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THE INHIBITION BY SACCHARIN AND CYCLAMATE OF PHOSPHOTRANSFERASE AND PHOSPHOHYDROLASE ACTIVITIES OF GLUCOSE-6-PHOSPHATASE

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SUMMARY

Saccharin and cyclamate were found to inhibit the glucose-6-phosphate phosphohydrolase and PP_i-glucose phosphotransferase activities of beef liver microsomal glucose-6-phosphatase (D-glucose-6-phosphate phosphohydrolase, EC 3.1.3.9). The extent of inhibition decreased with increasing pH in the range pH 4–8. At pH 5.6 inhibition by both compounds was competitive with respect to phosphate substrates and non-competitive with respect to glucose. K_i values for saccharin and cyclamate were, respectively, 6.5 and 26 mM for the phosphohydrolase reaction, and were 16 and 68 mM with respect to PP_i and 110 and 190 mM with respect to glucose for the phosphotransferase reaction. Of a variety of structural analogs of saccharin and cyclamate, saccharin was the most effective inhibitor tested.

INTRODUCTION

Saccharin (*o*-sulfobenzimide) and sodium or calcium salts of cyclamic acid (cyclohexylsulfamic acid) have been used as non-nutritive sweetening agents in food. The safety of these compounds as food additives has been questioned because of reported mutagenic and carcinogenic effects associated with the administration of saccharin [1–4], cyclamate [3–8], cyclamate–saccharin combinations [9], and cyclohexylamine [6, 9, 10], the major known metabolite of cyclamate [11–13]. However, specific metabolic effects of saccharin and cyclamate are largely unknown. The effects of these compounds on the glucose-6-phosphate phosphohydrolase (Reaction 1) and PP_i-glucose phosphotransferase (Reaction 2) activities of beef liver microsomal glucose-6-phosphatase (D-glucose-6-phosphate phosphohydrolase, EC 3.1.3.9) were examined and results of these studies are presented in this paper.

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

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

MATERIALS AND METHODS

Substrates, NADP, buffer salts, and sodium salts of saccharin and cyclamic acid were purchased from Sigma Chemical Co., St. Louis, Mo. Saccharin and cyclamate each exhibited a single spot on thin-layer chromatography. The cyclamate contained 0.16% cyclohexylamine as measured by gas–liquid chromatography. Saccharin was examined for impurities by high-pressure liquid chromatography and contained 0.034% *o*-toluenesulfonamide. Glucose-6-phosphate dehydrogenase was obtained from Boehringer Mannheim Corp., New York, N.Y. All additional chemicals were of reagent grade. Beef liver was obtained from Schaake Packing Co., Ellensburg, Wash.

Assays for glucose-6-phosphatase and PP_i-glucose phosphotransferase were as described by Nordlie and Arion [14]. Reaction mixture composition and further experimental details are given in the legends to tables and figures and in the text. Protein was determined by a modification (see ref. 15) of the biuret method [16] with bovine serum albumin as the reference standard. It was demonstrated in supplementary experiments that activities were linear with respect to incubation time and concentration of protein and were unaffected by variations of ionic strength in the range used in this study. All assays were carried out in duplicate. In studies of the effect of pH on enzyme activity, reaction mixtures were prepared in triplicate and the pH of one series was measured at 30 °C with a Beckman expanded scale meter equipped with microelectrodes. Velocities were normalized to 0.112 unit* of glucose-6-phosphate phosphohydrolase activity.

The enzyme preparation was obtained from beef liver which had been stored at −20 °C for ten months. The liver was thawed and homogenized at 4 °C with a Waring Blendor containing 5 ml of ice-cold 0.25 M sucrose solution per g liver. The homogenate was centrifuged at 17 300 × *g* for 15 min. The resultant supernatant fraction was centrifuged at 123 000 × *g* for 40 min. The sedimented microsomes were resuspended in approx. 1 ml of 0.25 M sucrose solution per g liver by means of a Potter–Elvehjem homogenizer. This enzyme preparation had a specific activity of 0.13 unit per mg protein and was stable for several months when stored at −20 °C.

RESULTS AND DISCUSSION

Effect of pH

Phosphohydrolase and phosphotransferase activities of beef liver microsomal glucose-6-phosphatase were measured in the absence and presence each of 20 mM saccharin and 30 mM cyclamate in the range pH 4–8. The data are depicted in Figs 1A and 1B. The extent of inhibition of both activities decreased progressively with increasing pH. Inhibition of the phosphotransferase activity was minimal above pH 6. In supplementary studies, a similar pattern of inhibition by saccharin and cyclamate was observed for inorganic pyrophosphatase activity, which also is catalyzed by hepatic glucose-6-phosphatase [17, 18]. Since the pK_a values for saccharin and

* One unit of glucose-6-phosphate phosphohydrolase activity is one micromole of glucose-6-*P* hydrolyzed per min at 30 °C in a reaction mixture (pH 5.6) containing, in 1.5 ml, 10 mM glucose-6-*P* and 40 mM sodium cacodylate.

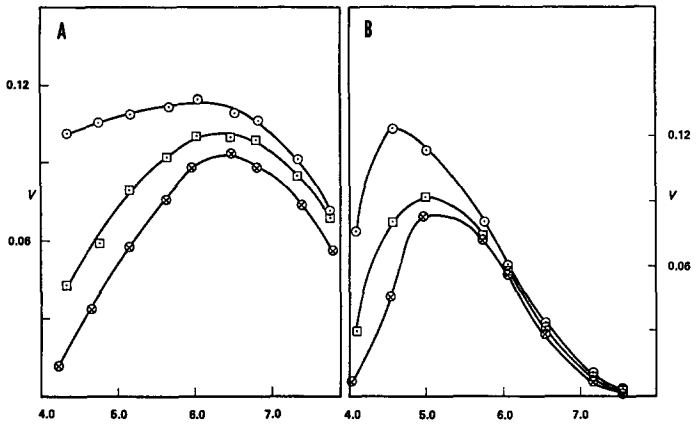

Fig. 1. (A) Effect of reaction mixture pH on glucose-6-phosphate phosphohydrolase activity (v) in the absence (○) and presence of 20 mM saccharin (⊠) or 30 mM cyclamate (□). All reaction mixtures contained, in 1.5 ml, 40 mM buffer (sodium acetate, pH 4–5; sodium cacodylate, pH 5.5–7; *N*-2-hydroxyethylpiperazine-*N*-ethanesulfonate, pH 7.5–8), and 8.7 mM glucose-6-*P*. Activity is expressed as μmoles of glucose-6-*P* hydrolyzed per min. (B) Effect of reaction mixture pH on PP_i-glucose phosphotransferase activity (v) in the absence (○) and presence of 20 mM saccharin (⊠) or 30 mM cyclamate (□). All reaction mixtures contained, in 1.5 ml, 40 ml buffer (sodium acetate, pH 4–5; sodium cacodylate, pH 5.5–7; *N*-2-hydroxyethylpiperazine-*N*-ethanesulfonate, pH 7.5–8), 10 mM PP_i, and 180 mM glucose. Activity is expressed as micromoles of glucose-6-*P* formed per min. In experiments described in both A and B, the actual reaction mixture pH was determined with a Beckman, model Century SS-1. pH meter equipped with microelectrodes.

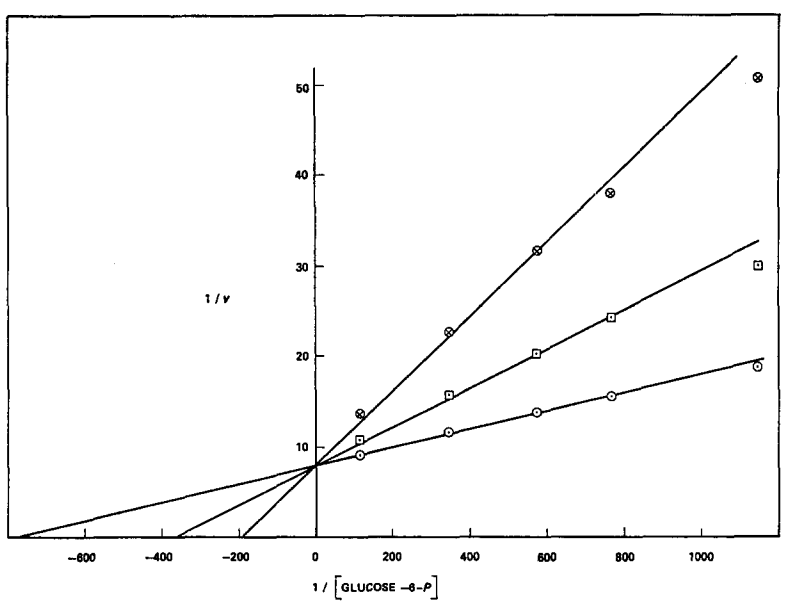

Fig. 2. Kinetics of inhibition of glucose-6-phosphate phosphohydrolase activity by saccharin and cyclamate. All assay mixtures, pH 5.6, contained, in 1.5 ml, 40 mM cacodylate buffer and indicated amounts of glucose-6-*P*. Activities were determined in the absence (○) and presence of 20 mM saccharin (⊠) or 30 mM cyclamate (□). Activities are expressed as described in Fig. 1A. Kinetic parameters evaluated from these data are: K_m (glucose-6-*P*) = 1.3 mM; K_i (saccharin) = 6.5 mM; and K_i (cyclamate) = 26 mM.

cyclamate are 2.2 (see Minegishi et al. [19]) and 1.9 [20], respectively, the pH dependency of inhibition may be attributable to an ionization of a group on the enzyme. The observed pattern of inhibition is similar to that reported for citrate, which inhibited glucose-6-phosphatase activities only below pH 7 [21]. In that study, the participation of an imidazolium group of a histidine residue in the enzyme was postulated to participate in enzyme–substrate complexation. This conclusion also was supported by studies with ^{32}P-labeled substrates [22]. The pH-dependent inhibition of glucose-6-phosphatase activities by saccharin and cyclamate thus is consistent with previous observations and interpretations.

Kinetics

Results of kinetic studies of inhibition by saccharin and cyclamate at pH 5.6 are depicted in Figs 2, 3A and 3B. Data are presented as conventional double reciprocal plots. Michaelis and inhibitor constants were calculated as described by Dixon and Webb [23, 24]. Both saccharin and cyclamate were competitive inhibitors toward glucose-6-*P* and PP_i and were non-competitive with respect to glucose. K_i (saccharin)

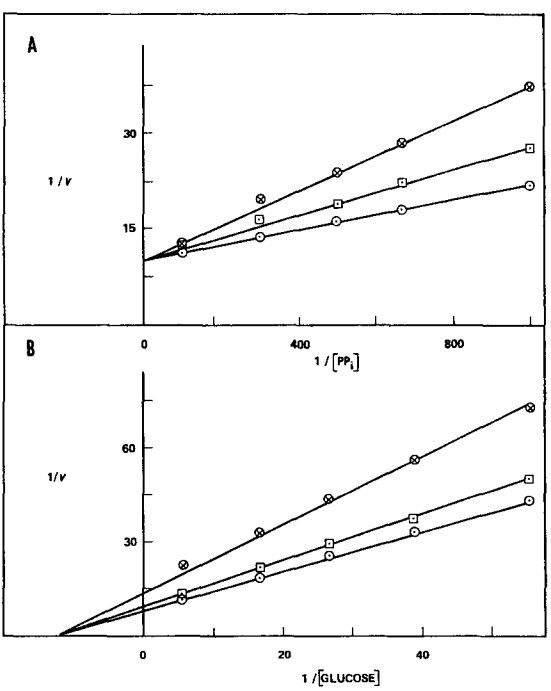

Fig. 3. Kinetics of inhibition of PP_i-glucose phosphotransferase activity by saccharin and cyclamate. (A) Inhibition with respect to PP_i. Assay mixtures, pH 5.6, contained, in 1.5 ml, 40 mM cacodylate buffer, 180 mM glucose, and indicated amounts of PP_i. Activities were determined in the absence (○) and presence of 20 mM saccharin (⊕) or 30 mM cyclamate (□). (B) Inhibition with respect to glucose. Assay mixtures, pH 5.6, contained, in 1.5 ml, 40 mM cacodylate 10 mM PP_i, and indicated concentrations of glucose. Activities were measured in the absence (○) and presence of 20 mM saccharin (⊕) or 30 mM cyclamate (□). Activities are expressed as described in Fig. 1B. Kinetic parameters evaluated from these data are: K_m (PP_i) = 1.2 mM; K_m (glucose) = 80 mM; K_i (saccharin) = 16 mM relative to PP_i and 110 mM with respect to glucose; K_i (cyclamate) = 68 mM with respect to PP_i and 190 mM relative to glucose.

values relative to glucose-6-*P*, PP_i, and glucose were 6.5, 16, and 110 mM, respectively. K_i (cyclamate) values were 26, 68, and 190 mM relative to glucose-6-*P*, PP_i, and glucose, respectively. In supplementary studies at pH values above 5.6, inhibition by saccharin and cyclamate was of the mixed or non-competitive type toward the phosphate substrates.

Since a single enzyme catalyzes both the hydrolysis of glucose-6-*P* and the PP_i-glucose phosphotransferase reaction in mammals (see Nordlie [25]), the K_i value for each inhibitor would normally be expected to be constant relative to each substrate tested. That relationship was not observed in the present study and also was not observed in a previous study [26] of phlorizin inhibition of these same activities. Klein [27] has observed such anomalies in the inhibition of pig kidney D-amino acid oxidase by benzoate where determination of K_i values from Lineweaver–Burk plots led to different values for various substrates. Pocker and Meany [28] also reported a similar phenomenon in studies with carbonic anhydrase.

Inhibitor specificity

The inhibitory effect of various structural analogs of saccharin and cyclamate was examined for the phosphohydrolase and phosphotransferase reactions. The results are compiled in Table I. The inhibitory effects are not attributable to increased

TABLE I

INHIBITOR SPECIFICITY

Reaction mixtures (pH 5.6) contained, in 1.5 ml, 40 mM sodium cacodylate, 0.1 ml enzyme preparation, 5 mM glucose-6-*P* (phosphohydrolase) or 5 mM PP_i and 90 mM glucose (phosphotransferase), and 30 mM inhibitor, except benzamide and sulfanilamide which were 8 mM.

Inhibitor tested	Inhibition (%)	
	Glucose-6-*P* phosphohydrolase	PP_i-glucose phosphotransferase
Benzamide	<5	<5
Sulfanilamide	<5	<5
Cyclohexylamine	<5	<5
Sulfamic acid	<5	<5
Aniline	<5	6
Glucosamine	<5	6
Sulfanilic acid	11	<5
p-Aminobenzoic acid	15	<5
Benzenesulfonic acid	26	10
Cyclamate	23	13
Cyclohexanecarboxylic acid	21	23
Benzoic acid	31	12
Saccharin	50	31

ionic strength since no inhibition of the activities was observed upon addition of NaCl up to a final concentration of 67 mM and there was only a 7% inhibition at 133 mM NaCl. Saccharin was the most effective inhibitor tested. Cyclamate inhibited both activities but no significant inhibition was obtained with cyclohexylamine, a

metabolite of cyclamate and an impurity in the commercial cyclamate preparation. *o*-Toluenesulfonamide, the impurity in the saccharin preparation, exhibited no inhibitory effect when tested at the concentration present in the saccharin solutions.

In contrast with the other substances tested, compounds which exhibitec inhibitory effects of 10% or greater contained both a cyclohexane or benzene ring anc an anionic group. Saccharin and cyclamate were competitive inhibitors toward phosphate substrates so these other inhibitors may act in a similar fashion. The presence of an anionic group may well be an essential structural feature of such inhibitors tc facilitate binding to the enzyme in a manner analogous to that for the phosphate substrates, which have been postulated [25] to interact with a metal cation and a positively charged imidazolium group at the enzymic active site.

Physiological significance

The physiological importance of these inhibitory effects is uncertain. The K values were determined at pH 5.6 where the extent of inhibition is greater than at pH 7. The observed K_i (saccharin) and K_i (cyclamate) values for the phosphohydrolase reaction exceed the average hepatic levels of these compounds, though higher concentrations may exist in certain subcellular regions. The assumed maximum safe daily dose of cyclamate for man is 2.4 mg/kg per day [29] or 168 mg (0.8 mmole)/day for a 70-kg person. Neither saccharin nor cyclamate accumulate appreciably in the body since approx. 90% of each compound is excreted within 24 h after ingestion [13, 19, 30]. Rats fed a diet containing 5% saccharin for 15 weeks had an average saccharin concentration of 47 ppm (0.25 mM) in blood and 22.4 ppm in the liver [31]. Cyclohexylamine, the major known metabolite of cyclamate, did not inhibit the glucose-6-phosphatase activities.

Several dietary and/or hormonal factors may modify the possible physiological significance of these inhibitory effects. The kinetic parameters and/or pH–activity profiles of these activities are altered under conditions of glucocorticoid administration, diabetes, and fasting (see Nordlie [25]). The effect of some of these factors on the inhibition by saccharin and cyclamate currently is being investigated in this laboratory.

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