Uridine Diphosphate Glucose Synthase from Calf Liver: Determinants of Enzyme Activity in Vitro†

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ABSTRACT: The reaction catalyzed by calf liver uridine diphosphate glucose synthase (pyrophosphorylase) (EC 2.7.7.9; UTP + glucose 1-phosphate = UDP-glucose + PP_i) is an example of an enzymic reaction in which a nucleoside triphosphate other than ATP is the immediate source of metabolic energy. Kinetic properties of the enzyme, acting in the direction of UDP-glucose formation, were investigated in vitro. The reaction was inhibited by UDP-glucose (0.072), P_i (11), UDP (1.6), UDP-xylose (0.87), UDP-glucuronate (1.3), and UDP-galacturonate (0.95). The numbers in parentheses indicate the concentration (m*M*) required for half-maximal inhibition under the conditions used. Other compounds tested, including ATP, ADP, and AMP, had no effect. Over a range of concentrations of UTP (0.04–0.8 m*M*) and UDP-glucose (0.05–0.3 m*M*), the reaction rate was more dependent on the concentration ratio [UDP-glucose]/[UTP] than on the absolute concentration of either compound. Comparison of the kinetic properties in vitro with estimates of metabolite levels in vivo suggests that (1) the enzyme operates in a range far from its maximal rate, and (2) the concentrations of glucose 1-phosphate and P_i and the ratio [UDP-glucose]/[UTP] may be the most important determinants of UDP-glucose synthase activity.

The adenine nucleotides ATP, ADP, and AMP provide the stoichiometric coupling between metabolic sequences of energy utilization and energy production. In most instances this coupling is direct: ATP is the product of such processes as oxidative phosphorylation, photophosphorylation, and glycolysis, and it is consumed directly in many biosynthetic reactions as well as a variety of energy transducing systems such as muscle contraction, transport across membranes, and bioluminescence. This special metabolic role for the adenylates has influenced the evolutionary design of the kinetic properties of enzymes interacting with the adenylate system (Atkinson, 1970). Kinetic studies of several such enzymes in vitro (Atkinson, 1969; Bigler and Atkinson, 1969; Brenner et al., 1970; Shen and Atkinson, 1970; Liao and Atkinson, 1971; Miller and Atkinson, 1972; Chulavatnatol and Atkinson, 1973; Shargool, 1973) have suggested the importance of the relative concentrations of the three adenylates, ATP, ADP, and AMP, in determining enzyme activity. This is exemplified by kinetic behavior in response to variation of the adenylate energy charge, defined as ([ATP] + 1/2[ADP])/([ATP] + [ADP] + [AMP]) (Atkinson, 1968).

In contrast to this direct coupling to ATP, many biosynthetic reactions are coupled to ATP indirectly through the mediation of a different nucleoside triphosphate. UDP-glucose synthase (pyrophosphorylase) (EC 2.7.7.9; UTP + glucose-1-P = UDP-glucose + PP_i) catalyzes such a reaction. This enzyme has been isolated from a variety of sources (for a review, see Turnquist and Hansen, 1973) and is responsible for the formation of UDP-glucose, an important intermediate in hexose interconversions and the biosynthesis of glycogen and other polysaccharides. This paper reports that the activity of UDP-glucose synthase from calf liver is altered by relatively few of the metabolites tested. The most important determinants of activity appear to be the concentrations of glucose-1-P and P_i and the concentration ratio [UDP-glucose]/[UTP].

Materials and Methods

Materials. Concentrations of UTP and UDP-glucose were checked by ultraviolet absorption, assuming a molar absorbance of 10^4 at 262 nm and pH 7 (P-L Biochemicals catalog No. 102, 1970). Glucose-1-P used was estimated to be 95% pure on the basis of a coupled enzymic assay (phosphoglucomutase, EC 2.7.5.1, and glucose-6-P dehydrogenase, EC 1.1.1.49). UDP-glucose (Calbiochem) was 94% pure as estimated from its oxidation by UDP-glucose dehydrogenase using a slight modification of assay I below. UTP displayed one major component after one-dimensional chromatography on polyethylenimine-cellulose thin-layer plates (W. H. Huisman, this laboratory). UDP-xylose, UDP-glucuronate, and UDP-galacturonate each showed one component after two-dimensional chromatography on polyethylenimine thin-layer plates (R. J. Sedo, this laboratory). Crystalline UDP-glucose synthase from calf liver was kindly provided by Dr. R. G. Hansen, Department of Chemistry, Utah State University, Logan, Utah.

Assays for UDP-glucose Activity. Assay I. UDP-glucose formation was coupled to its oxidation by UDP-glucose dehydrogenase (EC 1.1.1.22; Type VI from bovine liver, Sigma) so that NADH production could be followed spectrophotometrically at 340 nm (Knop, 1969). The reaction mixture (1 ml) contained 99 m*M* Tris-acetate (pH 7.8), 0.8 m*M* NAD^+, 0.016 new Sigma units of UDP-glucose dehydrogenase, 10 μg of bovine serum albumin, 2 m*M* magnesium acetate, 20–40 ng of UDP-glucose synthase, and UTP and glucose-1-P as indicated. After a short lag (less than 20 sec) a linear increase in absorption with time was observed

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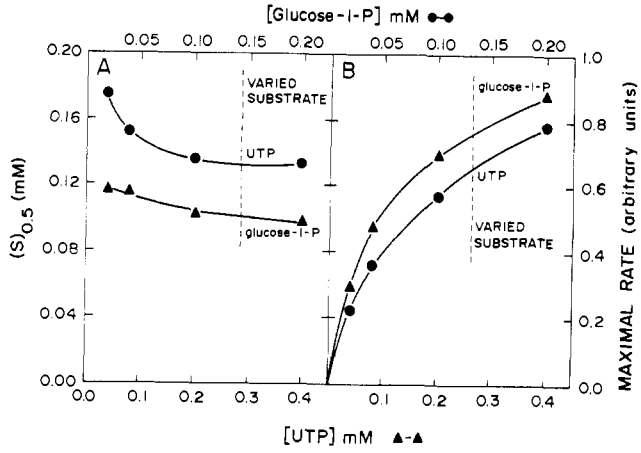

FIGURE 1: Dependence of kinetic parameters of UDP-glucose synthase on substrate concentrations. Assay I was used with 20–40 ng of synthase; concentrations of glucose-1-P and UTP as indicated. The maximal rate scale is normalized to allow comparison of six separate experiments.

for 5–20 min depending on the conditions. A rate of change of absorption of less than 0.015/min ensured that the synthase was rate limiting. Reaction temperature was 37°.

Assay II. The measurement of [^{14}C]UDP-glucose formation from [U-^{14}C]glucose-1-P was based on adsorption of the product on DEAE paper after hydrolysis of residual reactant glucose-1-P (Roberts and Tovey, 1970). The reaction mixture (0.1 ml) contained 99 m*M* Tris-acetate (pH 7.8), bovine serum albumin, 2 m*M* magnesium acetate (unless otherwise noted), 2–20 ng of enzyme, and UTP and [U-^{14}C]glucose-1-P (Amersham-Searle) as indicated. After incubation for 4 min at 37°, the reaction was terminated by the addition of 0.05 ml of 20 m*M* EDTA and placed on ice. *Escherichia coli* alkaline phosphatase, 30 μg in 0.01 ml (EC 3.1.3.1, Type IIIs, Sigma), was added followed by incubation at 37° for 90 min. A sample, 0.05 ml, was then placed on a ⅞-in. diameter DEAE paper disc (DE 81, Whatman) which was washed with three 80-ml volumes of water on a sintered glass filter. The dried disc was placed in a scintillation vial with 10 ml of a solution containing 4 g of Omniflor (New England Nuclear) per liter of toluene, and the radioactivity retained on the disc was determined with a liquid scintillation counter. The results of control experiments in which EDTA was added before enzyme varied with the batch of [U-^{14}C]glucose-1-P, but the radioactivity retained on the disc was never greater than 1% of the total radioactivity present in the assay. This blank value was reproducible in a given set of assays, and was always measured for each glucose-1-P concentration used. Linearity of UDP-glucose formation with time was established at the extremes of the conditions used; typically less than 5% conversion of glucose-1-P was observed.

For both assays, reaction mixtures were incubated for 2–4 min at 37° before starting the reaction by the addition of enzyme.

Dilution of Enzyme. Stock enzyme (1.8 mg/ml) was diluted immediately before use into Tris-acetate buffer (pH 7.8) containing bovine serum albumin which stabilized the enzyme activity. With 90 m*M* Tris-acetate (pH 7.8) and 200 μg/ml of albumin (20 μg/ml in the assay) and with 0.18 μg/ml of enzyme, the enzyme activity decreased slowly in the bulk solution stored on ice, but could be recovered simply by shaking the enzyme solution. All activity could be removed from bulk solution by low-speed centrifugation with a bench centrifuge and could be restored by agitating the solution, suggesting the formation of very large aggregates. This phenomenon was not observed when (1) 9 m*M* Tris-acetate (pH 7.8) and 20 μg/ml of albumin was the diluting buffer, (2) the temperature of the diluting buffer was 37°, or (3) the enzyme was diluted further to 9 ng/ml. These last two conditions are those of most assays, indicating that the enzyme was never present in the putative aggregated form during the measurement of its activity. However, following this finding the enzyme was routinely diluted into 9 m*M* Tris-acetate (pH 7.8) and 20 μg/ml of bovine serum albumin (giving 2 μg/ml in the assay).

Table I: Inhibitors of UDP-glucose Synthase Reaction.[a]

Addition	Relative Rate: No UDP-glucose	Relative Rate: 0.2 m*M* UDP-glucose
None	100	35
UDP-glucose (0.2 m*M*)	35	
PP_i (1 m*M*)	61	
P_i (5 m*M*)	64	12
UDP (1 m*M*)	70	29
UDP-xylose (1 m*M*)	48	18
UDP-glucuronate (2 m*M*)	45	23
UDP-galacturonate (1 m*M*)	39	22
UDP-*N*-acetylglucosamine (1 m*M*)	78	32
UDP-mannose (1 m*M*)	76	35
UDP-galactose (1 m*M*)	78	36
GDP-mannose (1 m*M*)	66	33
$(NH_4)_2SO_4$ (4 m*M*)		20
NH_4Cl (8 m*M*)		35

[a] Assay II was used with 0.5 m*M* [U-^{14}C]glucose-1-P (10 Ci/mol), 0.2 m*M* UTP, 2 μg/ml of bovine serum albumin, and either no UDP-glucose and 4–10 ng of enzyme, or 0.2 m*M* UDP-glucose and 20 ng of enzyme.

Computational Methods. Calculation of $(S)_{0.5}$[1] and maximal velocity from initial rate measurements was based on the method of Wilkinson (1961). With inhibition data, the fractional inhibition compared to the uninhibited control was substituted for initial velocity, leading to the computation of $(M)_{0.5}$ and maximal inhibition. The statistical formulation of Wilkinson is not rigorously applicable in this latter case but the trend of the error variation is similar to that of a substrate saturation curve, and this method was considered to give a useful objective estimate of inhibition parameters. For both $(S)_{0.5}$ and $(M)_{0.5}$ estimations, the above computational treatment was used only after a Hill slope (for inhibitors, the slope of log $[(v - v_{sat})/(V_{max} - v)]$ against log [inhibitor concentration]) close to unity had been observed.

Results

Enzymic Activity as a Function of Substrate Concentrations. $(S)_{0.5}$ values, at saturating levels of the other substrate, were in the range 0.1–0.4 m*M* for UTP and 0.05–0.1 m*M* for glucose-1-P, compared with reported Michaelis constants of 0.2 and 0.055 m*M*, respectively (Albrecht et al., 1966). Hill slopes with respect to UTP and glucose-1-P were never significantly different from unity. $(S)_{0.5}$ values

[1] Abbreviations used are: $(S)_{0.5}$, the concentration of substrate required under specified conditions for a velocity half of the maximal value under the same conditions; $(M)_{0.5}$, the concentration of modifier required for half-maximal effect under specified conditions.

Table II: Kinetic Parameters for Inhibitors of UDP-glucose Synthase Activity.[a]

Compound[b]	No UDP-glucose		0.2 m*M* UDP-glucose		UDP-glucose Inhibition	
	$(M)_{0.5}$[c] (m*M*)	Maximum Inhibition (%)	$(M)_{0.5}$ (m*M*)	Maximum Inhibition (%)	$(UDPG)_{0.5}$[d] (*μM*)	Maximum Inhibition (%)
UDP-glucose	0.072	100			72	100
PP_i	0.75	100				
P_i (5)	11	53	1.7	88	37	100
UDP (1)	1.6	82	2.8	63	120	91
UDP-xylose (1)	0.87	100	1.2	98	100	87
UDP-glucuronate (1)	1.3	100	1.1	70	72	83
UDP-galacturonate (1)	0.95	82	1.1	53	210	100

[a] Assay II was used with 0.5 m*M* [U-^{14}C]glucose-1-P (10 Ci/mol), 0.2 m*M* UTP, and 2 μg/ml of bovine serum albumin. [b] Numbers in parentheses indicate the concentration (m*M*) of inhibitor present in the measurement of UDP-glucose inhibition parameters (last two columns). These numbers do not apply to the first four columns. [c] $(M)_{0.5}$ is the inhibitor concentration required for half-maximal effect and maximum inhibition is the percentage inhibition extrapolated to infinite inhibitor concentration. Values for these parameters are calculated on the basis of a hyperbolic variation of percentage inhibition with inhibitor concentration (see Materials and Methods). [d] $(UDPG)_{0.5}$ is the concentration of UDP-glucose required for half-maximal effect in the presence of the indicated concentrations of the other inhibitors.

for both UTP and glucose-1-P were relatively insensitive to changes in the level of the other substrate but each increased slightly as the concentration of the other substrate was decreased (Figure 1). The maximal rates observed at saturating levels of either substrate varied hyperbolically with the concentration of the other (Figure 1).

Screening for Effectors of UDP-glucose Synthase. UDP and P_i have been reported to inhibit calf liver UDP-glucose synthase acting in the direction of UTP formation (Albrecht et al., 1966). The products of the physiological reaction, PP_i and UDP-glucose, were inhibitors of the enzyme from other sources (Tsuboi et al., 1969; Franke and Sussman, 1971; Knop and Hansen, 1970). In addition to these compounds, several nucleoside diphospho sugars inhibit UDP-glucose formation by the calf liver enzyme (Table I) as has been observed for the enzyme from *Lilium longiflorum* pollen (Hopper and Dickinson, 1972). At a concentration of 1 m*M*, however, only UDP-xylose, UDP-glucuronate, and UDP-galacturonate caused significant additional inhibition in the presence of 0.2 m*M* UDP-glucose, which is near the physiological level (Guynn et al., 1974; Burch et al., 1969; Bucher and Swaffield, 1966; Keppler et al., 1974; Keppler and Smith, 1974). Sulfate ion was also inhibitory (Table I).

A number of other compounds caused no significant activation or inhibition. Using assay I with 0.05 m*M* glucose-1-P and 0.1 m*M* UTP: GDP-glucose, *N*-acetylneuraminate, *N*-acetylmuramate, *N*-acetylglucosamine, *N*-acetylgalactosamine, glucose-6-P, fructose-6-P, glucose, α-ketoglutarate, 6-P-gluconate, aspartate, glutamate, and glutamine, all at 1 m*M* concentration. Using assay II with 0.5 m*M* glucose-1-P, 0.2 m*M* UTP, 4 m*M* magnesium acetate, 2 μg/ml of bovine serum albumin, and both with and without 0.2 m*M* UDP-glucose: 2 m*M* ATP and ADP, AMP, UMP, CTP, CDP, CMP, GTP, GDP, GMP, ITP, IMP, and galactose-1-P, all at 1 m*M* concentration. Using assay II with 0.5 m*M* glucose-1-P, 0.2 m*M* UTP, 2 μg/ml of bovine serum albumin, and 0.2 m*M* UDP-glucose: galactose, mannose, fructose, pyruvate, L-lactate, glucosamine, ribose, and glycogen (with respect to monomer), all at 10 m*M* concentration, and NAD^+, NADH, $NADP^+$, NADPH, fructose-1-P, and fructose-1,6-P_2, all at 1 m*M* concentration. Also, 1 m*M* fructose-1,6-P_2 preincubated with rabbit muscle aldolase gave no effect suggesting that dihydroxyacetone-P and glyceraldehyde-3-P were ineffective. Using assay II with 0.05 m*M* glucose-1-P, 0.2 m*M* UTP, 2 μg/ml of bovine serum albumin: 0.1 m*M* cAMP, and fructose-1,6-P_2, phosphoenolpyruvate, galactose-1-P, fructose-1-P, fructose-1-P, acetyl-CoA, CoA, DL-α-glycero-P, 2-P-glycerate, and 3-P-glycerate, all at 1 m*M* concentration.

The adenylates notably had no significant effect, either when tested singly as above, or when present together at 2 m*M* total concentration and energy charge values of 0.65 or 0.95, with or without 0.2 m*M* UDP-glucose. We found no inhibition with galactose-1-P at a concentration 20 times that of glucose-1-P. Oliver (1961) reported inhibition of UDP-glucose synthase preparations from brain and liver by galactose-1-P but the K_i values quoted (about 15 m*M* and about 9 m*M*, respectively) are high compared with the concentrations used here.

Concentrations of Reaction Products and the Value of the [UDP-glucose]/[UTP] Ratio as Determinants of Reaction Rate. Inorganic pyrophosphate at 2 m*M* caused a 75% decrease in V_{max} when either substrate was varied and approximately doubled both $(UTP)_{0.5}$ and $(glucose\text{-}1\text{-}P)_{0.5}$. The value for $(PP_i)_{0.5}$ was 0.75 m*M* (Table II). This is probably much higher than the physiological concentration of pyrophosphate (Guynn et al., 1974; Flodgaard, 1969).

Inhibition by UDP-glucose was studied in some detail. At 0.2 m*M* UTP, $(UDP\text{-}glucose)_{0.5}$ was 72 *μM* (Table II). When glucose-1-P was constant at 0.5 m*M* UDP-glucose caused an increase in $(UTP)_{0.5}$ with a slight decrease in the maximal rate (Figure 2). This dependence of $(UTP)_{0.5}$ on UDP-glucose concentration was approximately linear, and the same data revealed a similar relationship between $(UDP\text{-}glucose)_{0.5}$ and UTP concentration (not shown). Hill plots for both UTP and UDP-glucose had slopes of one. The initial rate data for the UDP-glucose inhibition experiments are shown in Figure 3, in which reaction rate is also plotted as a function of the concentration ratio [UDP-glucose]/[UTP]. It is apparent from this representation of the data that the rate is more directly related to the concentration ratio than to the absolute concentrations of UDP-glucose and UTP.

The results in Figure 2, except for the slight V_{max} effect, are what would be predicted if UTP and UDP-glucose competed for the same noninteracting sites. Thus we use a simple competition model in simulations for the sake of simplicity and generality since the V_{max} effect is too small to affect the results materially. The model consists simply of

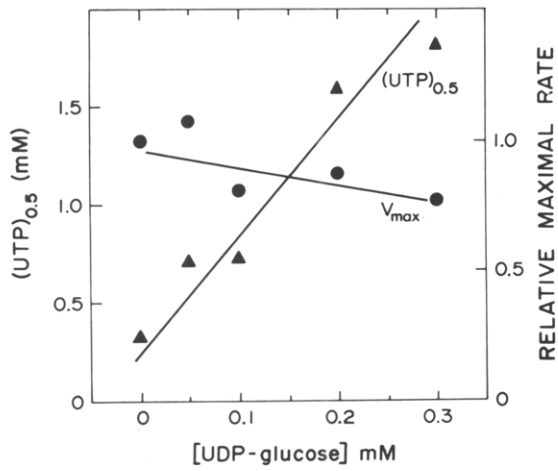

FIGURE 2: Dependence of $(UTP)_{0.5}$ and maximal rate for the UDP-glucose synthase reaction on UDP-glucose concentration. Assay II used with 0.5 mM [U-^{14}C]glucose-1-P (10 Ci/mol), 20 μg/ml of bovine serum albumin, 2–4 ng of enzyme, 0.04–0.8 mM UTP, UDP-glucose as indicated. Rates are normalized so that the uninhibited maximal rate is unity to allow comparison of three separate experiments. If a model of simple competitive inhibition is assumed, then $(UTP)_{0.5}$ at zero UDP-glucose is the dissociation constant for UTP and the slope of $(UTP)_{0.5}$ as a function of UDPglucose concentration is the dissociation constant for UTP devided by that for UDPglucose.

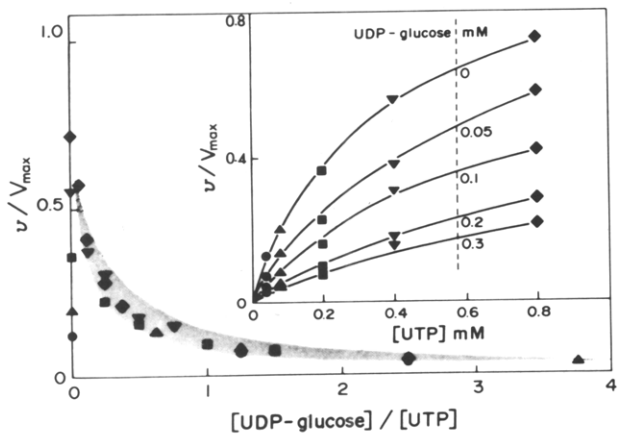

FIGURE 3: Inhibition of the UDP-glucose synthase reaction by UDP-glucose: reaction rate as a function of the ratio [UDP-glucose]/[UTP]. The data are from the same experiments as those in Figure 2. Each set of symbols corresponds to a constant UTP concentration as indicated in the inset. The shaded area is that defined by allowing UTP to vary only in the range 0.04–0.8 mM and UDP-glucose in the range 0.05–0.3 mM and calculating the expected range of velocities, using a model for simple competition between UTP and UDP-glucose (see text). Values for dissociation constants were estimated from Figure 2: UTP, 0.36 mM and UDP-glucose, 0.070 mM.

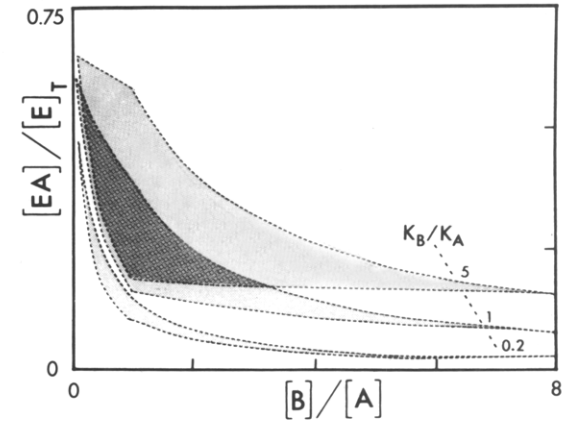

FIGURE 4: Model system with competition between substrate A and product B for an enzyme catalytic site. Concentrations of both A and B are allowed to vary between 0.25 and $2K_A$. Each shaded area, or "window", represents the range of values of $[EA]/[E]_T$, and thus of relative velocity v/V_{max}, that is possible for any value of [B]/[A] when the individual concentrations are varied within the indicated limits. The equation used is $[EA]/[E]_T = [A]/(K_A(1 + [B]/K_B) + [A])$. Each window corresponds to a different ratio of affinities for A and B, as indicated by the ratios of dissociation constants shown on the figure.

an enzyme site that binds substrate A and product B with dissociation constants K_A and K_B. Figure 4 shows the fraction of sites occupied by A as a function of the concentration ratio [B]/[A] on the basis of such a model. The reaction rate is presumably proportional to the concentration of the enzyme–A complex. Each shaded area or "window" is defined by placing limits on the concentration ranges allowed A and B, which are both constrained to the range 0.25 K_A to 2 K_A. The vertical width of the window is an indication of the relative importance of the absolute concentrations in determining reaction rate, since vertical displacement on the graph corresponds to changes in the concentrations of both ligands with the ratio of their concentrations held constant. Each window corresponds to a different ratio of affinities for A and B, as shown by the ratios of dissociation constants. It is evident from the changes in shape of the window that as this affinity ratio decreases, that is, as the product B becomes a more effective inhibitor, the ratio of the concentrations becomes a more important determinant of enzyme reaction rate. The narrower the window in the vertical dimension, the more nearly the rate of the reaction is independent of actual concentrations, and the more it depends on the product/substrate concentration ratio. An affinity ratio of about 0.2, corresponding to the bottom window in Figure 4, is close to the situation for UDP-glucose synthase, and a similar window, calculated from the experimental parameters, is shown superimposed on the experimental results in Figure 3. It is clear that, over a rather wide range of concentrations of UTP and UDP-glucose, the enzyme responds primarily to the ratio of these concentrations. Similar "windows" have been used in a general discussion of the importance of concentration ratios in enzyme regulation (Atkinson et al., 1975).

When the concentration of UTP was held at 1 mM, the value of $(glucose\text{-}1\text{-}P)_{0.5}$ was not greatly changed by UDP-glucose in the range 0.1–0.3 mM, but the maximal rate was decreased (60% decrease at 0.2 mM UDP-glucose). The value of $(UDP\text{-}glucose)_{0.5}$ decreased by 40% as the level of glucose-1-P was reduced by a factor of ten from 0.5 to 0.05 mM. This might be predicted, on the basis of competition between UTP and UDP-glucose, from the increase in $(UTP)_{0.5}$ at low sugar phosphate levels (Figure 1). It thus appears that the parameters $(UTP)_{0.5}$ and $(UDP\text{-}glucose)_{0.5}$ are strongly interdependent but neither is strongly influenced by the level of glucose-1-P, nor is $(glucose\text{-}1\text{-}P)_{0.5}$ greatly influenced by the concentration of either uridylate. The primary effect of variation in concentration of glucose-1-P in these experiments is to alter the maximal rate attainable when the catalytic site is saturated by UTP. Thus when an experiment analogous to that of Figures 2 and 3 was performed with 0.05 mM glucose-1-P, the results obtained were similar to those with 0.5 mM glucose-1-P if both experiments were normalized to the same uninhibited maximal rate. It thus seems that extrapolation from the experimentally more accessible levels of glucose-1-P (0.5–0.05

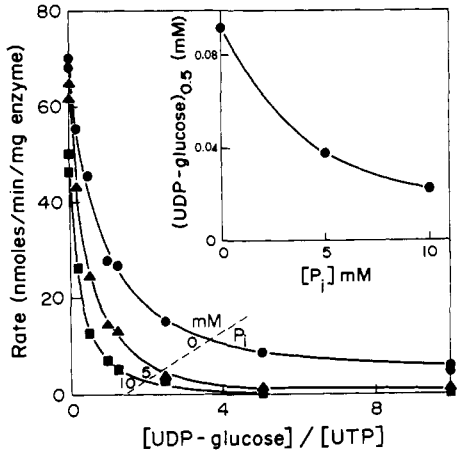

FIGURE 5: Effect of P_i on UDP-glucose inhibition. Assay II was used with 0.2 mM UTP, 0.5 mM [U-^{14}C]glucose-1-P (10 Ci/mol), 3.6 ng of enzyme, 20 μg of bovine serum albumin, and UDP-glucose to give the appropriate concentration ratios. The inset shows $(UDP\text{-}glucose)_{0.5}$, calculated from these results, as a function of P_i concentration.

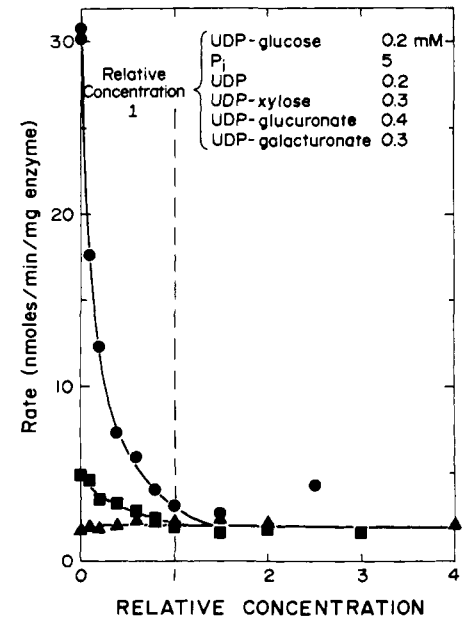

FIGURE 6: Multiple inhibition experiment with UDP-glucose synthase. Assay II was used with 0.2 mM UTP, 2 μg/ml of bovine serum albumin, 0.05 mM [U-^{14}C]glucose-1-P (100 Ci/mol), and 1.8 ng of enzyme. For the upper curve, the concentrations of possible inhibitors were varied simultaneously at a constant ratio such that at "relative concentration 1" on the abscissa the individual levels were those shown on the figure. The middle curve arises from a similar design except that the concentration of UDP-glucose was 0.2 mM throughout. The lowest curve was also similarly obtained; in this case both 0.2 mM UDP-glucose and 5 mM P_i were present throughout.

mM) to the lower levels thought to occur in the cell (Woods and Krebs, 1973; Gunn and Taylor, 1973; Guynn et al., 1974) may not be unreasonable.

Inhibitors of UDP-glucose Synthase and Their Interactions. Parameters characterizing the most effective inhibitors of the reaction are listed in Table II. Hill slopes never varied significantly from one. UDP-glucose and inorganic phosphate are seen to inhibit synergistically. This interaction between P_i and UDP-glucose is demonstrated visually in Figure 5 where increasing P_i concentration causes more effective UDP-glucose inhibition, which is seen as a sharper response to the ratio [UDP-glucose]/[UTP] in the region 0–2.

No striking synergism or antagonism was observed when mixtures of inhibitors were tested, except for the interaction between P_i and UDP-glucose. In another type of multiple inhibition experiment, an attempt was made to assess the relative importance of the different inhibitors (Figure 6). The uppermost curve results from simultaneous variation of all six inhibitors: the total inhibitor concentration was increased while the concentration ratios were kept constant. Relative concentration of 1 on the abscissa corresponds to estimates of physiological levels. Such information for UDP-xylose and UDP-galacturonate is lacking, and the values used are probably overestimates, but this does not materially alter the following argument. The middle curve is the result of maintaining UDP-glucose constant at 0.2 mM and varying the concentrations of the remaining inhibitors as just described. The lowest curve is similar except that in this case both P_i and UDP-glucose concentrations were fixed (5 and 0.2 mM, respectively). It is evident that nearly all of the inhibition observed at high inhibitor concentrations can be obtained with 0.2 mM UDP-glucose, and that addition of 5 mM P_i accounts for the remaining effect. If concentration 1 on the abscissa mimics the effects of these compounds in vivo, then it would appear that (1) the enzyme should operate in vivo at a very low fraction of its uninhibited rate and (2) this inhibition could be largely ascribed to the effects of the UDP-glucose/UDP ratio and to the concentration of orthophosphate.

Discussion

When the concentrations required for inhibition are compared with probable physiological concentrations, only the product UDP-glucose seems likely, among a rather large number of metabolites tested, to exert regulatory effects on UDP-glucose synthase in vivo. It should be noted that UDP was a much effective inhibitor (K_i = 150 μM) in the experiments of Albrecht et al. (1966) than in ours. The discrepancy may result from the fact that the two assays were run in opposite directions.

The significance to be attached to the pronounced inhibition of UDP-glucose synthase by orthophosphate is not obvious. It is not clear in general how important a regulatory parameter the concentration of phosphate may be, or under what metabolic conditions it varies and by how much.

It is generally assumed that pyrophosphate is held at very low concentration by the action of pyrophosphatase, and that this adds to the thermodynamic favorability of the reactions in biosynthetic or energy-storage sequences that produce pyrophosphate, as proposed by Kornberg (1962). Analytical values for pyrophosphate in vivo have covered a wide range, but recent results suggest a low level (Guynn et al., 1974; Flodgaard, 1969). Accordingly, we do not interpret our finding of pyrophosphate inhibition, with half-maximal effect at the high level of 750 μM, as being of any physiological significance. (This interpretation assumes that pyrophosphate and the enzyme are not strongly concentrated in the same intracellular compartment.)

As a product of the reaction, UDP-glucose must compete to some extent with reactant for the catalytic site. However, enzymes may be expected generally to avoid a significant degree of kinetic inhibition by products through evolving sites with higher affinity for substrate than for product. In some cases response to the concentration ratio of a product to a reactant is advantageous, however, and this can be at-

tained only if the product is bound more tightly than the reactant, as is shown by the model calculations summarized in Figure 4. Similar considerations apply to the response of many enzymes to the ratios of oxidized to reduced pyridine nucleotides or of ATP to ADP (Atkinson, 1970; Atkinson et al., 1975), and in those cases also the product is bound more firmly than the reactant. The resulting substrate inhibition is necessary for sensitive regulatory control, as is also shown in Figure 4. In general, any regulatory system must operate around a rate that is well below the maximal rate; otherwise there would be no flexibility for regulation.

In regulation of the rate of glycogen synthesis, the properties of UDP-glucose synthase must interact with those of glycogen synthase, which is at least partially under hormonal control (Larner and Villar-Palasi, 1971); of nucleoside diphosphate kinase, which regenerates UTP and is regulated by the adenylate energy charge (Thompson and Atkinson, 1971; W. Huisman, unpublished); and of the enzymes that produce or utilize glucose-1-P. In addition, UDP-glucose is involved in hexose interconversions and in the synthesis of structural macromolecules. Thus a clear picture of regulation of the production and use of UDP-glucose is not yet available. Results reported by Burch et al. (1969) are relevant to the question of regulation of UDP-glucose synthase in vivo. Injection of rats with fructose did not affect the rate of glycogen synthesis in the liver, but the concentrations of both UTP and of UDP-glucose were reduced by about 50%. In contrast, injection of dihydroxyacetone enhanced glycogen synthesis, and in this case only the concentration of UDP-glucose decreased, causing a decline in the UDP-glucose/UTP ratio. It is clear that the effects were not exerted directly on UDP-glucose synthase. The fact that after dihydroxyacetone injection the rate of glycogen synthesis increased even though the concentration of the immediate glucosyl donor UDP-glucose decreased suggests that the properties of glycogen synthase may have been affected. However, a major role for UDP-glucose synthase in the regulation of glucose incorporation into glycogen may be inferred from the observation that when the UDP-glucose/UTP ratio remained constant (although the actual concentrations changed) the flux through the pathway remained constant, whereas when the UDP-glucose/UTP ratio decreased the flux increased. These results are consistent with our observation that the enzyme in vitro responds to the UDP-glucose/UTP ratio rather than to the concentrations individually.

Newsholme and Start (1974), on the basis of relative activities of enzymes measured in tissue extracts, considered UDP-glucose synthase not to be rate limiting for glycogen synthesis. It is difficult, however, to place much confidence in conclusions as to whether enzymes are limiting under physiological conditions that are based on comparisons of activities obtained under standard, and generally near-optimal, assay conditions in the absence of modifiers. In this case, for example, our results suggest that the rate of the UDP-glucose synthase reaction under normal physiological conditions may be a very small fraction of the maximal activity, and that the reaction might very well be rate limiting under some conditions. More generally, it is probably not valid to classify enzymes as specifically rate limiting or nonrate limiting. Where, as in glycogen metabolism, several enzymes appear to have regulatory properties, it seems probable that each is rate limiting under some conditions and not under others.

Acknowledgment

We thank Dr. R. G. Hansen for a generous gift of purified uridine diphosphate glucose synthase.

References

Albrecht, G. J., Bass, S. T., Siefert, L. L., and Hansen, R. G. (1966), *J. Biol. Chem. 241,* 2968.
Atkinson, D. E. (1968), *Biochemistry 7,* 4030.
Atkinson, D. E. (1969), *Annu. Rev. Microbiol. 23,* 47.
Atkinson, D. E. (1970), *Enzymes, 3rd Ed. 1,* 461.
Atkinson, D. E. Roach, P. J., and Schwedes, J. S. (1975), *Adv. Enzyme Regul.* (in press).
Bigler, W. N., and Atkinson, D. E. (1969), *Biochem. Biophys. Res. Commun. 36,* 381.
Brenner, M., DeLorenzo, F., and Ames, B. N. (1970), *J. Biol. Chem. 245,* 450.
Bucher, N. L. R., and Swaffield, M. N. (1966), *Biochim. Biophys. Acta 129,* 445.
Burch, H. B., Max, P., Chyu, K., and Lowry, O. H. (1969), *Biochem. Biophys. Res. Commun. 34,* 619.
Chulavatnatol, M., and Atkinson, D. E. (1973), *J. Biol. Chem. 248,* 2712.
Flodgaard, H. (1969), *FEBS Lett. 2,* 209.
Franke, J., and Sussman, M. (1971), *J. Biol. Chem. 246,* 6381.
Gunn, J. M., and Taylor, C. B. (1973), *Biochem. J. 136,* 455.
Guynn, R. W., Veloso, D., Lawson, J. W. R., and Veech, R. L. (1974), *Biochem. J. 140,* 369.
Hopper, J. E., and Dickinson, D. B. (1972), *Arch. Biochem. Biophys. 148,* 523.
Keppler, D. O. R., Pausch, J., and Decker, K. (1974), *J. Biol. Chem. 249,* 211.
Keppler, D. O. R., and Smith, D. F. (1974), *Cancer Res. 34,* 705.
Knop, J. K. (1969), Master's Thesis, Michigan State University.
Knop, J. K., and Hansen, R. G. (1970), *J. Biol. Chem. 245,* 2499.
Kornberg, A. (1962), in Horizons in Biochemistry, Kasha, M., and Pullman, B., Ed., New York, N.Y., Academic Press, p 251.
Larner, J., and Villar-Palasi, C. (1971), *Curr. Top. Cell. Regul. 3,* 196.
Liao, C. L., and Atkinson, D. E. (1971), *J. Bacteriol. 106,* 37.
Miller, A. L., and Atkinson, D. E. (1972), *Arch. Biochem. Biophys. 152,* 531.
Newsholme, E. A., and Start, C. (1974), Regulation in Metabolism, New York, N.Y., Wiley, p 153.
Oliver, I. T. (1961), *Biochim. Biophys. Acta 52,* 75.
Roberts, R. M., and Tovey, K. C. (1970), *Anal. Biochem. 34,* 582.
Shargool, P. D. (1973), *FEBS Lett. 33,* 348.
Shen, L. C., and Atkinson, D. E. (1970), *J. Biol. Chem. 245,* 3996.
Thompson, F. M., and Atkinson, D. E. (1971), *Biochem. Biophys. Res. Commun. 45,* 1581.
Tsuboi, K. K., Fukunaga, K., and Petricciani, J. C. (1969), *J. Biol. Chem. 244,* 1008.
Turnquist, R. L., and Hansen, R. G. (1973), *Enzymes, 3rd Ed. 8,* 51.
Wilkinson, G. N. (1961), *Biochem. J. 80,* 324.
Woods, H. F., and Krebs, H. A. (1973), *Biochem. J. 132,* 55.