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THE COMPETITIVE INHIBITION OF GLUCOSE TRANSPORT IN HUMAN ERYTHROCYTES BY COMPOUNDS OF DIFFERENT STRUCTURES

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Abstract—By kinetic analysis we found that the transport protein for glucose in human erythrocyte membranes has different binding sites for competitive inhibitors. They all change the transport protein with the effect that it loses its affinity to glucose. Some of the competitive inhibitors alter the conformation of the transport protein, so that other ones cannot be bound. There are inhibitors, however, which do not affect the affinity of other competitive inhibitors. A schematic model of our assumption about the mechanism of the competitive inhibition of glucose transport is presented.

It is known from the inhibition of enzymatic reactions that competitive inhibitors have a structure similar to that of the substrate; this property enables them to compete for binding sites of enzymes. Identical mechanisms occur in transport processes. In recent years, it has been shown that some drugs (such as local anesthetics [1], steroids [2], cytochalasin [3], and some amino acid esters considered as antisickling agents [4, 5]), having no structural similarity to glucose, act as competitive inhibitors of glucose transport in human erythrocytes. Their mechanism was supposed to be based on a conformational change of the transport protein due to its interaction with the inhibitor molecules [6].

In this paper, we present results obtained with competitive inhibitors of structures related and unrelated to that of glucose. We wanted to find out what transport protein–inhibitor complexes can be formed: whether two simultaneously used inhibitors bind to the same site of the transport protein, or whether they have two different binding sites. Comparison of the experimental data with the results of our theoretical considerations clarified some aspects of the competitive inhibition of glucose transport.

MATERIALS AND METHODS

Blood of healthy donors was collected in an ACD solution (11 g sodium citrate, 35 g glucose, 4 g citric acid in 1000 ml of twice distilled water). After removal of the plasma by centrifugation, the erythrocytes were preloaded with 200 mM glucose by four washings in isotonic NaCl solution containing 200 mM glucose. 150 μl of these preloaded cells were incubated for 5 sec at 20° in 10 ml isotonic phosphate buffer pH 7.5, containing different concentrations of [^{14}C]glucose and in the first series of experiments one sort of inhibitor (I_1)†, in the second series besides the first inhibitor (I_1) another drug acting as inhibitor (I_2). The incubation was stopped by pouring the suspension into 80 ml ice-cold stopping solution (2 mM $HgCl_2$, 310 mM NaCl, 1.25 mM KI). After centrifugation, the erythrocytes were hemolysed in twice distilled water, the proteins precipitated according to Somogyi [7], and the radioactivity of the [^{14}C]glucose determined by liquid scintillation counting (for details see Lacko *et al.* [8]).

THEORETICAL CONSIDERATION

To find out whether two inhibitors bind to the same site or to two different sites of the transport protein, we derived equations by which one can calculate the uptake velocity of glucose in the presence of only one inhibitor (v_{I_1}) from the experimentally found uptake velocity ($v_{I_1I_2}$) in the presence of two inhibitors.

One equation was based on the assumption that both inhibitors bind to the same site, the other that each of both inhibitors has a specific site on the transport protein.

For both assumptions, we determined the dissociation constants (K_{I_1}) of the inhibitor–transport–protein complex (CI_1) by kinetic analysis; of course,

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† List of symbols: K_m, Michaelis constant of the carrier glucose complex; [S], glucose concentration in the medium (mM); [S_1], [S_2], different concentrations of glucose (mM); V_{max}, maximum transport velocity for glucose; [I_1], concentration of the first inhibitor (mM); [I_2], concentration of the second inhibitor (mM); v_{I_1}, transport velocity of the glucose uptake inhibited by the first inhibitor of the concentration [I_1]; v_{I_2}, transport velocity of the glucose uptake inhibited by the second inhibitor of the concentration [I_2]; $v_{I_1I_2}$, transport velocity of glucose uptake inhibited by both inhibitors of the concentrations [I_1] and [I_2]; K_{I_1}, dissociation constant of the inhibitor–transport–unit complex of the first inhibitor; K_{I_2}, dissociation constant of the inhibitor–transport–unit complex of the second inhibitor; C_{tot}, total transport protein; C, free transport protein; CS, transport protein glucose complex; CI_1, CI_2, transport protein inhibitor complex with the first and with the second inhibitor; CI_1I_2, transport protein inhibitor complex with both inhibitors.

we obtained two different K_I values. One of them was identical with the K_I-value, which we found out experimentally by kinetic analysis in the presence of only one inhibitor I_1.

Of both the assumptions, the one which leads to the correct K_I value reflects the kind of binding of inhibitors to the transport protein.

First case.

$$C_{tot} = C + CS + CI_1 + CI_2 \quad (1)$$

The transport velocity equation:*

$$\frac{1}{v_{I_1I_2}} = \frac{K_m + [S] + K_m\frac{[I_1]}{K_{I_1}} + K_m\frac{[I_2]}{K_{I_2}}}{V_{max}\cdot[S]} \quad (2)$$

The equation for the uptake velocity with one inhibitor only is:

$$\frac{1}{v_{I_1}} = \frac{K_m + [S] + K_m\frac{[I_1]}{K_{I_1}}}{V_{max}\cdot[S]} \quad (3)$$

$1/v_{I_1}$ can be calculated from the experimentally determined $1/v_{I_1I_2}$ values by subtraction of $K_m\cdot[I_2]/K_{I_2}\cdot V_{max}\cdot[S]$, if the conservation equation is valid (i.e. if no complex CI_1I_2 occurs). (K_m and V_{max} of glucose transport under our experimental conditions were determined previously [1].)

$$\frac{1}{v_{I_1}} = \frac{1}{v_{I_1I_2}} - \frac{K_m\cdot[I_2]}{K_{I_2}\cdot V_{max}\cdot[S]} \quad (4)$$

Equation (2) can be easily rearranged for the Dixon plot:

$$\frac{1}{v_{I_1I_2}} = \frac{K_m + [S] + K_m\frac{[I_2]}{K_{I_2}}}{V_{max}\cdot[S]} + \frac{K_m}{K_{I_1}\cdot V_{max}\cdot[S]}\cdot[I_1] \quad (5)$$

Determining the glucose uptake velocities at two constant glucose concentrations $[S_1]$ and $[S_2]$, and at different concentrations for the first inhibitor (constant concentration for the second inhibitor), one obtains two straight lines in the Dixon plot. The K_{I_1} value can be calculated from the point of intersection according to

$$-K_{I_1} = [I_1]\cdot\frac{1}{1+a} \quad \text{where} \quad a = \frac{[I_2]}{K_{I_2}} \quad (6)$$

as determined by solving both equations for $[S_1]$ and $[S_2]$.

Second case. In the second case, the experimentally found glucose uptake velocities $v_{I_1I_2}$ in presence of both inhibitors I_1 and I_2 can also be converted into velocities v_{I_1} found in the first experimental series with one inhibitor only (see equation 10).

The basis for the derivation of the equation under the assumption that each of both inhibitors has its specific site at the transport protein is the conservation equation where the ternary complex CI_1I_2 also occurs:

$$C_{tot} = C + CS + CI_1 + CI_2 + CI_1I_2 \quad (7)$$

and the transport velocity equation:

$$\frac{1}{v_{I_1I_2}} = \frac{K_m\left(1 + \frac{[I_1]}{K_{I_1}}\right)\cdot\left(1 + \frac{[I_2]}{K_{I_2}}\right)}{V_{max}\cdot[S]} + \frac{1}{V_{max}} \quad (8)$$

The equation for the uptake velocity with one inhibitor only is again:

$$\frac{1}{v_{I_1}} = \frac{K_m\left(1 + \frac{[I_1]}{K_{I_1}}\right)}{V_{max}\cdot[S]} + \frac{1}{V_{max}}; \quad (9)$$

$1/v_{I_1}$ can be determined by conversion from $v_{I_1I_2}$:

$$\frac{1}{v_{I_1}} = \frac{1}{K_{I_2} + [I_2]}\left(\frac{K_{I_2}}{v_{I_1I_2}} + \frac{[I_2]}{V_{max}}\right) \quad (10)$$

As can be shown easily by solving the equations expressing both straight lines of the Dixon plot, the point of intersection (distance from the ordinate) corresponds to the K_{I_1} value in the presence of I_1 (equation 9) as well as in the presence of I_1 and I_2 (equation 8):

$$-K_{I_1} = [I_1] \quad (11)$$

In this case, one can determine the K_{I_1} value (contrary to the first case) also directly graphically.

RESULTS

We investigated the influence on the glucose uptake of all inhibitors separately, as indicated in Table 1. From the Lineweaver–Burk plot (not shown) and the Dixon plots (examples see Figs 1(a) and 2(a)), the dissociation constants of the CI_1 complex were determined. In the second experimental series, the erythrocytes were incubated in a medium containing besides glucose and the first inhibitor an additional, second inhibitor I_2 in constant concentrations (Table 1, column 2). Again, we estimated the inhibition constants of the first inhibitor I_1 derived from the glucose uptake velocities by the Lineweaver–Burk plot (not shown) and the Dixon plot (for examples see Figs 1(b) and 2(b)). Figures 1(c) and 2(c) represent the Dixon plots of the glucose uptake velocities v_{I_1} after correction of the experimentally estimated $1/v_{I_1I_2}$ values (according to equation 4). These calculated values were plotted against the I_1-concentrations in the Dixon plots. From Fig. 1 it can be seen that the K_{I_1} values of (a) and (c) are in agreement, but not that of (b). Contrary to this, the K_{I_1} value of Fig. 2(a) corresponds to that of 2(b), but not to that of 2(c).

Table 1 summarizes the K_{I_1} values of all inhibitors tested. The K_{I_1} values for the first inhibitor of the inhibitor pairs (indicated in column 2 of Table 1A) are equal in columns 1 and 4. The latter were deter-

* During the short incubation time at the 'infinite trans situation' (i.e. if the erythrocytes are preloaded with 200 mM glucose), the transport proteins of the membrane are saturated with glucose on the inner face (for details see [8]). Under such conditions, the glucose uptake corresponds to the unidirectional influx; it is characterized by the Michaelis–Menten kinetics (for details see [11]), whereby K_m is the half saturation constant incorporating an additional parameter which refers to the exchange of the glucose molecules on the transport protein from the *cis*- and *trans*-side of the membrane.

Table 1. Comparison of the dissociation constants K_{I_1} of different inhibitors of glucose transport

A (Case 1) Single inhibitor	K_{I_1} (mM)*	Inhibitor pair†	K_{I_1} (mM)‡	K_{I_1} (mM)§	$\frac{K_{I_1}^*}{K_{I_1}^\ddagger}$ ‖
Maltose	8.0	Maltose + ethanol	13.0	8.0	0.6
Cellobiose	32.0	Cellobiose + maltose	52.0	32.0	0.6
L-TME	2.0	L-TME + maltose	3.5	2.0	0.6
B (Case 2) Single inhibitor	K_{I_1} (mM)*	Inhibitor pair†	K_{I_1} (mM)‡	K_{I_1} (mM)§	$\frac{K_{I_1}^*}{K_{I_1}^\ddagger}$ ‖
L-TME	2.0	L-TME + ethanol	2.0	1.2	1.6
L-TEE	1.0	L-TEE + ethanol	1.0	0.6	1.6
Octylglucoside	2.9	Octylglucoside + ethanol	2.9	1.8	1.6

* Experimentally determined, according to equation (3).
† Inhibitor pair in the medium, acting simultaneously during glucose uptake.
‡ Graphically determined from the Dixon plot.
§ Dissociation constants of the first inhibitor, calculated from the glucose uptake velocities in the presence of the first and the second inhibitor according to equation (4).
‖ The correct K_{I_1} value is divided by the apparent K_{I_1} value.

mined after correction of the experimentally found $v_{I_1I_2}$ values according to equation (4), proceeding on the assumption that both the inhibitors (column 2) were bound to the same binding site of the transport protein. Extracting the K_{I_1} values directly from the Dixon plot, one obtains the K_{I_1} values indicated in column 3; these are very different from the data in column 1. From column 5 it can be seen that under our experimental conditions the quotient between the data of columns 1 and 3 was always 0.6.

This is perfectly in agreement with the theory (see equation (6)). It is obvious that the point of intersection of both straight lines (Dixon plot—distance from the y-axis) must be multiplied by $1/(1 + [I_2]/K_{I_2})$. The concentration chosen for the second inhibitor was $[I_2] = 0.6\ K_{I_2}$ in all our experiments, so the results in Table 1A, column 3 must be multiplied by $1/(1 + 0.6) = 0.6$ to get the data in column 1.

The K_{I_1} values in Table 1B, column 3 for the first inhibitor of the inhibitor pairs in column 2 are in agreement with the K_{I_1} values in column 1, i.e. K_I values could be determined directly from the Dixon plots. Identical K_{I_1} values are obtained when the reciprocal values of the glucose uptake velocities $1/v_{I_1I_2}$ are converted in terms of $1/v_{I_1}$ values according to equation (10), based on the assumption that the two inhibitors have different binding sites to the transport protein. Correcting the $1/v_{I_1I_2}$ values according to equation (4) (valid in the case that both inhibitors have one and the same binding site), one obtains the K_{I_1} values, indicated in column 4. These values have to be multiplied by $1 + [I_2]/K_{I_2}$ — in our case by $1 + 0.6$—to get the correct K_{I_1} values in column 1.

DISCUSSION

The discussion of these results starts from the following points:

(1) If I_1 and/or I_2 binds to the transport protein, glucose cannot be bound (condition for the competitive inhibition kinetics) and vice versa.

(2) Inhibitor I_2 is also effective in the absence of I_1, and vice versa.

(3) In the presence of two inhibitors I_1 and I_2 two cases are possible: (a) for case 1: if I_2 binds to the transport protein, I_1 cannot bind, and vice versa. That means the ternary complex CI_1I_2 is not formed. (b) For case 2: if I_2 binds to the transport protein, I_1 can be bound with unchanged affinity too, and vice versa. The ternary complex CI_1I_2 is formed.

These remarkable results show that the affinity of the transport protein to one of the inhibitors either remains unchanged by the interaction with the other inhibitor (case 2), or else disappears completely (case 1). A partial increase or decrease of the affinity is inconsistent with the observed kinetics.

The results in case 1 (Table 1A) could also be explained by the usually supposed conception for the competitive inhibition: glucose, I_1 or I_2, respectively, bind to one and the same binding site, which is in any case occupied by only one molecule. If I_1 forms a complex with the transport protein, neither glucose nor I_2 can be bound, and vice versa. How-

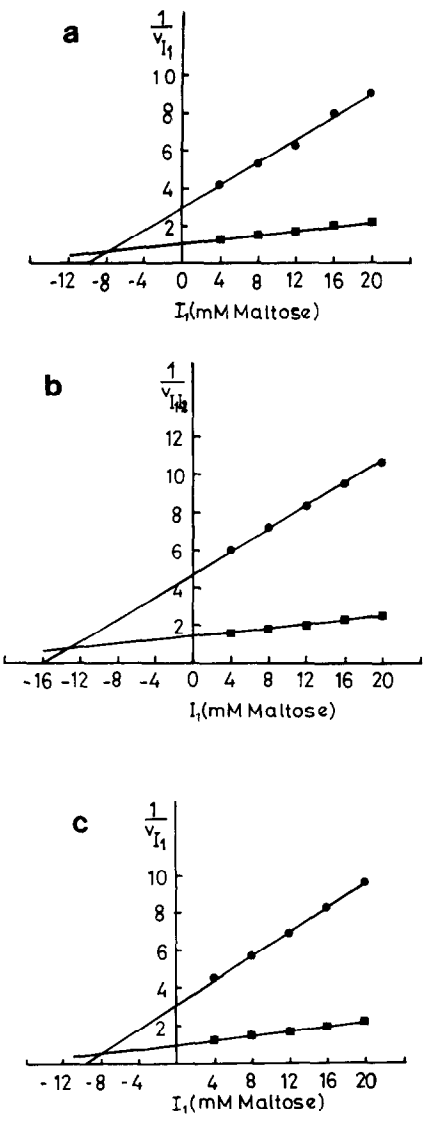

Fig. 1.

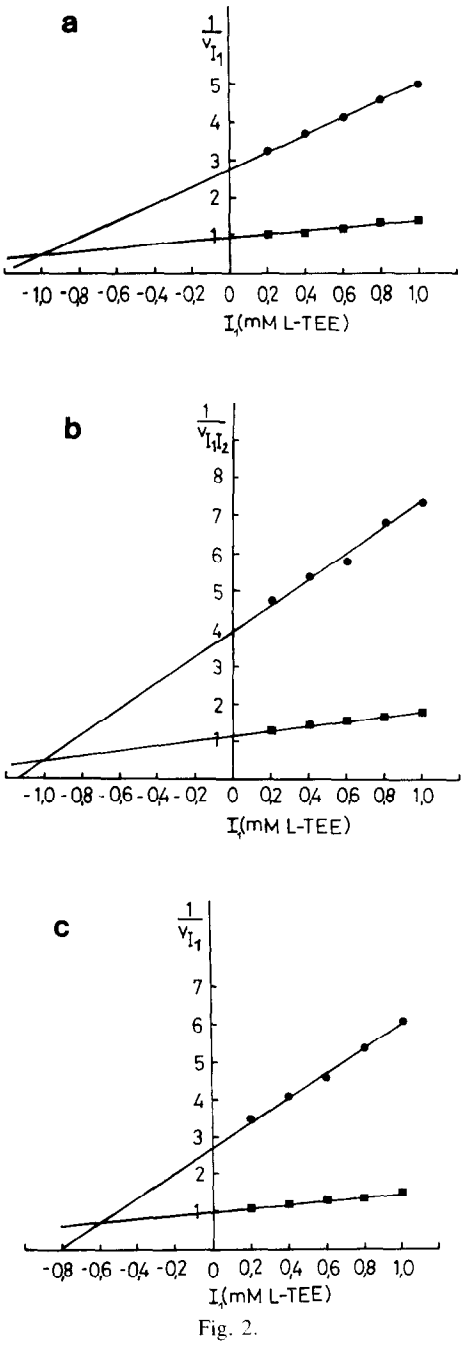

Fig. 2.

Figs. 1 and 2. Dependence of glucose uptake on the inhibitor concentrations I (Dixon plot). Erythrocytes (preloaded with 200 mM glucose in 0.9% NaCl solution) were incubated in isotonic phosphate buffer pH 7.5, containing two different glucose concentrations (● = 0.5 mM glucose and ■ = 2.5 mM glucose) and five different inhibitor concentrations. The reciprocal value of the glucose uptake velocity (sec mM^{-1}) was plotted against the inhibitor concentrations. (a) Maltose (concentrations between 4 and 20 mM) or L-TEE (concentrations between 0.2 and 1.0 mM), respectively, were dissolved in the buffer (only one inhibitor in the medium). (b) Maltose (concentrations as in (a) or L-TEE (concentrations as in (a)) respectively, were disolved with the second inhibitor ethanol of the constant concentration 514 mM (medium contained two inhibitors) in the buffer solution. (c) Experimental conditions as in (b); the $v_{I_1I_2}$ values of (b) were corrected according to equation (4).

ever, these results can be explained also by a conformational change, as described in case 2. In this case, the results showing that I_1 and I_2 have different binding sites, are not in line with the classical competition concept; when occupied by one or both inhibitors, binding of glucose is not possible.

It can be seen that ethanol, while inhibiting glucose binding, prevents also the binding of maltose to the transport system; this is also valid for maltose with respect to ethanol. What passes for the inhibitor pair ethanol–maltose agrees also for the inhibitor pair L-TME*–maltose. On the basis of the classical competition concept, also the inhibitor pair L-TME–ethanol should compete for the binding site. However, our results show that L-TME and ethanol can be bound to the transport protein simultaneously,

* Abbreviations: L-TME, tryptophanmethylester; L-TEE, tryptophanethylester.

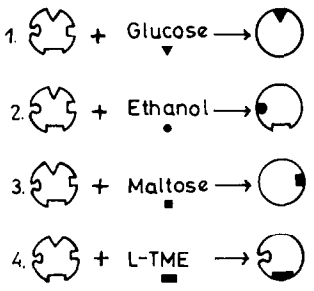

Fig. 3. Example of a schematic representation of the conformational change of the transport protein by some inhibitors: (1) if glucose interacts with the transport protein *C*, all the other binding sites disappear; (2) if ethanol interacts with *C*, the binding site for glucose and maltose disappears, that for L-TME remains; (3) if maltose interacts with *C*, the other three binding sites disappear; (4) if L-TME interacts with *C*, the binding site for glucose and maltose disappears, that for ethanol remains. ⬭ = Transport protein

forming a ternary complex. These findings can easily be explained by the assumption of conformational changes, caused by the interaction with the transport protein of glucose or of inhibitors, as shown schematically in Fig. 3. The members of the inhibitor pairs ethanol–L-TME and ethanol–octylglucoside, respectively, can also be bound simultaneously to the transport system. It is also unlikely that inhibitors structured quite differently from the substrate molecules compete for the same binding site. Many years ago, Wilbrandt [9] pointed out that an inhibition which kinetically appears as a competitive one, can be caused by binding of the unrelated inhibitor close to the substrate site and may then hinder the substrate to be bound to its site for steric reasons.

This explanation is not a probable one for the inhibition of the glucose uptake, because it would suppose that the binding sites of the various drugs, known as competitive inhibitors, are arranged around the glucose site. More acceptable is our conception of a conformational change of the transport protein, which—due to the interaction with the inhibitor—leads to a loss of affinity to glucose and—with some inhibitors—also to the second inhibitor as shown in Fig. 3. The conception of the conformational change does not alter under the assumption that one of the inhibitors is bound to the binding site of glucose (particularly if a similarly structured inhibitor is concerned). In Fig. 3 we described the more general case, where every inhibitor has its own binding site.

In this connection, it has to be pointed out that ethanol also interacts with the transport protein; this is important particularly in cases when ethanol is added to the medium because the drug investigated is not soluble in water [2, 10]. Thus it is not always possible to determine the K_i value from the Lineweaver–Burk and Dixon plots directly, even if ethanol is used in constant concentration for control.

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