Mechanism of L-glucose, raffinose, and inulin transport across intact blood-brain barriers

KATHRYN J. LUCCHESI AND ROBERT E. GOSSELIN
Department of Pharmacology and Toxicology, Dartmouth Medical School, Hanover, New Hampshire 03756

LUCCHESI, Kathryn J., and Robert E. GOSSELIN. Mechanism of L-glucose, raffinose, and inulin transport across intact blood-brain barriers. Am. J. Physiol. 258 (Heart Circ. Physiol. 27): H685–H705, 1990.—Brain capillary permeability-surface area products (PS) of hydrophilic solutes were evaluated in terms of a conventional two-compartment model. In rats whose blood-brain barrier (BBB) was presumed to be intact, metabolisminert carbohydrates with different molecular weights were injected in pairs to elicituate whether their transfer into the brain proceeds by diffusion through water- or lipid-filled channels or by vesicular transport. The distribution volume of 70 kDa dextran 10 min after intravenous injection was used as a measure of the residual volume of plasma in brain tissue after death. The two-compartment model yielded larger PS values for inulin and raffinose than for L-glucose, and the PS values of inulin and L-glucose were found to decrease as the labeling time was lengthened (10, 30, and 60 min). These observations were interpreted to mean that a rapidly equilibrating compartment was present between blood and brain, rendering the two-compartment model inadequate for computing true transfer rate constants. When multiple-time uptake data were reanalyzed using the three-compartment graphical analysis of Patlak, Blasberg, and Fenstermacher (J. Cereb. Blood Flow Metab. 3: 1–7, 1983), solutes of differing molecular size were found to enter the brain at approximately equal rates. This observation suggested that the predominant transport mechanism across an intact BBB is vesicular. Specifically, unidirectional transport is likely to be initiated by solute binding to the glycocalyx on the luminal surface of brain capillary endothelium. Apparently more inulin than L-glucose is adsorbed, which may account for its slightly faster transfer across the BBB. We suggest that this adsorptive surface is the location of the rapidly equilibrating compartment on the plasma side of the BBB.

rat; hydrophilic solutes; permeability-surface area products; adsorptive endocytosis; fast space; rapidly equilibrating compartment; vesicular transport; pinocytosis; brain plasma volume

THE BLOOD-BRAIN BARRIER (BBB), anatomically located at the luminal surface of cerebral capillary endothelial cells (29), retards the entry of most blood-borne hydrophilic solutes into the brain (1, 5, 9, 22). Nutrient amino acids and sugars, however, gain rapid entry because highly specialized, energy-requiring transport systems are present within the microvascular membranes to facilitate diffusion of these metabolically useful substrates (e.g., 23, 24). Because permeation rates of most lipidsoluble xenobiotics correlate fairly well with their octanol/water partition coefficients (27), solutes of this class are believed to enter the brain by solvation in and diffusion through the lipid membranes of cerebral capillary endothelial cells. For highly hydrophilic nonnutrient solutes, however, the mechanism of blood-to-brain transport is unknown. Because many clinically useful drugs belong to this class of solutes, information about transport mechanisms is highly desirable. In fact, much current research has focused on enhancing the delivery of chemotherapeutic agents to the central nervous system by transiently opening the BBB (e.g., 14), but these investigations have proceeded without particular regard to how transfer takes place across an intact barrier.

Published data based on direct measurements of solute transfer across a normal BBB are in poor agreement (1, 11, 22). Also, most studies of transcapillary transport of relatively impermeant solutes have been confined to single tracer flux experiments (1, 11, 22). Because the size of the perfused capillary bed is not usually measured or adequately controlled, transfer constants for single solutes cannot lead to reliable inferences about how solutes traverse the capillary wall. In the present study, simultaneous transfer rates were determined for pairs of hydrophilic nonelectrolytes for which specific, saturable carrier-mediated transport mechanisms into the brain are not available (19, 34). Unidirectional transfer rate constants across the BBB were evaluated in terms of permeability-surface area products (PS) computed on the basis of a conventional two-compartment model (the symbol PSac is used to designate these values). Because this model proved to be inadequate, transfer rate constants for inulin and L-glucose were also evaluated with a multiple-time graphical analysis (25). This analysis demonstrated that a three-compartment model provides a satisfactory description of hydrophilic solute transport across the intact BBB (these PS values are referred to as PSac). PS data obtained with pairs of hydrophilic solutes injected together suggest that for these tracers the major transport mechanism across the intact BBB may be vesicular.

Rationale of Experimental Design

Because it is impractical to measure or to stabilize the extent of the actively perfused capillary bed in the brain in situ, the current study was designed to measure the contemporaneous brain uptake of test solutes in pairs. When two solutes are injected together, it is assumed that the capillary surface area (S) available to both is...
identical. If $S_1 = S_2$, then $P_1S_1/P_2S_2 = P_1/P_2$, a ratio that reflects only the relative membrane permeability. As has been demonstrated before (21), this ratio is useful in attempting to identify which, if any, of the following hypothetical mechanisms accounts for the blood-to-brain transfer of the solute pairs.

**Free diffusion through water-filled channels.** For solutes that cross membranes by diffusion through water-filled channels, as is thought to occur in fenestrated capillaries, the PS ratio of two solutes should be equivalent to the ratio of their free diffusion coefficients ($D$), i.e., $P_1/P_2 = D_1/D_2$. This ratio will always be $<1$ if the less diffusible tracer is arbitrarily designated as solute 1; this convention has been used throughout this work.

**Restricted diffusion through water-filled channels.** Solutes with molecular dimensions that are appreciable relative to the transverse dimensions of the water-filled pores do not diffuse as rapidly in such pores as in an open solution. Solutes diameters of which are smaller than the width of the pore are not excluded, but diffusion may be retarded by steric hindrance and by wall drag (13) so that $P_1/P_2 < D_1/D_2$.

**Solute in and diffusion through lipid.** If the capillary wall behaves as a nonporous membrane, then lipid solubility may determine the rate at which a solute crosses the BBB. Under these circumstances, the PS ratio is likely to be similar to the ratio of the octanol/water partition coefficients.

**Fluid phase endocytosis.** If vesicles are formed by the engulfment of plasma at the luminal surface of capillary endothelium (micropinocytosis), any solute present in the plasma is swept into the vesicle. Solute transport through the endothelial cell occurs if the vesicle, by Brownian movement or by fusing with other vesicular structures within the cell, transfers and deposits its contents at the abluminal surface. Provided that the vesicle is not loaded by diffusion, the molecular diameter of the solute does not limit its transport rate. Thus, two test solutes with different molecular dimensions should cross capillary walls at rates that are proportional to their respective plasma concentrations. Under these circumstances, the PS ratio approaches unity. However, if preformed vesicles become solute loaded by diffusion through vesicular necks that open into the capillary lumen, then the following bounds are likely: $1.0 \geq P_1/P_2 > D_1/D_2$.

**Adsorptive-phase endocytosis.** Molecules that are bound to the plasma membrane before vesicular engulfment exhibit transport kinetics that are more complex than in the case of fluid-phase endocytosis. Rates of solute transfer are likely to be proportional to the amount of solute attached to the adsorbing surface, which in turn tends to be a hyperbolic function of the plasma concentration (12). Under these circumstances PS ratios $>1.0$ are possible.

**Materials and Methods**

**General Experimental Procedures**

Each adult male Sprague-Dawley rat (250–450 g) was given diethyl ether to induce light anesthesia. By the end of the surgical preparation, this ether had been largely excreted. Surgical anesthesia was produced with 20 mg/kg ip pentobarbital sodium and 60 mg/kg im ketamine, the latter administered in two separate injections (30 mg/kg each). Rats were tracheotomized and intubated with polyethylene tubing (PE-280). To measure blood pressure and to sample arterial blood for gases and isotopic analysis, both right and left femoral arteries were cannulated with PE-10. One femoral venous catheter was inserted to deliver injections of radioactive test solutes. Each catheter was connected to a 1-ml syringe prefilled with heparinized (200 U/ml) 0.9% saline. At the end of the surgical procedure, rats were given a mixture of 95% O2-5% CO2 to breathe. The gas input was connected to one arm of a “T” adapter; one arm was affixed to the tracheotomy tube while the other end of the “T” remained open. During the 45-min (approximate) experimental protocol, the animals appeared to be lightly anesthetized, as judged by the spontaneous respiratory rate and depth. Systemic arterial blood pressure was recorded from the femoral artery by means of a Statham pressure transducer connected to a Grass Instruments polygraph. Body temperature was monitored rectally and regulated with the heat from an overhead lamp. One or two femoral arterial blood samples (75 μl each) used to measure arterial CO2 tension ($P_{CO2}$), %O2 saturation, and pH were collected into heparinized Natelson capillary tubes (S/F Supplies), sealed anaerobically, and stored on ice. One of these samples was drawn immediately after surgery and the second immediately preceding the intravenous injection of radiolabeled solutes (see below). Arterial blood gases and pH were measured with a Corning blood gas analyzer model P170 within 1 h of sampling.

**Experimental Protocols for Brain Uptake (PS) Measurements**

Test solutes were administered to each group of rats by a bolus injection of a mixture of $^{14}$C- and $^3$H-labeled solutes dissolved in 70 μl of 0.9% saline into the femoral vein. The time of intravenous injection was denoted as $t = 0$, and brain uptake of labeled solutes proceeded for 10, 30, or 60 min. Timed arterial blood samples were collected into heparinized capillary tubes (S/F Supplies), sealed anaerobically, and stored on ice. One of these samples was drawn immediately after surgery and the second immediately preceding the intravenous injection of radiolabeled solutes (see below). Arterial blood gases and pH were measured with a Corning blood gas analyzer model P170 within 1 h of sampling.

**Measurement of Plasma Volume ($V_p$)**

Since dextran (70 kDa) is biochemically inert and is too large to enter red blood cells, the vascular space occupied by dextran is limited to plasma. Five rats received an intravenous bolus of $[14]$C-dextran into the femoral vein and were killed by decapitation 10 min after the bolus was given. To minimize contamination by solute in large blood vessels, the pial membranes and choroid plexuses were removed. The brain tissue radioactivity was determined as described below. $V_p$ was com-
puted as the quotient of $[1^4C]$dextran activity in brain tissue (μg) and in femoral arterial plasma (μl) at death.

**Tissue Processing**

**Brain.** Pial membranes and choroid plexuses were dissected off and discarded. The fresh brain was sectioned to preserve the following areas as discrete anatomic regions: right and left cerebral hemispheres (further subdivided into frontal, parietal, temporal, and occipital areas), a so-called sub cortex or anterior brain stem (extended caudal to include the inferior colliculi), the cerebellum, and posterior brain stem. The pieces (80-150 mg) were placed into preweighed 20-ml glass scintillation vials and reweighed. Two milliliters of tissue solubilizer (TS-1, RPI) were added to each scintillation vial. Tissue digestion was complete after 12 h at 50°C. Ten milliliters of Neutralizer Cocktail (RPI) were added to the solubilized brains, and the samples were set aside for at least 14 days to allow for the chemiluminescence in the $^3H$ channel to subside. Radioactivity was detected with a Beckman model LS3801 scintillation counter and converted to disintegrations per minute with appropriate quench monitoring, and efficiency and contamination corrections.

**Plasma.** Arterial blood samples were spun 10 min in a microhematocrit centrifuge; hematocrits were noted for the first and last samples. Known volumes of plasma (20 μl) were pipetted directly from the capillary tubes into 7-ml glass minivials. Fifty microliters of distilled water plus 5 ml Liquiscint (National Diagnostics) were added to each vial. Plasma samples were counted for disintegrations per minute immediately after processing.

**Oil-Water Partition Coefficients**

Butanol/water partition coefficients of L-[$1^4C$]glucose, [H]$^3$H]raffinose, and [H]$^3$H]inulin were measured. The concentration of each test solute in aqueous solution was first determined in triplicate 5-μl aliquots by liquid scintillation counting (dpm/ml). Next, 1-butanol was added, and the samples were vortexed for 3 min and allowed to completely separate (repeated twice). The concentration of each solute in butanol was determined (also in triplicate), and the ratio of disintegrations per minute per milliliter in butanol/water was computed. The values were transformed to octanol/water partition coefficients (O/W) according to the following empirical relationship

$$\log [O/W]_{octanol} = 1.43 \log [O/W]_{butanol} - 0.547 \quad (1)$$

The measurements and values computed in this study are presented in Table 1.

**Statistical Analyses**

All regional values for brain plasma volume, PS, and PS ratios were analyzed for differences by one-way analysis of variance (ANOVA). The method of least squares was used to fit straight lines to brain uptake data for the multiple-time graphical analysis. Analysis of covariance (COVAR) was used to detect differences in the slopes of these lines. The single-sample $t$ test was used to compare the PS ratios of various solute pairs with unity and with the ratios of their respective diffusion coefficients and octanol/water partition coefficients.

**Purification of Radiochemicals**

**Thin-layer chromatography.** The radiochemical purity of L-[$1^4C$]glucose and [H]$^3$H]raffinose was verified by thin-layer chromatography on silica- clad glass plates (Merck, Darmstadt Reagents) followed by qualitative autoradiography. The solvent system was ethyl acetate:2-propanol:water (6:3:1) as recommended by Ghebregzabher et al. (10) for effective separation of mixtures of saccharides.

**Dialysis and gel-column chromatography.** Purification of [$^3H$]inulin was by dialysis through Spectropore tubing which had a molecular weight cutoff of 3,500 Da. Gel-column chromatography over T-6 gel beads (Bio-Rad) was used to verify that dialysis had removed small labeled fragments (Fig. 1). The free diffusion coefficient ($D$) of newly dialyzed inulin was measured in 1% agar gel according to the method of Redwood et al. (28). Its value was 0.24 ± 0.02 × 10⁻⁵ (SD) cm²/s ($n = 4$). The purity of [$1^4C$]dextran was ascertained by its restriction to the void volume on the same column.

**Solutions, Isotopes, and Drugs**

The [$1^4C$]dextran, [H]$^3$H]raffinose, L-[$1^4C$]glucose, and (methoxy-$^3$H)inulin were purchased from New England Nuclear (NEN, Boston, MA). Pentobarbital (Nembutal) and ketamine (Vetalar) were obtained from Henry Schein (Port Washington, NY), and N-2-hydroxyethylpiperazine-N’,2-ethanesulfonic acid (HEPES) (H-3375) (Darmstadt Reagents) followed by qualitative autoradiography. The solvent system was ethyl acetate:2-propanol:water (6:3:1) as recommended by Ghebregzabher et al. (10) for effective separation of mixtures of saccharides.

**Methods of Computing Solute PS**

**Estimates based on single-time bolus injection technique.** For solutes of very limited permeability, e.g., PS < 10% of cerebral blood flow (8), the arterial plasma concentration (and not the blood flow) is the driving force for solute uptake by the brain. On the basis of a model composed of two compartments, namely blood plasma within the brain and the brain parenchyma, the transcapillary PS (μl·g⁻¹·min⁻¹) of each hydrophilic test solute was computed as

$$PS = A_b / \int_0^{t*} C_p(t)dt = A_b/AUC \quad (2)$$

where $A_b$ is the amount of test solute present in a gram of brain parenchyma at the time of death (t = $t*$), and AUC is the area under the curve relating arterial plasma concentration (C_p) to time from the time of injection (t = 0) to $t*$. As noted by Ohno et al. (22), Eq. 2 is obtained by integrating the usual equation of exchange between two compartments (dA_b/dt = PS[C_p - C_b]) under circumstances in which the brain solute concentration (C_b) is so much smaller than the concurrent plasma concentra-
HYDROPHILIC SOLUTES ACROSS INTACT BLOOD-BRAIN BARRIER

TABLE 1. Lipid water partition coefficients and free diffusion coefficients of test solutes

<table>
<thead>
<tr>
<th>Solute</th>
<th>n</th>
<th>Butanol/Water</th>
<th>Octanol/Water</th>
<th>D&lt;sub&gt;37&lt;/sub&gt; water, cm&lt;sup&gt;2&lt;/sup&gt;/s</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Glucose</td>
<td>9</td>
<td>3.62 (±0.33) x 10&lt;sup&gt;-2&lt;/sup&gt;</td>
<td>2.48 (±0.08) x 10&lt;sup&gt;-2&lt;/sup&gt;</td>
<td>0.90 x 10&lt;sup&gt;-5&lt;/sup&gt;</td>
</tr>
<tr>
<td>Raffinose</td>
<td>3</td>
<td>0.67 (±0.01) x 10&lt;sup&gt;-2&lt;/sup&gt;</td>
<td>0.22 (±0.01) x 10&lt;sup&gt;-2&lt;/sup&gt;</td>
<td>0.64 x 10&lt;sup&gt;-5&lt;/sup&gt;</td>
</tr>
<tr>
<td>Inulin</td>
<td>9</td>
<td>0.15 (±0.04) x 10&lt;sup&gt;-2&lt;/sup&gt;</td>
<td>0.03 (±0.003) x 10&lt;sup&gt;-2&lt;/sup&gt;</td>
<td>(0.21-0.26) x 10&lt;sup&gt;-5&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values of the octanol/water partition coefficients were computed from experimentally determined butanol/water partition coefficients by the relationship defined in Eq. 1 and are means ± SE of n separate determinations. Values of free-diffusion coefficients (D) were taken from the review by Crone and Levitt (7). Dialyzed [3H]inulin (see text) yielded a similar value of D (0.24 ± 0.02 x 10<sup>-5</sup> cm<sup>2</sup>/s, n = 4) when measured in 1% agar at 37°C by the method of Redwood et al. (28).

![Fractionation profile of [3H]inulin chromatographed before and after dialysis.](image)

**FIG. 1.** Fractionation profile of [3H]inulin chromatographed before and after dialysis. Stock [3H]inulin (NEN) chromatographs as a wide band that spans the fractionation limits (100-6,000 Da for carbohydrates) of this P-6 gel column (Bio-Rad). Small fragments (note overlap with L-glucose, 180 Da) and other larger labeled fragments (up to 3,500 Da) were completely removed by equilibrium dialysis.

**RESULTS**

**Physiological Parameters Measured in Test Rats**

Experimental animals consistently displayed stable mean arterial blood pressure (MABP) over time. From these continuous recordings, average values of MABP were grouped according to which test solute was injected. The inulin and L-glucose group was further subdivided according to the duration of the experiment. Mean values are summarized in Table 2, together with the blood pH and arterial PCO<sub>2</sub> measured in these rats. The average MABP was slightly higher in the 10- and 30-min inulin/L-glucose groups, but simple linear regression analysis revealed no significant correlations between the MABP and PS values of either tracer. No differences in the arterial PCO<sub>2</sub> or pH were present among test groups in Table 2. Most rats were slightly hypercapnic (mean arterial PCO<sub>2</sub> of 60.4 ± 1.9 mmHg), having been given 5% CO<sub>2</sub> to breathe. This CO<sub>2</sub> was administered in an attempt to dilate the cerebral vascular bed so as to maximize the number of perfused capillaries available for solute exchange (2, 30). Analysis by linear regression failed to reveal a significant correlation between solute PS and arterial PCO<sub>2</sub> in these rats. For example, in 13 experiments in which the L-glucose PS was measured after 10 min of labeling, the slope of PS<sub>2</sub> against PCO<sub>2</sub> was −0.008 (r = −0.63). As noted below, however, an apparent CO<sub>2</sub> effect could be demonstrated by allowing rats to breathe air rather than 5% CO<sub>2</sub>. Thus, in preliminary trials, significantly lower PS values were found in five rats breathing 95% O<sub>2</sub>-5% CO<sub>2</sub>.

**Regional V<sub>p</sub>, Entrapped in Cerebral Vasculature**

The observed distribution volume of [14C]dextran in brain regions (Fig. 2) served as a measure of the volume of plasma contained within the cerebral vasculature. When co-administered with [14C]dextran to four rats, the distribution volume of [3H]inulin was higher in every
95% oz-5% coz, *t, and $ denote 3 separate series of trials of brain region, indicating that inulin occupied a volume
of 5.6 pi/g. Plasma volumes in the brain presumably because of difficulties in removing the pial
systemic administration.

Distribution volume of [3H]inulin codetermined in 4 rats was signifi-
cantly higher than that of dextran in all brain regions, indicating that
inulin labels more than intravascular fluid volume in 10 min after
mined 10 min after intravenous bolus injection. Distribution volume of
larger
rho2
It is noted among cerebral regions. Specifically, ps2c values
in the temporal region were lower than those observed
free-diffusion coefficients (P < 0.0005) or the ratio of
octanol to water partition coefficients (P < 0.0005).

Thin-layer chromatography of the injected solutes verified
that these ratios were not spuriously high because of
contamination with monosaccharides.

The mean PSsc ratio of inulin/L-glucose was 1.78 ±
0.10. As with the raffinose/L-glucose pair, these values
were significantly higher than the ratios of free diffusion

TABLE 2. Physiological parameters measured in test rats

<table>
<thead>
<tr>
<th>Test Solutes</th>
<th>n</th>
<th>MARP, mmHg</th>
<th>Pb0, mmHg</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raffinose/L-glucose</td>
<td>3</td>
<td>108±14</td>
<td>78.3±7.5</td>
<td>7.26±0.03</td>
</tr>
<tr>
<td>Inulin/L-glucose*</td>
<td>5</td>
<td>131±6</td>
<td>64.8±3.2</td>
<td>7.26±0.02</td>
</tr>
<tr>
<td>Inulin/L-glucose†</td>
<td>6</td>
<td>127±5</td>
<td>65.4±1.3</td>
<td>7.25±0.01</td>
</tr>
<tr>
<td>Inulin/L-glucose‡</td>
<td>5</td>
<td>102±7</td>
<td>61.9±4.9</td>
<td>7.29±0.02</td>
</tr>
<tr>
<td>Dextran</td>
<td>6</td>
<td>108±7</td>
<td>55.9±5.2</td>
<td>7.30±0.02</td>
</tr>
<tr>
<td>Means ± SE</td>
<td>25</td>
<td>114±7</td>
<td>68.9±3.5</td>
<td>7.27±0.01</td>
</tr>
</tbody>
</table>

* is no. of animals. Not shown are %O2 saturation values of arterial blood, which was controlled at 98.6 ± 0.5% by allowing each rat to breathe
95% O2-5% CO2. *t, and $ denote 3 separate series of trials of 10, 30, and 60 min duration, respectively.

Regional PS values for these solutes were computed on basis of a two-
compartment model (PSSc). Bars represent means ± SE.

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>Raffinose/L-glucose</th>
<th>Inulin/L-glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frontal</td>
<td>1.06±0.04</td>
<td>1.47±0.17</td>
</tr>
<tr>
<td>Parietal</td>
<td>1.07±0.09</td>
<td>1.55±0.19</td>
</tr>
<tr>
<td>Occipital</td>
<td>1.05±0.03</td>
<td>1.75±0.20</td>
</tr>
<tr>
<td>Temporal</td>
<td>1.17±0.06</td>
<td>2.41±0.33</td>
</tr>
<tr>
<td>Subcortex</td>
<td>1.10±0.04</td>
<td>2.24±0.49</td>
</tr>
<tr>
<td>Colliculi</td>
<td>1.04±0.09</td>
<td>1.67±0.93</td>
</tr>
<tr>
<td>Brain stem</td>
<td>1.02±0.04</td>
<td>1.49±0.20</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>1.03±0.03</td>
<td>1.65±0.21</td>
</tr>
<tr>
<td>Means ± SE</td>
<td>1.07±0.02</td>
<td>1.78±0.10</td>
</tr>
</tbody>
</table>

n is number of rats.
and octanol/water partition coefficients and were also higher than unity ($P < 0.0005$ for all comparisons). Speculation that these unexpected findings might be the result of respiratory acidosis prompted a series of experiments in which two rats breathing $5\%$ CO$_2$-$95\%$ O$_2$ were compared with five rats breathing air. In these trials, 10-min $PS_{SC}$ values for inulin averaged 50% lower and for L-glucose 37% lower in the animals breathing air than in those breathing CO$_2$ ($P < 0.005$), but the $PS$ ratio was not significantly affected by the gas mixture ($P > 0.11$).

**PS Values of L-[14C]glucose and [3H]inulin Determined 30 and 60 min after Single Intravenous Bolus Injection**

Under steady-state conditions such as those present in these experiments (see Table 2), the $PS$ product of a solute should be time invariant. To investigate whether $PS_{SC}$ would yield constant $PS$ values, we measured $PS_{SC}$ for inulin and L-glucose at 30 and 60 min after the labeled solutes were injected. The data for the various brain regions in each series have been pooled and are shown in Fig. 4 together with the 10-min data. In the cerebrum, the $PS_{SC}$ values of inulin after 10 min of labeling were 2.5 times higher than at 30 or 60 min. Likewise, the $PS_{SC}$ values for L-glucose had fallen from 0.44 $\mu l \cdot g^{-1} \cdot min^{-1}$ at 10 min to 0.22 and 0.14 $\mu l \cdot g^{-1} \cdot min^{-1}$ at 30 and 60 min, respectively.

Ratios of regional $PS_{SC}$ values at each time point are seen in Table 4. This ratio fell from 1.78 ± 0.10 at 10 min to 1.27 ± 0.05 ($n = 6$) at 30 min but was again 1.80 ± 0.13 ($n = 5$) at 60 min. All ratios were significantly higher ($P < 0.0005$) than the ratio of $O/W$ partition coefficients (=0.01) of these two solutes. Similarly, $PS$ ratios were significantly higher than the ratio of free diffusion coefficients (=0.27). When the duration of labeling was 10 min, no regional differences in the $PS$ ratio of inulin/L-glucose were found. The pooled ratio was significantly greater than unity ($P < 0.0005$). Since some regional variability was noted at 30 and 60 min, the value in each brain region was considered separately. When the duration of labeling was 30 min, several brain regions, including frontal cortex, subcortex, brain stem, and cerebellum, yielded $PS$ ratios that were not significantly different from 1.0. A similar pattern evolved at 60 min. Overall, $PS_{SC}$ values of these two solutes fell as the labeling time was increased, and in most cases the $PS_{SC}$ ratio of inulin/L-glucose was >1.0.

**Multiple Time Graphical Uptake Analysis**

The data obtained in the 16 inulin/L-glucose experiments were reanalyzed according to the more general model suggested by Patlak et al. (25). In Fig. 5, the amount of test solute present in brain ($A_{eq}$) divided by its final plasma concentration, $C_p(t^*)$, at the time of death is the ordinate. The abscissa represents the area under the plasma concentration versus time curve (AUC) divided by $C_p(t^*)$. A straight line was fitted to the L-glucose (Fig. 5A) and inulin (Fig. 5B) data by the method of least squares. The slope of each line represents the unidirectional transfer constant from blood to brain for each test solute and is designated here as $PS_{SC}$. Because of the scatter in the 60-min inulin data (Fig. 5B) and the large effect that two of these 60-min experiments had on the computed intercept, the slope of the line for inulin was computed on the basis of the 10- and 30-min experiments only. The slope for L-glucose was the same whether it was computed from two or from three groups.

When slopes of lines for each tracer were computed separately for each brain region (see Table 5), no differences could be detected by analysis of covariance ($P = 0.59$). Thus values were pooled to determine one unidirectional transfer constant for each tracer. The result was $0.133 \pm 0.01 \mu l \cdot g^{-1} \cdot min^{-1}$ for inulin and $0.119 \pm 0.01 \mu l \cdot g^{-1} \cdot min^{-1}$ for L-glucose, representing our best estimates of $PS_{SC}$ for these two solutes. Others have reported values of 0.35, 0.36, 0.02 (22), 0.06 (33), and 0.15 (1) for inulin. The only reported figure for L-glucose is 1.0 (11), but if based on early uptake, this value may reflect loading of the “fast space” (see DISCUSSION) and be spuriously high. In the present study the $PS_{SC}$ ratio of inulin/L-glucose determined by graphical analysis was not significantly different from 1.0. This ratio, however, was significantly different from the ratios of the free diffusion and octanol/water partition coefficients ($P < 0.0005$).

The $y$-intercepts in Fig. 5 have been described as the “apparent initial volume of distribution” ($V_i$) of the test solute. As summarized in Table 5, no significant differences in $V_i$ among brain regions were detected for either

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**Table 4. Ratios of $PS_{SC}$ for [3H]inulin/L-[14C]glucose as a function of labeling time**

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>10 min ($n = 6$)</th>
<th>30 min ($n = 6$)</th>
<th>60 min ($n = 5$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frontal</td>
<td>1.47±0.17</td>
<td>0.97±0.12</td>
<td>1.53±0.36</td>
</tr>
<tr>
<td>Parietal</td>
<td>1.55±0.19</td>
<td>1.42±0.15*</td>
<td>2.22±0.45*</td>
</tr>
<tr>
<td>Occipital</td>
<td>1.75±0.20</td>
<td>1.50±0.18*</td>
<td>1.76±0.28*</td>
</tr>
<tr>
<td>Temporal</td>
<td>2.41±0.33</td>
<td>1.61±0.16†</td>
<td>2.34±0.38†</td>
</tr>
<tr>
<td>Subcortex</td>
<td>2.24±0.49</td>
<td>1.35±0.12*</td>
<td>2.02±0.38*</td>
</tr>
<tr>
<td>Colliculi</td>
<td>1.67±0.23</td>
<td>1.20±0.13</td>
<td>1.78±0.25†</td>
</tr>
<tr>
<td>Brain stem</td>
<td>1.48±0.20</td>
<td>1.04±0.10</td>
<td>1.65±0.42</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>1.65±0.51</td>
<td>1.17±0.11</td>
<td>1.46±0.29</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td>1.78±0.10†</td>
<td>1.27±0.05†</td>
<td>1.80±0.13†</td>
</tr>
</tbody>
</table>

$n$ is the no. of test rats. * Differs from 1 at $P < 0.025$ level of significance; † Differs from 1.0 at $P < 0.005$ level of significance.

---

**Figure 4.** Permeability-surface area product ($PS$) of [3H]inulin and L-[14C]glucose as a function of labeling time. Cerebral $PS_{SC}$ values of [3H]inulin and L-[14C]glucose are presented here as a function of labeling duration. When computed on basis of two-compartment model, $PS$ values for both solutes were lower as duration of labeling was increased, but they appeared to reach a minimum near 60 min. Error bars represent means ± SE of pooled cerebral regions.
HYDROPHILIC SOLUTES ACROSS INTACT BLOOD-BRAIN BARRIER

**Fig. 5.** Multiple time graphical analysis of L-[14C]glucose (A) and [3H]inulin (B) uptake into rat brain. Brain uptake data from experiments of varying length (10, 30, and 60 min) were plotted as suggested by Patlak et al. (25; cf. Eq. 4). Each point represents a single brain region. Slopes of lines (fitted by least squares) represent PS of L-[14C]glucose and [3H]inulin across the blood-brain barrier (BBB), and y-intercepts are equivalent to their initial distribution volumes in BBB complex (Vi). Numerical values are presented in Table 5. AUC, area under curve; A,A, amount of test solute in brain parenchyma at time of death; C,(t*), plasma concentration at time of death.

**TABLE 5.** Regional unidirectional transfer constants (PS) and initial distribution volumes (Vi) for [3H]inulin and L-[14C]glucose determined by graphical uptake analysis

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>Inulin</th>
<th></th>
<th>L-Glucose</th>
<th></th>
<th>PS Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frontal</td>
<td>0.073</td>
<td>19</td>
<td>0.116</td>
<td>13</td>
<td>0.63</td>
</tr>
<tr>
<td>Parietal</td>
<td>0.128</td>
<td>19</td>
<td>0.106</td>
<td>12</td>
<td>1.21</td>
</tr>
<tr>
<td>Occipital</td>
<td>0.188</td>
<td>17</td>
<td>0.099</td>
<td>12</td>
<td>1.89</td>
</tr>
<tr>
<td>Temporal</td>
<td>0.117</td>
<td>16</td>
<td>0.007</td>
<td>8</td>
<td>1.21</td>
</tr>
<tr>
<td>Subcortex</td>
<td>0.142</td>
<td>20</td>
<td>0.135</td>
<td>12</td>
<td>1.05</td>
</tr>
<tr>
<td>Colliculi</td>
<td>0.149</td>
<td>19</td>
<td>0.118</td>
<td>11</td>
<td>1.26</td>
</tr>
<tr>
<td>Brain stem</td>
<td>0.147</td>
<td>20</td>
<td>0.123</td>
<td>16</td>
<td>1.20</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>0.118</td>
<td>25</td>
<td>0.156</td>
<td>14</td>
<td>0.76</td>
</tr>
<tr>
<td><strong>Means ± SE</strong></td>
<td>0.132±0.013</td>
<td>19±1.7*</td>
<td>0.119±0.007</td>
<td>12±1.1*</td>
<td>1.15±0.013</td>
</tr>
</tbody>
</table>

*Significantly different from each other (P < 0.0005) by analysis of covariance and paired t tests.

**DISCUSSION**

The purpose of this study was to elucidate the pathways and mechanisms of hydrophilic solute transfer across the intact BBB. We cannot reconcile our findings with the hypothesis that these solutes entered the brain by diffusing across a lipid barrier or through water-filled channels, although both processes may have contributed to the transport. We know of no evidence that carrier-mediated transport is available for such relatively impermeant, nonnutrient molecules as L-glucose and inulin. Based largely on exclusion, vesicular transport is proposed as the major mechanism by which these intensely hydrophilic solutes were slowly transferred across the endothelial cells of intact brain capillaries; no other mechanism would appear to be capable of transporting inulin as fast as L-glucose. In this respect the intact BBB differs markedly from the BBB opened by osmotic shock (14). In the latter case these saccharides entered the brain rapidly and at differential rates consistent with diffusion through water-filled channels as the principal mode of transport (unpublished observations).

**Effect of Exogeneous CO₂**

For reasons discussed below, we believe that our two-compartment estimates of PS (PS) are higher than the true PS values of these test solutes, but as a semiphenometric measure of the solute permeation rate, PS does have some utility. For example, PS values of both L-glucose and inulin proved to be higher in rats breathing 5% CO₂-95% O₂ than in those breathing air. A plausible interpretation of this finding is that mild respiratory acidosis (Table 2) increased the number of actively perfused capillaries in the brain and therefore S in the PS product. Because the PS ratio (inulin/L-glucose) was not significantly different in the two groups, it seems unlikely that the permeabilities P were grossly affected by this level of exogenous CO₂. These inferences, however, remain tentative until more data are accumulated, but it is noteworthy that respiratory acidosis even more intense than in the present study did not affect BBB permeability to macromolecules in the absence of hypertension (2, 20). All PS values discussed below were measured in rats breathing 5% CO₂-95% O₂; as demonstrated in Table 2, these animals were normotensive.
Plasma Volumes in Excised Brain

Accurate estimates of the volume of blood plasma \( (V_p) \) entrapped within the vasculature of excised brain are essential for calculating \( PS_{2c} \) and for interpreting \( PS_{SC} \). The issue is especially critical when brain regions contain only small amounts of the test solute (see Eq. 3). In this study \( V_p \) has been equated to the 10-min regional distribution volume of 70 kDa \(^{14}C\) dextran, which is the largest molecule tested and which occupied a significantly smaller volume than did inulin. A significant difference in the distribution volumes of dextran and inulin has been observed as early as 1 min after intravenous injection (35). In using extracellular markers to determine \( V_p \), it is noteworthy that all of the recognized complicating factors (e.g., uptake or binding by blood cells, by endothelial cells, by glial or neuronal cells, extravasation, metabolic breakdown to smaller molecules, etc.) result in spuriously high, not low, estimates of the regional plasma volume. Therefore the tracer with the smallest distribution volume almost certainly provides the truest estimate of \( V_p \). The mean plasma volume determined with \(^{14}C\) dextran in the present study (5.6 \( \mu\)l/g) is lower than published values based on smaller extracellular markers (22, 36) but is almost identical to data obtained by Gjedde (11) with "protein-bound indium" and by Smith et al. (35) with 79 kDa dextran.

Evidence for a Rapidly Equilibrating Compartment Between Blood and Brain and Consequent Errors in Computing \( PS \)

Our demonstration that single-time estimates of solute \( PS \) are smaller the longer the labeling time (e.g., Fig. 5) is evidence that the conventional two-compartment model used to compute these values is invalid. In this model the two compartments are the cerebral plasma and the brain parenchyma. Ohno et al. (22) postulated the same two compartments, but they employed an iterative method to compute a hypothetical value for the volume of entrapped blood based on sucrose uptake data. Our regional estimates of brain plasma volume, based on direct measurements of the dextran space (Fig. 2), are smaller than those inferred from the computed blood volumes of Ohno et al. (22). Ziylan et al. (36) chose to employ in their two-compartment equations a different value of the entrapped blood volume for each test solute. The need to use an adjusted value for the blood volume is prima facia evidence that more than two compartments really exist. Thus the "blood volume" of Ohno and of Ziylan is believed to consist of the sum of the true blood volume and a space the size of which appears to differ from one solute to another. Although using an adjusted blood volume may serve operationally to combine two compartments, it is misleading in our opinion to refer to such a system as a two-compartment model.

From the observation that the y-intercepts \( (V_i) \) in the Patlak plots for inulin and L-glucose (Fig. 5, A and B) exceed the volume of entrapped blood plasma (Fig. 2), it is inferred that one or more compartments exist between blood and brain parenchyma and that both inulin and L-glucose have prompt access to at least some of these compartments. Thus, for each solute, \( V_i \) is composed of an intraluminal and an extraluminal component. Because both of these solutes are recognized extracellular markers (19, 34) and presumably do not bind to or enter blood cells, the intraluminal compartment must consist of entrapped blood plasma \( (V_p) \). Thus the volume of the extraluminal compartment \( (V_e) \) is

\[
V_e = V_i - V_p
\]  

For a plasma volume of 6 \( \mu\)l/g (computed from the dextran space), \( V_e \) for inulin and L-glucose are 13 and 6 \( \mu\)l/g, respectively. Although the rapidly equilibrating or "fast" space for inulin has been observed before (1, 33, 35), it was surprising that \( V_e \) proved to be twofold (L-glucose) to threefold (inulin) larger than the plasma volume.

Using two synthetic, nonmetabolizable amino acids, Blasberg et al. (3) also found that \( PS_{2c} \) decreased as the duration of uptake was lengthened. Based on this finding, the two-compartment model of Ohno et al. (22) was expanded to allow for the presence of rapidly equilibrating compartments in series and in parallel with blood and brain (25). If these compartments equilibrate with capillary blood fast enough, the result is essentially a three-compartment model. When analyzed in terms of this model, the amino acid data yielded a single, time-invariant estimate of \( PS \) for each test solute. According to Blasberg et al. (3), the two-compartment \( PS \) is related to the three-compartment \( PS \) as follows

\[
PS_{2c} = PS_{SC} + V_i \cdot C_p(\tau)/AUC
\]

A simpler and somewhat more informative equation is

\[
PS_{2c}/PS_{SC} = 1 + A_t/A_b
\]

where \( A_t \) (equals \( V_i \cdot C_p \)) is the amount of test solute in the fast compartment, and \( A_b \) is the amount in the brain parenchyma. Clearly the two \( PS \) estimates are identical if there is no fast compartment (\( V_e = A_t = 0 \)). Otherwise, \( PS_{2c} \) exceeds \( PS_{SC} \) unless \( A_b \gg A_t \), a condition that occurs only after a long infusion period with relatively impermeant solutes (4). Figure 6 illustrates how the \( PS_{2c} \)'s for inulin and L-glucose overestimate the true \( PS \)'s under various barrier conditions when the experimental duration is 10 min. With the intact BBB encoun-

FIG. 6. Deviations in \( PS_{2c} \) from true \( PS \) (\( PS_{SC} \)) resulting from presence of rapidly equilibrating compartments. Figure depicts various degrees of blood-brain barrier (BBB) permeability as represented by \( PS_{2c} \). Points represent mean values of \( PS_{2c} \) for each solute were entered. Points represent mean values of \( PS_{2c} \) observed in this study.
tered in the present series of experiments, the mean $PS_{2C}$ after 10 minutes of labeling was $\sim 380\%$ too high for L-glucose and $\sim 540\%$ too high for inulin (represented by the single point on each curve).

Others (3, 26) have also encountered difficulties in using two-compartment equations to compute the PS of small hydrophilic solutes at early times because of the presence of rapidly equilibrating compartments. However, the fast compartment is not the only reason why this methodology may yield spuriously high PS values. For example, if the volume of entrapped plasma is underestimated, the computed PS is falsely elevated (cf. Eqs. 2 and 3). Errors of this type are bound to occur when the correction for entrapped test solute involves substituting arterial hematocrits for the much smaller microvessel hematocrits (3, 26). To avoid this error in the present study, $V_a$ was equated to the dextran space and not computed from hematocrit data. Finally, small labeled fragments, reported to contaminate test solute mixtures and to yield artifactual PS values (26), were either not present or were removed by dialysis before their use as tracers in the present study.

**Anatomic Location of Rapidly Equilibrating Compartment**

Blasberg et al. (3) found that the extraluminal fast compartment available to their nonmetabolizable amino acids was sizeable ($V_e = 60 \mu l/g$). They inferred that it included the endothelial cells of the cerebral vasculature, the meninges, and ependyma and/or adjacent neural tissue. Unlike amino acids, the hydrophilic solutes used in the present study are generally recognized as extracellular markers (19, 34), and so it is not surprising that the $V_e$'s found for L-glucose and inulin are much smaller. Important questions, however, do remain about the location and even the nature of these fast compartments.

Two lines of inquiry suggest that $V_e$ is not a real tissue volume or a conventional pharmacokinetic compartment. First, if it were a true fluid space, there is no reasonable explanation for the observation that this space is larger for inulin (13 $\mu l/g$) than for L-glucose (6 $\mu l/g$). Zylan et al. (36) made similar observations with inulin and sucrose in rat brain but did not address this disparity. Because of their much smaller molecular size, glucose and sucrose should be able to diffuse into any space available to inulin. Indeed the inulin volume of distribution is somewhat smaller than that of sucrose in almost all tissues investigated (e.g., 17). Second, even if the BBB were located at the abluminal surface of capillary endothelial cells and if hydrophilic solutes could readily enter the intracellular water of these cells, the endothelial cell volume appears to be much too small to account for the values of $V_e$ observed.\(^1\)

\(^1\) For example, inulin $V_e$ is 13 $\mu l/g$, which is slightly greater than the volume of entrapped blood ($V_a = 10 \mu l/g$). If one assumes that all of this blood were present in capillaries with luminal diameters of 5 $\mu m$, lined by endothelial cells with an average thickness of 0.5 $\mu m$, then the ratio of aggregate endothelial cell volume to organ blood volume would have been $\sim 0.44$. This is much smaller than the $V_e$-to-$V_a$ ratio of 1.3. This estimate is conservative because much of the blood entrapped in an excised organ must reside in vessels considerably larger than capillaries. In a blood vessel with a diameter of 20 $\mu m$, for example, the endothelial cell volume is only one tenth of the entrapped blood volume.

These considerations lead us to the conclusion that the "fast compartment" for these hydrophilic solutes is not a tissue fluid space; rather it probably consists of binding sites to which the solutes are reversibly adsorbed. A likely location of these sites is in the glycoalyx that lines the luminal surface of endothelial cells. This glycoalyx consists of complex carbohydrates with exposed sialic acid residues (18, 31) that can presumably bind solute with low affinity by hydrogen bonding. Such an interaction provides an explanation for the larger apparent distribution volume of inulin, since inulin contains more hydrophilic residues than does L-glucose and so may be bound more tightly.

**Interpretation of PS Ratios**

As noted before (see *Rationale of Experimental Design*), an examination of unbiased PS ratios of solute pairs can lead to useful inferences about their mutual mode or modes of transport across capillary beds. Even though PS ratios should be unaffected by variations in the size (S) of the capillary bed, an attempt was made to stabilize the number of perfused capillaries by providing 5% $CO_2$ as a cerebral vasodilator. Although this $CO_2$ may have affected capillary permeability, preliminary data indicate that PS ratios were not different in rats breathing air.

With respect to PS ratios based on the two-compartment model, all means for raffinose/L-glucose and for inulin/L-glucose (Table 3) exceed unity. Because values of $PS_{2C}$ for these relatively impermeant solutes are spuriously high in short-term experiments because of the existence of a rapidly equilibrating compartment, the ratios are not easy to interpret. Presumably because of more extensive binding to the capillary wall, inulin, as noted above, has a larger fast space than does L-glucose (13 vs. 6 $\mu l/g$); by inference the value for raffinose is probably intermediate. To the extent that the larger solute has a larger $V_e$, ratios of $PS_{2C}$ are overestimates.

Ratios of PS values determined by the multiple-time graphical method (25) are free of this error. In the present study, least-squares fits to the pooled data yield straight lines that are nearly parallel (Fig. 5, A and B), and the same appears to be true in all brain regions. Thus, the $PS_{3C}$ ratio of inulin/L-glucose is close to unity (Table 5) and significantly higher than the ratio of their free-diffusion coefficients ($P < 0.0005$) or the ratio of their octanol/water partition coefficients ($P < 0.0005$). If one is prepared to assume that these two solutes employed the same mode or modes of transport across the BBB, it is reasonably certain that the principal mode was not diffusion through water-filled or lipid-filled channels.

Before accepting this conclusion, it is appropriate to search for possible bias in $PS_{3C}$ estimates. Whether PS values are predicated on a two-compartment (22) or a three-compartment (25) model, the experimental protocol assumes that all test solute that has crossed the BBB stays in the brain for the remainder of the test period. If backdiffusion were to occur, PS would be underestimated. In brain parenchyma, inulin and sucrose have rather similar equilibrium volumes of distribution (1, 17) and the same is probably true of L-glucose. Accordingly,
one would anticipate that backdiffusion, if present, would be greater for the more diffusible tracer, L-glucose, leading to a spuriously high PS ratio for inulin/L-glucose. In the multiple-time graphical analysis (25), backdiffusion would be revealed by downward curvature (3), but none is evident in the L-glucose or inulin lines (Fig. 5, A and B), even at 60 min. Furthermore, estimates of L-glucose concentration in the brain interstitial space at the end of 60 min are <20% of the concurrent plasma concentrations. Therefore, the PS values and the PS ratios in Table 5 are considered valid.

Conclusions

The conclusion that diffusion through water- or lipid-filled channels is not the principal mechanism by which these carbohydrates cross the intact BBB is consonant with electron microscopic evidence that junctions between neighboring endothelial cells are particularly tight in cerebral capillaries (6, 29). The generalization that BBB permeability is larger the higher the lipid water partition coefficient (9, 27) apparently does not apply to solutes as hydrophilic as L-glucose, raffinose, and inulin. That molecules as different in size as L-glucose and inulin yield essentially equal values of PS (Table 5) suggests micropinocytosis as a possible transport mechanism across the intact BBB. To what extent the physiological state of the rats used in these trials (e.g., mild respiratory acidosis, pentobarbital-ketamine anesthesia, etc.) influenced rates of pinocytic transport cannot be surmised, but it is noteworthy that hypertension, which allegedly promotes pinocytosis in capillary endothelium (32), was not present in these animals. Fluid-phase transport in vesicles of the size found in capillary endothelium is unlikely to discriminate among the solute molecules used here, but the endocytic engulfment of solute adsorbed on the endothelial glycoalyx (solid-phase transport) could yield differential rates of ingestion in favor of substances that are more extensively adsorbed. That more inulin was adsorbed is implicit in the finding that $V_i$ was larger for inulin than for L-glucose (Table 5). Perhaps the slightly higher $PS_{SO}$ of inulin is related to this difference. The scarcity of pinocytic vesicles in cerebral microvessels is consonant with the low permeation rates of all of these solutes when the blood-brain barrier is intact.

K. J. Lucchesi was a Fellow of the Albert J. Ryan Foundation during the conduct of these experiments.

Present address of K. J. Lucchesi: Yale University School of Medicine, Dept. of Pharmacology, Sterling Hall of Medicine, PO Box 3333, New Haven, CT 06510-8066.

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REFERENCES