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RABBIT INTESTINAL GLUCOSE-6-PHOSPHATE PHOSPHOHYDROLASE AND INORGANIC PYROPHOSPHATE–GLUCOSE PHOSPHOTRANSFERASE INHIBITION BY PHLORIZIN

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SUMMARY

The inhibition by phlorizin of rabbit intestinal D-glucose-6-phosphate phosphohydrolase (EC 3 1 3 9) and of PP_i–glucose phosphotransferase activity also catalyzed by this enzyme was investigated Studies were carried out with freshly prepared, unsupplemented microsomes and with microsomal suspensions to which various concentrations (0 05–0 30%, w/v) of the cationic detergent cetylhexadecylammonium bromide ("cetrimide") had been added prior to assay Inhibitions of both activities were found to rise progressively with increasing concentrations of phlorizin Kinetic studies indicated that inhibitions were noncompetitive with respect to all substrates tested, including glucose 6-phosphate, PP_i and glucose Pretreatment of microsomes with cetrimide accentuated the extent of inhibition by phlorizin of phosphotransferase activity but significantly ameliorated phlorizin-effected inhibition of glucose-6-phosphate phosphohydrolase activity Consistent with these observations, K_i values for phlorizin determined with the former activity decreased from a value of 5 5 mM, noted in the absence of detergent, to 1 3 mM following pretreatment of microsomes with 0 10% (w/v) cetrimide, while similar treatment caused an increase in K_i values determined with the phosphohydrolase activity from 1 5 mM (no detergent) to 5 0 mM with 0 10% (w/v) cetrimide These observations are interpreted as indicating the presence on the enzyme molecule of two effective, activity-specific inhibitor-binding sites with varying affinities for phlorizin and differing with respect to modification by cetrimide Bound inhibitor molecules are postulated to prevent the formation of phosphoryl–enzyme intermediates from binary enzyme–glucose 6-phosphate and enzyme–PP_i complexes

INTRODUCTION

In a recent publication from this laboratory SOODSMA *et al.*[1] described the detergent-sensitive, kinetically noncompetitive inhibition by phlorizin of rat kidney

microsomal D-glucose-6-phosphate phosphohydrolase (EC 3 1 3 9, Reaction 1) and PP_i–glucose phosphotransferase activity (Reaction 2) also catalyzed by this enzyme[2] More recently, ZERR AND NOVOA[3] have reported studies which also indicate the non-competitive nature of inhibition by this glycoside of this phosphohydrolase activity of rat kidney In contrast, however, the

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

$$PP_i + \text{glucose} \rightarrow \text{glucose-6-}P + P_i \quad (2)$$

latter workers observed that inhibition of rat liver glucose-6-phosphatase was kinetically competitive with respect to sugar phosphate substrate Because of this apparent variation in properties of the enzyme from these two tissues, the effects of phlorizin on glucose-6-phosphatase from a third major source—the small intestine—has become of particular interest We[4] recently described some catalytic properties of various synthetic and hydrolytic activities of this enzyme from microsomes of mucosa of rabbit small intestine The inhibitions by phlorizin of these intestinal activities have now been studied in some detail, as have the modifying effects thereon of the cationic detergent trimethylhexadecylammonium bromide ("cetrimide") which previously was found to potentiate significantly the inhibition by this glycoside of phosphotransferase activity of the kidney enzyme[1]. Results of these studies are described in this paper

MATERIALS AND METHODS

Enzymic assays and sources of most chemicals were as described previously[1,5,6] Rabbit intestinal microsomes were prepared and suspended in 0 25 M sucrose as described earlier[4] Freshly isolated preparations were used in all experiments Cetrimide was prepared as a 2% (w/v) aqueous solution and adjusted to pH 7 0 with dilute HCl Microsomal suspensions were preincubated at 0° in the presence of appropriate concentrations of this detergent solution, or with distilled water when cetrimide was absent, for a period of 15 min prior to assay for enzymic activities Reaction mixture compositions and other experimental details are given in legends to individual tables and figures, and in the text below All data have been normalized to 0 026 unit* of glucose-6-*P* phosphohydrolase activity

RESULTS

Inhibitions of both PP_i–glucose phosphotransferase and glucose-6-*P* phosphohydrolase activities were studied as a function of varied phlorizin concentrations The results of such studies which were carried out in the absence of detergent are depicted by open circles and solid lines in Figs 1A and 1B, respectively Inhibitions of both activities were found to increase progressively as a function of elevation in concentration of phlorizin Since the cationic detergent cetrimide has been found to potentiate quite significantly inhibition by phlorizin of PP_i–glucose phosphotransferase activity of kidney glucose-6-phosphatase[1], the above studies were repeated with microsomal

* One unit of glucose-6-phosphatase activity is 1 μmole of glucose-6-*P* hydrolyzed per min at 30° in a reaction mixture (pH 6 0) containing, in 1 5 ml, 10 mM D-glucose-6-*P* and 40 mM sodium cacodylate

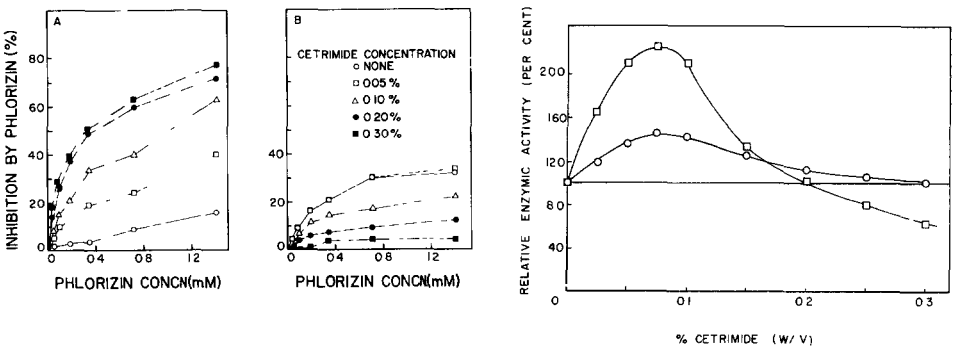


Fig 1 Effects of cetrimide on inhibition by phlorizin of (A) PP_1–glucose phosphotransferase and (B) glucose-6-*P* phosphohydrolase activities Microsomal suspensions were preincubated in the presence of the indicated concentrations of cetrimide, and 0 1-ml aliquots were then removed and incubated at 30° for 10 min with assay mixtures identical with those described in Fig 2, except for addition of the indicated concentrations of phlorizin Inhibitions in all cases were calculated relative to activity (defined as in Fig 2) observed in the absence of phlorizin The legend of cetrimide concentrations given in Fig 1B is in terms of concentrations (w/v) in microsomal suspensions, and applies to both (A) and (B)

Fig 2 Effects of varied concentrations of cetrimide on intestinal microsomal PP_1–glucose phosphotransferase (□) and glucose-6-*P* phosphohydrolase (○) activities Assay mixtures (pH 6 0) contained in 1 5 ml, 10 mM phosphate substrate, 180 mM glucose (transferase) and 40 mM sodium cacodylate buffer Fresh microsomal suspensions were preincubated at 0° for 15 min with the indicated concentrations of cetrimide, and 0 1-ml aliquots then were incubated for 10 min at 30° with the assay mixtures Activity = μmoles of glucose-6-*P* hydrolyzed (phosphohydrolase) or synthesized (phosphotransferase) per 10-min incubation at 30° Relative activity = 100 × (activity observed when the microsomes were preincubated with cetrimide — activity observed with water-pretreated microsomes)/(activity with water-pretreated microsomes)

preparations which had been supplemented with this detergent to final concentrations (w/v) of 0 05%, 0 10%, 0 20% and 0 30% Results of these studies are also presented, as broken or dashed lines, in Figs 1A and 1B As was noted in the absence of cetrimide, inhibitions were observed to increase as phlorizin concentrations were elevated However, in all instances the extent of inhibitions by unit concentrations of phlorizin of phosphotransferase activity were quite significantly increased as the concentration of cetrimide to which microsomes were exposed prior to assay was increased (Fig 1A) For example, 0 71 mM phlorizin produced 10% inhibition in the absence of cetrimide, while inhibition was increased to 24% with 0 05% cetrimide in microsomal suspensions, and to 40%, 60% and 63%, respectively, when cetrimide concentrations were increased to 0 10%, 0 20% and 0 30% (w/v) In marked contrast, inhibitions by phlorizin of glucose-6-*P* phosphohydrolase activity were progressively diminished as cetrimide levels were increased in this same range of concentrations (see Fig 1B)

The direct effects of cetrimide on enzymic activities are described in Fig 2 Maximal stimulations of both phosphotransferase activity (to 225% of control values) and phosphohydrolase activity (to 145% of control value) were observed when 0 075% (w/v) cetrimide was included in microsomal suspensions A decrease in extent of activation (phosphohydrolase) or an actual net inhibition (phosphotransferase) was noted with higher concentrations of this detergent Interestingly, even those concentrations of cetrimide which were supraoptimal for the production of direct increases in levels of enzymic activity (*i e* concentrations > 0 075% (w/v), see Fig 2) progressively modified the inhibitions by phlorizin of these same activities (see Figs 1A and 1B).

TABLE I

INHIBITION BY PHLORIZIN IN THE ABSENCE AND PRESENCE OF CETRIMIDE AS A FUNCTION OF ASSAY MIXTURE pH

Microsomal suspensions without or with cetrimide supplementation (0 10, w/v) were incubated in assay mixtures containing in 1 5 ml, 10 mM phosphate substrate, 180 mM glucose (phosphotransferase), 40 mM sodium cacodylate buffer (40 mM sodium cacodylate–sodium acetate buffer below pH 5 0) and either no or 0 57 mM phlorizin Assay mixtures were prepared in duplicate, activity was measured in one series and pH was determined with a Beckman expanded scale meter in the second series Percent inhibition = 100 × (activity without phlorizin — activity *plus* phlorizin)/(activity without phlorizin)

pH	*Inhibition (%)*			
	PP_i–glucose phosphotransferase		*Glucose-6-P phosphohydrolase*	
	Without cetrimide	*Plus cetrimide*	*Without cetrimide*	*Plus cetrimide*
4 1	30 5	46 4	15 1	17 7
4 7	23 1	48 6	20 2	18 6
5 1	16 6	50 7	25 2	18 0
5 6	15 5	49 1	29 2	16 4
6 0	21 6	41 8	35 7	16 0
6 6	12 3	32 4	22 0	15 9
7 0	12 5	60 3	15 5	19 3
7 4	—	—	10 2	8 1

Inhibitions of both phosphotransferase and phosphohydrolase activities were generally observed, with minor variations, over a rather broad range of pH values (pH 4 1–7 0), as indicated by data in Table I And at all pH values, inhibition by phlorizin of phosphotransferase activity was significantly higher when cetrimide-supplemented (0 1%, w/v) microsomal preparations were employed, while inhibition by phlorizin of glucose-6-*P* phosphohydrolase activity was ameliorated by the detergent (compare values in third and fifth vertical columns in Table I with corresponding values in the second and fourth columns)

Kinetic Studies

In order to provide further insight into the mechanism of action of phlorizin on the two activities and of modifications of this action by cetrimide, a series of kinetic studies was carried out at pH 6 0 Phosphohydrolase activity was measured as a function of glucose-6-*P* concentrations, which were varied in the range 1–10 mM In corresponding studies of the phosphotransferase activity, either glucose concentrations were varied from 30 to 180 mM with a constant PP_i level (10 mM), or PP_i concentrations were varied from 1 to 10 mM with a constant glucose level (180 mM) Studies were made with both untreated and cetrimide-supplemented microsomes, and activities were measured both in the absence and presence of phlorizin The resulting data were plotted in conventional double-reciprocal fashion[7], and K_m and K_i values were calculated as described by DIXON AND WEBB[8] Results of typical experiments, in which the inhibition by 0 57 mM phlorizin was studied with respect to glucose-6-*P*, glucose and PP_i, are described in Figs 3, 4A and 4B, respectively Kinetic parameters evaluated from these and similar studies are compiled in Table II In all instances, inhibition

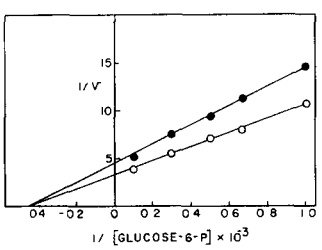
TABLE II

K_m VALUES, AND K_i VALUES FOR PHLORIZIN, DETERMINED IN THE ABSENCE AND PRESENCE OF CETRIMIDE FOR THE PHOSPHOHYDROLASE AND PHOSPHOTRANSFERASE ACTIVITIES OF INTESTINAL GLUCOSE-6-PHOSPHATASE

Assay mixtures (transferase, pH 6 0) contained in 1 5 ml, 40 mM sodium cacodylate buffer, 180 mM glucose when PP_i was varied (1–10 mM) and 10 mM PP_i when glucose was varied (30–180 mM) Glucose-6-*P* was varied from 1 to 10 mM Phlorizin, 0 57 mM, was found to be a noncompetitive inhibitor with respect to each reference substrate

Reference substrate	*No cetrimide*		*0 10% (w/v) cetrimide*	
	K_m *(mM)*	K_i *(phlorizin) (mM)*	K_m *(mM)*	K_i *(phlorizin) (mM)*
Glucose-6-*P*	2 1	1 5	0 6	5 0
PP_i	1 8	5 2	0 7	1 2
Glucose	70 0	5 7	70 0	1 4

by phlorizin was noncompetitive with respect to each substrate tested Cetrimide sharply reduced the K_m values for PP_i and glucose-6-*P*, but did not alter $K_{glucose}$ In the presence of detergent, the K_i (phlorizin) value was increased from 1 5 to 5 0 mM with respect to glucose-6-*P* hydrolysis and was decreased from 5 5 to 1 3 mM with respect to the phosphotransferase activity Similar inhibitor constant values were obtained with respect to both glucose and PP_i in the phosphotransferase reaction (See Table II) K_m values for glucose were unaffected by the detergent



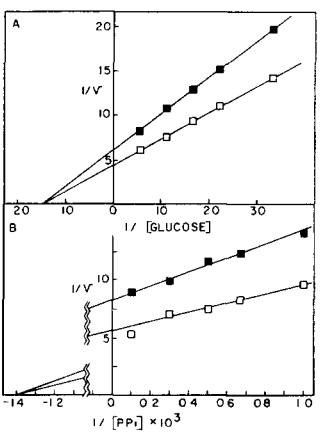


Fig 3 Kinetics of inhibition by phlorizin of glucose-6-*P* phosphohydrolase activity in the absence of cetrimide Assay mixtures (pH 6 0) contained in 1 5 ml, 40 mM sodium cacodylate buffer and the indicated glucose-6-*P* concentrations Activity (*v*, defined as in Fig 2) was measured in the absence (○) and presence (●) of 0 57 mM phlorizin The K_i value was calculated to be 1 5 mM (see text)

Fig 4 Kinetics of inhibition by phlorizin of PP_i-glucose phosphotransferase (A) Inhibition with respect to glucose Assay mixtures (pH 6 0) contained in 1 5 ml, 40 mM sodium cacodylate buffer, 10 mM PP_i and indicated glucose concentrations (B) Inhibition with respect to PP_i Assay mixtures (pH 6 0) contained in 1 5 ml, 40 mM sodium cacodylate buffer, 180 mM glucose and indicated levels of PP_i Microsomal preparations employed were supplemented, to 0 10% (w/v), with cetrimide In both instances, activities (*v*, defined as in Fig 2) were determined in the absence (□) and presence (■) of 0 57 mM phlorizin K_i values with respect to glucose and PP_i were 1 4 and 1 2 mM, respectively

DISCUSSION

The effects of phlorizin on PP_i–glucose phosphotransferase and glucose-6-*P* phosphohydrolase activities of rabbit intestine resembled generally those previously noted with the rat kidney enzyme by SOODSMA *et al.*[1] Inhibitions of both synthetic and hydrolytic activities of the enzyme from both sources were kinetically of the non-competitive type (see Figs 3 and 4 and RESULTS) Furthermore, the extent of phlorizin-effected inhibition of phosphotransferase activity was in both instances significantly potentiated by pretreatment of microsomal preparations with cetrimide The ameliorating effects of this same cationic detergent on the inhibition by phlorizin of glucose-6-*P* phosphohydrolase activity is described in detail for the first time in this paper (see Fig 1B, Tables I and II, and RESULTS) The same general effect (a reduction in inhibition by 0 57 mM phlorizin of glucose-6-*P* phosphohydrolase activity at pH 6 5 from 38% in the absence of cetrimide to 29% in the presence of 0 2% (w/v) cetrimide in microsomal suspensions) was, however, previously noted (see Fig 3 in ref 1), although the observation was not discussed

The intestinal and kidney preparations differ in that activities of the former appear generally less susceptible to inhibition by phlorizin than do those of the latter preparations K_i values for phlorizin depicted in Table II for the intestinal enzyme are considerably larger than corresponding values of 0 57 mM (both activities, cetrimide absent) and 0 22 mM (phosphotransferase, 0 2% cetrimide supplementation) obtained with the kidney preparation at pH 5 5 (ref 1) These differences may be explained by the fact that, in contrast with the kidney, the lumen of the small intestine, *in vivo*, is in continuous contact with bile salts and acids which are introduced from the gall bladder into the intestine, where they are reabsorbed *via* the enterohepatic circulatory pathway Deoxycholate and cholate previously have been found partially or completely to abolish sensitivity of activities of glucose-6-phosphatase to inhibition by phlorizin[1], and the intestinal preparations thus undoubtedly were moderately desensitized while still in the intestinal mucosal cell

SOODSMA *et al.*[1] previously have suggested that potentiation by cetrimide of inhibition by phlorizin of PP_i–glucose phosphotransferase activity of kidney microsomal glucose-6-phosphatase might involve an inactivation by the detergent of ineffective "silent" phlorizin binding sites on the enzyme, thus making added phlorizin molecules more available for interaction with the enzyme at the "effective" inhibitory sites The present data suggest that two effective phlorizin-binding sites with varying affinity for phlorizin and differing with respect to susceptibility to modification by cetrimide—one specifically affecting phosphotransferase activity and the second specific for glucose-6-*P* phosphohydrolase activity—may be involved The effectiveness of phlorizin-binding to the latter site appears to be diminished by cetrimide, as revealed by lowered extents of inhibition and increased K_i values for phlorizin noted in the presence of cetrimide with this activity (see Fig 1B and Tables I and II) In contrast, the enhancement of inhibition (Fig 1A and Table I) and lowering of K_i value for phlorizin (Table II) noted in the presence of various concentrations of cetrimide with the phosphotransferase activity indicate that the detergent treatment increases the affinity of this receptor site for phlorizin

On the basis of kinetic studies described above it is clear that phlorizin does not compete with substrates PP_i, glucose, or glucose-6-*P* for active enzymic sites In

terms of the mechanism previously proposed by ARION AND NORDLIE[9] to describe this multifunctional enzyme system*, it appears that the binding of two molecules of inhibitor per complete enzymic active site could produce the observed activity-discriminating effects either by (a) interfering individually with the formation of intermediate phosphoryl–enzyme complex from, respectively, enzyme–PP_i and enzyme–glucose-6-*P* complexes, or (b) by inhibiting the transfer of phosphoryl groups from the common phosphoryl–enzyme intermediate alternatively to (i) water (phosphohydrolase) or to (ii) glucose (phosphotransferase) If the latter possibility were true, the effects of cetrimide-produced modifications of phlorizin inhibition of PP_i phosphohydrolase should follow those of glucose-6-*P* phosphohydrolase, while if the mechanism considered in (a), above, were to prevail, inorganic pyrophosphatase activity would be predicted to respond to cetrimide-modification of phlorizin inhibition in a manner similar to that of PP_i–glucose phosphotransferase Preliminary experiments, carried out with kidney microsomes, indicate that cetrimide produces an increase in extent of inhibition by phlorizin of inorganic pyrophosphatase activity at pH 5 5 of 6 5 just as it does PP_i–glucose phosphotransferase, thus supporting the situation described under possibility (a), above, as the most likely stage for phlorizin to exert its inhibitory effect.

Further detailed studies are contemplated on the mechanism of action of phlorizin and cetrimide on activities of this enzyme since these compounds, acting in concert, appear from the present studies to constitute an extremely interesting model system permitting the control, in a predetermined, differential manner of the rates of synthesis and degradation of the key metabolic intermediate glucose-6-*P* by this multifunctional enzyme

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* On the basis of kinetic studies, ARION AND NORDLIE[9] have postulated a reaction mechanism for both synthetic and hydrolytic activities of classical microsomal glucose-6-phosphatase involving (a) formation of binary enzyme–glucose-6-*P* or enzyme–PP_i complexes, followed by (b) dissociation of glucose or P_i to produce a common phosphoryl enzyme intermediate, which then can (c) transfer the phosphoryl group alternatively to glucose (phosphotransferase) or to water (phosphohydrolase)