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LACK OF EFFECT OF CHARGE OF PHLORIZIN AND PHLORETIN ON MUTAROTASE ACTION

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Summary

Many transport phenomena are inhibited by the polyphenolic compounds phlorizin and its aglucone phloretin. We report here a hitherto unsuspected charge on the molecules at physiological pHs which may be of importance in interpreting their mode of action. The change in inhibition of mutarotase was studied with change of pH with these compounds, but no significant effect of charge was detected.

Introduction

Phlorizin, a classical inhibitor of glucose transport *in vivo* and *in vitro*, is known also to inhibit Na,K-ATPase (1), alkaline phosphatase (2), mutarotase (3) and anion transport (4). Both phlorizin and phloretin inhibit hexose transport (5,6,7).

The absorption spectra of phlorizin and phloretin change with changes in pH. This phenomenon was attributed to pH dependent keto-enol tautomerism by Lambrechts (8) and this interpretation has been frequently cited (9). However, we have observed, upon direct potentiometric titration of phlorizin and phloretin with alkali, a first acid dissociation constant for both around pH 7.0. Spectrophotometric changes of phlorizin and phloretin observed when pH is changed thus can be explained not by keto-enol tautomerism but by the different extinction coefficients of the molecules in their anionic and non-ionic forms. It follows, therefore, that in transport studies which have been carried out around physiological pHs, phlorizin and phloretin were present as both undissociated molecule and as anion, and this hitherto unsuspected charge must be taken into account in considering the interpretation of results.

In view of this fact, we undertook studies with the enzyme mutarotase (aldose 1-epinerase; EC 5.1.3.3) which catalyzes the mutarotation of glucose. Mutarotase was chosen for this study because its kinetics are simple and well

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known and inhibition studies may be carried out with high accuracy. It has been proposed that mutarotase is involved in glucose transport (10). Moreover, in a study with various analogues of phlorizin, Diedrich and Stringham proposed that the patterns of inhibition in transport studies are similar to those with mutarotase and that the enzyme therefore may serve at least as a model of the glucose transport carrier system (11). Fannin and Diedrich assumed that an affinity column comprising phloretin would bind the glucose carrier of kidney as well as other kidney proteins, but only the glucose carrier would be eluted with glucose. Only mutarotase was found to be eluted in this kind of experiment (12).

To determine the effect of the charge on phlorizin and phloretin on the degree of inhibition produced by these inhibitors, these compounds were used to inhibit mutarotase at pH 5.4 and 8.1. In this pH range, the mutarotase activity curve is substantially flat but the percentage of anion of the inhibitors varies almost two orders of magnitude.

Materials and Methods

Chemicals

Phlorizin was purchased from Nutritional Biochemicals Corporation and recrystallized twice from warm aqueous solution as its dihydrate form (m.p. 110°C). Phloretin was purchased from K&K Laboratories (Lot #17070) and recrystallized twice from alcohol-water mixture (dec. 260°C). Differential thermal analysis showed no water of hydration of phloretin. α-D-glucose was finely pulverized anhydrous Cerelose (Lot #2461) from Corn Products Co.. Hog kidney mutarotase was purchased from Boeringer Mannheim. Buffers at 0.05M were prepared from MES (pH 5.4, B/A = 0.4), HEPES (pH 8.1, B/A = 5) and MOPSO (pH 7.3, B/A = 6.3; pH 6.2, B/A = 1/4) which were purchased from Research Organics, Inc.. All buffers contained EDTA (5×10^{-4} M).

Apparatus

An Orion pH meter, Model No. 801 in conjunction with a semimicro pH combination electrode was used for pH measurements. Polarimetry was performed in a Cary 60 ORD spectropolarimeter. Spectrophotometric measurements were made with a Cary 118C spectrophotometer.

Procedure

In order to find the pK_1 of phloretin, 6.6 mg of phloretin were placed in 50 ml water and warmed to 85°C . Measurements of pH were made at about room temperature after one-half equivalent and two-thirds equivalent of NaOH were added, from which data the pK_1 was calculated. Phlorizin was titrated with standard NaOH and from the pH titration curve, pK_1 was obtained based on the calculated first half neutralization point.

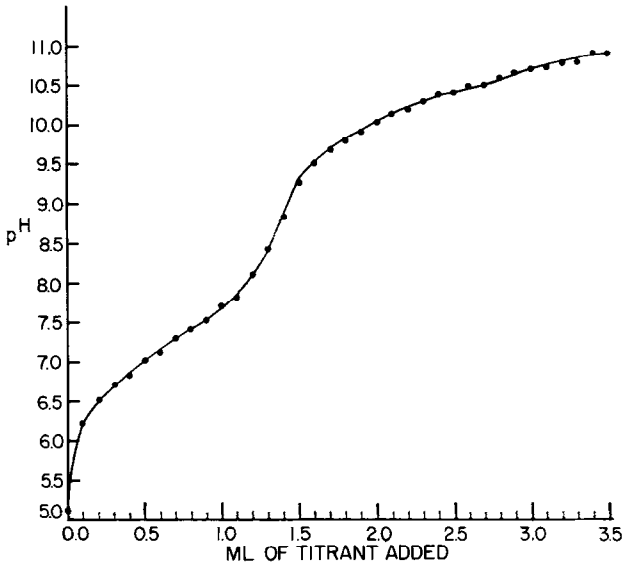

Figure 1. The potentiometric titration of 10 ml of 2.53 X 10^{-3}M phlorizin with 1.96 X 10^{-2}M NaOH.

Spectrophotometric determination of pK_1 was made through the concentration ratios of the monoanion and the neutral forms of phlorizin and phloretin. For both phlorizin and phloretin the monoanion form shows an absorption maximum at 320 nm; the neutral form at 284 nm. The molar extinction coefficients of the two forms at the two wavelengths were calculated from absorption spectra at pH values of 2.1 and 8.1 where only one of the two forms predominates. Between these pH limits, the spectra show isobestic points at 256 nm and 298 nm indicating the presence of only two species.

Polarimetric assays of mutarotase were carried out in a Cary 60 ORD instrument at a fixed wavelength of 400 nm or 589 nm. α-D-glucose was dissolved in buffer and within less than one minute, mutarotase was added. The solution was quickly introduced into the polarimeter tube which was kept at 24°C and measurements of optical rotation were started. The half time for the first order reaction and the inhibitor constant, K_i, were calculated as previously described (13).

Results and Discussion

Figure 1 shows a pH titration curve of 10.0 ml of 2.53 X 10^{-3} M solution of phlorizin with 1.96 X 10^{-2} M NaOH as the titrant. The calculated half-enutralization point at 0.645 ml titrant added leads to a pKa_1 value of 7.2. A set of four experiments show the pKa_1 values thus obtained are reproducible within 0.1 pH unit. The calculated first equivalence point agrees with the inflection point in the titration curves within 2%.

The values of pKa_1 obtained from calculations involving adding a half equivalent of NaOH and two thirds of an equivalent of NaOH to the phloretin as described above were respectively 6.9 and 7.0.

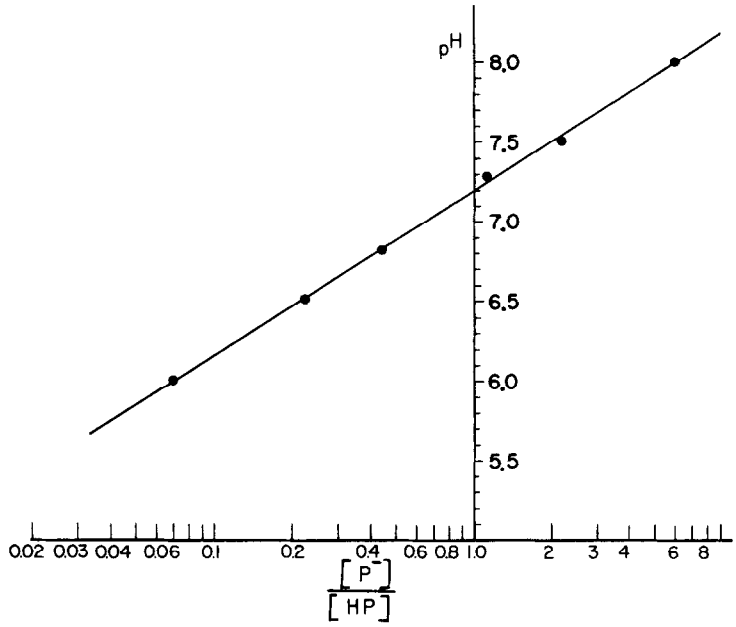

Figure 2. The variation in concentration ratios of mono-anion and neutral forms of phloriain with pH as calculated from the spectrophotometric titration curves at various pHs.

Figure 2 is a plot of pH of phlorizin solution vs. log $(P^-)/(HP)$ as derived from spectrophotometric data. The plot gives a straight line intercepting the pH axis at a value of 7.2. Thus the pKa_1 values obtained from spectrophotometric titration are in agreement with that from the titration curve.

Spectrophotometric titrations of phloretin lead to a pKa_1 value of 7.3. Hence, we conclude that around the physiological range of pH both phlorizin and phloretin can exist in anionic form and the ratio of the concentrations of the anionic forms and neutral molecules is highly pH dependent.

Figure 3 is a Lineweaver-Burke plot of mutarotase with α-D-glucose at pH 5.4 and pH 8.1. Table I gives the Michaelis-Menton constant (Km) and inhibitor constants (K_i) at these two pHs. V_{max} of mutarotase with α-D-glucose in the pH range 5.4 to 8.1 is presented in Table II.

The variation of K_i with pH from 5.4 to 8.1 is not significant with either inhibitor. Since at these two pHs there is close to two orders of magnitudes difference in concentration of anion, it must be concluded that the

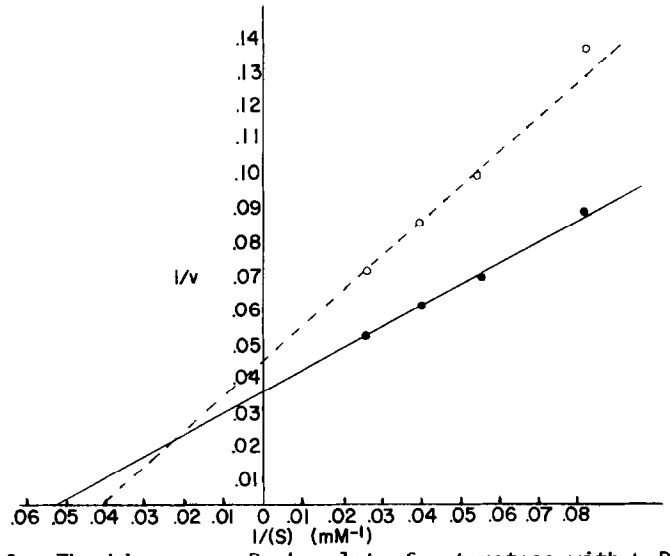

Figure 3. The Lineweaver-Burke plot of mutarotase with α-D-glucose. (------), pH 8.1; (————), pH 5.4.

TABLE I

The Variation of Km (Michaelis-Menton constant) and K_i (Inhibitor constant) for mutarotase and alpha-D-glucose with pH in the presence of phlorizin (0.47mM) and phloretin (0.066mM)

pH	Km (mMO)	K_i (mM)
5.4	19.2	.318 (phlorizin)
8.1	24.7	.451 (phlorizin)
5.4	19.2	.029 (phloretin)
8.1	24.7	.026 (phloretin)

TABLE II

The Variation of V_{max} for mutarotase and alpha-D-glucose with pH

pH	Relative V_{max}
8.1	.90
7.3	.91
6.2	1.00
5.4	.99

Concentration substrate : 0.46M.

charge on inhibitor does not significantly affect the capacity of either phlorizin or phloretin to inhibit mutarotase.

Since enzymes are highly specific, there is a suggestion here that the same factors which lead to the abnormally low pKas are involved in determination

of the effectiveness of binding of both compounds. Attempts to define the locus of the charge on the molecules are currently being undertaken by means of nuclear magnetic resonance.

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