Galactose-1-phosphate Uridylyltransferase: Rate Studies Confirming a Uridylyl-Enzyme Intermediate on the Catalytic Pathway†

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ABSTRACT: Steady-state initial rate measurements at pH 8.5 and 27° show that the galactose-1-P uridylyltransferase catalyzed interconversion of UDP-glucose and galactose-1-P with UDP-galactose and glucose-1-P follows ping pong bi bi kinetics. The following lines of supporting evidence are presented. Double reciprocal plots of initial rate data in both directions are families of parallel lines. The product inhibition patterns are in all cases those characteristic of the ping pong bi bi pathway. The reaction is subject to competitive substrate inhibition by all substrates at concentrations well above their K_m values. The exchange of ^{14}C from UDP-[U-^{14}C]glucose to glucose-1-P in the absence of cosubstrates follows the ping pong pathway with a maximum rate that is somewhat larger than the maximum overall reaction rate. The equilibrium constant calculated from the ping pong kinetic parameters is 2.1 in the direction of glucose-1-P formation, which is in good agreement with the value 2.1 obtained by direct measurement at pH 8.5 and 27°. The finding of ping pong kinetics confirms the kinetic competence of the uridylyl-enzyme intermediate recently isolated and of the proposed double displacement pathway. Using our measured equilibrium constant for this reaction and the published value for the UDP-galactose-4-epimerase reaction, we calculate that for the interconversion of galactose-1-P and glucose-1-P at pH 8.5 and 27° the equilibrium constant is 7.4 favoring glucose-1-P.

Galactose-1-P uridylyltransferase (EC 2.7.7.12) catalyzes the interconversion of UDP-glucose and galactose-1-P with UDP-galactose and glucose-1-P. It is a key enzyme in the Leloir pathway and is known to be defective or absent in galactosemia (Kalckar, 1960). It is also unique among nucleotidyltransferases utilizing phosphates as acceptors, in that it is the only one for which nucleoside di- or triphosphates are not nucleotidyl-donor substrates.

At the time they discovered and characterized UDP-glucose in connection with their studies on the conversion of galactose-1-P to glucose-6-P, Cardini *et al.* (1950) noted the possibility that galactose-1-P might be exchanged with the glucose-1-P moiety of the coenzyme. Subsequently, Kalckar *et al.* (1953) discovered galactose-1-P uridylyltransferase in yeast, and it was later detected in animals, bacteria, and plants (Maxwell *et al.*, 1955; Kurahashi, 1957; Pazur and Shadaksharaswamy, 1961). It has been purified to near homogeneity from *Escherichia coli* by Saito *et al.* (1967), who reported a dimeric structure and a dimer molecular weight of 8×10^4.

Of the Leloir pathway enzymes this is the only one whose mechanism of action has not been investigated. Nevertheless it is an interesting subject. Inasmuch as the uridylyl-donor substrates are nearly isosteric, differing only in configuration at glycosyl-C-4, and the reaction involves reversible cleavage of a pyrophosphoryl bond, the enzyme might be expected to have evolved in such a way as to follow a catalytic pathway which reflects the potential influences of these binding and chemical factors, that is, a double displacement pathway involving a covalent uridylyl-enzyme intermediate. In this paper we show that the kinetic pathway followed in this reaction is ping pong bi bi, which confirms the kinetic competence of the uridylyl-enzyme intermediate recently isolated (Wong and Frey, 1974), and we also discuss the possible significance of this in light of the fact that the chemically analogous UDP-glucose pyrophosphorylase reaction follows a different pathway.

Experimental Section

Enzymes, Coenzymes, and Substrates. Galactose-1-P uridylyltransferase was purified from a regulatory mutant of *E. coli*, ATCC-27797, by a modification of the published procedure (Saito *et al.*, 1967). Our preparations appeared to be slightly less active than that of Saito *et al.* (1967). Analytical disc gel electrophoresis showed a major band and a minor contaminant. The specific activities, when corrected to the substrate concentrations employed by Saito *et al.* (1967) in their assay methods, were about 170 units/mg of protein as compared with 209 reported by those workers. We also found the enzyme to be somewhat unstable, as reported (Saito *et al.*, 1967), so that the activities decreased gradually during these experiments. The enzyme did not contain detectable UDP-galactose-4-epimerase activity or other contaminants which would interfere with steady-state rate studies. It was judged to be acceptable for the present work.

UDP-glucose dehydrogenase, used as a coupling enzyme in initial rate measurements, was purified from beef liver through step 6 as described by Zalitis and Feingold (1969). UDP-galactose-4-epimerase was purified by the method of Wilson and Hogness (1964). The other coupling enzymes were purchased from commercial suppliers, as were TPN^+, DPN^+, glucose 1,6-diphosphate, galactose-1-P, glucose-1-P, UDP-galactose, UDP-glucose, UDP-[U-^{14}C]glucose, and UDP-[U-^{14}C]galactose. The ^{14}C nucleotide sugars were purified by paper chromatography with 5:2 95% ethanol–1 M ammonium acetate at pH 3.5.

Standard Activity Assay. Because of the above mentioned complications with stability, we related all rate measurements to a standard unit of activity. We arbitrarily defined the activi-

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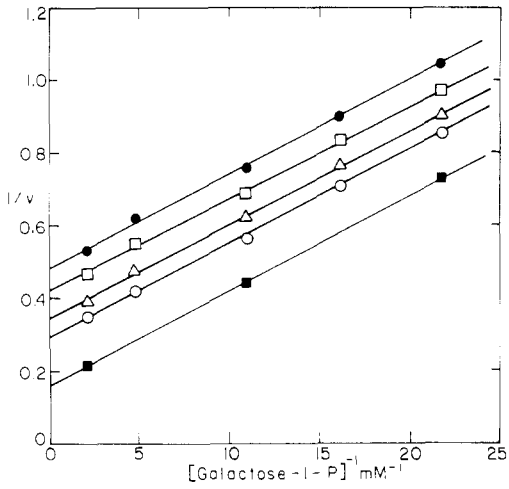

FIGURE 1: Double reciprocal plots of initial velocities of glucose-1-P formation at varying [galactose-1-P] and several fixed [UPD-glucose]. The rates were measured by method A. The UDP-glucose concentrations were: (●) 0.042 mM; (□) 0.051 mM; (△) 0.062 mM; (○) 0.082 mM; (■) 0.2 mM. The velocity units are mμmol of glucose-1-P per min produced by 1.8 milliunits of enzyme.

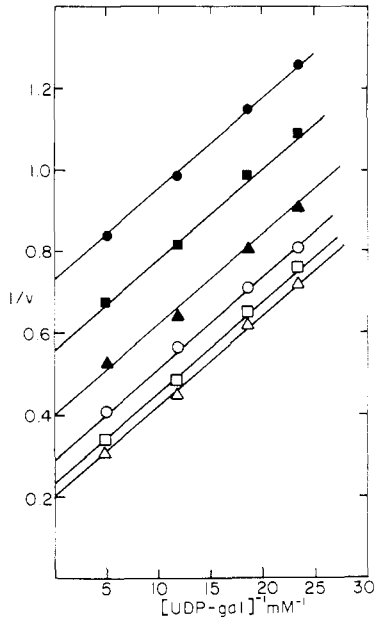

FIGURE 2: Double reciprocal plots of initial velocities of UDP-glucose formation at varying [UDP-galactose] and several fixed [glucose-1-P]. The rates were measured by method B. The glucose-1-P concentrations were: (●) 0.5 mM; (■) 0.073 mM; (▲) 0.125 mM; (○) 0.24 mM; (□) 0.49 mM; (△) 1.1 mM. The velocity units are mμmol of UDP-glucose per min produced by 2.4 milliunits of enzyme.

ty unit to be the production of 1 μmol of glucose-1-P per minute under standard assay conditions, which were: 0.20 mM galactose-1-P, 0.051 mM UDP-glucose, 8 mM cysteine, 0.326 mM TPN^+, 3.3 μM glucose-1,6-P_2, 95 mM sodium bicinate at pH 8.5, and excess phosphoglucomutase and glucose-6-P dehydrogenase as well as limiting galactose-1-P uridylyltransferase at 27°. These conditions differed from those of Saito *et al.* (1967), in that the substrate concentrations were lower and the assay was coupled instead of two step. The lower substrate concentrations were chosen for the standard assay in order to minimize substrate inhibition effects found in this work.

Initial Rate Measurements. Three methods were used for measuring initial rates. Method A was the same as the standard assay except that the substrate concentrations were varied as indicated in the figures. Method B measured the rate in the reverse direction under similar conditions, which were: 8 mM cysteine, 0.382 mM DPN^+, 95 mM sodium bicinate at pH 8.5, and excess UDP-glucose dehydrogenase at 27° as well as UDP-galactose and glucose-1-P at the concentrations indicated in the figures. In the studies on substrate inhibition by glucose-1-P the DPN^+ concentration was 0.624 mM. Product inhibition by glucose-1-P and UDP-glucose could not be evaluated by either of the above methods, so method C, a radiochemical method, was used for this purpose and for the exchange kinetics. The reaction conditions were 95 mM sodium bicinate at pH 8.5, 8 mM cysteine, and UDP-[^{14}C]sugar and sugar-1-P substrates at the indicated concentrations at 27°. The amount of enzyme used was adjusted to give up to 10% reaction in 5 min. After 5 min the reactions were stopped by adding 30 or 40 mg of charcoal, the mixtures were filtered through cotton plugs inserted into the tips of Pasteur pipets, and aliquots of the filtrates were assayed radiochemically by liquid scintillation counting. Initial rates were calculated from radiochemical data and the known specific radioactivities of the UDP-sugars.

All rates were measured in replicate, and the levels of enzyme used in any given series of measurements were varied in such a way as to give about the same experimental rate at all substrate concentrations. The rate data were then normalized to the same amount of enzyme and analyzed without weighting; the standard deviations from the mean in replicate rates were about the same at all substrate concentrations for any given set of data. The data for each saturation curve were computer fitted to the reciprocal form of the Michaelis–Menten equation by the linear least-squares method. Slope and intercept data in replots were good straight lines and were fitted in the same way.

Substrate Assays. The concentrations of all substrates were measured enzymatically; glucose-1-P as TPNH in the presence of phosphoglucomutase, TPN^+, and glucose-6-P dehydrogenase; galactose-1-P as glucose-1-P in the presence of excess UDP-glucose and galactose-1-P uridylyltransferase; UDP-glucose as DPNH in the presence of UDP-glucose dehydrogenase and DPN^+, and UDP-galactose as UDP-glucose in the pres-

TABLE I: Kinetic Parameters for Galactose-1-P Uridylyltransferase.

Parameters	Substrates			
	UDP-glucose (mM)	Galactose-1-P (mM)	UDP-galactose (mM)	Glucose-1-P (mM)
K_m	0.20 ± 0.016	0.303 ± 0.033	0.121 ± 0.037	0.157 ± 0.007
K_i	0.158 ± 0.009	0.434 ± 0.016	0.396 ± 0.016	0.337 ± 0.019
K_I	2.28 ± 0.01	5.46 ± 0.35		21.4 ± 0.3
V	V_f = 6.5 μmol min^{-1} unit^{-1}		V_r = 2.36 μmol min^{-1} unit^{-1}	

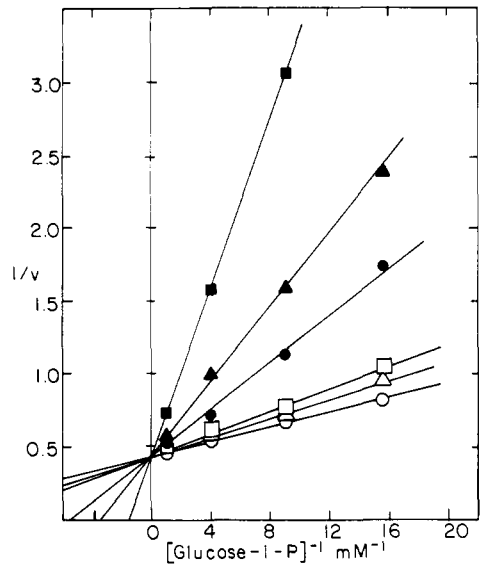

FIGURE 3: Competitive product inhibition by galactose-1-P with respect to glucose-1-P as the variable substrate. The rates were measured by method B at the indicated [glucose-1-P] and 0.09 mM UDP-galactose. The galactose-1-P concentrations were: (■) 0.94 mM; (▲) 0.36 mM; (●) 0.19 mM; (□) 0.044 mM; (△) 0.023 mM; (○) 0 mM. The velocity units are the same as in Figure 2.

ence of UDP-galactose-4-epimerase.

The equilibrium constant for the reaction was evaluated by measuring each of the reactant concentrations by the above methods in reaction mixtures first permitted to reach equilibrium enzymatically at pH 8.5 in 0.1 M sodium bicinate buffer and then heated to destroy enzyme activity.

Results

Steady-State Kinetics for Galactose-1-P Uridylyltransferase. Representative initial rate data are presented in Figures 1-6. The data shown are not necessarily the best sets obtained. They represent approximately half the data and are chosen to illustrate clearly both the kinetic pattern followed and the quality of data obtained in each of the three assay methods. They show that the kinetic pattern is ping pong bi bi with competitive substrate inhibition.

In each figure the *points* represent mean experimental rates from quadruplicate or triplicate determinations. The *lines* are calculated from the evaluated parameters and the designated equation to which the data are fitted. The slope and intercept replots, by which the data were fitted and parameters evaluated, are not shown, but in each case these were good straight line plots.

Figures 1 and 2 show that double reciprocal plots of initial rate data are families of parallel lines, both in the direction of glucose-1-P formation and in the direction of UDP-glucose formation. The data are fitted to eq 1, in which A and B refer to

$$\frac{1}{v} = \frac{1}{V} + \frac{K_a}{V[A]} + \frac{K_b}{V[B]} \qquad (1)$$

UDP-glucose and galactose-1-P in Figure 1 and UDP-galactose and glucose-1-P in Figure 2. The K_m values and maximum velocities are given in Table I. Figures 1 and 2 are consistent with the ping pong bi bi kinetic pattern (Alberty, 1956) involving a modified form of the enzyme as an intermediate. On chemical grounds the modified enzyme is expected to be a uridylyl-enzyme as indicated in eq 2 and 3, the proposed basic pathway.

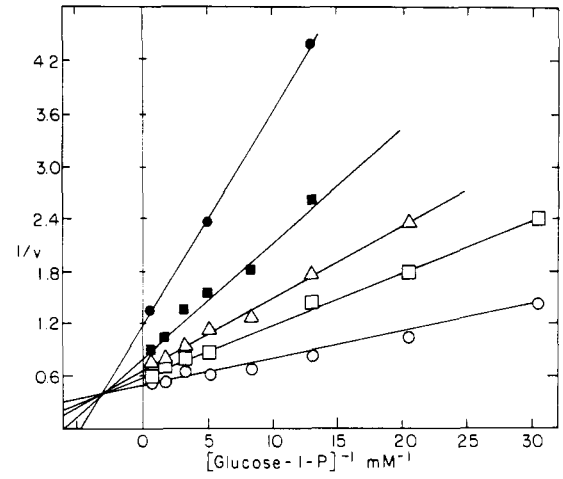

FIGURE 4: Noncompetitive product inhibition by UDP-glucose with respect to glucose-1-P as the variable substrate. The rates were measured by method C at the indicated [glucose-1-P] and 0.0714 mM UDP-[U-^{14}C]galactose. The specific radioactivity content of UDP-[U-^{14}C]galactose was 4.15×10^5 cpm per μmole. The UDP-glucose concentrations were: (●) 0.35 mM; (■) 0.16 mM; (△) 0.084 mM; (□) 0.045 mM; (○) 0 mM. The velocity units are the same as those in Figure 2.

$$\text{UDPGlu} + \text{E} \rightleftharpoons \text{UDPGlu}\cdot\text{E} \rightleftharpoons \text{uridylyl–E}\cdot\text{Glu-1-P} \rightleftharpoons \text{uridylyl–E} + \text{Glu-1-P} \qquad (2)$$

$$\text{uridylyl–E} + \text{Gal-1-P} \rightleftharpoons \text{uridylyl–E}\cdot\text{Gal-1-P} \rightleftharpoons \text{UDPGal}\cdot\text{E} \rightleftharpoons \text{UDPGal} + \text{E} \qquad (3)$$

Apparent parallel lines in Figures 1 and 2 cannot by themselves establish this pathway; however, several other lines of evidence are consistent with it and support the assignment. According to the steady-state rate equation for the ping pong bi bi pathway (Cleland, 1963, 1970) as applied to eq 2 and 3, this pathway is characterized by certain product inhibition patterns. We find the experimental patterns to be completely consistent with eq 2 and 3. In the direction of glucose-1-P formation UDP-galactose inhibits competitively with respect to UDP-glucose and noncompetitively with respect to galactose-1-P. In the reverse direction galactose-1-P inhibits competitively with respect to glucose-1-P and noncompetitively with respect to UDP-galactose and UDP-glucose inhibits noncompetitively with respect to glucose-1-P. A typical competitive product inhibition experiment is depicted in Figure 3, the data from which fit eq 4, where A is UDP-galactose, B is glucose-1-P,

$$\frac{1}{v} = \frac{1}{V}\left(1 + \frac{K_a}{[A]}\right) + \frac{K_b}{V}\left(1 + \frac{[P]}{K_{ip}} + \frac{K_{ia}[P]}{K_{ip}[A]}\right)\frac{1}{[B]} \qquad (4)$$

and P is galactose-1-P. Figure 4 depicts a noncompetitive inhibition pattern in which UDP-glucose is the product inhibitor. The data fit eq 5, where A is UDP-galactose, B is glucose-1-P,

$$\frac{1}{v} = \frac{1}{V}\left(1 + \frac{K_a}{[A]} + \frac{K_a[Q]}{K_{iq}[A]}\right) + \left(\frac{K_b}{V} + \frac{K_aK_{ib}[Q]}{VK_{iq}[A]}\right)\frac{1}{[B]} \qquad (5)$$

and Q is UDP-glucose. Equations 4 and 5 are the steady-state rate equations for initial rate derived by the method of King and Altman (1956))6) assuming eq 2 and 3 and the presence of one or the other product.[1] Figures 3 and 4 and other product

[1] The nomenclature and symbols for kinetic parameters are those of Cleland (1963, 1970).

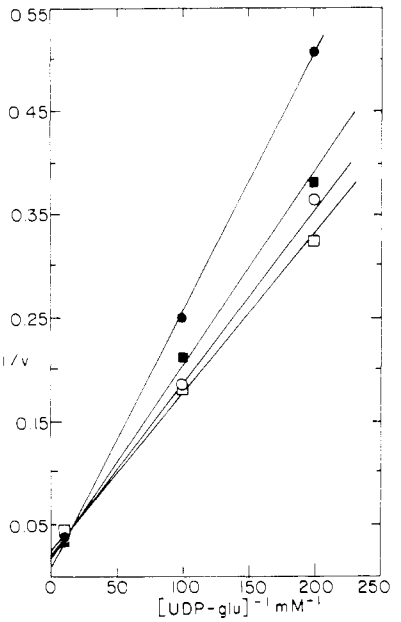

FIGURE 5: Competitive substrate inhibition by galactose-1-P. The rates were measured by method C at galactose-1-P concentrations: (●) 4.03 mM; (■) 1.61 mM; (○) 0.805 mM; (□) 0.403 mM. The velocity units are the same as those in Figure 1.

inhibition data of equal or superior quality are completely consistent with eq 2 and 3.

We collected enough data to evaluate all of the product inhibition constants from appropriate slope and intercept replots. From the least-squares intercepts of Figure 4 plotted *vs.* [UDP-glucose] according to eq 5, we calculated $K_{iUDPGlu}$ from the slope and known K_{UDPGal} and [UDP-galactose]. Then the least-squares slopes were replotted *vs.* [UDP-glucose] according to eq 5. The ratio of the slope of the latter replot to that of the former gave $K_{iGlu\text{-}1\text{-}P}$. The others were obtained by analogous methods.

In Table I are the K_m and K_i values for all substrates as well as the maximum velocities in both directions. The Haldane relationships calculated from them are given in Table II and compared with two experimental values for K_{eq}. The agreement is good, which verifies the quality of the data and supports the assignment of the pathway. Our experimental and calculated K_{eq} values do not agree with the published value of 1.1 (Kurahashi and Sugimura, 1960). The reason for the discrepancy is not clear. It may be due to different conditions or to the possibility that the partially purified enzyme available at that time was contaminated with an interfering enzyme.

Galactose-1-P uridylyltransferase is subject to competitive substrate inhibition at concentrations above K_m values. Such inhibition, found and analyzed in other systems which follow the basic ping pong pathway (Silverstein *et al.*, 1967; Garces and Cleland, 1969), is considered to be characteristic of ping pong pathways and to support their assignments (Mieyal and Abeles, 1972; Cleland, 1970). Figure 5 shows that galactose-1-P at high concentrations is inhibitory in a manner similar to that described for acceptor substrates in the case of sucrose phosphorylase (Silverstein *et al.*, 1967). It results from competition between UDP-glucose and galactose-1-P for binding to the free enzyme according to eq 2, 3, and 6, and the rate equation for glucose-1-P production is eq 7, in which A is UDP-glucose, B is galactose-1-P, and K_{Ib} is the substrate inhibition constant. This constant is evaluated by replotting the Figure 5 slopes *vs.* [galactose-1-P] (Silverstein *et al.*, 1967). The other substrates are also inhibitory at high concentrations, which can be attributed, in the case of glucose-1-P, to competition with UDP-galactose for free enzyme. Substrate inhibition by UDP-hexoses results from competition between UDP-hexoses and hexose 1-phosphates for binding to the uridylyl-enzyme intermediate (Cleland, 1970). The substrate inhibition constants are included in Table I, with the exception of that for UDP-galactose, which we did not measure although we detected inhibition at large concentrations. Our finding of substrate inhibition by all substrates extends the basic pathway (eq 2 and 3) to include eq 6, 8, 9, and 10 as well.

$$E + \text{Gal-1-P} \rightleftharpoons E \cdot \text{Gal-1-P} \quad (6)$$

$$\frac{1}{v} = \frac{1}{V} + \frac{K_b}{V[B]} + \frac{K_a}{V[A]}\left(1 + \frac{[B]}{K_{Ib}}\right) \quad (7)$$

$$E + \text{Glu-1-P} \rightleftharpoons E \cdot \text{Glu-1-P} \quad (8)$$

$$\text{uridylyl–E} + \text{UDPGlu} \rightleftharpoons \text{uridylyl–E} \cdot \text{UDPGlu} \quad (9)$$

$$\text{uridylyl–E} + \text{UDPGal} \rightleftharpoons \text{uridylyl–E} \cdot \text{UDPGal} \quad (10)$$

Any residual doubt that eq 2 and 3 represent the basic pathway is removed by the data in Figure 6. Figure 6 shows that the exchange of [^{14}C]glucose between UDP-glucose and glucose-1-P catalyzed by this enzyme (Wong and Frey, 1974) also follows the ping pong pathway and at a maximum rate comparable with the maximum overall rate, as required if eq 2 is compulsory. The data are fitted to eq 1, in which A is UDP-[U-^{14}C]glucose and B is glucose-1-P. The apparent maximum exchange rate at infinite [glucose-1-P] for each of the lines in Figure 6 is approximately 2.7 times larger than the apparent maximum velocity for the overall reaction at infinite [galactose-1-P] and the same UDP-glucose concentrations. Correspondingly, the maximum exchange velocity in Figure 6 is 18 μmol per min per activity unit as compared with 6.5 μmol per min per unit for V_f in Table I. The exchange rate is clearly large enough to support the assignment of eq 2 and 3 as the basic pathway. The probability is exceedingly small that a pathway other than eq 2 would give both parallel lines in Figure 6 and an exchange rate faster than that of the overall reaction.

TABLE II: Comparison of Haldane Relationships with Experimental K_{eq}.[a]

$\left(\frac{V_f}{V_r}\right)^2 \frac{K_{Glu\text{-}1\text{-}P}K_{UDPGal}}{K_{UDPGlu}K_{Gal\text{-}1\text{-}P}}$	$\left(\frac{V_f}{V_r}\right) \frac{K_{Glu\text{-}1\text{-}P}K_{iUDPGal}}{K_{UDPGlu}K_{iGal\text{-}1\text{-}P}}$	$\left(\frac{V_f}{V_r}\right) \frac{K_{iGlu\text{-}1\text{-}P}K_{UDPGal}}{K_{iUDPGlu}K_{Gal\text{-}1\text{-}P}}$	$\frac{K_{iGlu\text{-}1\text{-}P}K_{iUDPGal}}{K_{iUDPGlu}K_{iGal\text{-}1\text{-}P}}$	K_{eq}
2.25	1.97	2.23	1.95	2.22 1.97

[a] The Haldane relationships are calculated from the Table I parameters. K_{eq} refers to the reaction proceeding in the direction of glucose-1-P formation and was measured as described under the Experimental Section.

The fact that the exchange rate is larger than the overall rate shows that the enzyme distinguishes between glucose-1-P and galactose-1-P at acceptor saturation; therefore, the transition state for the rate-limiting step in the forward direction contains galactose-1-P or UDP-galactose. This indicates that the rate may not be limited solely by the rate of uridylyl-enzyme formation at saturation. The rate at which uridylyl-enzyme reacts with acceptor may be at least partially rate limiting. The interpretation of exchange rates compared with overall rates is discussed by Jencks (1969).

We conclude that the reaction follows the ping pong pathway, eq 2 and 3, and that the uridylyl-enzyme previously isolated (Wong and Frey, 1974) is kinetically competent as an intermediate.

Equilibrium Constants for Uridylyl Transfer and Hexose-1-P Interconversion. The measured kinetic parameters and overall equilibrium constant for this reaction together with the published K_{eq} for the UDP-galactose-4-epimerase reaction enable us to calculate the equilibrium constants for uridylyl transfer to the enzyme by uridylyl donors and the equilibrium constant for the conversion of galactose-1-P to glucose-1-P as well. The K_{eq} for uridylyl transfer to enzyme by UDP-glucose (eq 11a) is given by eq 11b. That for uridylyl transfer by UDP-

$$\text{UDP-glucose} + \text{E} \rightleftharpoons \text{uridylyl-E} + \text{glucose-1-P} \quad (11a)$$

$$K_{eq} = \frac{V_f K_{Glu\text{-}1\text{-}P}}{V_r K_{UDPGlu}} = 2.1 \quad (11b)$$

galactose (eq 12a) is given by eq 12b. The calculated constants

$$\text{UDP-galactose} + \text{E} \rightleftharpoons \text{uridylyl-E} + \text{galactose-1-P} \quad (12a)$$

$$K_{eq} = \frac{V_r K_{Gal\text{-}1\text{-}P}}{V_f K_{UDPGal}} = 0.96 \quad (12b)$$

refer to pH 8.5 and 27° and are 2.1 and 0.96, respectively, which shows that the stabilities of the uridylyl-enzyme intermediate and the pyrophosphoryl linkages in the substrates are similar.

From the equilibrium constant at pH 8.5 and 27° for the galactose-1-P uridylyltransferase reaction in the direction of glucose-1-P production, which is 2.1 in Table I, and that for the UDP-galactose-4-epimerase reaction in the direction of UDP-glucose formation, which is 3.5 at pH's 8.7 and 7.1 at 27° (Wilson and Hogness, 1964; Imae *et al.*, 1964), we calculate the equilibrium constant for the conversion of galactose-1-P to glucose-1-P to be 7.4, which corresponds to a standard free energy difference of 1.2 kcal/mol favoring glucose-1-P under these conditions. Therefore, the fact that UDP-glucose is favored in the epimerase reaction can be attributed principally to the difference in stabilities between the hexose 1-phosphates. This difference is somewhat larger than we expected. Published data are, however, consistent with it as follows. The standard free energy change for hydrolysis of ATP to ADP and P_i at pH 7.0, 25°, and 10 mM Mg^{2+} is −8.5 kcal/mol (Alberty, 1969), while that for the phosphorylation of galactose to galactose-1-P by ATP is +1.9 kcal/mol under similar conditions (Atkinson *et al.*, 1961a). From these data we calculate that the standard free energy change for hydrolysis of galactose-1-P is −6.6 kcal/mol. This is 1.6 kcal/mol more negative than −5.0 kcal/mol, the published value for the hydrolysis of glucose-1-P under similar conditions (Atkinson *et al.*, 1961b).

Discussion

We recently reported that a uridylyl-enzyme complex of galactose-1-P uridylyltransferase can be isolated and that the enzyme catalyzes certain exchange reactions as well (Wong and Frey, 1974). We proposed a double displacement pathway involving the uridylyl-enzyme as a compulsory intermediate. The kinetic competence of this pathway and of the uridylyl-enzyme intermediate have now been confirmed.

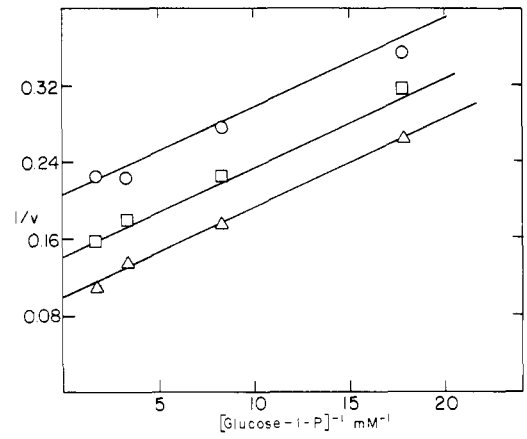

FIGURE 6: Steady-state kinetics for exchange of ^{14}C from UDP-[U-^{14}C]glucose (4.32×10^5 cpm/μmol) into glucose-1-P. The initial rates were measured by method C at the indicated glucose-1-P concentrations. The UDP-[U-^{14}C]glucose concentrations were: (○) 0.037 mM; (□) 0.059 mM; (△) 0.1 mM. The velocity units are mμmol of [U-^{14}C]glucose-1-P produced per min by 1.8 milliunits of enzyme.

The evidence so far accumulated validates several statements concerning the reaction pathway. The active site of the free enzyme binds uridylyl-donor substrates or, with less affinity, uridylyl-acceptor substrates, but it does not simultaneously bind both. A functional group, probably a nucleophile, in the active site reversibly cleaves the pyrophosphoryl linkage to form a uridylyl-enzyme intermediate in which the functional group is substituted for the hexose-1-P moiety. The hexose-1-P so formed must leave the site before the alternative hexose-1-P can enter and reverse the pyrophosphorolytic process. The uridylyl-enzyme intermediate binds uridylyl-acceptor substrates or, less tightly, uridylyl-donor substrates but not both at the same time.

The emerging picture is that of an active site conformationally and physicochemically competent to bind UDP-hexose substrates but not to form ternary complexes. There is at least one functional group near the center and suitably positioned to form a bond to the α-phosphorus atom of bound uridylyl-donor substrates. Concerning the identity of this group, nothing can as yet be said. Concerning the mechanism of the pyrophosphorolytic process, little can be said except that it probably proceeds by a direct displacement mechanism or an associative mechanism involving a pentacovalent phosphorus intermediate. A dissociative mechanism involving a metaphosphate ester intermediate is unlikely on chemical grounds (Benkovic and Schray, 1973).

The proposed pathway differs strikingly from that proposed for the UDP-glucose pyrophosphorylase reaction (eq 13). That

$$\text{UDP-glucose} + \text{PP}_i \rightleftharpoons \text{UTP} + \text{glucose-1-P} \quad (13)$$

reaction follows sequential kinetics through a ternary complex, ordered bi bi in the case of the erythrocyte enzyme (Tsuboi *et al.*, 1969). None of the exchange reactions characteristic of the ping pong pathway could be detected in studies on the yeast and mungbean enzymes (Neufeld *et al.*, 1957; Munch-Petersen, 1957). Of course, a uridylyl-enzyme might be involved as an intermediate on a pathway connecting central ternary complexes; however, radiochemical tracer studies on the highly pu-

rified calf liver UDP-glucose pyrophosphorylase uncovered no evidence for such an intermediate (Gillett *et al.*, 1971). The possibility that a uridylyl-enzyme might be involved cannot be definitely excluded by negative evidence; nevertheless, the weight of the available evidence does not support such a proposal. In the absence of additional information it appears that the uridylyl moiety may be transferred directly between the uridylyl donors and acceptors within the ternary complexes.

The two reactions are closely analogous in the chemical sense, yet the reaction pathways differ in every respect so far examined. While this conceivably could be a chance occurrence, we believe that the evolution of these pathways along different lines was probably determined in large part by substrate binding requirements.

In the case of galactose-1-P uridylyltransferase, for which the uridylyl donors are electrostatically identical and nearly isosteric, the catalytic center evolved either with a single binding site or with overlapping sites for uridylyl acceptors. In the absence of a second acceptor binding site it was essential for the enzyme also to provide a suitable functional group to accomplish the pyrophosphorolytic process reversibly. In the case of UDP-glucose pyrophosphorylase the uridylyl donors and acceptors are electrostatically and sterically quite different, such that the uridylyl acceptors could not be well accommodated by a single acceptor binding site. Consequently the active center evolved with two nonoverlapping acceptor binding sites, one for pyrophosphate and one for glucose-1-P. With both acceptor and donor present in a ternary complex, it would not be essential for the enzyme to provide a functional group to mediate group transfer by covalent catalysis, although the possibility that it may cannot as yet be definitely excluded.

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Supplementary Material Available

Figures 7-11 containing additional product and substrate inhibition data will appear following these pages in the microfilm edition of this volume of the journal. Photocopies of the supplementary material from this paper only or microfiche (105 × 148 mm, 24× reduction, negatives) containing all of the supplementary material for the papers in this issue may be obtained from the Journals Department, American Chemical Society, 1155 16th Street, N.W., Washington, D. C. 20036. Remit check or money order for $3.00 for photocopy or $2.00 for microfiche, referring to code number BIO-74-3889.

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