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THE NATURE OF MODIFICATIONS BY VARIOUS ANIONS OF SYNTHETIC AND HYDROLYTIC ACTIVITIES OF MULTIFUNCTIONAL GLUCOSE-6-PHOSPHATASE

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SUMMARY

The in vitro effects of 13 inorganic and organic salts on Glc-6-*P* phosphohydrolase, carbamyl-*P*:glucose phosphotransferase, and PP_i:glucose phosphotransferase of multifunctional D-Glc-6-*P* phosphohydrolase (EC 3.1.3.9) have been studied systematically.

Identical concentrations of NaCl, KCl, LiCl and CsCl were equally effective as inhibitors, when studied with individual activities.

In marked contrast, when the cation (Na^+) was maintained constant and modifying affects of salts studied as a function of varied anion (Cl^-, acetate, cacodylate, *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonate, I^-, NO_2^-, NO_3^-, HSO_3^-, SO_4^{2-} and F^-), a heterogeneity of effects—various degrees of inhibition, activation, or no change —were noted with equivalent salt concentrations.

These same general patterns of response were noted at pH 6.0 as well as pH 7.0.

NaCl was found to function as an effective inhibitor competitive with respect to phosphate substrates in both phosphohydrolase and phosphotransferase reactions. K_i = 70 mM with both activities at pH 7.0.

It is concluded from these findings, and additional earlier observations described in the literature, that these alterations in activity levels are manifest through the action of anions, rather than by monovalent cations or generalized ionic strength effects. Further, it is suggested that many such anions may compete with phosphate substrates (themselves divalent anions) for a positive functional group, possibly protein-bound divalent cation, at the enzyme's active site.

Physiological, metabolic regulatory implications of these observations are discussed briefly.

INTRODUCTION

Various inorganic ions are potentially of great significance in the regulation of cellular metabolism at the enzymic level [1]. The importance of monovalent and diva-

Abbreviation: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid.

lent cations as metabolic regulators recently has been emphasized by Stadtman [1]. However, like generalized regulatory roles for various monovalent and divalent anions have thus far not received a similar amount of attention.

The enzyme glucose-6-phosphatase (D-Glc-6-*P* phosphohydrolase, EC 3.1.3.9) not only occupies a sensitive, key position in hepatic carbohydrate metabolism, but also is now known to possess a potent capacity for Glc-6-*P* synthesis (e.g., Eqn 2 and 3) as well as hydrolysis (Eqn 1) [2, 3]. The initial step in catalysis of various reactions by this enzyme appears to involve ionic interaction of anionic species of phosphate substrates (Glc-6-P^{2-}, carbamyl-P^{2-}, and PP_i^{2-}, for example) with a negatively charged group (presumably protein-bound divalent cation) [4] at the enzyme's active site [2, 3]. Inhibition by anions of activities of this complex enzyme thus is not only of potential interest from the metabolic, regulatory standpoint, but appears likely from mechanistic considerations.

And, indeed, a number of complex anions previously have been found to affect synthetic and/or hydrolytic activities of this multifunctional enzyme [5–15]. The possible physiological relevance for metabolic regulation by two such inhibitory anions (P_i and HCO_3^-) has been emphasized elsewhere by the senior author and colleagues [2, 3, 5, 8] and by others [6, 7].

In some earlier studies from this laboratory relating to catalytic and biological properties of synthetic and hydrolytic activities of glucose-6-phosphatase, ionic strength routinely was controlled with supplemental NaCl [16–19](for example). Hanson et al. [16] and very recently the present authors (see below), noted that apparent K_m values for phosphoryl substrates increased directly with ionic strength thus effected.

All of the above considerations have prompted us to examine systematically the effects of a variety of salts on both Glc-6-*P* phospophydrolase (Eqn 1) and certain phosphotransferase activities (Eqns 2 and 3) of this enzyme.

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

$$\text{Carbamyl-}P + \text{glucose} \rightarrow \text{Glc-6-}P + NH_3 + CO_2 \quad (2)$$

$$PP_i + \text{glucose} \rightarrow \text{Glc-6-}P + P_i \quad (3)$$

Results of these studies, which reveal generalized inhibitions by many anions, competitive with respect to phosphate substrates and possibly brought about through ion-pairing with protein-bound divalent cation at the enzyme's active site [2–4], are described here. The possible physiological relevance of these observations is discussed briefly.

MATERIALS AND METHODS

Materials

Sources of most reagents and methods of assay of substrates were as previously described [11, 20]. All salts and acids used were either "analyitcal grade" or "ACS certified grade". KCl, CsCl, $NaHSO_3$ and cacodylic acid were purchased from Fisher Scientific Co., Fair Lawn, N.J. NaCl, NaI, $NaNO_3$, $NaNO_2$ and acetic acid were obtained from Mallinckrodt Chemical Works, St. Louis, Mo. LiCl and Na_2SO_4 were products of J. T. Baker Chemical Co., Phillipsburg, N.J. Acetic acid, cacodylic

acid, and *N*-2-hydroxyethylpiperazine-N^1-ethanesulfonic acid (HEPES) were adjusted to pH 6.0 or 7.0 with 6 M NaOH.

Enzyme preparation partially purified from rat liver microsomal fraction by the method of Nordlie and Arion [11, 21] was employed. The preparation had a specific activity of 0.21 units*/mg protein.

Enzymic activity assays

Enzymic activities routinely were carried out at 30 ± 0.1 °C in a thermostatically controlled water bath equipped with tube-shaking mechanism. Except for the kinetic studies, assay mixtures contained, in 1.5 ml, 40 mM sodium cacodylate (pH 6.0) or 40 mM sodium HEPES buffer (pH 7.0), 2 mM phosphoryl substrate, 180 mM D-glucose (phosphotransferase), 0.23 mg of partially purified enzyme preparation protein, and a sufficient amount of designated salt to give the desired final ionic strength.

The concentrations of relevant ionic species of the various organic salts and phosphate substrates in solution were calculated on the basis of known pK_a values [20, 22–24], and total ionic strength, I, was calculated from such information with the aid of the relationship, $I = 1/2\ \Sigma_i m_i z_i^2$ (where m is the gram-formula weights per l and z is the valence of each of i ions involved).

All assay mixtures were thermally equilibrated for 3 min before addition of the enzyme to initiate the reaction. Enzymic reactions routinely were for 10 min. The pH of assay mixtures prepared simultaneously with those used for measurement of enzymic activities was determined in all cases with a Beckman expanded scale meter; pH was shown not to change significantly during 10 min of incubation of assay mixtures with enzyme.

Hydrolase and phosphotransferase activities were measured by previously described procedures [21, 25]. Supplemental studies were carried out to show that the salts used did not interfere with analytical procedures employed. Activity in all cases was a linear function of period of incubation.

RESULTS

Effects of various salts on hydrolytic and synthetic activities of glucose-6-phosphatase at pH 7 and pH 6

The effects of various supplemental amounts of several inorganic and organic salts on hydrolytic and synthetic activities of the enzyme are described in Figs 1 and 2. Data in Fig. 1, A and B, were obtained at pH 7.0 with Glc-6-*P* phosphohydrolase and carbamyl-*P*:glucose phosphotransferase, respectively. The chlorides of sodium, potassium, lithium, and cesium all decreased both activities progressively as salt concentrations were increased. Effects with the four salts were either completely (phosphotransferase) or nearly totally (phosphohydrolase) indistinguishable from one-another. $NaNO_3$ affected both activities in a quite similar fashion. However, sodium acetate and sodium cacodylate produced effects contrasting dramatically with those produced by the other salts tested. With these latter two salts, a slight (phosphotransferase) or

* One unit of enzymic activity is that amount catalyzing the hydrolysis of 1 μmole of Glc-6-*P* per min with saturating substrate concentration at pH 7.

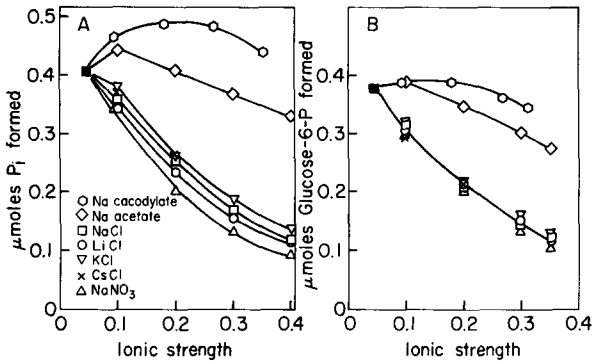

Fig. 1. Effects of several concentrations of various salts on Glc-6-*P* phosphohydrolase (A) and carbamyl-*P*:glucose phosphotransferase (B) activities at pH 7.0. All reaction mixtures contained, in 1.5 ml, 40 mM sodium HEPES buffer, 2.0 mM phosphate substrate, 180 mM D-glucose (transferase only), and sufficient supplemental salt to give the indicated total ionic strength. Ionic strength of basic, unsupplemented assay mixtures is indicated by closed squares. Additional information is given in A, and is applicable to both A and B. See text for other details.

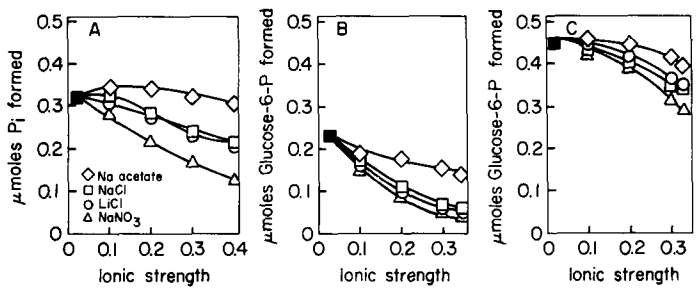

Fig. 2. Effects of several concentrations of various salts on Glc-6-*P* phosphohydrolase (A), PP_i:glucose phosphotransferase (B), and carbamyl-*P*:glucose phosphotransferase (C) at pH 6.0. All reaction mixtures contained, in 1.5 ml, 40 mM sodium cacodylate buffer, 2 mM phosphate substrate, 180 mM D-glucose (phosphotransferase) and sufficient indicated salt to give the designated final total ionic strength. Other details are as in Fig. 1, or as indicated on the figure.

relatively pronounced (Glc-6-*P* phosphohydrolase) activation was observed with lower concentrations of supplemental such compounds, while inhibition occurred at higher levels.

The effects of varied concentrations of certain of these salts also were tested at pH 6.0. This lower assay pH allowed the inclusion of a consideration of effects with PP_i–glucose phosphotransferase (Fig. 2B), which is relatively low in activity at neutral pH [2, 11], along with Glc-6-*P* phosphohydrolase (Fig. 2A) and carbamyl-*P*:glucose phosphotransferase (Fig. 2C). All three of these activities were inhibited progressively at this pH by increasing levels of NaCl, LiCl, and $NaNO_3$. The effects with sodium acetate contrasted with those due to the preceding salts, as at higher pH (cf. Fig. 1, A and B), although the activation of phosphohydrolase at the lower pH value was more modest than at pH 7. Activation of PP_i–glucose phosphotransferase was not observed, but acetate was relatively less effective in depressing this activity than were equilvalent amounts of the other three salts tested.

TABLE I

INHIBITIONS OF Glc-6-*P* PHOSPHOHYDROLASE AND CARBAMYL-*P*:GLUCOSE PHOSPHOTRANSFERASE BY SOME ADDITIONAL SODIUM SALTS

Assay mixtures were basically as in Fig. 1, except that salts were added as indicated. The ionic strength (*I*) of unsupplemented assay mixtures was 0.045; this value was raised to 0.10 or 0.20 by supplementation with designated salt. Enzymic activity is expressed as μmoles Glc-6-*P* formed (phosphotransferase) or hydrolyzed (phosphohydrolase) per 10 min. NaF ($I = 0.1$ or 0.2) produced nearly complete (>90%) inhibition of phosphotransferase. This compound interfered with the phosphohydrolase colorimetric assay for P_i.

Supplemental salt	Final *I* of assay mixture	Observed enzymic activity	
		Glc-6-*P* phosphohydrolase	Carbamyl-*P*:glucose phosphotransferase
None	0.045	0.48	0.39
NaCl	0.10	0.43	0.32
NaI	0.10	0.42	0.33
$NaNO_2$	0.10	0.45	0.28
$NaHSO_3$	0.10	0.38	0.20
Na_2SO_4	0.10	0.48	0.38
HEPES (sodium salt)	0.10	0.50	0.40
NaCl	0.20	0.30	0.21
NaI	0.20	0.29	0.22
$NaNO_2$	0.20	0.35	0.11
$NaHSO_3$	0.20	0.27	0.09
Na_2SO_4	0.20	0.43	0.30
HEPES (sodium salt)	0.20	0.50	0.40

The studies in Fig. 1 were extended to include a consideration of an additional six salts. Initial ionic strength of assay mixtures (0.045) was increased to 0.10 or 0.20 by supplementation with the indicated sodium salts (see Table I). Inhibition was seen in all but one instance (sodium salt of HEPES); and such inhibition was more extensive at the higher than at the lower levels of added salt. Sodium HEPES appears to approximate the ideal, inert buffer, an effect (activation) of less than 4% being observed with either activity.

The significance of these observations is considered further in Discussion.

Kinetics of inhibition by NaCl of Glc-6-P phosphohydrolase and carbamyl-P:glucose phosphotransferase

The results of kinetic analyses of inhibitions of Glc-6-*P* phosphohydrolase and carbamyl-*P*:glucose phosphotransferase are presented in Fig. 3, A and B, respectively. NaCl was chosen as a representative, physiologically prominent salt for these studies. Phosphoryl substrate concentration was held at either 1.5 or 4 mM, as indicated, and activity determined in the presence of 10, 20, 30, 40 or 50 mM supplemental NaCl. Data obtained were plotted according to the method of Dixon and co-workers [26, 27]. Extrapolations of experimental plots intersected in the second quadrant in both cases, consistent with NaCl's functioning as a classical, reversible competitive inhibitor with respect to phosphate substrates. A K_i value of 70 mM was determined with both activities [26, 27].

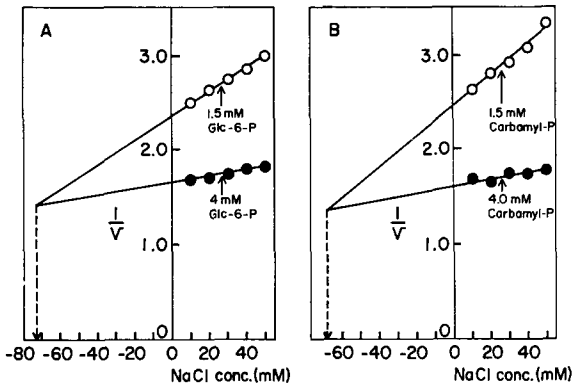

Fig. 3. Kinetics of inhibition by NaCl of Glc-6-*P* phosphohydrolase (A) and carbamyl-*P*:glucose phosphotransferase (B). All assay mixtures (pH 7.0), contained, in 1.5 ml, 40 mM HEPES buffer, 1.5 or 4.0 mM phosphate substrate as indicated on figure, 180 mM D-glucose (B only), 0.23 mg enzyme preparation protein, and designated amounts of supplemental NaCl. Data are plotted according to the method of Dixon and co-worker [26, 27]. Velocity, *v*, is expressed as μmoles of Glc-6-*P* hydrolyzed (A) or formed (B) per 10 min. K_i values, indicated by arrows to the axis of abscissas, were approx. 70 mM in both A and B.

DISCUSSION

Experimental observations which are described here* would appear to support specifically the modifying effects of a variety of anions on hydrolytic and synthetic activities of multifunctional glucose-6-phosphatase–phosphotransferase. The series of tested salts in which the anion was kept constant (Cl^-) and cation varied (Na^+, K^+, Li^+, or Cs^+) produced remarkably uniform, concentration-dependent effects on both hydrolytic and synthetic activities (Fig. 1). (In contradistinction, mM levels of these same chloride salts produce highly varying effects with pyruvate kinase, which has an absolute requirement for monovalent anion [28].)

In marked contrast, when the cation (Na^+) was kept constant and anion (Cl^-, acetate, cacodylate, NO_2^-, NO_3^-, I^-, HSO_3^-, SO_4^{2-}, F^-, or HEPES) varied, a striking heterogeneity of modifying effects was noted with equivalent amounts of these salts (see Figs 1 and 2 and Table I). Activation, inhibition, or no effect was noted, depending on the nature of the anionic component and concentration of the salt studied.

Thus, modifications in enzymic activity levels vary with the nature of the anion studied, but are invariant with the several monovalent cations considered.

The heterogeneity of effects noted with the variety of salts tested tends to eliminate modifications in manifest enzymic activity brought about generally through variations in overall ionic strength of assay mixtures, although additional effects of this nature also could be present but masked by the overriding, specific anionic effects.

It should be pointed out also that while the monovalent cations considered here do not appear to be involved in inhibition or activation, a particular divalent

* The present studies were designed to assess the nature of previously noted modifications of activities of glucose-6-phosphatase–phosphotransferase by mM levels of NaCl [16] and other salts. They do not, nor were they intended to, eliminate the possibility of additional effects which conceivably could be caused by very low (i.e. μM) levels of monovalent cations.

cation (Mg^{2+}) recently has been found in this laboratory (Johnson, W. T., Colilla, W. and Nordlie, R. C., unpublished observations) to exert highly specific, substrate-discriminant effects on a variety of activities of this complex enzyme. The mechanism in this case, however, appears to be one of complexation of the inorganic ion with phosphate substrate rather than interaction with enzyme.

Mechanistic considerations

The kinetic studies in Fig. 3, A and B, indicate that NaCl acts as a competitive inhibitor with respect to both Glc-6-*P* and carbamyl-*P*.These observations rationalize an earlier noted increase in apparent K_m values for phosphoryl substrates (ATP and phosphoenolpyruvate) with this enzyme correlative with elevations in ionic strength of assay mixtures [16]. NaCl was employed routinely in these earlier studies to change ionic strength, and although it was not recognized at the time, the salt (specifically Cl^-; see below) was acting as a competitive inhibitor. V was relatively unaffected.

Earlier observations of Arion and Nordlie [29] that variations in ionic strength (supplemental NaCl was employed to this end) did not appear to affect PP_i–glucose phosphotransferase also are understandable in these terms. In these early studies, phosphate substrate was maintained at a very high, saturating level. Changes in apparent K_m for PP_i due to the presence of a competitive inhibitor (Cl^-) would not serve to alter demonstrable activity levels under these conditions.

Inhibitions competitive with phosphate substrates, as noted here with NaCl, previously have been observed with the anions bicarbonate [8] and P_i [5–7]. Additionally, the anions molybdate, iodide, arsenate, and borate have been shown to inhibit phosphotransferase and/or phosphohydrolase activity of the enzyme [9–15]. The present studies bring to 15 the total number of anions found to inhibit this enzyme.

Inhibition by anions competitive with respect to phosphate substrates is generally consistent with the mechanism of action of this complex enzyme proposed by the senior author [2, 3]. The enzyme appears to contain as a functional group protein-bound divalent cation [4]. It has been proposed, as depicted in Fig. 4, I→II, that the initial step in catalysis by this enzyme is the binding, through ionic interaction, of phosphate substrates (doubly negatively charged anions themselves [20]) to this positive center in the active site [2, 3]. As indicated in Fig. 4, a competition between such anionic substituted phosphate substrates (I→II) and various other anions (I→III) for this positive binding site (through ion pairing) is proposed to account for the observed inhibitions generally produced by anions.

Inhibitions of the type proposed and described here previously have been demonstrated for certain other enzymes. Racker [30] has shown that glutathione reductase is inhibited by chloride ions. Similarly, a number of anions (including P_i, Cl^-, and SO_4^{2-}) are known to inhibit phosphoglucomutase by competing with ribose-1-*P* for a positively-charged functional group in the enzyme's active site [31].

Some physiological implications

Activation, as seen specifically with lower levels of cacodylate and acetate (Fig. 1), is not presently understood mechanistically, but may well involve conformational alterations in the membrane-bound enzyme (see [32] and [3] for a consideration of interrelationships between membrane morphology and catalytic behavior of glucose-6-phosphatase–phosphotransferase). Most significant in this regard, we believe, is

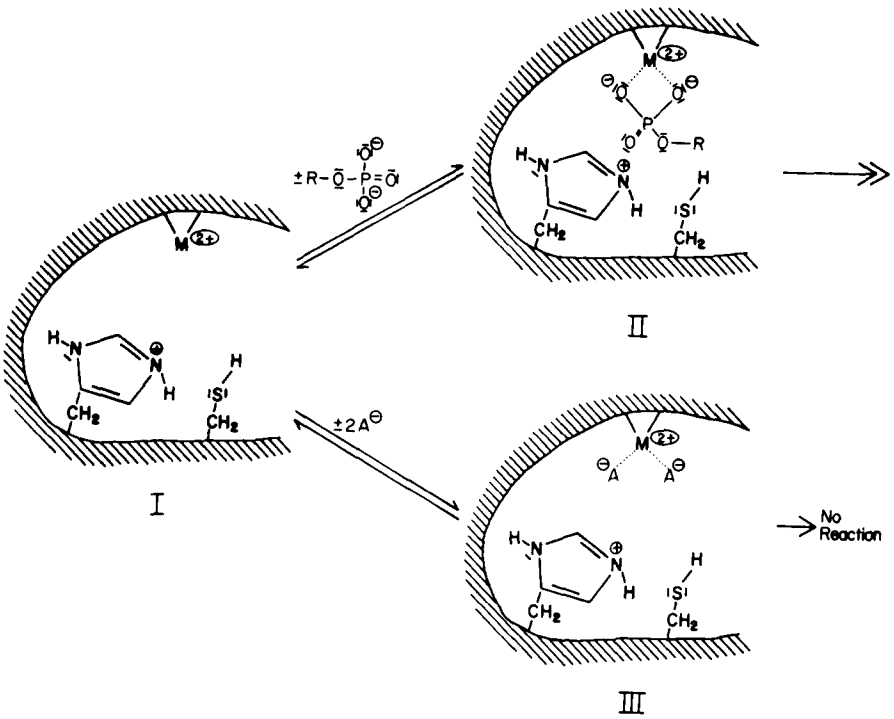

Fig. 4. Proposed mechanism of inhibition by anions of activities of glucose-6-phosphatase–phosphotransferase. Generalized anion A^- (Cl^-, HCO_3^-, I^-, P_i^{2-}, etc.) is suggested to compete with phosphate substrate R–O–PO_3^{2-} (Glc-6-*P*$^{2-}$, carbamyl-*P*$^{2-}$, PP_i^{2-}, etc.) for positively charged, protein-bound divalent cation at the active center of the enzyme (I). Alternatively, active enzyme–phosphate substrate Complex II or inactive enzyme–inhibitor Complex III may be formed through ionic bonding. The hypothesized mechanism is based on experimental observations on inhibitions described in the text, and on additional information and concepts regarding the nature of the enzyme's active site and mechanism of action as considered in detail by Nordlie elsewhere [2, 3]. See also Discussion.

the discrimination with respect to the various enzymic activities which these two anions manifest. Both, at the lower tested levels (see Fig. 1), activate phosphohydrolase discriminantly in comparison with phosphotransferase. Such activity–discriminant modulation previously has been seen with long-chain acyl coenzyme A esters [33] and phlorizin acting in concert with cetyltrimethylammonium bromide [34]; the latter favored phosphydrolase as compared with phosphotransferase, while the former discriminated in favor of transferase activity. Thus differential control of activities of the enzyme for Glc-6-*P* formation and Glc-6-*P* hydrolysis is possible. Such possibilities for discriminant control are essential from the regulatory, physiological point of view (see [3] for details in this regard).

Although the K_i value for Cl^-, as determined here (see Fig. 3) to be about 70 mM, may appear quite high, none-the-less, the level of this ion normally present in hepatic cells (approx. 40 m*) is sufficient to exert a significant inhibitory effect. For exam-

* Normal rat liver Cl^- content is reported [35] as 26 mequiv/kg moist tissue. This value corresponds with 43 mM, assuming heterogeneous distribution of this ion in liver cell water consisting of 60% of liver by wt.

ple, if it is assumed [36] that the level of Glc-6-*P* in the hepatic parenchymal cell is 0.13 mM and that K_m for this compound is 1.8 mM, then it may be calculated (see Nordlie et a . [36]) that 40 mM Cl^- produces 34% inhibition of the rate of Glc-6-*P* hydrolysis. Considered along with possible inhibitions adidtionally [3] by such other cellular metabolites as P_i [5–7], HCO_3^- [8], ATP [36], PP_i [11], and carbamyl-*P* [25], inhibitions by anions may become a very significant modulating influence regulating the activities of this enzyme.

The present observations highlight the need for extreme caution when possible effects of various cations on activities of this enzyme are being considered. Experimental controls adequately taking into account the possible modifications in enzymic activity due to anions requisitely co-introduced into assay mixtures along with subject cations must in all instances be included in experimental protocol.

Finally, it appears from these studies that sodium HEPES is ideally suited as a buffer, at applicable pH (7.0, for example) for study of this enzyme. As indicated in Table I, little or no significant effect, positive or negative, was exerted by this compound on either Glc-6-*P* phosphohydrolase or phosphotransferase activity. Apparently, this relatively bulky anion is unable, because of steric problems, to approach the enzyme's active site sufficiently closely to exert inhibitory effects.

NOTE ADDED IN PROOF

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It appears likely from data presented here that competitive inhibition by residual deoxycholate (an anion) may explain the fact that apparent K_m values for phosphate substrates noted with enzyme preparations involving treatment with this detergent [2, 3] are larger than those observed when the cationic detergent cetyltrimetyhlammonium bromide is employed to disperse microsomal suspensions [37].

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