by organic phosphates in general than do the muscle phosphorylases, but the latter do exhibit the phenomenon.} \]

\[ \text{N. B. Madsen and S. Shechosky, unpublished observations.} \]
A slight inhibition of phosphorylase a by glycerophosphate (16) has long been known. We have found that glycerophosphate, pyrophosphate, and UDP compete with P_i in the case of phosphorylase b with \( K_i \) values of 32, 16, and 15 mM, respectively, all of which are higher than those found for liver phosphorylase. On the other hand, such compounds as ATP, ADP, and glucose-6-P, which cause a weak competitive inhibition of the liver phosphorylase, have been shown to exhibit strong inhibitions of phosphorylase b in which the kinetics has been altered to a complex form (25, 26). Thus, while the inhibition by the latter compounds may play a role in the control of the phosphorylase b activity in the muscle, that role may be entirely different in the liver. We have previously suggested, however, that the cumulative effect of weak inhibitions by all the organic phosphates in the liver cell to which the phosphorylase might be exposed may add up to a large, relatively constant lowering of the activity of the enzyme in vivo (1).

The inhibition by UDP-glucose is quite strong in relation to its intracellular concentration (1), and this inhibition increases at low glycogen concentration so that, especially when a fasted animal is refed, this inhibition may constitute a significant control mechanism. This possibility has also been discussed more extensively in our earlier communication (1).

Some comparisons of the kinetics of liver phosphorylase with that of the muscle phosphorylases have been made above where it seemed appropriate. The \( K_m \) for glucose-1-P and either phosphorylase a or b, at saturating levels of glycogen and AMP, has been variously reported to be from 2 to 6 mM (16,27), a range of values which has been confirmed repeatedly in this laboratory (24, 25). Lowry et al. reported considerably lower values for phosphorylase a, however (7). We find that when the \( K_m \) of glucose-1-P and liver phosphorylase is determined under exactly the same conditions as for the muscle enzyme, the values range from 0.2 to 0.4 mM, an order of magnitude less than for the muscle enzymes. The apparent \( K_m \) values for P_i are 2- to 3-fold less than for the muscle enzymes. We feel that these differences are real, and, as noted above, they are also reflected in a presumably related phenomenon, the greater sensitivity of the liver enzyme to inhibition by organic phosphates, as evidenced by the lower \( K_i \) values.

The apparent \( K_m \) values for glycogen and the liver phosphorylase are of the same order of magnitude reported for the muscle enzymes when measured under similar conditions (16, 17, 26, 27), although Lowry et al. (7) found much lower values for phosphorylase a. The dissociation constant for glycogen and free enzyme, which we have found to be 13 to 15 mM for liver phosphorylase, was previously determined by direct physical methods to be 12 to 15 mM for phosphorylase a (17). Both enzyme systems need further investigation to prove that this agreement is not fortuitous.

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Kinetics of Purified Liver Phosphorylase
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