Biochimica et Biophysica Acta, 406 (1975) 505–515

BBA 77110

INHIBITION OF GLUCOSE TRANSPORT INTO BRAIN BY PHLORIZIN, PHLORETIN AND GLUCOSE ANALOGUES

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(Received May 7th, 1975)

SUMMARY

An indicator dilution technique with $^{22}Na^{+}$ as the intravascular marker was used to measure unidirectional transport of D-[6-^{3}H]glucose from blood into the isolated, perfused dog brain. 18 compounds which are structurally related to glucose were tested for their ability to inhibit glucose transport. The data suggest that no single hydroxyl group is absolutely required for glucose transport, but rather that glucose binding to the carrier probably occurs through hydrogen bonding at several sites (hydroxyls on carbons 1, 3, 4 and 6). In addition, α-D-glucose has higher affinity for the carrier than does β-D-glucose.

A separate series of experiments demonstrated that phlorizin and phloretin are competitive inhibitors of glucose transport into brain; however, phloretin is partially competitive and inhibits at lower concentrations than does phlorizin. Inhibition by phlorizin and phloretin is mutually competitive, indicating that these compounds compete for binding to the glucose carrier. Comparison with the results reported in the literature for similar studies using the human erythrocyte demonstrates a fundamental similarity between glucose transport systems in the blood-brain barrier and erythrocyte.

INTRODUCTION

A comparison of the results of kinetic experiments indicates that the mechanism for glucose transport from blood to brain may be similar to the mechanism for glucose transport in the erythrocyte [1]. Additional evidence for their similarity is provided by studies which demonstrate that neither system can transport glucose against a concentration gradient [2, 3]. It has also been suggested that inhibition of transport by phlorizin as well as by several glucose analogues is similar in the two systems [4, 5]. The present study is a detailed analysis of the effects of phlorizin, phloretin and glucose analogues on glucose transport from blood to brain. Part of this work was described previously [6].

METHODS

The method used to prepare and maintain the isolated canine brain has been described in another report [7]. Important in this study is the fact that arterial blood perfuses brain tissue only and that all venous blood is collected through a single outlet located at the confluence of venous sinuses.

The perfusate was compatible donor blood that had been diluted with dextran to a hematocrit of about 24–28 % and conditioned [8]. Blood with low glucose concentrations was obtained from donor dogs that had received 10–20 I.U. of regular insulin when blood conditioning was begun. In an earlier study, we demonstrated that increased insulin levels have no effect on unidirectional glucose transport into the brain [9]. The viability of the preparation was assessed at frequent intervals from both electroencephalographic recordings and oxygen consumption data. The brain was removed and weighed at the end of each experiment.

Indicator dilution injections

The rate of unidirectional glucose transport into the perfused dog brain was quantified by using the indicator dilution technique. This technique has been described in detail elsewhere [9]; however, the procedure will be briefly summarized here. The 50-μl injectate contains 2 μCi of $^{22}Na^+$ (the intravascular marker) and 10 μCi of D-[6-^{3}H]glucose (the test molecule). A 50-μl syringe is used to inject the isotope mixture directly into the common carotid artery near the internal carotid bifurcation. The injection requires less than 0.5 s. Sampling starts 3 s after the injection and 30 consecutive venous blood samples are collected at 1-s intervals for later analysis of radioactivity. Immediately after the thirtieth sample, arterial and venous blood is collected for glucose analysis. The blood flow rate is determined by measuring the volume of a 1-min collection of venous blood.

The data treatment used [9] permits calculation of the maximal glucose extraction from the blood, E, for each indicator dilution injection that is made. The rate of unidirectional glucose transport into the brain, v, is calculated from $v = (E - 0.036)A\ F_p/W$ where A is the arterial plasma glucose concentration, F_p is the plasma flow rate, W is the brain weight, and 0.036 is a correction for the estimated rate of simple diffusion of glucose into the brain [9].

Inhibition of glucose transport by glucose analogues

83 indicator dilution injections were made on nine isolated brains perfused at plasma flow rates between 0.45 and 0.52 ml/g of brain per min and at arterial glucose concentrations between 4.4 and 5.9 mM.

The isotopes in the injectate were diluted with either 0.9 % (w/v) NaCl (control injections) or with a solution of one of the glucose analogue inhibitors. The final concentration of inhibitor in the injectate was 500 mM in all cases. Several hours were allowed for the solutions to reach mutarotational equilibrium except when α- or β-D-glucose was used. In these cases, 500 mM solutions were made by adding the isotope solutions directly to a preweighed quantity of crystalline α-D-glucose or β-D-glucose. Indicator dilution injections were then made within 1 min of mixing. There is little mutarotation of the isomers during this time interval [10]. A series of 10–14 indicator dilution injections were performed on each isolated brain. The first and last injections of each series were controls which were used to assure reproducibility of results.

Inhibition of glucose transport by phlorizin and phloretin

The kinetics of unidirectional glucose transport were determined on 23 isolated brains in the absence (control) or in the presence of phlorizin or phloretin in the perfusate. The dual perfusion system, described previously was used in all experiments [8, 9]. Blood from the control oxygenator contained normal glucose levels (4.5–6.5 mM) and was used for brain perfusion except during 3-min pulses with blood from the experimental oxygenator. The glucose levels in the experimental oxygenator were varied while the concentration of phlorizin or phloretin was held constant. Therefore, the rate of glucose transport could be determined over a range of blood glucose levels at various fixed inhibitor concentrations. Further details regarding this procedure are available in earlier reports [1, 9].

Experiments were performed on three brains to determine whether inhibition by phlorizin and phloretin was mutually competitive. In these experiments, the blood glucose concentration in the experimental oxygenator was maintained at 1.97 mM $\pm$ 0.06 S.E. and indicator dilution injections were performed in the presence of phlorizin (1.5 mM), phloretin (0.20 mM), or both (1.5 and 0.20 mM, respectively). The average rate of unidirectional glucose transport was then determined for each of the three conditions studied.

MATERIALS

Phlorizin and phloretin were obtained from K and K Laboratories and recrystallized once. Purity was checked by paper chromatography in 3 % acetic acid and by thin-layer chromatography on silica gel using 25 % (v/v) *n*-propanol in chloroform as the solvent. Phlorizin is converted to a more polar compound when stored in alcohol for an extended period of time. Therefore, all solutions were made up fresh each day. D-[6-^{3}H]Glucose was obtained from New England Nuclear and ^{22}NaCl from Amersham/Searle. D-Mannose, D-fructose, and 2-deoxy-D-glucose were obtained from Calbiochem and 1,5-anhydro-D-glucitol from NK Laboratories, Jersey City, N.J. 5-Thio-D-glucose was obtained from Aldrich Chemicals. All other compounds were obtained from Sigma. Each sugar was examined for glucose contamination by gas chromatography of silylated derivatives on an SE-30 column [11]. Silylation was acheived by adding 0.5 ml of Tri-Sil-Z (Pierce Chemicals) to 10 μl of a 1 M aqueous solution of sugar. The only compounds that were measurably contaminated with glucose were 1,5-anhydro-D-glucitol (2.4 %) and D-fucose (1 %).

RESULTS

Inhibition by glucose analogues

The rates of glucose transport in the presence of various glucose analogues are shown in Table I. The inhibitors are listed in order of most to least effective in their ability to reduce unidirectional uptake of [^{3}H]glucose. Although the concentration of inhibitor in the injectate was 500 mM, we have previously estimated that the peak concentration in the blood in the brain capillaries would be about 0.5 % of the injectate concentration [12]. This is due to the dilution of the injectate by the blood.

Inhibition by phlorizin or phloretin

The control data consist of values for glucose transport, v, obtained over a

TABLE I

The rate of [^{3}H]glucose transport, v, was determined in the presence of the compounds listed. Values are shown as averages ± S.E. The average capillary glucose concentration for this study was 4.94 mM ±0.06 S.E.

Inhibitor	N	(μmol v/g per min)	Percent of control
α-D-Glucose	5	0.340±0.029	53
α, β-D-Glucose	5	0.371±0.026	58
2-Deoxy-D-glucose	3	0.391±0.013	61
3-*O*-Methyl-D-glucose	3	0.398±0.007	62
β-D-Glucose	5	0.402±0.026	63
D-Galactose	3	0.432±0.024	67
D-Mannose	3	0.444±0.016	69
1,5-Anhydro-D-glucitol	3	0.495±0.016	77
D-Xylose	3	0.513±0.035	80
D-Fucose	3	0.542±0.016	85
5-Thio-D-glucose	8	0.550±0.021	86
D-Ribose	3	0.564±0.014	88
L-Arabinose	3	0.579±0.017	90
i-Inositol	3	0.582±0.012	91
L-Fucose	3	0.601±0.024	94
D-Sorbitol	6	0.623±0.013	97
L-Glucose	4	0.642±0.038	100
D-Fructose	3	0.646±0.041	101
0.9 % NaCl (control)	14	0.641±0.013	100

range of blood glucose concentrations with no inhibitor present. These data, shown in Fig. 1 as double reciprocal plots, were fitted by computer [9] to the Michaelis-Menten equation:

$$v = \frac{V\bar{A}}{K_m + \bar{A}} \tag{1}$$

where $\bar{A}$, the average of the arterial and venous glucose concentrations, is an approx-

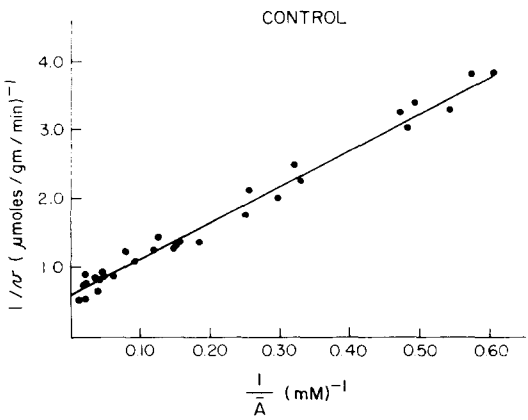

Fig. 1. Control data. Double reciprocal plot of the rate of glucose transport into brain, v, versus the average capillary glucose concentration, $\bar{A}$, for experiments performed on four isolated brains with no inhibitors added. The line was derived from a least-squares fit to Eqn 1.

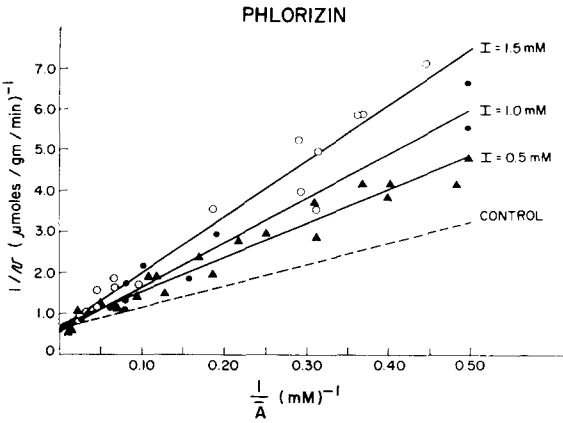

Fig. 2. Double reciprocal plots of the rate of glucose transport, v, versus the average capillary glucose concentration, $\bar{A}$, at three different concentrations of phlorizin, I, in the blood. The dashed line represents the control data (Fig. 1). All lines were derived from a least-squares fit of the corresponding data to Eqn 1. Kinetic constants ±S.E. for $I = 0.5$ mM were $K_m = 12.4$ mM ±1.7, $V = 1.49$ μmol/g per min±0.16, three brains ($N = 24$); for $I = 1.0$ mM, $K_m = 19.9$ mM±5.1, $V = 1.83$ μmol/g per min±0.35, three brains ($N = 13$); for $I = 1.5$ mM, $K_m = 21.6$ mM±3.6, $V = 1.57$ μmol/g per min±0.22, four brains ($N = 17$).

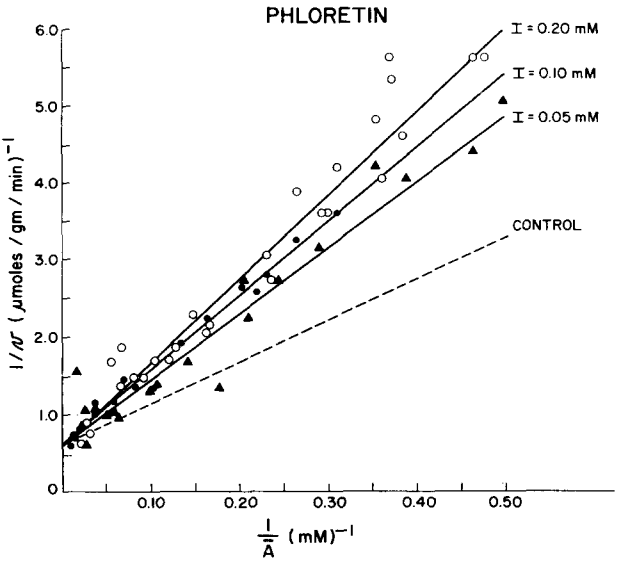

Fig. 3. Double reciprocal plots of the rate of glucose transport, v, versus the average capillary glucose concentration, $\bar{A}$, at three different concentrations of phloretin, I, in the blood. The dashed line represents the control data (Fig. 1). All lines were derived from a least-squares fit of the corresponding data to Eqn 1. Kinetic constants ±S.E. for $I = 0.05$ mM were $K_m = 14.3$ mM ±3.3, $V = 1.70$ μmol/g per min±0.29, four brains ($N = 20$); for $I = 0.10$ mM, $K_m = 15.6$ mM±1.6, $V = 1.66$ μmol/g per min ±0.37, two brains ($N = 17$); for $I = 0.20$ mM, $K_m = 17.8$ mM±2.5, $V = 1.68$ μmol/g per min±0.19, three brains ($N = 28$).

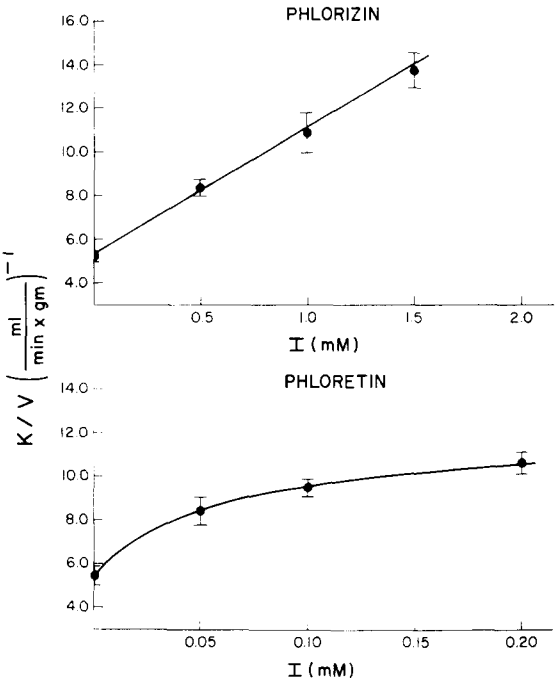

Fig. 4. Plot of the slopes of the double reciprocal plots in Figs 1, 2, and 3 versus the inhibitor concentrations. Values are shown ±S.E.

imation of the average glucose concentration in the capillary. The calculated values for the kinetic constants were $K_m = 8.65$ mM±0.70 S.E. and $V = 1.65$ μmol/g of brain per min±0.09 S.E. These agree with values obtained in other studies [1, 9].

Each group of data obtained in the presence of a constant inhibitor concentration was initially fitted to Eqn 1. Double reciprocal plots of these data (Figs 2 and 3) indicate that the slope but not the intercept is affected by the inhibitor. Thus, both compounds are competitive inhibitors of glucose transport into brain. However, phloretin, the aglycone, is effective at much lower concentrations than phlorizin. A further analysis of the inhibition pattern is shown in Fig. 4 where the slopes of the double reciprocal plots in Figs 2 and 3 are plotted as a function of the inhibitor concentration. These slope replots indicate that phlorizin is a fully competitive inhibitor while phloretin is a partially competitive inhibitor [13].

In order to calculate the inhibition constant, K_i, the phlorizin data were fitted by computer to an equation that describes fully competitive inhibition.

$$v = \frac{V\bar{A}}{K_m\left(1+\frac{I}{K_i}\right)+\bar{A}} \tag{2}$$

This analysis yielded the following values for the kinetic constants: $K_m = 9.1$ mM± 0.9 S.E., $V = 1.6$ μmol/g per min±0.1 S.E., and $K_i = 1.11$ mM±0.12 S.E. ($N = 84$).

Partially competitive inhibition can be described by the following equation [13]:

$$v = \frac{V\bar{A}}{K_m \frac{\left(1+\frac{I}{K_{in}}\right)}{\left(1+\frac{I}{K_{id}}\right)} + \bar{A}} \tag{3}$$

In this equation, the effect of I is described by two inhibition constants K_{in} and K_{id}. Because of the one additional unknown kinetic constant, we were not successful in fitting our phloretin data to this equation.

Inhibition when phlorizin and phloretin are present together

When a single competitive inhibitor, J, is present at a constant concentration the apparent Michaelis constant K_{app} can be calculated as follows: $K_{app} = K_m \cdot c_{ij}$, where c_{ij} is a constant that is related to the inhibitor's concentration and its K_i values (see Eqns 2 and 3). The analogous equation when two competitive inhibitors (J and K) are present is $K_{app} = K_m \cdot c_{ijk}$. If the two inhibitors are not mutually competitive (i.e. they can bind to the carrier at the same time) then $c_{ijk} = c_{ij} \cdot c_{ik}$. However, if the two inhibitors do compete with each other, then $c_{ijk} < c_{ij} \cdot c_{ik}$.

In the present study, c_{iplz}, c_{iplt}, and $c_{iplz+plt}$ were determined using the K_m and V from the control data, the observed $\bar{A}$ and v values in the presence of the inhibitors and the following equation:

$$c_i = \frac{\bar{A}\,(V-v)}{vK_m} \tag{4}$$

Table II shows that $c_{iplz+plt}$ is 2.54±0.31 S.E. The product of $c_{iplz} \cdot c_{iplt}$ is 4.68±1.11 S.E. The difference between these two values is significant ($p < 0.05$) and we therefore conclude that the binding of phlorizin and phloretin to the carrier is mutually competitive.

TABLE II

The rate of glucose transport into brain, v, was determined in the presence of constant inhibitor concentrations and constant capillary glucose concentrations, $\bar{A}$. c_i was calculated from Eqn 4. Values are shown±S.E. The standard error for c_i was calculated using the usual methods for propagation of errors.

Inhibitor	N	$\bar{A}$ (mM)	(μmol v/g per min)	c_i
Phlorizin (1.5 mM)	3	2.06±0.13	0.148±0.023	2.41±0.50
Phloretin (0.20 mM)	6	1.95±0.07	0.172±0.006	1.94±0.22
Phlorizin (1.5 mM)+ Phloretin (0.20 mM)	6	2.15±0.10	0.147±0.006	2.54±0.31

DISCUSSION

Current evidence suggests that it is the capillary endothelial cells which prevent the free diffusion of polar solutes between blood and brain [14, 15]. This theory is consistent with previous observations that the properties of the so-called blood-brain barrier are similar to those of a cell membrane [4, 14–17]. Therefore, studies of solute transport between blood and brain are analogous to studies of solute transport across the membranes of other cells such as erythrocytes, intestinal epithelial cells and microorganisms. The isolated perfused brain preparation is an ideal system for studying solute transport at the capillary endothelial cell membrane because the perfusate flow rate and composition can be controlled. The present study is an extension of our previous studies on the mechanism of glucose transport across the blood-brain barrier [1, 9, 12, 18].

Stereospecificity of glucose transport

Our results (Table I) are similar to those obtained in less comprehensive studies of glucose transport into brain [2–4, 19, 20]. Because 3-*O*-methyl-D-glucose is a competitive inhibitor of glucose transport [12], it is assumed that the sugars used in the present study also are competitive inhibitors and that their ability to inhibit glucose transport is related to the affinity of the compound for the glucose binding site on the carrier.

Our results are similar to the results obtained in studies using human erythrocytes (Table III). Although the erythrocyte data were obtained by a variety of techniques [5, 21–23], there is as much agreement between our study and any one of the erythrocyte studies as there is between any two erythrocyte studies.

Most evidence [21, 24, 25] suggests that β-D-glucose has higher affinity than α-D-glucose for the erythrocyte glucose carrier, although the results of one study [23]

TABLE III

Sugars are ranked according to decreasing affinity for the glucose carrier (i.e. number 1 represents the best inhibitor, 2 is second best, etc.). Only those compounds used by other investigators which were also used in our study are considered.

Dog brain	Human erythrocyte		
	LeFevre [5]	Barnett et al. [21]	Kahlenberg and Dolansky [22]
1. α, β-D-Glucose	2	2	1
2. 2-Deoxy-D-glucose	1	1	2
3. 3-*O*-methyl-D-glucose		3	4
4. D-Galactose	4	8	5
5. D-Mannose	3	4	3
6. 1,5-Anhydro-D-glucitol		7	9
7. D-Xylose	5	6	6
8. D-Fucose	8	5	7
9. 5-Thio-D-glucose			8
10. D-Ribose	7		12
11. L-Arabinose	6	9	10
12. L-Fucose	9		11
13. L-Glucose	10		13

were interpreted as showing that the α anomer is more easily transported. Our data indicate that the glucose carrier in brain has slightly greater affinity for α-D-glucose than for the β anomer, but equal affinities for the two anomers were reported for rat brain [4]. Thus, the system for glucose transport across the blood-brain barrier may not be identical to that in the human erythrocyte.

Based on our study, several preliminary conclusions about the structural features required for the binding of a sugar to the glucose carrier can be drawn. Inhibition of glucose transport by 1,5-anhydro-D-glucitol and *i*-inositol and lack of inhibition by sorbitol suggest that glucose is normally transported in the pyranose ring conformation. It appears that no single hydroxyl group is absolutely required for binding since elimination or epimerization at any single asymmetric carbon does not abolish the ability of the analogue to inhibit glucose transport. The hydroxyl on C-1 probably is involved in binding since elimination (1,5-anhydro-D-glucitol) or anomerization (α vs β) at this position decreases the inhibitory potency. The hydroxyl on C-2 is not involved in binding since there is no change in the rate of transport when it is eliminated (2-deoxy-D-glucose). However, epimerization from the equatorial to the axial conformation (D-mannose) decreases binding probably due to steric hindrance. Either addition of a methyl group to the hydroxyl on C-3 or epimerization at C-4 results in a decrease in the ability to inhibit transport, but we cannot determine whether this is due to steric hindrance or to interference with a hydrogen bond which is normally present. The hydroxyl on C-6 is probably involved in binding since elimination of the hydroxymethyl group on C-5 of glucose (D-xylose) reduces inhibitory potency. In addition, elimination of either the hydroxyl on C-6 of D-galactose (D-fucose) or of the hydroxymethyl group on C-5 of D-galactose (L-arabinose) causes a further decrease in the ability to inhibit glucose transport. Finally, replacement of the oxygen of the glucopyranose ring with an hydroxymethyl group (*i*-inositol) or with sulfur (5-thio-D-glucose) results in compounds with low affinity for the glucose carrier. This low affinity is probably due to steric hindrance and/or absence of hydrogen bonding to the substituted groups. Thus, the ring oxygen of D-glucose is required for optimal binding of glucose to the carrier.

Interaction of phlorizin and phloretin with the glucose carrier

Our results (Figs 2 and 3) indicate that both phlorizin and its aglycone, phloretin, are competitive inhibitors of glucose transport from blood to brain and that phloretin is at least 10 times more effective than phlorizin. The data show that phloretin is a partially competitive inhibitor and that phlorizin is apparently fully competitive.

The hyberbolic nature of the phloretin slope replot (Fig. 4) could be explained by the limited solubility of phloretin in aqueous solutions; however, we have determined that phloretin is completely soluble in our perfusate at concentrations of at least 0.60 mM. This is greater than the solubility limit in aqueous solutions (0.25 mM) reported by LeFevre and Marshall [26]. These authors also showed that erythrocytes have a high capacity for binding phloretin since the distribution ratio between red cells and buffered medium was 35–50 : 1. Therefore, it is likely that, in our experiments, the plasma phloretin concentrations were considerably lower than the whole blood phloretin concentrations (0.05–0.20 mM). In addition, several studies have shown that phlorizin is completely excluded from erythrocytes [26, 27]. This suggests

that the actual plasma phlorizin levels attained in our study were about 30 % higher than the values given (Figs 2 and 4). Consequently, the actual K_i for phloretin is much lower and the K_i for phlorizin is slightly higher than the values calculated from our data. Recently published data [30] is in general agreement with these findings.

Phlorizin and phloretin are also competitive inhibitors of glucose transport into the erythrocyte [28, 29]. It is not clear whether these compounds are fully or partially competitive inhibitors in the red cell, but it is likely that phloretin acts partially since in other respects this carrier system is similar to the one in the blood-brain barrier.

Our hypothesis for the interaction of phlorizin and phloretin with the glucose carrier in the blood-brain barrier is an extention of Alvarado's scheme [31] of two binding loci, one for sugars and one for phenols. Binding of phloretin to the phenol site hinders, but does not eliminate, glucose binding to the sugar site either by partially blocking the sugar receptor or by causing a slight conformational change at the glucose site [31]. Since the β-glucoside, phlorizin, is a fully competitive inhibitor of glucose transport, it must bind directly to the sugar binding site. The affinity of phlorizin (K_i = 1.11 mM) for this site is probably greater than that of β-D-glucose itself (estimated K_i = 3.4 mM*). Therefore, the phenol portion of the molecule enhances phlorizin binding possibly due to an affinity for a second site adjacent to the sugar receptor. The fact that inhibition by phlorizin and phloretin is mutually competitive could be interpreted to mean that this second site is the phloretin receptor. An alternative interpretation, however, is that these phenols do not compete directly for the same site but rather that the observed mutual competition is due solely to the effect of phloretin on the sugar receptor. Thus, phloretin would decrease phlorizin binding to the sugar site just as it decreases glucose binding to the sugar site.

ACKNOWLEDGEMENTS

The authors thank Dr W. W. Cleland for the use of computer programs used in fitting kinetic equations and gratefully acknowledge the technical assistance of Mr Paul Conway, Mr James Fitzpatrick, Mr Wilbert Heiman, Mr Bruce Levin, Mr Alton Mitmoen, Mr Bill Patterson, Miss Karla Raab, Mr Mark Saffitz, and Mrs Susan Zimbric. This investigation was supported by Grants NS05961 and 6M01932 from the National Institutes of Health and by University Surgical Associates. A. L. Betz is the recipient of a Medical Scientist Training Program award.

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* The K_i for β-D-glucose was estimated using Eqn 2, the apparent K_m and V from the control data (Fig. 1), and v in the presence of β-D-glucose (Table I). The corresponding $\bar{A}$ for the β-D-glucose data was 4.85 mM ±0.11 S.E. and I was estimated to be 0.5 % of 500 mM which is 2.5 mM (see Results). The fact that the K_i is less than the apparent K_m for glucose transport is to be expected since this transport system does not conform to the kinetics predicted on the basis of a symmetric mobile-carrier model [1].

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