Glucose-6-phosphatase Activity in the Retina of the Awake Rat

Norman P. Blair, William E. Shaw, and Beatrice Yue

In the 2-deoxyglucose technique, the rate of glucose utilization in small areas of the central nervous system is measured using a standard operational equation that assumes negligible glucose-6-phosphatase activity. Hoping to apply this technique in the mammalian retina, we sought to identify the extent of this enzyme's activity in vivo. [2-3H] glucose but not [U-14C] glucose loses its label during metabolism and returns to the glucose precursor pool in the presence of glucose-6-phosphatase. Accordingly, a decline of 3H/14C in the retinal glucose pool with time indicates glucose-6-phosphatase activity. We injected a mixture of [2-3H] glucose and [U-14C] glucose into the internal carotid artery of 10 awake rats via a previously inserted catheter. Plasma samples were collected and the eyes enucleated at timed intervals. The eyes were immediately frozen, freeze-dried and dissected to obtain retina. Radiolabeled glucose was separated using ion exchange and paper chromatography prior to scintillation counting. The 3H/14C ratio was found to decline at a statistically significant rate of about 2.5% per minute, indicating glucose-6-phosphatase activity. However, an estimate of the turnover of retinal glucose suggests that glucose-6-phosphatase dephosphorylates a minimal percentage of the glucose entering the glycolytic pathway, allowing application of the standard operational equation to the mammalian retina in vivo. Invest Ophthalmol Vis Sci 30:2268–2271, 1989

Much could be learned about retinal function in health and disease by measuring the rate of glucose utilization in small areas of the retina. This has been achieved in the brain using the 2-deoxyglucose (2-DG) technique developed by Sokoloff and associates.1 The study of 2-DG uptake has previously been used to make inferences about retinal metabolism,2-4 but without methodology to rigorously quantitate the local retinal metabolic rate of glucose.

In the 2-DG technique, the radiolabeled glucose analog 2-DG is transported into the tissue and phosphorylated identically and in proportion to glucose. However, while glucose-6-phosphate is isomerized to fructose-6-phosphate and proceeds through the glycolytic pathway, this isomerization is impossible for 2-deoxyglucose-6-phosphate. Consequently, while glucose-6-phosphate is metabolized to products that can escape from the tissue, labeled 2-deoxyglucose-6-phosphate accumulates and can autoradiographically provide a record of the amount of 2-DG phosphorylated. The utilization rate of glucose in small areas can be determined from this information via an operational equation.1

To use this technique, the magnitude of glucose-6-phosphatase (G-6-Pase) activity must be known. G-6-Pase hydrolyzes glucose-6-phosphate or 2-deoxyglucose-6-phosphate back to glucose or 2-DG. If G-6-Pase activity is substantial, the amount of 2-deoxyglucose-6-phosphate that accumulates in the tissue after introduction of a bolus of 2-DG would be less than the total amount phosphorylated, leading to an underestimate of the rate of glucose utilization using the standard operational equation. Moreover, this dephosphorylation of glucose-6-phosphate by G-6-Pase is of theoretical interest. It constitutes a futile cycle: that is, ATP is consumed in the phosphorylation of glucose but is not regenerated upon dephosphorylation. There has been controversy as to the existence of nontrivial G-6-Pase activity in the brain.5-8 While several workers have identified G-6-Pase in retinal tissue,9-13 significant activity has not been demonstrated previously in the retinas of awake mammals. We sought to identify G-6-Pase activity in the retinas of awake rats.

Materials and Methods. Theory: Investigators have previously evaluated G-6-Pase activity in vivo by measuring the differential loss of tritiated and carbon-14 glucose from the retinal glucose pool.5,6 In the normal glycolytic pathway [2-3H] glucose metabolized from glucose-6-phosphate to fructose-6-phosphate loses its 3H label eventually as [3H]H2O, while the 14C label of [U-14C] glucose metabolized to fructose-6-phosphate is retained. Since fructose-6-phosphate is readily isomerized back to glucose-6-phosphate, G-6-Pase activity returns fructose-6-phosphate back to the glucose pool as glucose with the 14C labels still attached but the 3H labels missing (Fig. 1). Thus, decline in the 3H/14C ratio in the retinal glucose pool, caused by the return of 14C glucose but not 3H glucose, is used to evaluate G-6-Pase activity.

Administration of labeled glucose: Ten normal male hooded rats (300–350 g, Charles River, Wilmington, MA) were anesthetized with 0.006 mg/kg of fenzytinol citrate and 12 mg/kg of dropiprelit (Innovar) and immobilized in plaster casts before catheterization of the tail and internal carotid arteries. The carotid catheter was inserted into the external carotid artery approximately 1 cm from the carotid bifurca-
PATHWAY OF RADIOLABELED GLUCOSE ISOTOPES IN PRESENCE OF GLUCOSE-6-PHOSPHATASE ACTIVITY

<table>
<thead>
<tr>
<th>Glucose</th>
<th>Glucose-6-Phosphate</th>
<th>Fructose-6-Phosphate</th>
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<tbody>
<tr>
<td>1 H-C-OH</td>
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<td>2 HO-C-OH</td>
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<td>5 HO-C-H</td>
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<td>6 CH(OH)</td>
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**Fig. 1.** Potential metabolic cycles of [2-3H] and [U-14C] glucose from precursor pool to fructose-6-phosphate and back into glucose precursor pool via glucose-6-phosphatase. [2-3H]glucose-6-phosphate loses its radioactive moiety as [3H]H2O upon conversion to fructose-6-phosphate, while [U-14C] glucose, arbitrarily labeled at position number 1, retains its radioactivity. HEX = hexokinase, ATP = adenosine triphosphate, G6PISO = glucose-6-phosphate isomerase, G6PASE = glucose-6-phosphatase, P1 = phosphate.

...tion and passed retrograde into the distal common carotid. The external carotid was ligated around the catheter, and branches adjacent to the incision were ligated, enabling injection of a bolus into the retinal vasculature via the internal carotid.

A mixture of 50 μCi [U-14C] glucose and 5 μCi [2-3H] glucose was prepared and adjusted to approximate the plasma glucose concentration (6 mM) using unlabeled D-glucose in a 0.9% saline solution. Six hours after anesthesia (by then dissipated), this mixture was rapidly injected into the carotid catheter. Blood samples from the tail artery were collected in prechilled heparinized hematocrit tubes at timed 1 min intervals. These were maintained in crushed ice until the experiment was terminated and promptly centrifuged to obtain the plasma. Experiments were terminated at times ranging from 2 to 7 min, at which time a lethal dose of pentobarbital sodium (180 mg/kg) was injected into the carotid catheter. The left eye was immediately enucleated and plunged into isopentane chilled to near its freezing point in liquid nitrogen. The time between pentobarbital injection and enucleation averaged 15–30 sec.

**Isolation of radiolabeled retinal glucose:** The frozen eyes were freeze-dried (Tis U Dry, FTS Systems, Stone Ridge, NY) for at least 7 days, after which dissection of the retina was carried out under a microscope. Separation of the retina from the underlying choroid was easily accomplished in the freeze-dried eyes, but variable amounts of pigment epithelium adhered to the retina, and were removed as much as possible. The retinas were then sonicated (Model W...
resulted from its theoretical value of unity. The decline of NR did not differ significantly from zero.

To our knowledge, this study provides the first evidence of measurable G-6-Pase activity in the retinas of awake mammals. If NR had remained constant, we could have concluded that G-6-Pase activity in vivo is not detectable under our experimental conditions, even though it has been identified in vitro and histochemically. However, we did identify a statistically significant linear decline indicating enzyme activity. While the actual theoretic decline may be mathematically complex, we fit our data using linear regression because it is a good first approximation and has previously been used by investigators in this field.

To clarify the significance of retinal G-6-Pase activity in vivo, we compared the amount of radiolabeled glucose dephosphorylated by G-6-Pase during any minute studied (GD) to that utilized (GU). We found that the quantity of glucose in 10 rat retinas was 16 nmol/mg (dry wt). The rate of glucose utilization in the rat retina has been shown to be 11 nmol/min/mg retina (dry wt). It follows that about 69% per min is utilized. Let GI be the amount of 14C glucose in the retina at the beginning and G2 the amount at the end of any minute in the interval studied. Because approximately 69% of GI is lost as GUs indicating that an almost trivial amount of the glucose entering the glycolytic pathway is dephosphorylated.

We conclude that G-6-Pase activity, while detectable in vivo, is of a magnitude that allows application

375, Ultrasonics, Inc., Plainview, NY) in 0.6 M perchloric acid, and complete recovery of the supernatants was achieved by centrifugation using Centrex microfilters (Schleicher and Schuell, Keene, NH).

The retinal supernatants and perchloric acid (0.6 M) extracts of the plasma samples and injectates were then analyzed for labeled glucose by the method of Nelson and coworkers. Bulk labeled anionic and cationic metabolites were removed from the extracts using AG 1-X8 formate (200–400 mesh) and AG 50-X8 H+ (100–200 mesh) ion-exchange columns (Biorad, Richmond, CA). The samples were evaporated to dryness to remove [3H]H2O and chromatographed using 20 X 20 cm chromatography paper (Whatman 3MM, WR Balston, England) in a solvent system consisting of isobutyric acid, water, and 18N ammonium hydroxide (66:33:1 by volume). The labeled glucose fraction was eluted from 2 cm bands whose position corresponded to the migration of a D-glucose standard chromatographed in parallel. The samples were assayed for 3H and 14C using a scintillation counter (Beckman LS 7000, Arlington Heights, IL) calibrated with [3H] toluene and [14C] toluene standards.

**Data analysis:** The 3H/14C ratios of the radiolabeled retinal and plasma extracts were normalized to that of the injectate used in each experiment. These normalized ratios for the retina and plasma are referred to as NR and NP, respectively. NR and NP were plotted as a function of time and by linear regression were fit to a line with a corresponding statistical significance.

**Determination of retinal glucose content:** Five normal male hooded rats (300–350 g, Charles River) were anesthetized with 75 mg of ketamine hydrochloride. Their eyes were partially dissected so that the blood supply near the optic nerve remained intact and were enucleated before sacrifice with an intraperitoneal injection of 50 mg of pentobarbital sodium. The 10 eyes were frozen within seconds of interruption of their blood supply and then freeze-dried. The retinas were then dissected, weighed as a group and sonicated in 0.6 M perchloric acid. After neutralization, the supernatant was completely recovered, evaporated, and resuspended to a known volume whose glucose concentration was determined (Beckman Glucose Analyzer 2).

**Results and Discussion.** NR declined at a rate of about 2.5% per minute over the several minute interval studied (Fig. 2). The equation that best fit the data was:

\[ NR = 1.082 - 0.025 \times \text{time (min)}, \quad P < 0.035 \quad (1) \]

The y-intercept value of 1.082 did not differ statistically from its theoretical value of unity. The decline of NP did not differ significantly from zero.

**Fig. 2. Time course of the 3H/14C ratio in glucose purified from retina after intracarotid injection of a mixture of [2-3H] and [U-14C] glucose. Each value was normalized to the 3H/14C ratio of the injectate, and represents a study eye from one rat. The best fit line was drawn by linear regression.**
of the 2-DG technique's standard operational equation to the mammalian retina. Moreover, G-6-Pase activity does not lead to a significant futile cycle. These conclusions are similar to those of Nelson and coworkers,8 who showed a statistically significant decline in the rat brain 3H/14C ratio of about 1% per minute.

Key words: glucose-6-phosphatase, retinal energy metabolism, 2-deoxyglucose technique, glucose utilization, glucose content

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References


Furosemide-Sensitive Cl Transport in Bovine Retinal Pigment Epithelium

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Bovine retinal pigment epithelium (RPE)--choroid explants were sealed in an Ussing chamber. Typical preparations produced a transepithelial voltage (V_e) of 12 mV (retina side positive) and had an electrical resistance (R) of 300 ohm-cm². These values can be attributed to the RPE. Furosemide and ouabain reduced the V_e without affecting R when applied to the apical side of the RPE, but had no effect upon V_e and R when applied to the choroidal side. Acetazolamide had no effect upon V_e and R when applied to either side of the tissue. In Cl-free medium, ouabain reduced V_e without affecting R, while furosemide had no effect upon V_e and R. In Na-free medium, ouabain and furosemide had no effect upon V_e and R. Unidirectional isotope flux studies performed under open circuit conditions showed a net retina-to-choroid Cl flux that was abolished by furosemide. These results indicate that bovine RPE possesses a furosemide-sensitive Cl transport system. Invest Ophthalmol Vis Sci 30:2271–2274, 1989

Transport properties of retinal pigment epithelium (RPE) from a variety of species have been studied by a number of investigators.1-6 Although the RPE from these species seem to possess generally similar trans-