BBA 76051

AN EXAMPLE OF MUTUAL COMPETITION BETWEEN TRANSPORT INHIBITORS OF DIFFERENT KINETIC TYPE: THE INHIBITION OF INTESTINAL TRANSPORT OF GLUCALOGUES BY PHLORETIN AND PHLORIZIN

V. E. COLOMBO AND G. SEMENZA

Department of Biochemistry of the Swiss Federal Institute of Technology (E.T.H.), Zurich (Switzerland)

(Received May 18th, 1972)

SUMMARY

1. Kinetic analysis allows the detection of mutual competition between transport inhibitors of similar or of different kinetic type.

2. Phlorizin, a fully competitive and phloretin, a fully non-competitive inhibitor compete mutually for the transport system(s) of glucalogues in hamster small intestine. This observation suggests common step(s) in the mechanism of inhibition by phloretin and phlorizin.

INTRODUCTION

Phlorizin is a fully competitive inhibitor of the Na^+-dependent transport of glucalogues at the brush border pole of the small intestinal cylindrical cells[1], whereas its aglycone, phloretin, produces a decrease in the apparent maximum transport velocity, without affecting the apparent K_m (ref. 2). Despite the fact that the two types of inhibition by these compounds have different kinetics, hypotheses have been put forward on the mechanism of phlorizin inhibition, which imply similarity or overlap between the modes of action of phlorizin and phloretin:

(i) Phlorizin binds at two sites, *viz.* at the glucalogue carrier and at the phloretin binding site (the resulting higher binding energy would explain the unusually small K_i of phlorizin[2,3]. (ii) The aglycone of phlorizin (liberated by the brush border-bound phlorizin hydrolase[4,5]) would be the actual inhibitor[5] rather than phlorizin itself. A third possibility is, of course, that phlorizin and phloretin inhibit intestinal sugar transport through unrelated mechanisms.

Hypothesis (i), and probably Hypothesis (ii) also, imply that phlorizin and phloretin should compete with each other, in spite of their different kinetic type of inhibition. To the best of our knowledge, however, such competition was never subjected to a quantitative kinetic test*, nor, in fact, were ever the appropriate kinetic equations made available. In the following we provide the equations for

* A first step in this direction is reported in ref. 3.

testing whether two transport inhibitors, fully competitive and/or fully non-competitive compete mutually or not. It will be seen that, whenever the two inhibitors compete mutually, Dixon plots (1/initial flux *vs* variable concentrations of one inhibitor) in the presence and in the absence of a second inhibitor, always yield *parallel lines*, irrespective of whether the two inhibitors belong to the same kinetic type or not.

Experiments on the inhibition of small intestinal sugar transport by phloretin and phlorizin clearly show that these two inhibitors compete mutually.

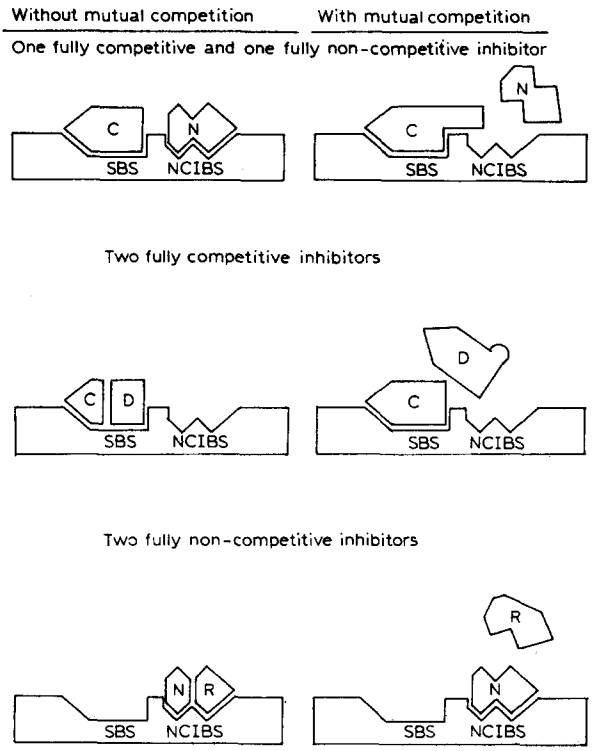

Fig. 1. Schematic model of mutual competition or lack of mutual competition between fully competitive and fully non-competitive inhibitors. SBS, substrate binding site; NCIBS, non-competitive inhibitor binding site. C, D, fully competitive inhibitors; N, R, fully non-competitive inhibitors.

THEORY

Assumptions

In deriving the equations of Table I the following assumptions were made: (a) The carrier binds one substrate molecule. (b) The rate-limiting step in the unidirectional flux is the translocation of the carrier through the membrane (or its physical equivalent), so that carrier, substrate and inhibitor can be considered as being always in equilibrium at either face of the membrane. (c) In the steady state there is no build-up of carrier anywhere in the membrane (*i.e.* the total

velocity of translocation of carrier molecules in its various forms is the same in both directions). (d) The permeability coefficients (P, or their physical equivalents) for a given carrier form are equal in both directions.

Definition of symbols

C and D were fully competitive inhibitors; N and R fully non-competitive inhibitors; S the substrate; X the carrier. Their concentrations are indicated by small letters. X_t was the total carrier concentration. $J_{mc}^{s',i'=0}$ was the initial unidirectional flux of substrate from the mucosal incubation medium into the intestinal cells across the brush border membrane.

The K values at the cis side (incubation medium) were defined as follows:

$$K_S = \frac{x \cdot s}{xs} = \frac{xn \cdot s}{xsn} = \frac{xr \cdot s}{xsr}$$

$$K_N = \frac{x \cdot n}{xn} = \frac{xs \cdot n}{xsn} = \left[\frac{xc \cdot n}{xcn}\right] = \left[\frac{xr \cdot n}{xrn}\right] = \left[\frac{xd \cdot n}{xnd}\right]$$

$$K_C = \frac{x \cdot c}{xc} = \left[\frac{xn \cdot c}{xnc}\right] = \left[\frac{xd \cdot c}{xdc}\right] = \left[\frac{xr \cdot c}{xcr}\right]$$

$$K_R = \frac{x \cdot r}{xr} = \frac{xs \cdot r}{xsr} = \left[\frac{xc \cdot r}{xcr}\right] = \left[\frac{xn \cdot r}{xrn}\right] = \left[\frac{xd \cdot r}{xdr}\right]$$

$$K_D = \frac{x \cdot d}{xd} = \left[\frac{xn \cdot d}{xnd}\right] = \left[\frac{xc \cdot d}{xdc}\right] = \left[\frac{xr \cdot d}{xdr}\right]$$

When the inhibitors are mutually competitive, the K_s values of the equilibria in square brackets were equal to ∞. The magnitudes referring to equilibria at the trans (cellular) side were defined similarly and indicated by a dash ($'$). The permeability coefficients (or their physical equivalents) were indicated by P and are indexed appropriately. P_{XSN} and P_{XSR} were assumed to be zero ("fully" non-competitive inhibition), whereas none of the other P values was assumed to be necessarily equal to zero*. This additional assumption can be made, the corresponding term then vanishing from the equations of Table I.

Dixon plots

The rate equations for the initial unidirectional fluxes (*i.e.* at zero trans concentrations of substrate and inhibitors) are given in Table I in the form requested for Dixon plots ($1/J_{mc}^{s',i'=0}$ *vs* either n or c). They provide a simple kinetic test to decide whether the inhibitors considered compete mutually or not: whatever the kinetic type of their inhibition, if the two inhibitors compete mutually, the line obtained in Dixon plots in the presence of a constant concentration of a second inhibitor is always parallel to that obtained with the variable inhibitor alone. (compare eqns III and IV with eqn I and eqns V and VI with eqn II) On the other hand, if the two inhibitors do not compete mutually, the lines obtained cross at the left side of the $1/J_{mc}^{s',i'=0}$ axis, the crossing point being either above, or on,

* When P_{XC} or P_{XD} are > 0, C or D, respectively, can of course be regarded as substrates.

Biochim. Biophys. Acta, 288 (1972) 145–152

TABLE I

INITIAL UNIDIRECTIONAL FLUXES OF THE SUBSTRATE S IN THE PRESENCE OF ONE OR TWO FULLY COMPETITIVE OR FULLY NON-COMPETITIVE INHIBITOR(S) (ZERO TRANS CONCENTRATIONS OF SUBSTRATE AND INHIBITORS):

$I/J_{mc}^{s',i'=0} = f(i)$

One inhibitor		
Fully competitive inhibitor C	$\frac{I}{J_{mc}^{s',i'=0}} = Z + cW$	(I)
Fully non-competitive inhibitor N	$\frac{I}{J_{mc}^{s',i'=0}} = Z + nY$	(II)
Two mutually competing inhibitors		
Two fully competitive inhibitors C and D: constant d, variable c	$\frac{I}{J_{mc}^{s',i'=0}} = Z + \frac{\frac{K_S d}{sK_D}\left(1 + \frac{P_{XD}}{P_X}\right)}{P_{XS}X_t} + cW$	(III)
One fully competitive inhibitor C, one fully non-competitive inhibitor N: constant n, variable c	$\frac{I}{J_{mc}^{s',i'=0}} = Z + \frac{\frac{n}{K_N}\left[1 + \frac{K_S}{s}\left(1 + \frac{P_{XN}}{P_X}\right)\right]}{P_{XS}X_t} + cW$	(IV)
constant c, variable n	$\frac{I}{J_{mc}^{s',i'=0}} = Z + \frac{\frac{K_S c}{sK_C}\left(1 + \frac{P_{XC}}{P_X}\right)}{P_{XS}X_t} + nY$	(V)
Two fully non-competitive inhibitors N and R: constant r, variable n	$\frac{I}{J_{mc}^{s',i'=0}} = Z + \frac{\frac{r}{K_R}\left[1 + \frac{K_S}{s}\left(1 + \frac{P_{XR}}{P_X}\right)\right]}{P_{XS}X_t} + nY$	(VI)

TABLE I (CONTINUED)

Two non-mutually competing inhibitors

Two fully competitive inhibitors C and D: constant d, variable c

$$\frac{1}{J_{mc}^{s',i'=0}} = Z + \frac{\frac{K_S d}{sK_D}\left(1 + \frac{P_{XD}}{P_X}\right)}{P_{XS}X_t} + c\left[W + \frac{\frac{K_S d}{sK_D}\left(1 + \frac{P_{XCD}}{P_X}\right)}{K_c P_{XS}X_t}\right] \quad \text{(VII)}$$

One fully competitive inhibitor C, one fully non-competitive inhibitor N: constant n, variable c

$$\frac{1}{J_{mc}^{s',i'=0}} = Z + \frac{\frac{n}{K_N}\left[1 + \frac{K_S}{s}\left(1 + \frac{P_{XN}}{P_X}\right)\right]}{P_{XS}X_t} + c\left[W + \frac{\frac{K_S n}{sK_N}\left(1 + \frac{P_{XCN}}{P_X}\right)}{K_c P_{XS}X_t}\right] \quad \text{(VIII)}$$

constant c, variable n

$$\frac{1}{J_{mc}^{s',i'=0}} = Z + \frac{\frac{K_S c}{sK_C}\left(1 + \frac{P_{XC}}{P_X}\right)}{P_{XS}X_t} + n\left[Y + \frac{\frac{K_S c}{sK_C}\left(1 + \frac{P_{XCN}}{P_X}\right)}{K_C P_{XS}X_t}\right] \quad \text{(IX)}$$

Two fully non-competitive inhibitors N and R: constant r, variable n

$$\frac{1}{J_{mc}^{s',i'=0}} = Z + \frac{\frac{r}{K_R}\left[1 + \frac{K_S}{s}\left(1 + \frac{P_{XR}}{P_X}\right)\right]}{P_{XS}X_t} + n\left[Y + \frac{\frac{K_S r}{sK_R}\left(1 + \frac{P_{XRN}}{P_X}\right)}{K_N P_{XS}X_t}\right] \quad \text{(X)}$$

Abbreviations used:

$$\frac{1 + \frac{P_{XS}}{P_X} + 2\frac{K_S}{s}}{P_{XS}X_t} \equiv Z, \qquad \frac{\frac{K_S}{s}\left(1 + \frac{P_{XC}}{P_X}\right)}{K_C P_{XS}X_t} \equiv W, \qquad \frac{1 + \frac{K_S}{s}\left(1 + \frac{P_{XN}}{P_X}\right)}{K_N P_{XS}X_t} \equiv Y.$$

or below the i axis, depending on the P values involved. This test is further illustrated in Fig. 2.

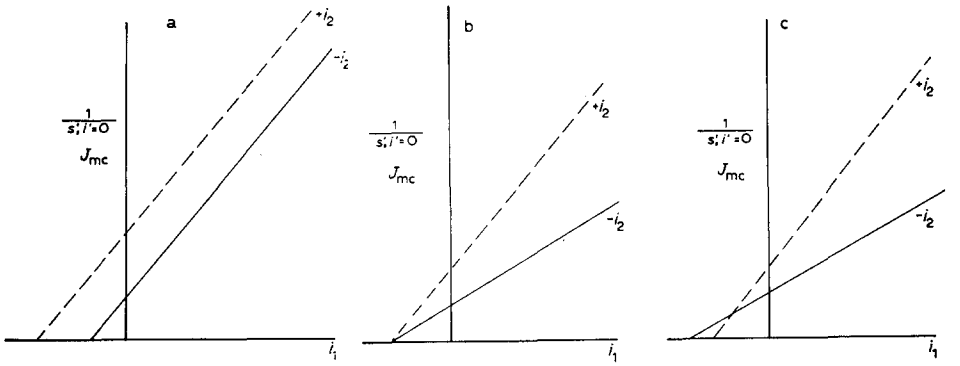

Fig. 2. Dixon plots. $(1/\int_{mc}^{s',i'=0}$ *vs* variable concentrations of one inhibitor i_1, with or without another inhibitor i_2 at a constant concentration). (a) Mutual competition between inhibitors. (b) and (c) two examples of lack of mutual competition between inhibitors.

METHODS AND MATERIALS

The intestinal sugar transport was measured *in vitro* in Krebs–Henseleit buffer[6], using 6-deoxy-D-glucose (2.0 mM) as substrate, with [^{3}H]mannitol (0.25 μM) as a marker for the extracellular space*. Everted small-intestinal rings from hamsters were mounted in appropriate frames exposing to the medium the mucosal surface alone[7]. At short incubations the uptake was an accurate approximation of the unidirectional flux[7]; the extracellular space amounted to 10% at most. Preincubation (at 37 °C, for 0–30 min) was carried out in Krebs–Henseleit buffer containing 0–10 mM phloretin. (The stock solution of phloretin was 180 mM in acetone; equivalent amounts of acetone were added to the controls). At the beginning of the incubation proper, 6-deoxy-D-glucose and mannitol were added, as well as phlorizin at the concentrations indicated. (Phlorizin was not present during the preincubation, because of the phlorizin hydrolase activity of the small intestine, refs 4 and 5). Incubation at 37 °C lasted 15 min. Preliminary experiments had showed that the uptake of 6-deoxy-D-glucose was linear up to this time at least.

During both preincubation and incubation the tissues were supplied continuously with O_2 (95%) and CO_2 (5%), which prevented any damage — either morphological or functional — to the tissue[8]. At the end of the incubation, the tissue was rinsed with cold Krebs–Henseleit buffer, punched[7] and weighed. After deproteinization with $Ba(OH)_2$– $ZnSO_4$, 6-deoxy-D-glucose was measured according to the method of Dische and Shettles[9]. [^{3}H]mannitol was determined in a scintillation counter; the scintillation liquid was 0.05% butyl-PBD [2-(4′-*tert*-butylphenyl)-5-(4″-biphenyl)-1,3,4-oxadiazole] in toluene. The data were expressed as μmoles substrate taken up per min per ml tissue water, assuming that the latter amounted to 80% of the fresh weight[10].

* The absorption of mannitol in most of hamster small intestine is negligible (P. Ruf and G. Semenza, *Biochim. Biophys. Acta*, to be submitted for publication), as compared to that of 6-deoxy-D-glucose. For the purpose of the present investigation, therefore, mannitol can be regarded as a marker for extracellular space.

6-Deoxy-D-glucose was supplied by Koch–Light Lab., Colnbrook, Bucks; phlorizin by Fluka, Bucks, and was recrystallized twice from water; phloretin was obtained from K and K Lab., Inc., Plainview, N.J., and [^{3}H]mannitol from The Radiochemical Centre, Amersham, Bucks. All other chemicals were reagent grade and were obtained from commercial sources.

TABLE II

6-DEOXY-D-GLUCOSE UPTAKE IN THE SMALL INTESTINE OF ADULT HAMSTER IN THE PRESENCE OF 1 mM PHLORETIN; PREINCUBATION 30 MIN

The data are corrected for the extracellular space and are expressed as μmoles taken up per min per ml tissue water. (Intestine mounted in frames, ref. 7). Average of 4 determinations $\pm$ S.E.

	Uptake	*Inhibition* (%)
Controls	0.614 $\pm$ 0.012	—
+ Phloretin (reagent grade)	0.111 $\pm$ 0.007	82.0
+ Phloretin (pretreated in 3 M HCl at 100 °C for 100 min)	0.092 $\pm$ 0.017	85.1

RESULTS AND DISCUSSION

The batch of phloretin used was first tested for the possible contamination with traces of the much more active phlorizin. If the inhibition brought about by phloretin was due in part to contaminating phlorizin, treatment of the phloretin preparation with 3 M HCl for 100 min at 100 °C should decrease its inhibiting power. Table II shows that this is not the case.

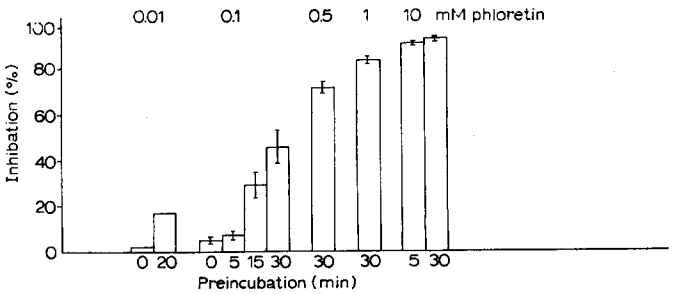

Fig. 3. Inhibition of 6-deoxy-D-glucose uptake in the small intestine of adult hamsters at various phloretin concentrations and after preincubations of different lengths of time in the presence of phloretin.

The onset of inhibition is much greater in the case of phlorizin than in that of phloretin[11]. The time course of phloretin was therefore checked by preincubation experiments of increasing length of time (Fig. 3). For the mixed tests, the concentration of phloretin chosen was 0.5 mM and the time of preincubation 30 min. Due to the slow onset of phloretin inhibition, it was chosen to carry out the mixed inhibition tests at one phloretin concentration and at variable phlorizin concentrations.

The results of mixed inhibition tests are reported in Fig. 4. It is clear that the line obtained with phlorizin in the presence of phloretin is parallel to that obtained with phlorizin alone. (The slope with phlorizin and phloretin is 0.54; the one with phlorizin alone is 0.57).

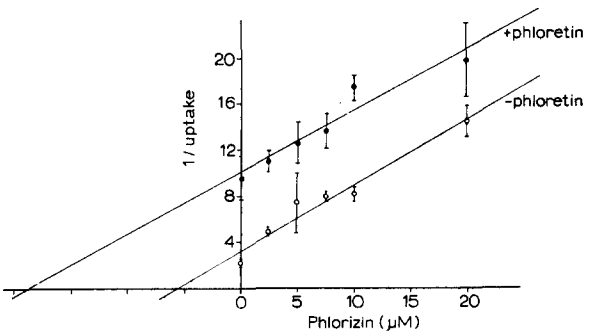

Fig. 4. Inhibition of 6-deoxy-D-glucose uptake in the small intestine of adult hamsters by phlorizin in the presence (●) or in the absence (○) of 0.5 mM phloretin. Dixon plots; $n = 5$; the lines are regression lines; the bars indicate the S.E.

Phlorizin being a fully competitive inhibitor[1] and phloretin probably a fully non-competitive inhibitor[2], the relevant equations are I and IV (Table I). It should be pointed out, however, that parallel lines in this plot allow the diagnosis of mutual competition irrespective of the kinetic type of the individual inhibitors. (see under Theory). This point is of importance, in view of the present uncertainty as to the kinetic type of phloretin inhibition[2] and as to the chemical mechanism of phlorizin inhibition[5]. The mutual competition between phloretin and phlorizin suggests common step(s) in their mechanisms of inhibition, and is thus compatible with, but does not necessarily prove, Hypotheses (i) and (ii) quoted in the Introduction.

To the best of our knowledge, this is the first reported example of competition between inhibitors of different kinetic type.

ACKNOWLEDGEMENTS

This work was supported by the Swiss National Science Foundation, Bern.

REFERENCES

1 F. Alvarado and R. K. Crane, *Biochim. Biophys. Acta*, 56 (1962) 170.
2 D. F. Diedrich, *Arch. Biochem. Biophys.*, 117 (1966) 248.
3 F. Alvarado, *Biochim. Biophys. Acta*, 135 (1967) 483.
4 P. Malathi and R. K. Crane, *Biochim. Biophys. Acta*, 173 (1969) 245.
5 D. F. Diedrich, *Arch. Biochem. Biophys.*, 127 (1968) 803.
6 H. A. Krebs and K. Henseleit, *Z. Physiol. Chem.*, 210 (1932) 33.
7 G. Semenza, *Biochim. Biophys. Acta*, 173 (1969) 104.
8 P. Béguin, Diplomarbeit, ETH-Z, 1971.
9 Z. Dische and L. B. Shettles, *J. Biol. Chem.*, 175 (1948) 595.
10 R. K. Crane and P. Mandelstam, *Biochim. Biophys. Acta*, 45 (1960) 460.
11 F. Ponz and M. Lluch, *Rev. Esp. Fisiol.*, 20 (1955) 267.