In situ kinetics of glucose transport across the blood-retinal barrier in normal rats and rats with streptozocin-induced diabetes

S. R. Ennis, J. E. Johnson,* and E. L. Pautler

Glucose transport across the blood-retinal barrier (BRB) was investigated in situ by means of a modification of the single-injection technique developed for studying transport across the blood-brain barrier. A retinal uptake index was calculated from the fractional extraction for $^{14}$C-glucose divided by the fractional extraction for the $^{3}$H$_{2}$O reference. The use of the Eadie-Hofstee transformation to determine the $K_{m}$ of carrier-mediated transport revealed at least two transport systems for the unidirectional glucose flux across the BRB. Control data showed a relatively high-affinity system with a $K_{m}$ of 0.24 mM and a lower affinity system with a $K_{m}$ of 7.81 mM. Both these transport systems have been shown to be sensitive to phloretin but not to phloridzin or acetazolamide. It has also been shown that glucose transport is unaffected by Na$^{+}$ or K$^{+}$ concentrations but that calcium does have a significant effect on the lower affinity transport system for glucose. We have also demonstrated that the $K_{m}$ values of both transport systems are significantly increased by 2 months of streptozocin-induced diabetes. (INVEST OPHTHALMOL VIS SCI 23:447-456, 1982.)

Key words: blood-retinal barrier, retinal pigment epithelium, glucose transport, kinetics, phloretin, ion dependence, streptozocin-induced diabetes

The concept of the blood-retinal barrier (BRB) is used to describe the restricted movement of substances between the retina and the blood supply. The primary anatomic basis for the BRB are the tight junctions that surround both the retinal pigment epithelium (RPE) and the endothelial cells of the retinal vessels. The BRB limits movement of substrates into the retina and probably requires specific transport systems to maintain adequate supplies to meet the metabolic needs of the retina. Since D-glucose is the major metabolic substrate for the retina, the mechanism by which it moves into the retina under normal and pathologic conditions warrants investigation.

The present study reports on an in situ method for evaluating the kinetics of D-glucose transport across the total BRB, which was a modification of the single-injection technique developed by Oldendorf and Cremer et al. Oldendorf's procedure consists of a simultaneous carotid injection of a bolus of $^{14}$C-labeled test substance and reference triitated water, which is carried to the brain by the blood. Our modification of the basic technique is to use a continuous carotid perfusion to carry the bolus to the eye. This modification allows for precise control of the compo-

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EXPERIMENTAL APPARATUS

Fig. 1. Perfusion system for in situ determination of glucose transport across the BRB. The system consists of a Gilson peristaltic pump to pump perfusate into a rat through a carotid cannula. The perfusate first passes through a tube within a tube in order to warm the perfusate to 34° C. A side channel containing a series of three-way valves allows for the introduction of the test bolus into the rat. At the appropriate time one of the three-way valves is turned to divert the perfusate into the side channel. This allows introduction of the bolus without any interruption in perfusate flow.

sition of the perfusate and permits the use of steady-state conditions in determining the kinetics of D-glucose transport by the BRB. According to Lund-Anderson,7 the use of steady-state conditions will eliminate a major source of error encountered in determining kinetic constants with a non-steady-state approach.

Materials and methods

Experimental arrangement. The pigmented rats (Long-Evans strain) of both sexes, weighing 200 to 400 gm, used in this study were from established colonies maintained by our laboratory. Rats were anesthetized with an intraperitoneal injection (50 mg/kg) of sodium pentobarbital. Tracheotomy was established, and the left and right jugulars and right carotid were exposed. The right jugular was cannulated toward the head with PE205 polyethylene tubing to allow for drainage of perfusate. Rats were heparinized with 1000 U/kg through a temporary right jugular cannula toward the heart. The rat was then moved to the experimental apparatus (Fig. 1), and the right carotid was cannulated toward the head with PE50 polyethylene tubing. Immediately after the carotid cannulation the rat was connected to the perfusion system. The circulating water bath and water jacket arrangement shown in Fig. 1 were used to warm the perfusate to 34° C.

Perfusion solutions. The composition of the perfusion solution (mM concentrations) was 0.71 Na₂HPO₄, 1.45 Na₂H₂PO₄, 22.7 NaHCO₃, 120 NaCl, 3.62 KCl, 0.69 MgSO₄, and 2.04 CaCl₂. The perfusate solution was heparinized to a concentration of 20 U/ml and was bubbled with 95% O₂-5% CO₂ to provide oxygen to the tissue and to maintain a pH of 7.4. The concentrations of D-glucose in the perfusate used were trace (0.003), 1, 2, 3, 5, 10, 20, 50, and 100 mM. To correct for osmolarity changes arising from the use of increasing concentrations of glucose, mannitol was added to the perfusate. This was done for all glucose concentrations except 50 and 100 mM, so that the final calculated osmolarity of the perfusate was 335 mOsm. The bolus solution used to measure retinal uptake for a given D-glucose concentration was the same as the perfusate except that it contained 4 μCi of ³H₂O and 0.80 μCi of uniformly labeled [¹⁴C]D-glucose in 0.8 ml. All radionuclides used in this study were obtained from New England Nuclear.

D-Glucose extraction. After carotid cannulation, a 10 min perfusion was begun and the rat was euthanized. The purpose of the perfusion was to allow the D-glucose in the perfusate to reach a new steady state across the BRB. This amount of equilibration time has been shown to be adequate for D-glucose to reach a new steady state after a sudden change in plasma D-glucose concentration across the blood-brain barrier. After 10 min of perfusion the bolus was introduced into the carotid cannula by diversion of the perfusate flow into a side channel containing the bolus. As a consequence, no disruption in the flow or rate of flow of perfusate into the rat or in intraocular hemodynamics will result from injection of the bolus directly into the carotid. Ten seconds after the bolus entered the rat, the retina was removed by inserting a pair of curved forceps under the eye, rotating 90 deg, and squeezing the retina out through a slit in the cornea. The retina and a sample of the bolus were prepared for analysis of radioactivity.

Sample analysis. Retina from D-glucose extraction experiments were solubilized in 1 ml of Protocol (New England Nuclear), and 10 ml of Aquasol II (New England Nuclear) were added. The samples were then analyzed for ³H and ¹⁴C activity in a Beckman LS9000 liquid scintillation counter.

Diabetic rats. Rats were fasted 16 hr before the injection of streptozocin (Sigma Chemical Co.), 65...
mg/kg body weight, via heart puncture. Streptozocin was dissolved just prior to use in acidified 0.9% saline (pH 4.5) at a concentration of 50 mg/ml. Diabetes was established by the demonstration of polyuria, glucosuria, and impaired growth in the experimental animals. Serum glucose was measured by the glucose oxidase method on a Beckman glucose analyzer.

Electroretinograms (ERGs) and perfusion pressure of perfused rat. For ERG recordings, rats were dark-adapted for 24 hr and then anesthetized with an intraperitoneal injection of 50 mg/kg sodium pentobarbital. The rat eyes were then covered to prevent light-adaptation, and the above-described surgery was performed. The pupils were dilated with 1.0% tropicamide. Differential recordings were made by cotton wicks threaded through PE50 polyethylene tubing filled with saline connecting to an Ag-AgCl wire. One of these electrodes was inserted behind the palpebral conjunctiva at a distance of 2 to 3 mm, and the other was placed on the cornea. A needle electrode under the scalp was connected to ground. Responses were recorded on a Grass 70A polygraph after preamplification by a Grass P16 DC amplifier. A Grass PS2 photostimulator placed 1 m from the eye was employed as light source. To obtain c-wave data, stimuli of relatively long duration (20 sec) were provided by high-frequency flicker at the rate of 50 flashes/sec.

We found that a perfusion rate of 18 ml/min and a perfusate temperature of 34° C were necessary to maintain the ERG of the perfused rat. Fig. 2 shows representative ERGs from a perfused and an intact rat during a 20 sec light stimulus. All ERG components are present in the perfused rat, even though the PH component is somewhat reduced in amplitude. The reduction in the PII component may be due to the limited oxygen-carrying capacity of the perfusate.

The pressure in the perfusion apparatus was measured with a Narco Bio-Systems pressure transducer. The Gibson pump used has a peristaltic action so that the equivalent of a systolic and diastolic pressure was measured. The perfused rat had a systolic pressure of 137 mm Hg and a diastolic pressure of 107 mm Hg. In vivo carotid systolic pressure in the pentobarbital-anesthetized rat averages 129 mm Hg and the diastolic averages 91 mm Hg.

Calculations. Analysis of the radioactive content of retinas for D-glucose allows for the calculation of a retinal uptake index percentage as follows:

\[
I_R = \frac{\text{tissue } ^{14}C/\text{tissue } ^{3}H}{\text{bolus } ^{14}C/\text{bolus } ^{3}H} \times 100
\]

Rearrangement of this equation gives

\[
I_R = \frac{\text{tissue } ^{14}C}{\text{bolus } ^{14}C} \times \frac{\text{tissue } ^{3}H}{\text{bolus } ^{3}H} \times 100
\]

The \( I_R \) is equivalent to the measured fractional extraction of glucose (\( E_{\text{glucose}} \)) divided by the measured fractional extraction of the highly diffusible reference (\( E_{\text{H}_{2}O} \)):

\[
I_R = \frac{E_{\text{glucose}}}{E_{\text{H}_{2}O}} \times 100
\]

The retinal uptake index for glucose is corrected for any of the bolus solution trapped in the retinal capillaries by subtracting an uptake index for inulin. The retinal uptake index for inulin is determined in a separate group of experiments and the average of 9.0 is used thereafter:

\[
I_R = \frac{E_{\text{inulin}}}{E_{\text{H}_{2}O}} \times 100
\]
Fig. 3. A, Uptake and efflux of $^3$H$_2$O across the BRB. Points are means ± S.E.M. See Results in text for mean and number of rats done at each point. B. Inot of $^{14}$C-glucose (left ordinate) plotted against time after the leading edge of the bolus reaches the carotid artery. Values are means ± S.E.M. Also shown is the fractional extraction of glucose (right ordinate) plotted vs time after injection. These extraction fractions for $^{14}$C-glucose are calculated from the product of retinal uptake index and $^3$H$_2$O extraction fraction for each point.

The retinal uptake of $^3$H$_2$O and $^{14}$C-glucose were also studied by varying the retinal sampling time between 5 and 20 sec. From the earlier description of the retinal uptake index, $E_{glucose} = I_R \cdot E_{H_2O}$. The fractional extraction of glucose was calculated by multiplying the $I_R$ by the fractional extraction of water determined at 5, 7.5, 10, 15, and 20 sec after the leading edge of the bolus enters the carotid artery.

The rate of total unidirectional flux of glucose into the retina is defined by

$$ V = (E_{glucose}) \cdot (F) \cdot (S_t) $$ (5)

where $F$ is the rate of combined choroidal and retinal perfusate flow and $S_t$ is the tracer concentration of $^{14}$C-glucose in the bolus. Since $E_{glucose} = I_R \cdot E_{H_2O}$, equation 5 may be restated as

$$ V = I_R \cdot E_{H_2O} \cdot F \cdot S_t $$

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$$ V = (E_{glucose}) \cdot (F) \cdot (S_t) $$ (5)

Fig. 4. Eadie-Hofstee plot of $I_{tot}$ vs $I_{tot}/G$ for glucose transport across the BRB in control rats. $K_m$ values for each of the straight-line segments were determined from the slope of the best linear fit by regression analysis on equation 13.

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$$ V = (E_{glucose}) \cdot (F) \cdot (S_t) $$ (5)

We have followed the mathematical consideration given by Pardridge for the kinetics of competitive inhibition of transport across the blood-brain barrier. The transport of $^{14}$C-glucose across the BRB may be modeled in terms of the Michaelis-Menten equation plus a linear term ($d$) for the nonsaturating portion of transport due to diffusion:

$$ V_i = \frac{V_{max} \cdot S_t}{K_m + S_t} + d $$

$$ V = (E_{glucose}) \cdot (F) \cdot (S_t) $$ (5)

where $I_{RT}$ is the retinal uptake index (including diffusion) determined with only tracer level glucose in the bolus. In all cases the value of $I_{RT}$ is the $I_R$ obtained with 0.003 mM glucose in the bolus.

The effect of a competitive inhibitor on tracer $^{14}$C-glucose transport may be written in a similar fashion to equation 8 as

Combination of equations 6 and 7 describing unidirectional flux gives

$$ V_T = I_{RT} \cdot E_{H_2O} \cdot F \cdot S_t = \frac{V_{max} \cdot S_t}{K_m + S_t} + d $$ (8)

where $I_{RT}$ is the retinal uptake index (including diffusion) determined with only tracer level glucose in the bolus. In all cases the value of $I_{RT}$ is the $I_R$ obtained with 0.003 mM glucose or tracer level in the bolus.
Table I. Retinal uptake index for glucose in rats: effects of inhibitors on glucose uptake.

<table>
<thead>
<tr>
<th>Glucose (mM)</th>
<th>Control</th>
<th>n</th>
<th>Phloretin (0.1 mM)</th>
<th>n</th>
<th>Phloridzin (0.75 mM)</th>
<th>n</th>
<th>Acetazolamide (1.0 mM)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.003</td>
<td>63.5 ± 9.0</td>
<td>13</td>
<td>43.2 ± 1.1</td>
<td>5</td>
<td>56.7 ± 3.4</td>
<td>5</td>
<td>64.4 ± 8.7</td>
<td>6</td>
</tr>
<tr>
<td>1.0</td>
<td>45.6 ± 3.9</td>
<td>12</td>
<td>40.6 ± 5.9</td>
<td>10</td>
<td>41.3 ± 3.9</td>
<td>6</td>
<td>46.1 ± 4.6</td>
<td>5</td>
</tr>
<tr>
<td>2.0</td>
<td>44.2 ± 5.0</td>
<td>12</td>
<td>38.7 ± 5.7</td>
<td>6</td>
<td>35.0 ± 2.5</td>
<td>6</td>
<td>48.2 ± 2.8</td>
<td>6</td>
</tr>
<tr>
<td>3.0</td>
<td>43.7 ± 3.5</td>
<td>9</td>
<td>36.9 ± 3.7</td>
<td>4</td>
<td>35.6 ± 3.4</td>
<td>6</td>
<td>44.5 ± 1.0</td>
<td>4</td>
</tr>
<tr>
<td>5.0</td>
<td>41.8 ± 3.9</td>
<td>16</td>
<td>31.6 ± 2.1</td>
<td>4</td>
<td>33.0 ± 3.2</td>
<td>5</td>
<td>39.2 ± 5.8</td>
<td>5</td>
</tr>
<tr>
<td>10.0</td>
<td>39.3 ± 3.0</td>
<td>18</td>
<td>31.0 ± 3.1</td>
<td>6</td>
<td>26.3 ± 2.5</td>
<td>5</td>
<td>32.6 ± 2.2</td>
<td>5</td>
</tr>
<tr>
<td>20.0</td>
<td>31.6 ± 2.7</td>
<td>10</td>
<td>31.0 ± 3.1</td>
<td>6</td>
<td>23.6 ± 4.4</td>
<td>5</td>
<td>30.7 ± 3.9</td>
<td>5</td>
</tr>
<tr>
<td>50.0</td>
<td>27.3 ± 4.7</td>
<td>23</td>
<td>23.9 ± 1.9</td>
<td>6</td>
<td>22.5 ± 3.0</td>
<td>5</td>
<td>27.9 ± 2.6</td>
<td>6</td>
</tr>
<tr>
<td>100.0</td>
<td>22.4 ± 3.1</td>
<td>10</td>
<td>19.4 ± 4.5</td>
<td>6</td>
<td>18.5 ± 2.4</td>
<td>5</td>
<td>22.1 ± 4.5</td>
<td>6</td>
</tr>
</tbody>
</table>

*Values are given as means ± S.D.; n = number of animals.

where \( I_{RG} \) is the retinal uptake index (including diffusion) determined with increasing concentrations of G (glucose), the inhibitor; G is the concentration of unlabeled glucose in the bolus; and \( K_e \) is the inhibitor concentration required to reduce the rate of transport by half at a given substrate concentration.

For the purposes of the present study, self-inhibition is defined as the inhibition of tracer level 14C-glucose by unlabeled "cold" glucose. The net unidirectional flux (\( V_m \)) during conditions of self-inhibition eliminates diffusion from the determination of kinetic constants and is given by

\[
V_m = \frac{I_{RG} \cdot E_{H2O} \cdot F \cdot S_t}{V_{max} \cdot S_t} = \frac{V_{max} \cdot S_t}{K_m + S_t} + \frac{K_m}{K_m + S_t} + S_t
\]

where \( V_m = V_T - V_G \) and \( I_{RM} = I_{RG} - I_{RG} \).

A plot of \( I_{RG}/G \) should be a straight line, where the y intercept = \( \frac{V_{max}}{K_m + S_t} \cdot \frac{E_{H2O} \cdot F}{(K_m + S_t) \cdot E_{H2O} \cdot F} \) and the slope = \( (K_m + S_t) \cdot (K_m + S_t) \).

\[
V_m = \frac{I_{RM} \cdot E_{H2O} \cdot F \cdot S_t}{V_{max} \cdot S_t} = \frac{V_{max} \cdot S_t}{K_m + S_t} + \frac{K_m}{K_m + S_t} + S_t
\]

\[
I_{RM} = \frac{I_{RM} \cdot E_{H2O} \cdot F}{G} - \frac{I_{RM} \cdot (K_m + S_t)}{G}
\]

Results

**Fractional extractions of \( ^3H_2O \) and \( ^14C \)-glucose.** The fractional extraction of \( ^3H_2O \) as a function of time is shown in Fig. 3, A. The fractional extraction of \( ^3H_2O \) 10 sec after the leading edge of the bolus enters the carotid artery is 0.097% ± 0.007% (mean ± S.E.M.) (n = 14). The fractional extractions at 5 and 7.5 sec are 0.064% ± 0.008% (n = 16) and 0.085% ± 0.007% (n = 15), respectively. These values are increasing with time because the bolus is in the process of passing through the retinal and choroidal circulation. After 10 sec of circulation time the bolus has...
completely passed through the eye and efflux of $^3$H$_2$O and $^{14}$C-glucose begins. The fractional extraction of $^3$H$_2$O at 15 and 20 sec of circulation time are 0.061% ± 0.005% (n = 10) and 0.047% ± 0.006% (n = 11), respectively. The fractional extractions of $^3$H$_2$O for each of the five sampling times were multiplied by the I$_R$ values at these time periods to determine the associated fractional extraction of $^{14}$C-glucose. These values are shown in Fig. 3, B, along with the values for I$_R$ (±S.E.M.). The glucose concentration for these experiments was 0.1 mM. Between 5 and 10 sec of circulation time, the I$_R$ decreases because the rate of $^3$H$_2$O uptake is faster than that of $^{14}$C-glucose. Between 10 and 20 sec the I$_R$ increases because the bolus has completely passed through the eye and the efflux of $^3$H$_2$O occurs at a faster rate than that of $^{14}$C-glucose. The lines shown in Fig. 3 were drawn by best fitting these points by linear regression analysis. The data in Fig. 3, B, show that a retinal sampling time of 10 sec gives a good approximation of the maximal extraction of $^{14}$C-glucose. Again, the efflux of label does not become significant until after 10 sec of circulation time. Therefore this sampling time was used for the remainder of the study.

**Glucose transport by the BRB.** Control data for glucose transport across the BRB are presented in Fig. 4. The data in this plot were generated by increasing the concentration of unlabeled glucose (G) in the perfusate and the bolus solution used to determine I$_R$. The I$_R$ values for the various glucose concentrations are shown in Table I for control and the effect of inhibitors on glucose transport. I$_{RM}$ data are calculated from I$_{RM}$ = I$_{RT}$ - I$_{RG}$ (see Materials and methods), which eliminates diffusion from the determination of K$_m$ values.

Fig. 4 shows that at least two transport systems for glucose transport across the BRB are apparent from our data. The K$_m$ values for each of the straight line segments were obtained from the slope of the best fit of I$_{RM}$ vs. I$_{RM}$/G data on equation 13. The lower line segment represents a relatively high-affinity transport system with a K$_m$ of 0.24 mM. The upper line segment, a lower affinity transport system, has a K$_m$ of 7.81 mM. Thomson has provided theoretical support for the interpretation of two transport systems from an upward deflection in an Eadie-Hofstee plot that has been corrected for diffusion.

The control experiments were repeated 1½ years after their initial determination.
Table II. $K_m$ values for glucose transport across the BRB in rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Bolus treatment</th>
<th>$K_{Na}$ (mM)$^c$</th>
<th>$K_{Na}$ (mM)$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>None</td>
<td>0.24 ± 0.07</td>
<td>7.81 ± 0.95</td>
</tr>
<tr>
<td></td>
<td>0.1 mM phloretin</td>
<td>5.01 ± 0.67</td>
<td>28.3 ± 2.96</td>
</tr>
<tr>
<td></td>
<td>0.75 mM phloridzin</td>
<td>0.60 ± 0.21</td>
<td>7.21 ± 0.79</td>
</tr>
<tr>
<td></td>
<td>1.0 mM acetazolamide</td>
<td>0.13 ± 0.12</td>
<td>7.38 ± 1.04</td>
</tr>
<tr>
<td></td>
<td>55% Na$^+$ replacement</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>100% K$^+$ removal</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>100% Ca$^{++}$ removal</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Diabetic 2 months after induction</td>
<td>None</td>
<td>5.54 ± 0.06</td>
<td>30.5 ± 5.56</td>
</tr>
</tbody>
</table>

$^a$ Derived from Eadie-Hofstee plot analyzed for $K_m$ of separate line segments.

$^b$ Indicates the manipulation of the composition of the bolus used to measure retinal uptake of glucose. In all cases the composition of the perfusate was the same as presented in the text.

$^c$ Values are given as means ± S.E.M. P values were determined by a modified t statistic. NS = not significant at $p > 0.001$.

Table III. Retinal uptake index for glucose in rats: effects of ions on glucose uptake.

<table>
<thead>
<tr>
<th>Glucose (mM)</th>
<th>Control</th>
<th>Na$^+$ replacement</th>
<th>K$^+$ removal</th>
<th>Ca$^{++}$ removal</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.003</td>
<td>63.5 ± 9.0</td>
<td>63.4 ± 11.2</td>
<td>51.4 ± 6.4</td>
<td>68.1 ± 6.6</td>
<td>4</td>
</tr>
<tr>
<td>1.0</td>
<td>45.6 ± 3.9</td>
<td>40.9 ± 2.0</td>
<td>41.2 ± 3.1</td>
<td>40.6 ± 3.6</td>
<td>5</td>
</tr>
<tr>
<td>2.0</td>
<td>44.2 ± 5.0</td>
<td>41.3 ± 1.9</td>
<td>39.7 ± 1.1</td>
<td>38.8 ± 3.6</td>
<td>5</td>
</tr>
<tr>
<td>3.0</td>
<td>43.7 ± 3.5</td>
<td>40.4 ± 3.1</td>
<td>37.9 ± 3.3</td>
<td>35.7 ± 3.2</td>
<td>6</td>
</tr>
<tr>
<td>5.0</td>
<td>41.8 ± 3.9</td>
<td>35.9 ± 3.1</td>
<td>34.0 ± 2.9</td>
<td>31.1 ± 1.7</td>
<td>6</td>
</tr>
<tr>
<td>10.0</td>
<td>39.3 ± 3.0</td>
<td>35.9 ± 3.1</td>
<td>34.0 ± 2.9</td>
<td>31.1 ± 1.7</td>
<td>6</td>
</tr>
<tr>
<td>20.0</td>
<td>31.6 ± 2.7</td>
<td>26.6 ± 3.4</td>
<td>28.6 ± 2.7</td>
<td>25.9 ± 1.0</td>
<td>6</td>
</tr>
<tr>
<td>30.0</td>
<td>28.0 ± 3.4</td>
<td>25.1 ± 2.6</td>
<td>26.4 ± 3.3</td>
<td>22.1 ± 2.0</td>
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<tr>
<td>50.0</td>
<td>27.3 ± 4.7</td>
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<td>21.6 ± 4.2</td>
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<td>6</td>
</tr>
<tr>
<td>100.0</td>
<td>22.4 ± 3.1</td>
<td>21.6 ± 4.2</td>
<td>21.6 ± 4.2</td>
<td>22.2 ± 3.6</td>
<td>6</td>
</tr>
</tbody>
</table>

$^*$ Values are given as means ± S.D.; n = number of animals.

with no significant change in the calculated $K_m$ values of glucose transport. This replication demonstrates the reliability of the technique. As a result, the data from these separate determinations have been combined and account for the larger n value shown for each glucose concentration for the control curve. A summary of the apparent $K_m$ values for glucose transport under various experimental conditions is presented in Table II.

Effect of inhibitors on glucose transport.

The effect of adding 0.1 mM phloretin to the bolus is shown in Fig. 5. Phloretin, an established inhibitor of facilitated diffusion of glucose, significantly increased the $K_m$ (p < 0.001) for both transport systems revealed by control data. The apparent $K_m$ for the higher affinity system was increased to 5.01 mM, while the $K_m$ for the lower affinity system increased to 28.3 mM. The increase in $K_m$ values is as expected for the effect of a competitive inhibitor on carrier-mediated transport.

Phloridzin, at bolus concentration 0.75 mM, had no statistically significant effect on glucose transport by the total BRB. The $K_m$ values for the higher and lower affinity systems were 6.0 and 7.21 mM, respectively. This is in agreement with the results of studies on the effect of phloridzin in either isolated RPE cells$^{11}$ or RPE tissue studies$^{12}$ reported by our laboratory.

Acetazolamide at a concentration of 1.0
Effect of ions on glucose transport. The retinal uptake indices for the effect of ions on glucose uptake are given in Table III. Replacement of 55% of the total Na⁺ in the bolus with lithium chloride resulted in no statistically significant modification of glucose transport. Km₁ (high-affinity system) was 0.23 mM, while Km₂ was 6.44 mM. The reduction of the K⁺ concentration in the bolus from 3.62 mM to zero also had no statistically significant effect on the kinetics of glucose transport (see Table II).

Removal of 100% of the calcium in the bolus produced the only statistically significant ion effect in our study. The plot of these data is shown in Fig. 6. The Km of the high-affinity system was not significantly different at 0.33 mM. Only the Km of the lower affinity system was statistically different at 3.62 mM (p < 0.02).

Effect of streptozocin-induced diabetes on glucose transport. Glucose transport was also studied in diabetic rats 2 months after the administration of streptozocin. The diabetic state was confirmed by the observation of polyuria, glucosuria, and impaired growth in the experimental animals. Serum glucose levels averaged 407 ± 107 mg/dl.

After 2 months of the diabetic state, glucose transport appeared to be significantly altered (Table IV, Fig. 7). Km₁ was increased to 5.54 mM (p < 0.001), while Km₂ also increased to 30.5 mM (p < 0.01). The physiologic consequence of these changes is that a higher blood glucose concentration is required in the diabetic rat to allow the same quantity of glucose to be transported into the retina as occurs at a lower blood glucose concentration in normal rats.

Discussion

Conflicting reports on the mechanism of glucose transport by isolated RPE have appeared in the literature. Zadunaisky and Degani¹⁶ found a net flux of 3-O-methyl-glucose in the choroid to retina direction and that the unidirectional fluxes were altered by phloridzin. However, Miller and Steinberg¹⁷ were unable to detect a net flux of 3-O-methyl-glucose or to demonstrate inhibition by phloridzin. An earlier report from our laboratory¹⁸ indicated a carrier-mediated transport

<table>
<thead>
<tr>
<th>Glucose (mM)</th>
<th>Control (mM ± S.D.)</th>
<th>Diabetic (mM ± S.D.)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.003</td>
<td>63.5 ± 9.0</td>
<td>43.6 ± 4.3</td>
<td>5</td>
</tr>
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<td>1.0</td>
<td>45.6 ± 3.9</td>
<td>41.3 ± 2.9</td>
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<td>2.0</td>
<td>44.2 ± 5.0</td>
<td>39.7 ± 3.3</td>
<td>5</td>
</tr>
<tr>
<td>3.0</td>
<td>43.7 ± 3.5</td>
<td>39.7 ± 3.3</td>
<td>9</td>
</tr>
<tr>
<td>5.0</td>
<td>41.8 ± 3.9</td>
<td>36.4 ± 3.4</td>
<td>6</td>
</tr>
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<td>10.0</td>
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<td>34.1 ± 2.1</td>
<td>6</td>
</tr>
<tr>
<td>20.0</td>
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<tr>
<td>30.0</td>
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<td>5</td>
</tr>
<tr>
<td>100.0</td>
<td>22.4 ± 3.1</td>
<td>17.3 ± 1.6</td>
<td>10</td>
</tr>
</tbody>
</table>

*Values are given as means ± S.D.; n = number of animals.
Glucose transport by the BRB

of glucose that was sensitive to 0.75 mM phloridzin. We now believe that this phloridzin inhibition of glucose transport was due to the high concentration of inhibitor used, which may have allowed for a significant conversion of phloridzin to phloretin by a glucosidase, as occurs in intestinal epithelia. We have repeated these experiments on the isolated RPE mounted as a membrane and found that 0.1 mM phloretin did significantly alter the unidirectional fluxes but that 0.1 mM phloridzin did not do so. This differential effect of phloretin over phloridzin has been taken to be indicative of a facilitated diffusion for glucose transport in intestinal epithelium. A recent study on isolated bovine retinal capillaries indicates that glucose transport occurs by a stereospecific carrier-mediated transport system that can be inhibited by phloretin, phloridzin, and cytochalasin B.

Only a few studies have been done in vivo on D-glucose transport across the BRB. All these studies suggest that D-glucose moves through the BRB by a saturable, stereospecific, carrier-mediated mechanism. However, these in vivo studies did not report on the effects of inhibitors and specific ion concentrations on D-glucose transport.

The present studies were undertaken to determine whether an in situ perfusion technique, in which the ERG of the eye is maintained, would verify previous results and enhance understanding of D-glucose transport across the BRB. The unidirectional flux for D-glucose as measured by the internal standard technique is a composite flux across both the RPE and the retinal capillaries. The technique does not allow experimental isolation of either the RPE or the retinal capillaries. The technique has been presented evidence for a carrier-mediated transport system for D-glucose translocation across the composite BRB and have found that glucose transport could be inhibited by phloretin but not by phloridzin or acetazolamide. Glucose transport has also been shown to be unaffected by Na+ or K+ concentrations. Again, the only significant ion effect we observed on glucose transport by the BRB resulted from removal of calcium from the bolus. The RPE has been shown to have a calcium concentration that exceeds that of muscle and is as great as that in platelets. Further, it has been proposed by many authors that Ca++ may serve as an activator for glucose transport in a variety of cell types.

**Effect of streptozocin-induced diabetes on D-glucose transport.** Evidence for structural changes in RPE during early streptozocin-induced diabetes in rats has been uncovered by the work of Grimes and Laties. They found an increase in the area of the basolateral cell surface of the RPE. In trying to further elucidate the consequences of this morphologic change, Kirber et al. found that fluorescein was detectable in the cytoplasm of RPE cells of streptozocin-induced diabetic rats but that horseradish peroxidase was still excluded from the RPE and the retina. Further, these investigators were unable to detect any change in the permeability of the retinal blood vessels with either fluorescein or horseradish peroxidase, indicating that the tight junctions of both the RPE and endothelial cells were intact 3 to 5 weeks after induction of diabetes. From these studies it is clear that a permeability defect of the BRB occurs before any detectable change in the tight junctions.

We have provided evidence for another permeability defect in early streptozocin-induced diabetes in rats that causes significant alteration of transport of D-glucose across the BRB. The increased Km values for both transport systems may be an adaptation to the high circulating blood glucose levels in the diabetic rat. This adaptation may serve to protect the retina from the metabolic and osmotic effects of an increase in blood glucose.

In summary, our data indicate at least two carrier-mediated transport system for D-glucose translocation across the BRB. In addition, we have shown that the kinetics of the apparent low-affinity transport system may be altered by removal of calcium from the bolus used to measure unidirectional flux. Finally, we have demonstrated that the Km values of glucose transport are significantly
increased by 2 months of streptozocin-induced diabetes.

REFERENCES