

## **Reexamination of Glucose-6-Phosphatase Activity in the Brain in Vivo: No Evidence for a Futile Cycle**

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**Abstract.** *Glucose-6-phosphatase activity in the rat brain in vivo was estimated by measuring the differential loss of tritium and carbon-14 from the glucose pool labeled by a mixture of [2-<sup>3</sup>H]glucose and [U-<sup>14</sup>C]glucose. The results provide no evidence of significant dephosphorylation of glucose-6-phosphate and do not support the hypothesis of a futile cycle involving glucose-6-phosphatase activity in the brain.*

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It is widely believed that the mammalian brain is not a gluconeogenic organ and contains negligible glucose-6-phosphatase (G6Pase) activity (1). The presence of G6Pase in the brain has been demonstrated by histochemistry (2, 3), but quantitative assays of the enzyme activity in cerebral tissues in vitro have usually shown it to be a small fraction of that of known gluconeogenic organs, such as liver and kidney (4, 5), and supported the view that G6Pase activity has little, if any, role in the carbohydrate metabolism of the brain. This belief was recently challenged by Huang and Veech (6), who reported that there was sufficient G6Pase activity in the rat brain to maintain a steady-state hydrolysis of glucose-6-phosphate (G6P) equal to at least 35 percent of its rate of formation by hexokinase-catalyzed phosphorylation of glucose. Since the brain has a very high rate of glucose utilization (7), a rate of dephosphorylation of G6P equal to one-third the rate of glucose phosphorylation by hexokinase would represent a level of G6Pase activity approaching that found in the rat liver after feeding (8).

Huang and Veech injected a mixture of [2-<sup>3</sup>H]glucose and [U-<sup>14</sup>C]glucose into one carotid artery of rats, removed the brains by freeze-blowing (9) at various times up to 5 minutes later, separated free glucose from the tissue, and deter-

mined its <sup>3</sup>H/<sup>14</sup>C ratio by liquid scintillation counting. The principle behind these procedures is as follows. Any [2-<sup>3</sup>H]glucose metabolized as far as fructose-6-phosphate in the glycolytic pathway loses most of the <sup>3</sup>H label, but [<sup>14</sup>C]fructose-6-phosphate retains the <sup>14</sup>C (8). Some fructose-6-phosphate is converted back to G6P by reversal of the reaction catalyzed by hexosephosphate isomerase, and, if there is G6Pase activity, the glucose moiety is returned to the free glucose pool with its <sup>14</sup>C but without <sup>3</sup>H. The <sup>3</sup>H/<sup>14</sup>C ratio in the free glucose pool should then decline progressively with time if there is G6Pase activity. Huang and Veech reported such a decline, suggesting previously unsuspected levels of G6Pase activity in the brain.

A key issue is the purity of the free glucose pool in which they measured the <sup>3</sup>H/<sup>14</sup>C ratio. Most products of glucose metabolism beyond the G6P step lose the <sup>3</sup>H but not the <sup>14</sup>C label; if any of these products were to contaminate the glucose fraction, they would lead to low <sup>3</sup>H/<sup>14</sup>C ratios. In their experiments Huang and Veech relied on Dowex 1-formate and Dowex 1-borate column chromatography of perchloric acid extracts of the brain tissue for purification of the glucose. In a subsequent study (10) they added derivatization of the glucose fraction by hexokinase-catalyzed phosphorylation.

We repeated the experiments of Huang and Veech (6) but with special efforts to ensure the purity of the glucose fraction in which the <sup>3</sup>H/<sup>14</sup>C ratio was measured. Normal male Sprague-Dawley rats (330 ± 9 g) were anesthetized with pentobarbital (30 mg/kg, intraperitoneally), and one femoral artery and one external carotid artery were cath-

eterized in each animal. The carotid catheter was inserted 5 mm past the carotid bifurcation into the common carotid artery. Both catheters were secured by ligatures. In one group of seven rats the internal carotid artery contralateral to the side of catheterization was ligated as in the studies of Huang and Veech (6). Because these animals occasionally exhibited neurological and behavioral defects at the time of the experiment, a second group of eight animals was similarly prepared except that the contralateral internal carotid artery was not permanently ligated but was encircled with a loose ligature that was temporarily pulled taut during injection of the labeled glucose through the carotid catheter to ensure bilateral distribution of tracer.

Approximately 24 hours after surgery a mixture of 50 μCi of [2-<sup>3</sup>H]glucose, 5 μCi of [U-<sup>14</sup>C]glucose (11), and 5.1 mM glucose in 0.05 ml of 0.9 percent saline was injected through the carotid catheter. At various times between 2 and 7 minutes after the injection the brain was removed by freeze-blowing (9). Several blood samples were drawn from the femoral artery at various times during the interval between the injection of labeled glucose and freeze-blowing.

The frozen brains were powdered under liquid nitrogen in a cryostat at -35°C and perchloric acid (0.6M) extracts of the brain tissue and plasma were prepared. Acidic metabolites were removed from the neutralized supernatant fractions by passage through columns containing 2 ml of Dowex AG 1-X8 formate (200 to 400 mesh). After adjustment of the pH of the effluent to 4.0, basic metabolites were removed by cation-exchange chromatography on columns with 2 ml of Dowex AG 50-X8 H<sup>+</sup> (200 to 400 mesh). The final effluent contained approximately 96 percent of the glucose originally present in the perchloric acid extracts. Tritiated water released by the metabolism of [2-<sup>3</sup>H]glucose was also present.

The extracts, now depleted of all anionic and cationic metabolic products of glucose metabolism, were evaporated to dryness to eliminate the [<sup>3</sup>H]H<sub>2</sub>O and chromatographed as 4-cm bands on Whatman 3MMChr paper in the ascending direction with a solvent system consisting of isobutyric acid, water, and concentrated ammonium hydroxide (66:33:1 by volume). [<sup>14</sup>C]Glucose standards were chromatographed in parallel lanes on the same chromatographic papers. Bands migrating to the same position relative to the solvent front (*R<sub>F</sub>*) as authentic [<sup>14</sup>C]glucose were eluted with water; this eluate contained 25 to 40

percent of the total  $^{14}\text{C}$  applied to the chromatogram. Half of each of these samples was assayed for  $^3\text{H}$  and  $^{14}\text{C}$  concentrations by liquid scintillation counting calibrated with internal [ $^3\text{H}$ ]toluene and [ $^{14}\text{C}$ ]toluene standards; the other halves were converted to G6P by incubation with adenosine triphosphate, yeast hexokinase, and  $\text{MgCl}_2$  and chromatographed on paper as before. Bands migrating with the same  $R_F$  as authentic [ $^{14}\text{C}$ ]G6P were eluted with water and assayed to determine the  $^3\text{H}/^{14}\text{C}$  ratios.

The  $^3\text{H}/^{14}\text{C}$  ratios of the plasma and brain extracts were normalized to the  $^3\text{H}/^{14}\text{C}$  ratio of the injectant used in each experiment. The  $^3\text{H}/^{14}\text{C}$  ratios of the injectants were determined in samples purified by the same procedures used for the plasma and brain extracts to eliminate radiochemical impurities that might have contaminated the injected labeled glucose. Best-fitting straight lines representing the normalized  $^3\text{H}/^{14}\text{C}$  ratios of the labeled glucose and its G6P derivative with respect to time were calculated by least-squares regression.

The results for the two groups did not differ significantly and therefore were pooled for statistical analysis. The glucose fractions separated from plasma showed a slight, progressive decline in the  $^3\text{H}/^{14}\text{C}$  ratio below that of the injectant over the 2- to 7-minute interval after injection (Fig. 1A); this decline probably reflects the consequences of glucose metabolism throughout the body. The glucose fractions from the brain showed a

slightly greater progressive decline in  $^3\text{H}/^{14}\text{C}$  ratios, but the difference was not statistically significant (Fig. 1B), indicating no differential loss of  $^3\text{H}$  and  $^{14}\text{C}$  in the glucose pool in the brain.

To ensure that the  $^3\text{H}/^{14}\text{C}$  ratios represented uncontaminated glucose, portions of the fractions derived from plasma, brain, and injectants were derivatized to G6P by hexokinase-catalyzed phosphorylation. The labeled G6P was separated by paper chromatography and assayed to determine the  $^3\text{H}/^{14}\text{C}$  ratios. The fractions from plasma so treated showed a statistically insignificant, time-dependent trend toward reduced  $^3\text{H}/^{14}\text{C}$  ratios (Fig. 2A), and the fractions from brain tissue exhibited no greater change in  $^3\text{H}/^{14}\text{C}$  ratio with time (Fig. 2B). In contrast to the results of Huang and Veech (6), there was no evidence of differential loss of  $^3\text{H}$  and  $^{14}\text{C}$  from glucose in the brain after intracarotid injection of [ $2\text{-}^3\text{H}$ ]glucose and [ $\text{U-}^{14}\text{C}$ ]glucose.

It appears that Huang and Veech inadequately purified the fraction extracted from brain tissue which they designated as glucose and in which they measured the  $^3\text{H}/^{14}\text{C}$  ratio. The labeled glucose in brain tissue gives rise to many metabolic products, most of which lose the  $^3\text{H}$  but not  $^{14}\text{C}$ . Experiments in our laboratory have confirmed their results but have demonstrated that the sequential column chromatography of perchloric acid extracts of brain tissue on Dowex AG 1-X8 formate and Dowex AG 1-X8 borate does not isolate glucose uncontaminated

by other labeled products. The eluate from the Dowex AG 1-X8 borate column does have a low  $^3\text{H}/^{14}\text{C}$  ratio but contains several labeled components other than glucose when chromatographed on paper. At 7 to 8 minutes after the injection, 40 to 50 percent of its  $^{14}\text{C}$  content are in contaminants that contain little tritium, but the  $^3\text{H}/^{14}\text{C}$  ratio in its glucose component is similar to that of the injectant. When the eluate from the Dowex AG 1-X8 borate column is chromatographed on a cation-exchange column, the  $^3\text{H}/^{14}\text{C}$  ratio in the effluent is increased. Treatment of the effluent with glucose oxidase, which converts glucose to gluconic acid, and separation of this derivative by anion-exchange chromatography raises the  $^3\text{H}/^{14}\text{C}$  ratio to that of the plasma and close to that of the injectant. Hexokinase-catalyzed phosphorylation of the eluate from the Dowex AG 1-X8 borate column is inadequate to ensure purity of the glucose; hexokinase is less specific than glucose oxidase and would phosphorylate any fructose or glucosamine that might be present in that fraction.

Our results do not support the finding of significant G6Pase activity in the brain in vivo. The activity of the small amount of enzyme that is present is limited by intracellular compartmentation. In the cell, G6Pase is present on the inner surfaces of the cisterns of the endoplasmic reticulum (ER) (3, 12); the G6P is formed in the cytosol. In tissues with high gluconeogenic activity there is a specific

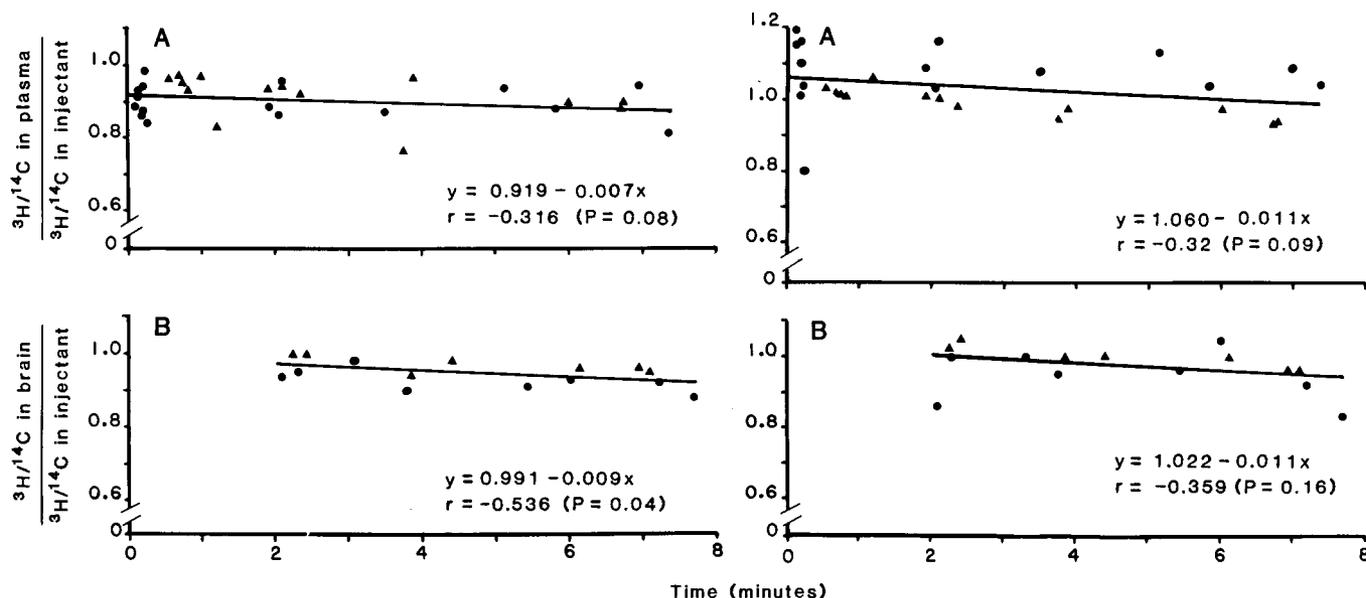


Fig. 1 (left). Time course of  $^3\text{H}/^{14}\text{C}$  ratios in glucose purified from plasma (A) and brain (B) after intracarotid injection of a mixture of [ $2\text{-}^3\text{H}$ ]glucose and [ $\text{U-}^{14}\text{C}$ ]glucose. Symbols: ( $\blacktriangle$ ) results for animals with carotid ligation contralateral to side of carotid catheterization and ( $\bullet$ ) results for animals with contralateral carotid obstruction only during the injection. The  $r$  represents the product-moment correlation coefficient of the normalized  $^3\text{H}/^{14}\text{C}$  ratio on the ordinate with respect to time;  $P$  represents the probability value of the correlation coefficient. Fig. 2 (right). Time course of  $^3\text{H}/^{14}\text{C}$  ratios in G6P derivatized from the labeled glucose isolated from plasma (A) and brain (B) in Fig. 1. The symbols are explained in the legend to Fig. 1.

carrier in the ER that transports the substrate across the membrane to the phosphatase (12, 13). Karnovsky *et al.* (14) found that this carrier is absent in the brain and that G6P gains access to the phosphatase only by slow diffusion across the ER membrane. This would further slow the phosphohydrolytic activity of whatever G6Pase is present in the brain.

The report of Huang and Veech (6) has led to debate on the role of G6Pase in the brain and to speculations about futile cycles in cerebral tissue (15). It has also been used to argue against the validity of the deoxyglucose method for measuring local utilization of glucose in the brain in animals and humans (6, 14), a method that in its earliest form assumed negligible loss of deoxyglucose-6-phosphate in the brain during the experimental period (16). It now seems that such speculations and extrapolations are without foundation.

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