Differential Effects of Cu^{2+} on Carbamoyl Phosphate:Glucose Phosphotransferase and Glucose-6-phosphate Phosphohydrolase Activities of Multifunctional Glucose-6-phosphatase†

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ABSTRACT: The in vitro effects of Cu^{2+} on carbamoyl-P:glucose phosphotransferase and glucose-6-P phosphohydrolase of rat hepatic D-glucose-6-P phosphohydrolase (EC 3.1.3.9) have been investigated at pH 7.0 with a number of membranous enzyme preparations. Cu^{2+} was found to exert an activity-discriminating effect with respect to synthetic and hydrolytic activities. With enzyme preparations partially purified from rat liver microsomes, micromolar levels of Cu^{2+} (0 to 30 μM) exerted a biphasic effect on carbamoyl-P:glucose phosphotransferase, causing a 16% stimulation at a concentration of 5 μM with return to control values at 30 μM. However, glucose-6-P phosphohydrolase was progressively inhibited at all Cu^{2+} concentrations, with 50% inhibition occurring at 30 μM concentration. Most significantly, elevations in μM levels of Cu^{2+} progressively increased the ratio of phosphotransferase to phosphohydrolase by a factor maximally approaching two with 30 μM Cu^{2+}. Similar differential effects on synthetic and hydrolytic activities also were noted with mannose-6-P as substrate, and with enzyme preparations including intact nuclei, nuclear membrane preparations, and microsomal suspensions (the latter two preparations both without and with deoxycholate supplementation), indicating that the discriminant Cu^{2+} effect is independent of enzyme source and state of membrane integrity. Studies with μM levels of other divalent and trivalent cations support Cu^{2+} specificity. Detailed kinetic studies were carried out to gain insight into the mechanism whereby Cu^{2+} produces these activity-discriminating modifications. Cu^{2+} had no effect on the K_m for glucose in the phosphotransferase reaction nor on V_{max} for glucose-6-P hydrolysis, but did alter apparent K_m values for carbamoyl-P and glucose-6-P and V_{max} for phosphotransferase. Plots of reciprocals of v for both transferase and hydrolase against Cu^{2+} concentration were nonlinear. These and other observations are consistent with a kinetic mechanism in which Cu^{2+} interacts with two forms of the enzyme, the free enzyme and binary enzyme-phosphoryl substrate complexes, to form an inactive enzyme-Cu complex in each instance. Cu^{2+}-promoted displacement of the product of the phosphotransferase reaction (glucose-6-P) from the enzyme is proposed to account for noted stimulation of synthetic activity by low (<10 μM) levels of Cu^{2+}. The interaction of Cu^{2+} with free enzyme to form the inactive enzyme-Cu complex is suggested to contribute to the diminution of phosphotransferase seen at higher (>10 μM) levels of Cu^{2+}, and also explains the competitive inhibition of phosphohydrolase noted at all Cu^{2+} concentrations studied. R. C. Nordlie ((1974) *Curr. Top. Cell. Regul. 8,* 33) previously has suggested that the ratio, *phosphotransferase/phosphohydrolase activities of glucose-6-phosphatase,* may be important in the "tuning" of blood glucose levels through modifications in the direction and net rate of flux of glucose between hepatocyte and blood. Increases in the phosphotransferase/phosphohydrolase ratio, as noted here with Cu^{2+}, predictably should lead to enhanced uptake of glucose from the blood by the liver with ensuing hypoglycemia and elevated hepatic glycogen levels. These latter effects indeed have been seen by others in a variety of metabolically perturbed conditions which, significantly, promoted elevations in serum and liver Cu^{2+} levels. The possible physiological relevance of observations described in this paper is thus rather strongly suggested.

The multifunctional nature of glucose-6-phosphatase (D-glucose-6-phosphate phosphohydrolase; EC 3.1.3.9) is now well-established and is discussed in several reviews (Nordlie, 1969, 1971, 1974, 1976; Nordlie and Jorgenson, 1976). This enzyme possesses the ability to both catalyze the hydrolysis of glucose-6-P (reaction 1) and the synthesis of glucose-6-P from a phosphoryl donor and glucose. The most potent of the synthetic activities at physiological pH is carbamoyl-P:glucose phosphotransferase (reaction 2) (Nordlie, 1971; Herrman and Nordlie, 1972; Lueck and Nordlie, 1970).

$$\text{glucose-6-P} + H_2O \rightarrow \text{glucose} + P_i \qquad (1)$$

$$\text{carbamoyl-P} + \text{glucose} \rightarrow \text{glucose-6-P} + NH_3 + CO_2 \qquad (2)$$

Glucose-6-P is a key metabolic intermediate in gluconeogenesis, glycogenolysis, and glycolysis. The hydrolysis of this compound by glucose-6-P phosphohydrolase, which is the common terminal step in gluconeogenesis and glycogenolysis, is important in the production of blood glucose, while the synthesis of glucose-6-P is a key reaction in glucose utilization. Since glucose-6-phosphatase-phosphotransferase is capable of catalyzing two opposing reactions directly involved in glucose production and utilization, it is highly desirable to have differential regulation of the hydrolytic and synthetic activities of this enzyme if both types of activities are to be of physiological significance.

The modifying effects of divalent cations on enzymic reaction are potentially important in the regulation of cellular metabolism (Stadtman, 1970). Previous studies in this laboratory indicated that Mg^{2+} may exert an activity discriminating effect on glucose-6-phosphatase-phosphotransferase

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through selective complexation of cation with phosphoryl substrate causing inhibition of carbamoyl-P:glucose phosphotransferase but having no effect on glucose-6-P phosphohydrolase (Johnson et al., 1974). As a part of our ongoing program of studies dealing with the physiological functions and discriminant regulation of this complex enzyme, the effects of a variety of possible regulatory cations were investigated. From these studies it was found that micromolar levels of Cu^{2+} also have activity discriminating effects on the hydrolytic and synthetic activities of glucose-6-phosphatase. The potential importance of Cu^{2+} for the directive regulation of glucose-6-phosphatase–phosphotransferase serves as a basis for the present investigation in which the effect of Cu^{2+} has been systematically studied to determine a feasible mechanism for the action of this cation on the enzyme, and its possible role in carbohydrate metabolism.

Materials and Methods

Chemicals. Sources of chemicals were as follows: sodium salts of glucose-6-P, $NADP^+$, deoxycholic acid, and ATP; lithium salt of carbamoyl-P; Trizma base (2-amino-2-hydroxymethyl-1,3-propanediol); Hepes (*N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid); and DNase I from Sigma; D-glucose ("Analytical Reagent") from Mallinckrodt; $CuCl_2 \cdot 2H_2O$ ("Certified Reagent") and Folin Ciocalteau reagent from Fisher; and glucose-6-P dehydrogenase from Boehringer. Distilled water passed through a Research Model Amberlite ion-exchange resin and then redistilled in glass was used in the preparation of all reagents. Substrates and buffers were made up to 90% of the final volume and the pH was adjusted to 7.0 with dilute solutions of either NaOH or HCl as required before making the solutions up to final volume. All substrates, with the exception of carbamoyl-P, were stored as solutions at −20 °C and their concentrations were determined as previously described (Nordlie and Arion, 1966). Because of its lability, carbamoyl-P was freshly prepared in ice-cold water immediately before its introduction into the reaction mixtures (Lueck and Nordlie, 1970).

Methods. Young adult, male, albino rats were purchased from Sprague-Dawley, Madison, Wis., and maintained, ad libitum, on commercial chow (Purina) and tap water. When the animals' weights were 170–250 g, they were decapitated and bled, and the livers were rapidly removed, blotted, and placed in the appropriate ice-cold homogenizing solution, after which they were homogenized and fractionated as desired. Microsomes and partially purified microsomal suspensions, which are moderately enriched over microsomes, were prepared as described previously (Nordlie and Arion, 1966). Nuclei were isolated according to the procedure of Blobel and Potter (1966) as adapted for higher volumes (Kashnig and Casper, 1969), and nuclear membranes were prepared from isolated nuclei as outlined by Berezney et al. (1972). Microsomes and partially purified microsomal suspensions were stored at −20 °C. Before use, the frozen preparations were thawed in ice-water and diluted (see protein concentrations in tables and legends to figures) with 0.25 M sucrose at 0 °C. Nuclei and nuclear membranes were freshly prepared before each experiment and suspended in 0.25 M sucrose–TKM (0.05 M Tris-HCl (pH 7.5)–0.025 M KCl–0.005 M $MgCl_2$) also at 0 °C. When deoxycholate supplementation was required, the preparations were assayed 15 min after supplementation (Gunderson and Nordlie, 1975). Protein was determined by the modified biuret method (Nordlie and Arion, 1966) or by the method of Lowry et al. (1951).

Reactions were initiated by the addition of a 0.1-mL aliquot of enzyme preparation to the reaction mixture and were allowed to proceed for 10 min, with shaking, at 30 ± 0.1 °C before stopping with either 10% trichloroacetic acid (phosphohydrolase) or 12% perchloric acid (phosphotransferase). Assays were linear with respect to enzyme concentration and time. All reaction mixtures contained 40 mM Hepes at pH 7.0 (other components of reaction mixtures are indicated in legends to figures). Carbamoyl-P:glucose phosphotransferase was monitored by measuring the amount of glucose-6-P produced and glucose-6-P phosphohydrolase was assessed by measuring P_i liberated (Nordlie and Arion, 1966). Experimental lines presented as double-reciprocal plots (Lineweaver and Burk, 1934) or Dixon plots (Dixon, 1953) were constructed by inspection. Michaelis constants and maximal velocities were determined from the intercepts on the abscissa and ordinate, respectively, of experimental double reciprocal plots (Dixon and Webb, 1964a). All studies were done in duplicate and repeated a second and usually a third time. Representative experiments are presented.

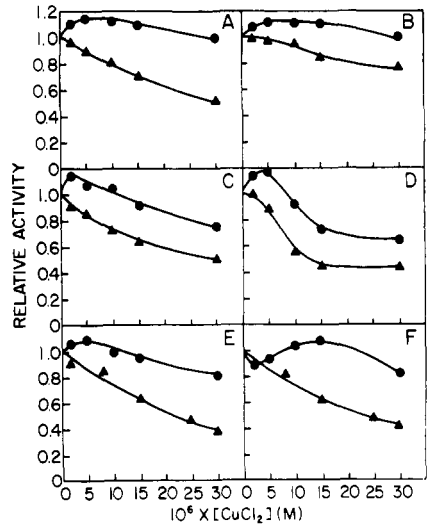

FIGURE 1: The effect of various concentrations of Cu^{2+} on carbamoyl-P:glucose phosphotransferase (●) and glucose-6-P phosphohydrolase (▲). Assay mixtures, pH 7.0, contained in 1.5 mL, 40 mM Hepes buffer, 10 mM carbamoyl-P (phosphotransferase), or 10 mM glucose-6-P (phosphohydrolase), 90 mM (A thru D) or 180 mM (E and F) D-glucose (phosphotransferase only), and $CuCl_2$ at the indicated concentrations. Relative activity is defined as the ratio of initial velocity in the presence of $CuCl_2$ to initial velocity in the absence of $CuCl_2$, where initial velocity was expressed as μmol of glucose-6-P (phosphotransferase) or P_i (phosphohydrolase) formed/min per 1.5 mL of reaction mixture. The various enzyme preparations used, the amount of protein in 1.5 mL of reaction mixture, and the initial velocities in the absence of $CuCl_2$ were: (A) partially purified preparation, 0.14 mg, phosphotransferase = 0.036, phosphohydrolase = 0.035; (B) intact nuclei, 0.45 mg, phosphotransferase = 0.018, phosphohydrolase = 0.019; (C) nuclear membrane with 0.05% deoxycholate supplementation, 0.01 mg, phosphotransferase = 0.0061, phosphohydrolase = 0.0054; (D) nuclear membrane with no deoxycholate supplementation, 0.01 mg, phosphotransferase = 0.0022, phosphohydrolase = 0.0044; (E) microsomes with 0.2% deoxycholate supplementation, 0.20 mg, phosphotransferase = 0.066, phosphohydrolase = 0.042; (F) microsomes with no deoxycholate supplementation, 0.20 mg, phosphotransferase = 0.014, phosphohydrolase = 0.022.

Results and Discussion

Differential Effects of Cu^{2+} on Carbamoyl-P:Glucose Phosphotransferase and Glucose-6-P Phosphohydrolase. The modifying effects of varying concentrations of Cu^{2+} on carbamoyl-P:glucose phosphotransferase and glucose-6-P phosphohydrolase activities of partially purified microsomal suspensions are shown in Figure 1A. Cu^{2+}, included in the assay

TABLE I: The Effect of Cu^{2+} on the Ratio of Carbamoyl-P:Glucose Phosphotransferase to Glucose-6-P Phosphohydrolase.[a]

Cu^{2+} Concn (μM)	Phosphotransferase/phosphohydrolase					
	Partially purified prep	Microsomes		Nuclei	Nuclear	Membrane
		+DOC[b]	−DOC		+DOC[c]	−DOC
0	1.04	1.54	0.61	0.94	1.13	0.91
5	1.28	1.82	0.63	1.06	1.42	1.40
15	1.58	2.32	1.00	1.23	1.61	1.50
30	1.89	3.38	1.16	1.21	1.70	1.33

[a] Assay mixture pH 7.0, contained in 1.5 mL of 40 mM Hepes, 10 mM phosphate substrate, and 90 mM glucose (phosphotransferase only). Sufficient $CuCl_2$ was added to each assay mixture to achieve the indicated Cu^{2+} concentration. [b] 0.2% w/v. [c] 0.05% w/v.

mixtures (see the legend to Figure 1 for details) at the indicated concentrations, produced a differential, discriminant effect on the phosphotransferase and phosphohydrolase activities of glucose-6-phosphatase. The response of carbamoyl-P:glucose phosphotransferase to added Cu^{2+} was biphasic, with a small, but significant (16%) stimulation occurring at an optimum Cu^{2+} concentration of 5 μM and return to control values occurring at 30 μM Cu^{2+}. In contrast to the phosphotransferase, increasing Cu^{2+} concentrations progressively inhibited glucose-6-P phosphohydrolase with 30 μM Cu^{2+} causing 50% inhibition of this activity.

Although Cu^{2+} may have produced only a small stimulation with respect to carbamoyl-P:glucose phosphotransferase, the ratio of carbamoyl-P:glucose phosphotransferase to glucose-6-P phosphohydrolase was quite extensively altered by Cu^{2+} and may be the most significant indication of this ion's potential directive role in carbohydrate metabolism. Results in vertical columns 1 and 2 in Table I indicate that, as Cu^{2+} concentrations were increased progressively to 30 μM, the ratio of phosphotransferase to phosphohydrolase increased, also progressively, to a value 82% above control values for partially purified microsomal suspensions, indicating the greater sensitivity of glucose-6-P phosphohydrolase to Cu^{2+} inhibition.

The importance of interrelationships between the origin and conformation of membranes and the enzymic activity of membrane bound glucose-6-phosphatase–phosphotransferase has recently been established (Nordlie, 1974; Gunderson and Nordlie, 1975). For this reason, the differential effect of Cu^{2+} on carbamoyl-P:glucose phosphotransferase and glucose-6-P phosphohydrolase was studied in intact nuclei, nuclear membrane preparations, with and without deoxycholate supplementation, and microsomes, with and without deoxycholate supplementation. The results, shown in Figure 1B–F, indicate that the phosphotransferase of all preparations tested exhibited a biphasic response to Cu^{2+} while the phosphohydrolase was progressively inhibited. However, subtle differences were seen among the various preparations relating to the nature of the response of phosphotransferase and phosphohydrolase to Cu^{2+}. For instance, intact nuclei (Figure 1B) exhibited a broad optimum for Cu^{2+} stimulation of carbamoyl-P:glucose phosphotransferase, whereas the nuclear membrane preparations, with and without deoxycholate supplementation (Figure 1C and D, respectively) had a sharply defined optimum for the Cu^{2+} stimulation of this activity. Also, Cu^{2+} was a less potent inhibitor of glucose-6-P phosphohydrolase in intact nuclei than in nuclear membrane preparations. Differences were also noted between the responses of deoxycholate supplemented and unsupplemented microsomes to Cu^{2+} (Figure 1E and F, respectively). Although the phosphohydrolase of microsomes was inhibited to the same extent regardless of deoxycholate supplementation, the optimum Cu^{2+} concentration for stimulation of the phosphotransferase was lowered from 15 to 5 μM by supplementation with deoxycholate.

As shown in Table I (vertical columns 3–7), the ratio of phosphotransferase to phosphohydrolase from intact nuclei, nuclear membrane preparations, and microsomes also increased in the presence of added Cu^{2+}, similar to partially purified microsomal suspensions. However, the degree of increase over control values varied among the preparations. Cu^{2+} concentrations of 30 μM produced 119 and 90% increase in the ratio of phosphotransferase to phosphohydrolase in deoxycholate supplemented and unsupplemented microsomes, respectively, while increasing this ratio only 50 and 46% respectively in deoxycholate supplemented and unsupplemented nuclear membrane preparations and 30% in intact nuclei.

Even though minor differences occurred in the nature of the responses of carbamoyl-P:glucose phosphotransferase and glucose-6-P phosphohydrolase to added Cu^{2+}, the biphasic response of phosphotransferase, inhibition of phosphohydrolase, and increase in the ratio of phosphotransferase to phosphohydrolase by micromolar levels of Cu^{2+} in all the preparations tested indicate that the differential effect of Cu^{2+} on glucose-6-phosphatase–phosphotransferase is a general effect qualitatively and semiquantitatively independent of membrane integrity. The subtle differences outlined above do, however, emphasize the importance of membrane conformation in the regulation of this membrane bound enzyme.

Specficity of the Cu^{2+} Effect. The effects of low (0 to 30 μM) concentration of the chloride salts of Cd^{2+}, Cr^{3+}, Fe^{3+}, Zn^{2+}, Ni^{2+}, Fe^{2+}, and Mn^{2+} on carbamoyl-P:glucose phosphotransferase and glucose-6-P phosphohydrolase were studied using partially purified microsomal suspensions under experimental conditions identical to those used with Cu^{2+}. Of the ions tested, Ni^{2+}, Mn^{2+}, Cr^{3+}, and Fe^{3+} had no effect on either activity, while the effects of the other ions varied somewhat: Zn^{2+} inhibited both phosphotransferase (10% at 30 μM) and phosphohydrolase (23% at 30 μM), Fe^{2+} had no effect on phosphotransferase but inhibited phosphohydrolase (10% at 30 μM), and Cd^{2+} also had no effect on phosphotransferase yet inhibited phosphohydrolase modestly (20% at 30 μM). Thus, among the ions tested, Cu^{2+} was unique in causing a biphasic response in the micromolar range with respect to carbamoyl-P:glucose phosphotransferase and for this reason was the subject of a more detailed mechanistic study.

Kinetic Studies. Since micromolar levels of Cu^{2+} produced the described effects with millimolar substrate levels, it appeared unlikely that divalent cation–substrate complexation, as observed previously with 2–15 mM Mg^{2+} (Johnson et al., 1974), was involved. Hence, systematic kinetic studies using partially purified microsomal suspensions were performed at

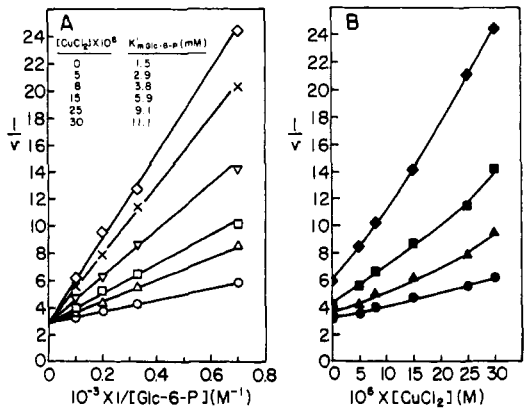

FIGURE 2: Kinetic plots of the inhibition by Cu^{2+} of glucose-6-P phosphohydrolase. Panel A is a plot of reciprocal initial velocity (v) against reciprocal of molar concentrations of D-glucose-6-P in the absence (○) and presence of 5 μM (△), 8 μM (□), 15 μM (▽), 25 μM (X), and 30 μM (◇) $CuCl_2$. Panel B is a plot of reciprocal of initial velocity against $CuCl_2$ concentration with D-glucose-6-P concentrations held constant at 10 mM (●), 5 mM (▲), 3 mM (■), and 1.43 mM (◆). In both A and B, v is expressed as μmol of P_i produced per 1.5 mL of reaction mixture per 10 min. Reaction mixtures in both A and B also contained, in 1.5 mL, 40 mM Hepes (pH 7.0) and 0.13 mg of protein (partially purified preparation).

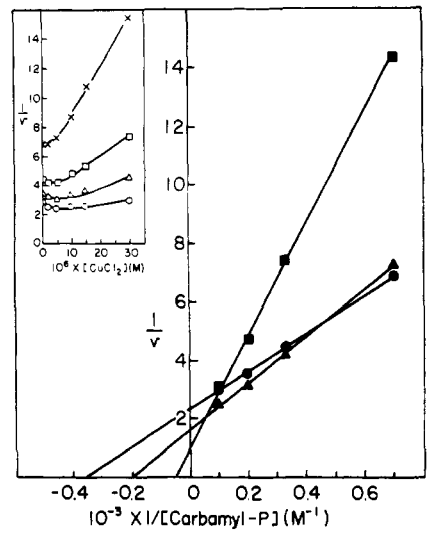

FIGURE 3: Kinetics of the effect of Cu^{2+} on carbamoyl-P:glucose phosphotransferase, studied with respect to varied carbamoyl-P concentration. Reciprocals of initial velocity (v) are plotted against the reciprocals of molar concentration of carbamoyl-P in the absence (●) and presence of 5 μM (▲) and 30 μM (■) $CuCl_2$. The insert shows a plot of the reciprocal of v against the concentration of $CuCl_2$ for 10 mM (○), 5 mM (△), 3 mM (□), and 1.43 mM (X) carbamoyl-P. Reaction mixtures also contained, in 1.5 mL, 90 mM D-glucose, 40 mM Hepes (pH 7.0), and 0.13 mg of protein (partially purified preparation). Initial velocity is expressed as μmol of glucose-6-P produced per 1.5 mL of reaction mixture per 10 min.

pH 7.0 to determine the mechanistic nature of the effects Cu^{2+} exerts on phosphohydrolase and phosphotransferase.

Glucose-6-phosphohydrolase. The results of kinetic studies of the inhibition of glucose-6-P phosphohydrolase by Cu^{2+} are shown in Figure 2. When the data were plotted in conventional double-reciprocal fashion (Figure 2A) using several fixed concentrations of Cu^{2+}, a linear pattern of inhibition was obtained that was competitive with respect to glucose-6-P, with V_{maxHyd}[1] = 0.036 μmol of P_i produced per 1.5 mL of reaction mixture per min. Also, the Michaelis constant value for glucose-6-P (K_{mG6P}) was significantly and progressively increased from 1.5 mM in the absence of Cu^{2+} to 11.1 mM in the presence of 30 μM Cu^{2+}. This phenomenon was further analyzed by plotting the reciprocals of initial velocity against Cu^{2+} concentrations at several fixed glucose-6-P concentrations to ascertain whether the competitive inhibition of the phosphohydrolase by Cu^{2+} was linear or nonlinear (Cleland, 1970). Data plotted in this manner (Figure 2B) were nonlinear with the deviation from linearity increasing with decreasing glucose-6-P concentrations.

Curvilinearity in the plot of $1/v$ against Cu^{2+} concentration is suggestive of two possible types of interactions which could lead to inhibition of the phosphohydrolase. The first of these is that Cu^{2+} may react with glucose-6-P to form an inactive Cu-glucose-6-P complex (Webb, 1963). However, this possibility can be discounted since inhibition in this case would only occur if the concentration of Cu^{2+} were of the same order of magnitude as that of glucose-6-P (Dixon and Webb, 1964b), and, under the experimental conditions of these studies, inhibition occurred even though Cu^{2+} concentrations were $^1/_{1000}$ of those of glucose-6-P. Also, if an inactive complex were formed between Cu^{2+} and glucose-6-P, the plots of $1/v$ against 1/glucose-6-P concentration would be nonlinear in the presence of Cu^{2+} (Webb, 1963), which was not the case in these studies (Figure 2A). The second, and more likely explanation for curvilinear plots of $1/v$ against Cu^{2+} concentration is the interaction of Cu^{2+} with multiple, reversibly connected forms of glucose-6-phosphatase (see Cleland, 1963).

Carbamoyl-P:Glucose Phosphotransferase. The results of similar kinetic studies on the effects of Cu^{2+} on carbamoyl-P:glucose phosphotransferase are given in Figures 3 and 4. Figure 3 shows as double-reciprocal plots the effects of Cu^{2+} with carbamoyl-P as the variable substrate with glucose held constant at 90 mM. In contrast to the phosphohydrolase, in which Cu^{2+} displayed a competitive pattern with respect to phosphate substrate (Figure 2A), *both* the slope and intercept (not just the slope; Figure 2A) were affected by added micromolar levels of Cu^{2+} and intersection was obtained in the first quadrant. The phosphotransferase activity was stimulated by 5 μM Cu^{2+} at concentrations of carbamoyl-P greater than 2.2 mM but was inhibited by this level of Cu^{2+} with carbamoyl-P levels less than 2.2 mM. The apparent maximal velocity (V'_{maxTrf}) in the presence of 5 μM Cu^{2+} was 0.062 and in the absence of Cu^{2+} was 0.044 μmol of glucose-6-P formed per min per 1.5 mL of reaction mixture. And although 5 μM Cu^{2+} stimulated phosphotransferase, this level of Cu^{2+} increased the apparent Michaelis constant for carbamoyl-P (K'_{mCP})

[1] Definitions: K_{mG6P}, the Michaelis constant for glucose-6-P [that concentration of glucose-6-P for which the velocity of the glucose-6-P phosphohydrolase reaction is one-half the maximal velocity (V_{maxHyd})]; K'_{mCP} and K'_{mGlc}, the apparent Michaelis constants for carbamoyl-P and glucose [that concentration of carbamoyl-P or glucose for which the velocity of the carbamoyl-P:glucose phosphotransferase reaction is one-half the apparent maximal velocity for infinite carbamoyl-P concentration and finite glucose concentration (V'_{maxTrf}) or one-half the apparent maximal velocity for infinite glucose concentration and finite carbamoyl-P concentration (V''_{maxTrf})]; K_{mGlc} and K_{mCP} are the Michaelis constants for glucose and carbamoyl-P [the concentration of glucose and carbamoyl-P for which the velocity of the carbamoyl-P:glucose phosphotransferase reaction is one-half the maximal reaction velocity for infinite levels of both substrates (V_{maxTrf})]. The partially purified preparation used in all kinetic experiments had a specific activity of 0.24 unit/mg protein, where 1 unit of enzyme activity is that amount catalyzing the hydrolysis of 1 μmol of glucose-6-P per 1.5 mL of reaction mixture per min at pH 7.0 and 10 mM glucose-6-P; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid; TKM, 0.05 M Tris-HCl (pH 7.5)-0.025 M KCl-0.005 M $MgCl_2$; Tris, tris(hydroxymethyl)aminomethane.

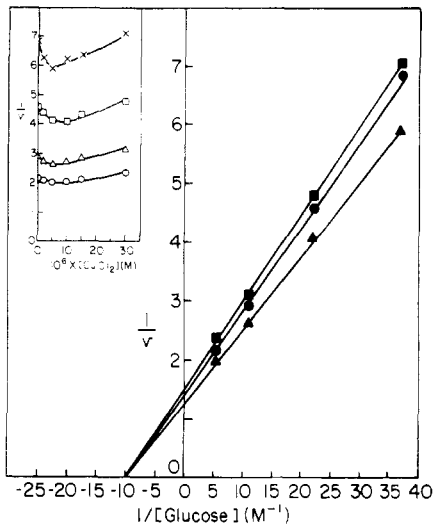

FIGURE 4: Kinetics of the effect of Cu^{2+} on carbamoyl-P:glucose phosphotransferase, studied with respect to varied glucose concentration. Reciprocals of initial velocity (v) against the reciprocal of molar concentration of D-glucose in the absence (●) and presence of 5 μM (▲) and 30 μM (■) $CuCl_2$. The insert shows a plot of the reciprocal of v against concentration of $CuCl_2$ for 180 mM (○), 90 mM (△), 45 mM (□), and 27 mM (X) D-glucose. Reaction mixtures also contained, in 1.5 mL, 10 mM carbamoyl-P, 40 mM Hepes (pH 7.0), and 0.13 mg of protein (partially purified preparation). Initial velocity is expressed as in Figure 3.

from 2.8 to 5 mM. Higher levels of Cu^{2+} (30 μM) inhibited transferase with carbamoyl-P levels below 10 mM, stimulated activity with carbamoyl-P concentrations above 10 mM, increased V'_{maxTrf} more than twofold (from 0.044 to 0.10 μmol of glucose-6-P formed per min per 1.5 mL of reaction mixture), and increased K'_{mCP} to 20 mM. As shown in the inset to Figure 3, the effects of Cu^{2+} on carbamoyl-P:glucose phosphotransferase were nonlinear for several concentrations of carbamoyl-P with glucose fixed at 90 mM when $1/v$ was plotted against Cu^{2+} concentration (Dixon, 1953).

With glucose as the variable substrate and carbamoyl-P fixed at 10 mM, Cu^{2+} produced a noncompetitive pattern in which both the slope and ordinate intercept of the double-reciprocal plots were affected by added Cu^{2+} (Figure 4). Whereas the apparent maximal velocity was increased by both 5 and 30 μM Cu^{2+} with carbamoyl-P as the variable substrate (Figure 3), the apparent maximal velocity (V'''_{maxTrf}) was *either increased* in the presence of 5 μM Cu^{2+} *or decreased* in the presence of 30 μM Cu^{2+} (maximal velocities expressed as μmol of glucose-6-P formed per min per 1.5 mL of reaction mixture were 0.072 with no added $CuCl_2$, 0.081 with 5 μM $CuCl_2$ added, and 0.068 with 30 μM $CuCl_2$ added). The apparent Michaelis constant for glucose (K'_{mGlc}) was 105 mM and remained unchanged in the presence of Cu^{2+}. Plots of $1/v$ against $CuCl_2$ concentration (inset Figure 4) again indicate that the effect of Cu^{2+} on carbamoyl-P:glucose phosphotransferase is nonlinear for the several tested concentrations of glucose with carbamoyl-P held constant at 10 mM.

Since the apparent Michaelis constant for one substrate involved in the phosphotransferase reaction is dependent on the concentration of the second substrate (Lueck et al., 1972), the effects of low (5 μM), activity enhancing levels of Cu^{2+} on the kinetic parameters for phosphotransferase were determined for finite levels of one substrate and an infinite level of the second. Primary plots (Florini and Vestling, 1957) of $1/v$ against 1/carbamoyl-P concentration, with glucose at several fixed levels, and $1/v$ against 1/glucose concentration, with carbamoyl-P at several fixed levels, were constructed from data obtained in the absence and presence of 5 μM Cu^{2+} (data not shown). Extrapolations of these primary plots converged at a common point below the x axis (i.e., in the third quadrant), consistent with previous observations (Lueck et al., 1972). The "complex constant" ($K_{CP:Glc}$), calculated from the x-coordinate values of the points of convergence of the primary plots, was increased by 5 μM Cu^{2+} to 312 mM^2 from a value of 250 mM^2. Apparent maximal velocities obtained from the y-axis intercepts of the primary plots were then used to construct secondary plots (Florini and Vestling, 1957) of reciprocals of apparent maximal velocity against reciprocal glucose concentration, with carbamoyl-P infinite, or reciprocal carbamoyl-P concentration, with glucose infinite (data not shown). Comparison of secondary plots representing data in the presence and absence of 5 μM Cu^{2+} indicated that, with glucose at an infinite level, 5 μM Cu^{2+} increased the Michaelis constant for carbamoyl-P (K_{mCP}) from 5.2 to 9.0 mM. However, with an infinite level of carbamoyl-P, this concentration of Cu^{2+} had no effect on the Michaelis constant for glucose (K_{mGlc}), which remained at 125 mM. The maximal reaction velocity of phosphotransferase (V_{maxTrf}), measured at infinite levels of both substrates, was increased by 5 μM Cu^{2+} to 0.125 from a value of 0.100 μmol of glucose-6-P formed per min per 1.5 mL of reaction mixture.

Activities with Mannose-6-P. Results of these studies (Table II) indicate that Cu^{2+}-induced modifications are not unique to carbamoyl-P:glucose phosphotransferase and glucose-6-P phosphohydrolase activities of the enzyme. As with phosphotransferase with carbamoyl-P, mannose-6-P:glucose phosphotransferase was affected differentially by Cu^{2+}. Low concentrations of Cu^{2+} activated when mannose-6-P level was high; the activation was abolished and inhibition ensued when Cu^{2+} levels were increased and/or mannose-6-P levels were reduced. Mannose-6-P phosphohydrolase, like glucose-6-P phosphohydrolase, was inhibited by all levels of Cu^{2+} with no stimulation in evidence. Dixon plots (Dixon, 1953) of these data (not shown) were curvilinear and concave upward, as with the earlier studied reactions (Figure 2B and insets to Figures 3 and 4).

Mechanistic Considerations. Any mechanism proposed to describe the effects of Cu^{2+} must be congruous with the kinetic mechanism already established for phosphotransferase and phosphohydrolase activities of glucose-6-phosphatase (Lueck et al., 1972; Arion and Nordlie, 1964; Hass and Byrne, 1960; Segal, 1959). Further, the mechanism must reconcile the following experimental observations reported here: (a) Cu^{2+} exerts differential, discriminant effects on glucose-6-phosphatase–phosphotransferase, enhancing carbamoyl-P:glucose phosphotransferase activity at low Cu^{2+} levels, inhibiting the same activity at higher levels, and inhibiting glucose-6-P phosphohydrolase activity at all Cu^{2+} concentrations (see Figure 1); (b) Cu^{2+} increases both the maximum velocity for phosphotransferase and the Michaelis constant values for carbamoyl-P at both finite and infinite levels of glucose (see Figure 3 and above text), but has no effect on K_{mGlc} (see above text); (c) Cu^{2+} exhibits a competitive pattern of inhibition with respect to glucose-6-P in the phosphohydrolase reaction, with resultant increase in K_{mG6P} and no change in V_{maxHyd} (Figure 2A); and (d) plots of reciprocals of v against Cu^{2+} concentration are curvilinear for both hydrolase and transferase (Figure 2B, and inserts to Figures 3 and 4).

Such a mechanism is shown in Figure 5 where reactions 1–5 constitute the kinetic mechanism previously proposed to describe uninhibited activities of the multifunctional enzyme

TABLE II: Effects of Cu^{2+} on Mannose-6-P:Glucose Phosphotransferase and Mannose-6-P Phosphohydrolase.[a]

$CuCl_2$ concn (μM)	Mannose-6-P:Glucose phosphotransferase Mannose-6-P concn		Mannose-6-P phosphohydrolase Mannose-6-P concn		Phosphotransferase / Phosphohydrolase Mannose-6-P concn	
	10 mM	2 mM	10 mM	2 mM	10 mM	2 mM
0	0.241 (1.00)	0.130 (1.00)	0.272 (1.00)	1.172 (1.00)	0.89	0.76
2	0.257 (1.06)	0.126 (0.96)	0.269 (0.98)	0.156 (0.91)	0.95	0.81
5	0.236 (0.97)	0.109 (0.83)	0.238 (0.87)	0.126 (0.73)	0.99	0.86
10	0.207 (0.85)	0.073 (0.56)	0.216 (0.79)	0.089 (0.51)	0.96	0.82
20	0.161 (0.66)	0.050 (0.38)	0.164 (0.60)	0.053 (0.31)	0.98	0.94

[a] Assay mixtures, pH 7.0, contained in 1.5 mL of 40 mM Hepes buffer, 10 mM mannose-6-P, 0.20 mg of partially purified enzyme preparation, 180 mM D-glucose (phosphotransferase only), and indicated levels of $CuCl_2$. Activity is expressed as μmol of glucose-6-P formed (phosphotransferase) or μmol of mannose-6-P hydrolyzed (phosphohydrolase) per 10 min. Values in parentheses are relative activities based on a presumed value of 1.00 with each vertical series in the absence of Cu^{2+}.

(Lueck et al., 1972; Arion and Nordlie, 1964). Basically, this mechanism involves initial interaction of enzyme alternatively with phosphate substrates RP and glucose-6-P leading to binary enzyme–phosphoryl substrate complexes (reactions 1 and 4, Figure 5), dissociation of these complexes to produce phosphoryl enzyme (reactions 2 and 3, Figure 5), and transfer of this phosphoryl group alternatively to water (phosphohydrolase, reaction 5) or to glucose (or other phosphoryl acceptor) (reverse of reaction 3, Figure 5) followed by release of glucose-6-P from the enzyme (reverse of reaction 4, Figure 5) to complete the phosphotransferase process.

The possibility of inhibition simply through interaction of Cu^{2+} with phosphoryl substrates has been eliminated on the basis of comparative levels of inhibitor (μM) and substrate (mM) (see above). Further, inhibition through binding of Cu^{2+} to a single form of the enzyme is contraindicated by curvilinearity of Dixon plots (Figure 2B, inserts 3 and 4).

A mechanism involving Cu^{2+} acting as a modifier capable of combining *either* with free enzyme *or* with the enzyme–phosphoryl substrate complex is, however, consistent with all the data presented here and reported earlier, and is included in Figure 5 as reactions 6, 7, and 8. Combination of Cu^{2+} with free enzyme at a modifier site (reaction 8, Figure 5) is postulated to yield an enzyme–Cu complex that is much less reactive with phosphoryl substrates than is the free enzyme and, therefore, Cu^{2+} reduces the affinity of glucose-6-phosphatase for phosphoryl substrates as observed experimentally (see Figures 2B and 3, and above text). Cu^{2+} also is postulated to interact with the enzyme–phosphoryl substrate complexes causing thereby a dissociation of these complexes to yield the enzyme–Cu complex and phosphoryl substrate (reactions 6 and 7, Figure 5). Because glucose-6-P is a product of phosphotransferase reactions, this latter displacement from the enzyme of product would tend to *enhance* the rate of phosphotransferase, as experimentally observed at low Cu^{2+} levels (Figures 1, 3, and 4 and above text). The net effect of added Cu^{2+} on phosphotransferase would then be determined as the resultant difference between enhancement of activity through Cu^{2+}-promoted displacement of glucose-6-P (reaction 6, Figure 5) and inhibition caused by Cu^{2+} lowering the level of free enzyme available to carbamoyl-P in the initial steps (reactions 1, 7, and 8, Figure 5). Because glucose-6-P is both a substrate and a product for the several activities of the enzyme, activity-discriminant, differential responses to Cu^{2+} ensue. Specific correlations of this mechanism with experimental data for glucose-6-P phosphohydrolase, carbamoyl-P:glucose phosphotransferase, and activities with mannose-6-P are made in turn below.

Glucose-6-P Phosphohydrolase. Because the free enzyme, enzyme–Cu complex, and the enzyme–glucose-6-P complex are all reversibly connected, inhibition through the interaction of Cu^{2+} with free enzyme or the enzyme–glucose-6-P complex would increase the slope of the double-reciprocal plot as shown in Figure 2A (see Cleland, 1963). When glucose-6-P is saturating, the enzyme–Cu complex would not be formed from free enzyme and Cu^{2+} since saturation by glucose-6-P would favor formation of the binary enzyme–glucose-6-P complex (reaction 4, Figure 5). Furthermore, assuming $k_{11} \gg k_{12}$, the formation of the enzyme–Cu complex through the interaction of Cu^{2+} with the enzyme–glucose-6-P complex, at saturating levels of glucose-6-P, would have no net effect on the level of the enzyme–glucose-6-P complex because the enzyme–Cu complex would not achieve kinetically significant levels. Therefore, the inhibition of glucose-6-P phosphohydrolase by Cu^{2+} would be reversed by an infinite glucose-6-P level as shown in Figure 2A. Assuming that the affinity of the enzyme–glucose-6-P complex for Cu^{2+} (reaction 6, Figure 5) is greater than that of free enzyme for Cu^{2+} (reaction 8, Figure 5), low concentrations of Cu^{2+} would favor the formation of the enzyme–Cu complex via the interaction of the enzyme–glucose-6-P complex with Cu^{2+} (reaction 6, Figure 5). At nonsaturating concentrations of glucose-6-P, this would lower the level of the enzyme–glucose-6-P complex and also the level of free enzyme, causing inhibition of the phosphohydrolase. At higher Cu^{2+} concentrations, the formation of the enzyme–Cu complex would also occur through the interaction of Cu^{2+} and free enzyme (reaction 8, Figure 5), which, at nonsaturating levels of glucose-6-P, would cause inhibition by channeling free enzyme into the inactive enzyme–Cu complex. Therefore, since Cu^{2+} combines at two reversibly connected points in the reaction sequence and causes inhibition at both points, Cu^{2+} inhibition of glucose-6-P phosphohydrolase would be nonlinear (see Cleland, 1963) as illustrated in Figure 2B.

Steady-state rate equations describing the mechanism shown in Figure 6 were derived according to the method of Fromm (1970), assuming $k_{13} \ll k_{12}$ and $k_{15} \ll k_{14}$ so that the affinity

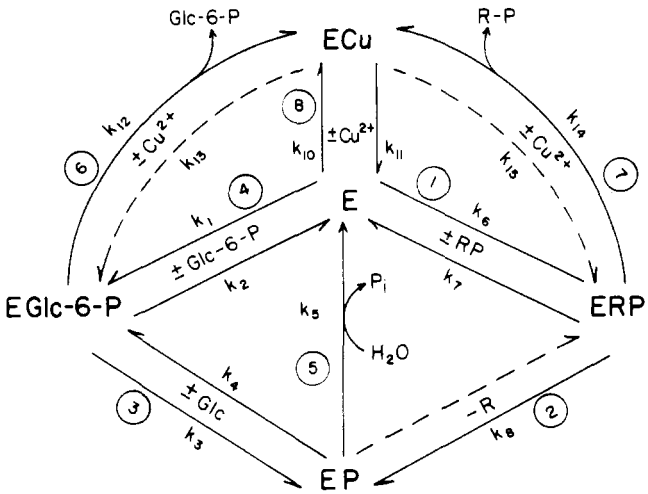

FIGURE 5: Proposed reaction mechanism describing the effects of Cu^{2+} on glucose-6-phosphatase-phosphotransferase. E denotes enzyme, EP represents the phosphoryl-enzyme intermediate, and RP is carbamoyl-P (or other phosphoryl donor). When high energy compounds such as carbamoyl-P are used as substrate, the reaction step between ERP and EP is irreversible. This step is reversible for low energy phosphoryl donors such as mannose-6-P. Individual reactions are numbered (encircled) for ease of reference in the text.

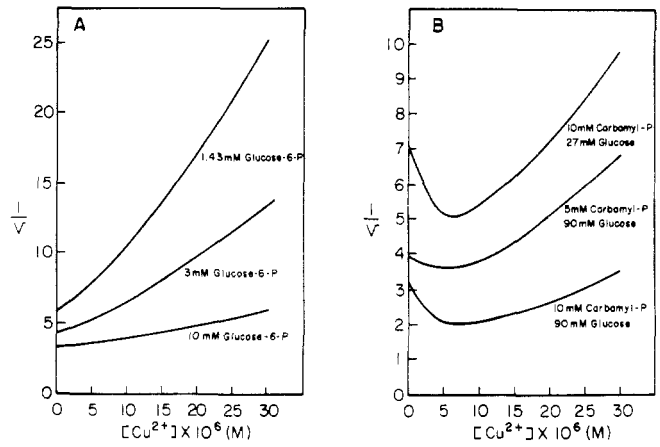

FIGURE 6: Simulation of glucose-6-P phosphohydrolase (A) and carbamoyl-P:glucose phosphotransferase (B) activities in the presence of varied levels of Cu^{2+}. Calculations were performed using rate eq 3 and 4 of the Appendix with values of the kinetic constants used in the calculations based on criteria from the text (see Mechanistic Considerations). These values were $K_{13} = 1.6 \times 10^5\ M^{-1}$, $K_{14} = 2 \times 10^9\ M^{-2}$, $K_{15} \approx 0$, $K_1 = 1.1 \times 10^7\ M^{-1}$, $K_2 = 3 \times 10^{12}\ M^{-2}$, $K_3 = 3.8 \times 10^6\ M^{-1}$, $K_4 = 1.1 \times 10^{11}\ M^{-2}$, $K_5 = 2 \times 10^7\ M^{-1}$, $K_6 = 9 \times 10^{11}\ M^{-2}$, $K_7 = 1 \times 10^{17}\ M^{-3}$, $K_8 = 3.8 \times 10^6\ M^{-1}$, $K_9 = 1.1 \times 10^{11}\ M^{-2}$, $K_{10} = 5 \times 10^7\ M^{-1}$, $K_{11} = 6 \times 10^{12}\ M^{-2}$, $K_{12} = 8 \times 10^{17}\ M^{-3}$. Other kinetic constants were (see text and Figure 2A): $V_{max:Trf} = 1.00$ μmol of glucose-6-P formed per 10 min per 1.5 mL reaction mixture, $K_{mCP} = 5$ mM, $K_{mGlc} = 125$ mM, $K_{CP:Glc} = 250\ (mM)^2$, $V_{max:Hyd} = 0.36$ μmol of P_i formed per 10 min per 1.5 mL reaction mixture, and $K_{mG6P} = 1.5$ mM. Concentration of glucose-6-P, carbamoyl-P, and glucose used in the simulation were as indicated in the figure.

of the enzyme–Cu complex for substrate is very low. Equation 3, shown in the Appendix, describes the behavior of glucose-6-P phosphohydrolase in the presence and absence of Cu^{2+}. This equation is consistent with data shown in Figure 2 in that the value for K_{mG6P} is increased by Cu^{2+}, V_{maxHyd} is not affected by Cu^{2+} assuming (see above) $k_{11} \gg k_{12}$, and the reciprocal of initial velocity is a nonlinear function of Cu^{2+}. The complexity of this mechanism prevents the evaluation of inhibitor constants for Cu^{2+} for either phosphohydrolase or phosphotransferase.

Carbamoyl-P:Glucose Phosphotransferase. A similar analysis may be applied to the reaction sequence for carbamoyl-P:glucose phosphotransferase. Cu^{2+} combines with both free enzyme (reaction 8, Figure 5) and the enzyme–carbamoyl-P complex which is reversibly connected to free enzyme (reaction 7, Figure 5). Therefore, as seen in Figures 3 and 5A, the slope of the double-reciprocal plot with carbamoyl-P as the variable substrate would be increased and the affinity of enzyme for substrate would be decreased by Cu^{2+} because the formation of the enzyme–Cu complex via either free enzyme or the enzyme–carbamoyl-P complex lowers the level of free enzyme (Cleland, 1963).

Although the Cu^{2+} inhibition of the phosphotransferase would be reversed by saturating concentrations of carbamoyl-P, the maximal velocity would be increased. When carbamoyl-P is saturating, formation of the enzyme–Cu complex through the Cu^{2+}-promoted dissociation of the enzyme–carbamoyl-P (reaction 7, Figure 5) complex is insignificant because of the reversible steps connecting these two complexes with free enzyme. With saturating levels of carbamoyl-P, then, the production of glucose-6-P in the presence of Cu^{2+} would occur by two paths: the normal dissociation of the enzyme–glucose-6-P complex, governed by k_2 (reaction 4, Figure 5); and the Cu^{2+}-promoted dissociation of the enzyme–glucose-6-P complex, governed by k_{12} (reaction 6, Figure 5). At low Cu^{2+} concentrations and levels of carbamoyl-P sufficient to prevent the interaction of Cu^{2+} with free enzyme, as shown in Figure 3, the enzyme–Cu complex would be formed primarily through the interaction of Cu^{2+} with the enzyme–glucose-6-P complex with the consequential production of glucose-6-P (reaction 6, Figure 5). The observed stimulation of carbamoyl-P:glucose phosphotransferase, therefore, is a result of this interaction with $k_{12} > k_2$. As the Cu^{2+} concentration is increased (substrate levels remaining constant), the formation of the inactive enzyme–Cu complex through the interaction of Cu^{2+} and free enzyme, competitive with carbamoyl-P (reactions 8 and 1, Figure 5), becomes more significant, inhibition predominates over stimulation, and net inhibition of the phosphotransferase occurs. This dual enhancement-inhibition effect of Cu^{2+} on phosphotransferase results in the nonlinearity in the plots of reciprocal velocity against Cu^{2+} concentration. (See insets of Figures 3 and 4.)

The rate equation describing the effect of Cu^{2+} on carbamoyl-P:glucose phosphotransferase (eq 4 in the Appendix) is more complex than the one describing the activity of glucose-6-P phosphohydrolase in the presence of Cu^{2+}, but it is consistent with the data shown in Figures 3 and 4, and as described in the text. In the presence of Cu^{2+}, the maximal reaction velocity (V_{maxTrf}), Michaelis constant for carbamoyl-P (K_{mCP}), Michaelis constant for glucose (K_{mGlc}), and complex constant ($K_{CP:Glc}$) all are multiplied by factors (i.e., values in brackets in eq 4) whose numerators and denominators contain Cu^{2+} concentration terms, raised to the first, second, and third powers. Experimental increases in maximal reaction velocity values, Michaelis constant values for carbamoyl-P, and the values of the "complex constant" in the presence of Cu^{2+} and this ion's lack of effect on the Michaelis constant values for glucose (see Figures 3 and 4, and the above text) imply that the following relationships occur between kinetic constants: $[1 + K_1(Cu) + k_2(Cu)^2]$, $[1 + K_5(Cu) + K_6(Cu)^2 + K_7(Cu)^3]$, $[1 + K_{10}(Cu) + K_{11}(Cu)^2 + K_{12}(Cu)^3] > [1 + K_3(Cu) + K_4(Cu)^2]$; $[1 + K_3(Cu) + K_4(Cu)^2] = [1 + K_8(Cu) + K_9(Cu)^2]$; and $[1 + K_1(Cu) + K_2(Cu)^2] > [1 + K_3(Cu) + K_4(Cu)^2 + (K_{mGlc}/(Glc))(1 + K_8(Cu) + K_9(Cu)^2)]$. These

relationships between the kinetic constants were used in the simulation described below and in the legend to Figure 6. Furthermore, the reciprocal of initial velocity would be a nonlinear function of Cu^{2+} concentration, but Lineweaver–Burk plots would be linear as shown in Figures 3 and 4.

Simulation Studies. To further test the validity of the proposed mechanism, data were simulated for several concentrations of phosphoryl substrates and glucose using rate equations (eq 3 and 4 in the Appendix) derived for phosphohydrolase and phosphotransferase on the basis of the mechanism in Figure 5. As shown in Figure 6, the curves generated from these equations (using the kinetic constants stated in the legend to Figure 6, which satisfy the relationships discussed above) are qualitatively similar to experimental curves obtained using partially purified microsomal suspensions (Figure 2B and insets in Figures 3 and 4). Both the biphasic response of carbamoyl-P:glucose phosphotransferase to Cu^{2+} and the progressive inhibition of glucose-6-P phosphohydrolase by increasing Cu^{2+} levels are congruous with the derived rate equations.

Possible Physiological Significance of Cu^{2+} Effects. Copper content in adult rat liver has been reported as 4.84 μg/g wet liver (Gregoriadis and Sourkes, 1967). Assuming homogenous distribution in hepatic cell water, which is presumed to constitute 70% of the total wet liver weight, a concentration of 110 μM is obtained. Whether liver Cu^{2+} is bound is unknown. However, in human serum, 90% of Cu^{2+} is bound (Henkin, 1971). If a similar amount of Cu^{2+} is bound in the cell, the experimental range of free Cu^{2+} to which glucose-6-phosphatase is sensitive may well be within the physiological range of unbound cellular Cu^{2+}.

Possibly, Cu^{2+} may play a directive role in carbohydrate metabolism, as suggested by data presented above. Inhibition by Cu^{2+} of glucose-6-P hydrolysis would tend to lower rates of hepatic glycogenolysis, gluconeogenesis, and net release of glucose into the blood. Such a mechanism is consistent with reports in the literature wherein are described a lowering of blood glucose (Ussolzew, 1935) and an increase in liver glycogen (Voinar and Galakhova, 1962) following injection of copper salt solutions; a reduction in hepatic copper content concomitant with an increase in blood glucose in alloxan diabetes (Kovtunyak and Tsapok, 1973); and both a decrease in blood sugar level and an increase in liver glycogen content, along with an increase in hepatic copper, following blockage of thyroid function with 6-methylthiouracil (Kovtunyak et al., 1970). Possibly, Cu^{2+}-effected alterations in the ratio of transferase:hydrolase activity of glucose-6-phosphatase described here might also be of further relevance in this regard. These concepts, speculative at present, are in need of further experimental examination.

Appendix

Steady-state rate equations derived according to the method of Fromm (1970) describing the effect of Cu^{2+} on glucose-6-P phosphohydrolase and carbamoyl-P:glucose phosphotransferase are shown below.

$$\frac{1}{v} = \frac{1}{V_{\text{maxHyd}}} \times \left\{\frac{K_{\text{mG6P}}}{(\text{G6P})}[1 + K_{13}(\text{Cu}) + K_{14}(\text{Cu})^2] + K_{15}(\text{Cu}) + 1\right\} \quad (3)$$

where

$$K_{13} = \frac{k_{10}(k_2 + k_3) + k_{11}k_{12}}{k_{11}(k_2 + k_3)}$$

$$K_{14} = \frac{k_{10}k_{12}}{k_{11}(k_2 + k_3)}$$

$$K_{15} = \frac{k_{12}k_5(\text{H}_2\text{O})}{k_{11}[k_3 + k_5(\text{H}_2\text{O})]}$$

$$K_{\text{mG6P}} = \frac{k_5(\text{H}_2\text{O})(k_2 + k_3)}{k_1[k_5(\text{H}_2\text{O}) + k_3]}$$

$$V_{\text{maxHyd}} = \frac{k_3k_5(\text{H}_2\text{O})E_0}{k_3 + k_5(\text{H}_2\text{O})}$$

The subscript Hyd indicates the glucose-6-P phosphohydrolase reaction.

$$\frac{1}{v} = \frac{1}{V_{\text{maxTrf}}}\left[\frac{1 + K_3(\text{Cu}) + K_4(\text{Cu})^2}{1 + K_1(\text{Cu}) + K_2(\text{Cu})^2}\right] \times \left\{1 + \frac{K_{\text{mCP}}}{(\text{CP})}\left[\frac{1 + K_5(\text{Cu}) + K_6(\text{Cu})^2 + K_7(\text{Cu})^3}{1 + K_3(\text{Cu}) + K_4(\text{Cu})^2}\right] + \frac{K_{\text{mGlc}}}{(\text{Glc})}\left[\frac{1 + K_8(\text{Cu}) + K_9(\text{Cu})^2}{1 + K_3(\text{Cu}) + K_4(\text{Cu})^2}\right] + \frac{K_{\text{CP:Glc}}}{(\text{CP})(\text{Glc})}\left[\frac{1 + K_{10}(\text{Cu}) + K_{11}(\text{Cu})^2 + K_{12}(\text{Cu})^3}{1 + K_3(\text{Cu}) + K_4(\text{Cu})^2}\right]\right\} \quad (4)$$

where

$$K_1 = \frac{k_2k_{14} + k_8k_{12}}{k_2 + k_8}$$

$$K_2 = \frac{k_{14}k_{12}}{k_2k_8}$$

$$K_3 = \frac{k_{12}(k_8 + k_{11}) + k_{14}(k_2 + k_6)}{k_{11}(k_2 + k_8)}$$

$$K_4 = \frac{k_{12}k_{14}}{k_{11}(k_2 + k_8)}$$

$$K_5 = \frac{(k_7 + k_8)(k_{10}k_2 + k_{11}k_{12}) + k_2k_{14}k_{11}}{k_{11}k_2(k_7 + k_8)}$$

$$K_6 = \frac{k_{10}k_{12}(k_7 + k_8) + k_{14}(k_{10}k_2 + k_{11}k_{12})}{k_{11}k_2(k_7 + k_8)}$$

$$K_7 = \frac{k_{10}k_{12}k_{14}}{k_{11}k_2(k_7 + k_8)}$$

$$K_8 = \frac{k_{11}k_{12}[k_8 + k_5(\text{H}_2\text{O})] + k_5(\text{H}_2\text{O})[k_{14}(k_2 + k_3) + k_8k_2] + k_{11}k_3k_{14}}{k_{11}(k_2 + k_3)[k_8 + k_5(\text{H}_2\text{O})]}$$

$$K_9 = \frac{k_{12}k_{14}k_5(\text{H}_2\text{O})}{k_{11}(k_2 + k_3)[k_8 + k_5(\text{H}_2\text{O})]}$$

$$K_{10} = \frac{k_{10}(k_7 + k_8)(k_2 + k_3) + k_{14}k_{11}(k_2 + k_3) + k_{12}k_{11}(k_7 + k_8)}{k_{11}(k_7 + k_8)(k_2 + k_3)}$$

$$K_{11} = \frac{k_{10}k_{12}(k_7 + k_8) + k_{10}k_{14}(k_2 + k_3) + k_{11}k_{12}k_{14}}{k_{11}(k_7 + k_8)(k_2 + k_3)}$$

$$K_{12} = \frac{k_{10}k_{12}k_{14}}{k_{11}(k_7 + k_8)(k_2 + k_3)}$$

$$V_{\text{maxTrf}} = \frac{k_2k_8E_0}{k_2 + k_8}$$

$$K'_{\text{mCP}} = \frac{k_2(k_7 + k_8)}{k_6(k_2 + k_8)}$$

$$K_{\text{mGlc}} = \frac{(k_2 + k_3)[k_8 + k_5(H_2O)]}{k_4(k_2 + k_8)}$$

$$K_{\text{CP:Glc}} = \frac{k_5(H_2O)(k_2 + k_3)(k_7 + k_8)}{k_4k_6(k_2 + k_8)}$$

The subscript Trf indicates the carbamoyl-P:glucose phosphotransferase reaction.

K_{mG6P}, K_{mCP}, K_{mGlc}, and $K_{\text{Glc:CP}}$ represent the Michaelis constants for glucose-6-P, carbamoyl-P, glucose, and the complex constant for the phosphotransferase reaction, respectively. These constants, as well as V_{maxHyd} and V_{maxTrf}, in terms of rate constants as derived in this study are identical with those previously defined (Lueck et al., 1972).

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