Liver Microsomal Glucose 6-Phosphatase, Inorganic Pyrophosphatase, and Pyrophosphate-Glucose Phosphotransferase

II. KINETIC STUDIES*

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Recently, we (1) described experiments which strongly supported the common identity of glucose 6-phosphatase, inorganic pyrophosphatase, pyrophosphate-glucose phosphotransferase, and mannose 6-phosphate-glucose phosphotransferase activities present in a preparation obtained by ammonium sulfate fractionation of deoxycholate-dispersed rat liver microsomes. Previously, Segal, Washko, and Lee (2, 3) and Hass and Byrne (4) concluded, from studies of the glucose-14C-glucose 6-phosphate exchange reaction and apparent glucose inhibition of sugar phosphate hydrolysis catalyzed by microsomal glucose 6-phosphatase, that the reaction mechanism involved formation of a binary enzyme-glucose 6-phosphate complex, followed by dissociation of glucose from the enzyme, leaving a phosphoryl enzyme intermediate which then could transfer the phosphoryl group either to water (hydrolysis) or to glucose-14C (exchange). Their data differed, however, in that Segal et al. found that glucose appeared to be a noncompetitive inhibitor of the hydrolysis reaction while Hass and Byrne observed an apparent inhibition which was not of the "classical" type.

The newly found phosphotransferase reactions afforded an independent, analytically highly sensitive means of supplementing previous kinetic studies (2-4) of glucose 6-phosphatase. Experiments indicating that the previously postulated glucose 6-phosphatase mechanism also applies to the phosphotransferase reactions, and confirming Segal's (2) experimental observations, are described in this paper.

EXPERIMENTAL PROCEDURE

Sources of materials, preparation of rat liver microsomes, analytical procedures, and methods for measuring enzymic activities have been described in an earlier paper (1). The enzyme preparation used in these studies was obtained by fractional ammonium sulfate precipitation of deoxycholate (0.2%, w/v)-dispersed rat liver microsomes, as outlined previously (1). The preparation, precipitating between 40 and 50% ammonium sulfate saturation (calculated for 25°), had a specific activity of 0.35 unit2 per mg of protein. Phosphomannose isomerase and phosphogluco isomerase activities could not be detected in the preparation under the phosphotransferase assay conditions. Aliquots (0.083 unit) of enzyme were included routinely in 1.5-ml reaction mixtures, and incubations were carried out for 10 minutes at 30°, with shaking. Reaction mixtures contained 60 amoles of sodium citrate buffer and substrates as indicated in legends to figures; the pH was 6.0 in all instances. Supplementary experiments indicated that rates for all reactions studied were linear with time for at least 15 minutes under these conditions. Experiments in which 0 to 200 amoles of NaCl were added per 1.5 ml of reaction mixture indicated that variations in ionic strength in the range over which phosphoryl donor compounds were varied in the kinetics experiments were without effect on the activities. Kinetic data are expressed as conventional (5) double reciprocal plots. The accuracy of data obtained in various experiments was cross-checked by simultaneous assay of selected hydrolysis and transphosphate reaction mixtures in an individual experiment. All rate equations describing the reactions discussed below were derived by using the schematic method of King and Altman (6). In every instance, except when glucose-6-P hydrolysis in the presence of added glucose was considered (Equation 7; for all equations discussed in the text, see the "Appendix"), terms containing product concentration were disregarded.

RESULTS

In Figs. 1 through 4 are presented the results of kinetic studies of the PP1-glucose (Equation 1) and mannose-6-P-glucose (Equation 2) exchange reactions. One unit of enzymic activity is that amount catalyzing the hydrolysis of 1 amole of glucose 6-phosphate per 1.5 ml of reaction mixture per minute under previously defined conditions (1).

2 Reference reaction mixtures of constant composition were assayed concurrently with each experiment. It was found that the enzyme preparation, stored in small aliquots and thawed at 0° just prior to assay, showed no variation of activity over a period of 2 months.
tion 2) phosphotransferase reactions. Initial reaction velocities were measured in several series of reaction mixtures in which glucose concentrations were held at various constant levels and phosphoryl donor concentrations varied over the indicated ranges (Figs. 1 and 3), and for other series of reaction mixtures in which phosphoryl donor concentrations were maintained at several constant concentrations and glucose levels varied (Figs. 2 and 4). Secondary plots of y axis intercepts obtained from Figs. 1 through 4 against reciprocals of concentrations of substrate held at various constant levels are presented in Fig. 5. Maximal reaction velocities for both the PP\textsubscript{i}-glucose and mannose-6-P-glucose phosphotransferase reactions were equal (note the common intercept on the y axis in Fig. 5). Michaelis constants evaluated as negative reciprocals of x axis intercepts on the secondary plots are recorded in the legend to Fig. 5. Michaelis constants also may be calculated directly from data in primary plots (Figs. 1 through 4), since all lines in each experiment converge at a common point on the x axis. These values, determined as the negative reciprocals of x axis intercepts, were as follows: for the PP\textsubscript{i}-glucose phosphotransferase reaction (from Figs. 1 and 2, respectively),

$$K_{PPi} = 4.44 \times 10^{-3} \text{M}$$

and

$$K_{Glc6P} = 8.00 \times 10^{-2} \text{M}$$

for the mannose-6-P-glucose phosphotransferase reaction (from Figs. 3 and 4, respectively),

$$K_{mannose-6-P} = 2.61 \times 10^{-2} \text{M}$$

and

$$K_{Glc6P} = 8.33 \times 10^{-2} \text{M}.$$ 

The consistency of Michaelis constants calculated from the data in Figs. 1 through 4 and in Fig. 5 attest to the accuracy of the kinetic results.

Fig. 6 depicts a reaction mechanism which was found to be consistent with these kinetic findings, and includes as Reactions 4, 3, and 5 the mechanism previously formulated by Segal (2) and by Hass and Byrne (4) to describe glucose-6-P hydrolysis and glucose-6-P-glucose-14C phosphotransferase reactions. This scheme is written in terms of the PP\textsubscript{i}-glucose phosphotransferase reaction to facilitate discussion, but, with minor modifications, it also describes other phosphoryl donor-sugar phosphotransferase reactions. For example, a scheme depicting the mannose-6-P-glucose phosphotransferase reaction is obtained if the PP\textsubscript{i} term is replaced by mannose-6-P and E-PP\textsubscript{i} is replaced by E-mannose-6-P.

4 The abbreviations Glc, d-glucose; Glc-6-P, glucose 6-phosphate; trf, transferase; Glc-6-Pase, glucose 6-phosphatase; and PP\textsubscript{i}ase, inorganic pyrophosphatase, are used as subscripts in conjunction with Michaelis constants (K) and maximal reaction velocities (V\textsubscript{max}) only.
6-P in Fig. 6. While resynthesis of mannose-6-P (or glucose-6-P or other sugar 6-phosphate) would proceed readily from E-P1 plus sugar (that is, the reaction E-P1 + sugar → sugar-P + E is freely reversible), net PP1 synthesis from E-P1 + P1 would be negligible because of the "high energy" nature of the phosphohydrate compound. Hence, that reaction to which the rate constant \( k_s \) applies has been indicated by a dashed line in the scheme in Fig. 6 written specifically in terms of PPi-involving reactions. Rate Equation 3, pertaining to the PPi-glucose phosphotransferase reaction, was obtained by steady state consideration of the mechanism indicated in Fig. 6. A rate equation identical in form with Equation 3 can be derived to describe the mannose-6-P-glucose phosphotransferase reaction when the substitutions indicated above are made. This equation (not shown) and Equation 3 are in agreement with the observed increase both in the slope and in \( y \) axis intercepts accompanying decreases in concentrations of substrate held at various constant levels (for example, glucose in Figs. 1 and 3).

While Fig. 6 in its entirety describes the PP1-glucose phosphotransferase reaction, the left half of this diagram (i.e. Reactions 1, 2, 3, and 4) describes glucose 6-phosphatase (including glucose-6-P glucose \(^{14}C\) exchange) and the right half of the diagram (Reactions 1, 2, and 5) depicts the inorganic pyrophosphatase reaction. Rate Equations 4 and 5 were obtained for those reactions in Fig. 6 specifically describing, respectively, glucose-6-P hydrolysis (Equation 4) and PPi hydrolysis (Equation 5) in the absence of added glucose or other phosphoryl acceptor. In Fig. 7 are presented Lineweaver-Burk (5) plots of data obtained from studies of the rates of hydrolysis of glucose-6-P in the absence and presence of several concentrations of glucose. Similar plots of the results of studies of the rates of hydrolysis of PPi and mannose-6-P in the absence and presence of 6.00 \( \times 10^{-3} \) M glucose are presented in Fig. 8. Maximal reaction velocities evaluated from the \( y \) axis intercepts of these lines in Figs. 7 and 8 describing the hydrolysis of the various phosphate compounds in the absence of glucose (the values are recorded in the legends to Figs. 7 and 8) were found to be equal and, furthermore, were equal to the maximal reaction velocities for the phosphotransferase reactions extrapolated to infinite substrate concentrations (values are recorded in the legend to Fig. 5). Further, the Michaelis constant for PPi hydrolysis (4.44 \( \times 10^{-3} \) M), evaluated as the negative reciprocal of the \( x \) axis intercept of the plot of data obtained in the absence of glucose, depicted in Fig. 8, was identical with \( K_{PPi} \) (also 4.44 \( \times 10^{-3} \) M) calculated for the PPi-glucose phosphotransferase reaction (see Figs. 1 and 5). By equating the expressions for \( V_{max} \) for the phosphotransferase reaction (Equation 3d) with \( V_{max} \) for the PPi hydrolysis reaction in absence of glucose (Equation 5b), it may be shown that \( k_a = k_b \). This same relationship also follows from the experimental observation that \( K_{PPi+Trf} \) equals \( K_{PPi} \) determined for the hydrolysis reaction in the absence of glucose (see Equations 3a and 5a). By substitution of \( k_a \) for \( k_b \) in Equations 3b and 3c, it may be shown that \( K_{PPi+Trf} \times K_{PPi} = K_{PPi+GlUE} \), which is consistent with experimentally observed convergence of plots in Figs. 1 through 4 at the \( x \) axis. When \( k_2 \) is substituted for \( k_i \) in Equation 4a, it may be shown that \( K_{EGL+P} = k_i/k_1 \); that is, that the Michaelis constant for the glucose-6-P hydrolysis reaction actually is the dissociation constant for the enzyme-glucose-6-P complex. Furthermore, it also may be shown that when maximal reaction velocities for uninhibited inorganic pyrophosphatase (Equation 5b) and glucose 6-phosphatase (Equation 4b) reactions are equal, as experimentally observed (Figs. 7 and 8), \( k_a = k_b \).

A combination of all the reactions depicted in the scheme shown in Fig. 6 describes PPi hydrolysis in the presence of added glucose. Rate Equation 6 describing this reaction was derived by the King-Altman (6) method after substitution of \( k_i \) for \( k_1 \). All rate equations presented for reactions involving the participation of PPi, also are applicable for corresponding reactions described when the PPi and E-PPi terms in Fig. 6 are replaced, respectively, by mannose-6-P and E-mannose-6-P, since experimental data obtained in the various studies with either PPi or mannose-6-P as phosphoryl donor were similar in that (a) primary plots described in Figs. 1 through 4 all converged, in each experiment, at a common point on the \( x \) axis, (b) Michaelis constants determined for phosphoryl donors in the phosphotransferase
and $k_b$ for $k_a$. This equation is identical in form with the rate equation for classical noncompetitive inhibition (7). The results of experimental studies of PPi (and mannose 6-P) hydrolysis in the absence and presence of added glucose (Fig. 8) are in agreement with this equation.

Rate Equation 7 was derived by a consideration of those reactions in Fig. 6 (Reactions 4, 3, and 5) which describe glucose-6-P hydrolysis in the presence of added glucose. This equation may be rearranged to a form identical with that derived by Hass and Byrne (4) to describe "nonclassical" type of inhibition of glucose 6-phosphatase by glucose. Hass and Byrne (4) have pointed out that this equation would predict apparent noncompetitive inhibition only under those special conditions in which $k_b/k_1$ was equal to $K_{Glc-6-P}$. However, they calculated on the basis of their experimental data that $k_2/k_1 = 8.3 \times 10^{-4}$ M while $K_{Glc-6-P} = 6.1 \times 10^{-5}$ M, and pointed out that this inequality was consistent with the disagreement between their experimental data and the values that would have been expected for pure noncompetitive inhibition. In the present studies, however, data have been obtained which substantiate the equality $K_{Glc-6-P} = k_2/k_1$ (see the discussion above). When this relationship is substituted into Equation 7 and terms are rearranged, Equation 8 is obtained. This equation predicts that added glucose will appear to function as a noncompetitive inhibitor of glucose-6-P hydrolysis; experimental results depicted in Fig. 7 confirm this prediction.

The Michaelis constant for glucose in the phosphotransferase reactions (see Figs. 1 through 4 and Fig. 5) was found to equal the $K_I$ obtained by calculations (7) with added glucose considered as a noncompetitive inhibitor of glucose-6-P hydrolysis (Fig. 7), or of PPi, or mannose-6-P hydrolysis (Fig. 8). These experimental observations are consistent with theory in that when $k_2 = k_3$ and $k_2 = k_3$, the expression for $K_I$ in the transferase reaction (Equation 3b) and for $K_I$ (where $I$ is glucose) in the hydrolysis reaction rate equation (Equations 8a and 7a) become identical. From the observation that $K_{Glc-6-P} = k_2/k_1$, it follows that $k_2 = k_1 > k_3$ (Segal (5) previously has shown that if the assumption of the inequalities $k_3 < k_2$ and $k_2 < k_3$ is made, his rate equation derived on the basis of a proposed reaction mechanism for glucose 6-phosphatase (identical with Reactions 4, 3, and 5 in Fig. 6) assumes a form which may be shown to be identical with Equation 8.

**DISCUSSION**

Excellent agreement was found between experimental data for the various hydrolysis and phosphotransferase reactions studied and theoretical relationships predicted by steady state rate equations derived on the basis of the mechanism depicted in Fig. 6. The present results are consistent with those obtained in Segal's (2, 3) kinetic studies of glucose 6-phosphatase in that (a) we both observed apparent noncompetitive inhibition of glucose-6-P hydrolysis by added glucose, (b) this apparent inhibition was found to be due to a competition between added glucose (or glucose-14C (2)) and water for the phosphoryl group originating in glucose-6-P, and (c) the data of both groups of workers were consistent with a reaction mechanism involving a phosphoryl-enzyme intermediate. Furthermore, these same observations have been made, and identical conclusions reached, when PPi, or mannose-6-P was used in place of glucose-6-P as phosphoryl donor in the system. These findings are taken as additional evidence consistent with our original contention that the glucose 6-phosphatase, inorganic pyrophosphatase, and PPi-glucose phosphotransferase reactions are catalyzed by one, common enzyme.

The mechanism depicted in Fig. 6 is not, of course, the only reaction scheme which could be formulated for this system. However, Segal (2) and Hass and Byrne (4) previously have discussed kinetic reasons why such other mechanisms as one not involving kinetically significant binary enzyme-phosphoryl donor complexes, and a mechanism involving two distinct glucose-binding sites on the enzyme, are not applicable to the glucose 6-phosphatase system. Their arguments apply also to the present, expanded system and will not be repeated here.

It is interesting to note also that the general rate equation (Equation 3) describing phosphotransferase activity by an enzyme also exhibiting hydrolytic activity is applicable to other
transphosphorylase whose mechanisms involve phosphoryl-
enzyme intermediates, but which catalyze no significant hy-
drosis reactions. In such systems, where $k_1$ is taken as zero, 
Reaction 5 drops from the scheme depicted in Fig. 6, and the 
rate equation then takes the form indicated in Equation 9. 
This equation predicts that the lines in plots analogous to those 
in Fig. 1 through 4 would, under these circumstances, be parallel 
as was found to be the situation, for example, with brain hexo-
kinase (8).

While the physiological significance of phosphotransferase 
activity of glucose 6-phosphatase remains obscure, a number of 
possibilities exist. The Michaelis constant for glucose, 0.08 m, 
is equivalent to 1440 mg per ml, which is quite high compared 
with normal blood sugar levels. However, other studies of 
glucose metabolism in liver cells give some indication that this 
value is not so unreasonably high as to rule out a significant 
physiological role for the phosphotransferase on this basis. For 
example, a $K_m$ of 0.009 m for glucose utilization for glyco-
synthesis and for CO$_2$ formation has been demonstrated with 
liver slices (9); liver cells have been shown to be freely perme-
able to glucose (10). Liver glucokinase (11–13) recently has been 
suggested to play an important role in glucose metabolism under 
certain conditions. This has a Michaelis constant for 
glucose of between 0.01 and 0.04 m (11). Maximal reaction 
velocity was obtained with approximately 0.1 m glucose with the 
glucokinase (11), while we observed an initial reaction velocity 
that was approximately 60% of $V_{max}$ for the phosphotransferase 
with this concentration of glucose and saturating levels of PP$_i$. 
Significant, also, is the fact that $V_{max}$ for the phosphotransferase 
equals the $V_{max}$ for glucose-6-1' hydrolysis, an activity which has 
been assigned a significant role in regulation of mammalian car-
bohydrate metabolism. Morton (14) has suggested that, in vivo, 
the hydrolytic function of lysosomal acid phosphatases may be 
restricted and transferase reactions also catalyzed by these 
enzymes may be favored, since these phosphatases are associated 
with lipoprotein structures. A similar speculation may be made 
regarding glucose 6-phosphatase, which also is intimately as-
associated with lipoproteins (15).

Since a number of sugars (and a polyol) have been demon-
strated to serve as phosphoryl acceptors in the phosphotrans-
ferase system, it is possible that under appropriate conditions 
the interconversion of various sugar phosphates or polyol phos-
phates by transphosphorylation may be important.

Finally, the depression of net glucose-6-P hydrolysis by elev-
gated glucose levels (4), or by PP$_i$, which acts as a competitive 
inhibitor of glucose-6-P hydrolysis (1), also might be of impor-
tance in the regulation of carbohydrate metabolism.

**SUMMARY**

Kinetic studies of the inorganic pyrophosphate-glucose and 
mannose 6-phosphate-glucose phosphotransferase reactions 
catalyzed by a liver microsomal glucose 6-phosphatase prepara-
tion (1) have been carried out. Glucose inhibition of pyrophos-
phatase, mannose 6-phosphate, and glucose 6-phosphate hydrolysis 
also was studied. The results were in agreement with a mecha-
nism, previously proposed for glucose 6-phosphatase by Segal 
(2) and by Hass and Byrne (4), which involves (a) formation of 
a binary enzyme-phosphoryl donor complex, (b) a dissociation 
leaving a phosphoryl enzyme intermediate, and (c) transfer of 
phosphoryl group from enzyme either to water (hydrolysis) or to 
glucose or other hexose (phosphotransferase). These results are 
in complete agreement with our earlier conclusions (1) that 
intrigousyrophosphatase, inorganic pyrophosphate-glucose 
phosphotransferase, and other similar reactions are catalyzed by 
liver glucose 6-phosphatase.

**APPENDIX**

$$PP_i + glucose \rightarrow glucose-6-P + P_i$$  
$$Mannose-6-P' + glucose \rightarrow glucose-6-P + mannose$$

$$V_{max(Trf)} = 1 + \frac{K_{PP_i}}{PP_i} + \frac{K_{Glc}}{Glc} + \frac{K_{PP_i+Glc}}{PP_i+Glc}$$

$$E_0 = \text{total enzyme concentration, and the subscript Trf indicates the PP}_{i}\text{-glucose phosphotransferase reaction.}$$

$$V_{max(Glc-6-Pase)} = \frac{1 + K_{Glucose-P}}{Glc-6-P}$$

$$V_{max(Piase)} = \frac{1 + K_{PP_i}}{PP_i}$$

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Since concentration of water $> \text{substrate concentrations}$ employed, it may be regarded as a constant. To simplify notation, $k_5$ is used throughout this paper in place of $k_5' (H_2O)$. 

7 Since concentration of water $> \text{substrate concentrations}$ employed, it may be regarded as a constant. To simplify notation, $k_5$ is used throughout this paper in place of $k_5' (H_2O)$. 

8 Apparent $V_{max}$ for the PP$_i$-sugar phosphotransferase 
reaction, determined in the presence of 0.01 m PP$_i$ with varied concentrations of s-glucose, 2-deoxy-s-glucose, s-glucoamine, s-erythritol, or s-galactose, were found to be identical (R. C. Nordlie, unpublished observations). Phosphoryl transfer from glucose-6-P to 3-deoxy-s-glucose (M. Nemer and J. Ashmore, unpublished observations cited by Ashmore and Weber (16)) also has been observed.
where \( I = \) glucose, \( K_{PP_i} \) is as defined in Equation 5a, 
\( V_{\text{max}(PP_i)ase} \) is as defined in Equation 5b, and

\[
K_f = \frac{k_5 + k_6}{k_4} \tag{6a}
\]

\[
\frac{V_{\text{max}(Glc-6-Pase)}}{v} = \frac{K_{Glc-6-P}}{(Glc-6-P)} + \frac{k_1(I)}{K_f} + \frac{1}{K_f} \tag{7}
\]

where \( I = \) glucose, \( K_{Glc-6-P} \) is as defined in Equation 4a, 
\( V_{\text{max}(Glc-6-Pase)} \) is as defined in Equation 4b, and

\[
K_I = \frac{k_4 + k_5}{k_4} \tag{7a}
\]

\[
\frac{V_{\text{max}(Glc-6-Pase)}}{v} = \frac{K_{Glc-6-P}}{(Glc-6-P)} \left( 1 + \frac{K_f}{I} \right) + \frac{1}{K_f} \tag{8}
\]

\[
V_{\text{max}(Trf)} = 1 + \frac{K_{PP_i}}{K_{Glc}} \tag{9}
\]

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REFERENCES
