

Invalidity of Criticisms of the Deoxyglucose Method Based on Alleged Glucose-6-Phosphatase Activity in Brain

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Abstract: The observations made by Sacks et al. [*Neurochem. Res.* 8, 661–685 (1983)] on which they based their criticisms of the deoxyglucose method have been examined and found to have no relationship to the conclusions drawn by them. (1) The observations of Sacks et al. (1983) of constant concentrations of [¹⁴C]deoxyglucose and [¹⁴C]deoxyglucose-6-phosphate, predominantly in the form of product, reflects only the postmortem phosphorylation of the precursor during the dissection of the brain in their experiments. When the brains are removed by freeze-blowing, the time courses of the [¹⁴C]deoxyglucose and [¹⁴C]deoxyglucose-6-phosphate concentrations in brain during the 45 min after the intravenous pulse are close to those predicted by the model of the deoxyglucose method. (2) Their observation of a reversal of the cerebral arteriovenous difference from positive to negative for [¹⁴C]deoxyglucose and not for [¹⁴C]glucose after an intravenous infusion of either tracer is, contrary to their conclusions, not a reflection of glucose-6-phosphatase activity in brain but the consequence of the different proportions of the rate constants for efflux and phosphorylation for these two hexoses in brain and

is fully predicted by the model of the deoxyglucose method. (3) It is experimentally demonstrated that there is no significant arteriovenous difference for glucose-6-phosphate in brain, that infusion of [³²P]glucose-6-phosphate results in no labeling of brain, and that the blood-brain barrier is impermeable to glucose-6-phosphate. Glucose-6-phosphate cannot, therefore, cross the blood-brain barrier, and the observation by Sacks and co-workers [*J. Appl. Physiol.* 24, 817–827 (1968); *Neurochem. Res.* 8, 661–685 (1983)] of a positive cerebral arteriovenous difference for [¹⁴C]glucose-6-phosphate and a negative arteriovenous difference for [¹⁴C]glucose cannot possibly reflect glucose-6-phosphatase activity in brain as concluded by them. Each of the criticisms raised by Sacks et al. has been demonstrated to be devoid of validity. **Key Words:** Deoxyglucose method—Glucose-6-phosphate—Blood-brain barrier permeability—Brain—Carbohydrate metabolism—Glucose utilization—Glucose-6-phosphatase. Nelson T. et al. Invalidity of criticisms of the deoxyglucose method based on alleged glucose-6-phosphatase activity in brain. *J. Neurochem.* 46, 905–919 (1986).

The 2-deoxy-D-glucose (DG) method was first reported almost 10 years ago (Sokoloff et al., 1974; Kennedy et al., 1974, 1975) and described in detail 3 years later (Sokoloff et al., 1977). It was designed for use with quantitative autoradiography to measure local rates of glucose utilization in the anatomical components of the CNS. Because under normal physiological conditions glucose is the main substrate of cerebral energy metabolism and is consumed in stoichiometric proportion to oxygen utilization (Kety, 1957), glucose utilization in such

physiological conditions represents essentially a measure of the overall energy metabolism of the brain, and changes in functional activity are reflected in corresponding changes in glucose utilization. The ability of the DG method to measure the rates of glucose utilization simultaneously in all the functional and anatomic components of the CNS in conscious animals makes it particularly useful to map local functional activity, and it has been extensively used since its introduction to identify regions of altered functional activity in a variety

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Abbreviations used: DG, 2-deoxy-D-glucose; DG-6-P, 2-deoxy-D-glucose-6-phosphate.

of physiological and pharmacological states (Sokoloff, 1981, 1982). In these applications the method has generally performed successfully and effectively, and it has provided values for cerebral glucose utilization that are consistent with the known rates of cerebral energy metabolism established by other methods.

Sacks et al. (1983) recently published a sharp denunciation of the DG method that challenges its validity as a method to measure cerebral glucose utilization and purports to invalidate the model and basic principles on which it is founded. Specifically, their main points of criticism are as follows:

1. They observed that following an intravenous tracer pulse of labeled DG, the concentrations and proportions of [^{14}C]DG and [^{14}C]2-deoxy-D-glucose-6-phosphate ([^{14}C]DG-6-P) in the brain remained more or less constant, with [^{14}C]DG-6-P accounting for approximately 90% of the total radioactivity in brain. This is contrary to the theoretical basis of the method which predicts first a rising [^{14}C]DG concentration in brain followed by a decline while the [^{14}C]DG-6-P concentration progressively increases (Sokoloff et al., 1977).

2. They observed that following intravenous injections of [^{14}C]DG or [^{14}C]glucose, there were first positive cerebral arteriovenous differences for both compounds, indicating net brain uptake, followed by a reversal of the arteriovenous difference for deoxyglucose, indicating net loss from the tissue, but no such reversal with glucose. They interpreted this observation as evidence of deoxyglucose-6-phosphohydrolase activity which caused loss of product from the tissue.

3. Without providing any new relevant experimental data Sacks et al. (1983) cited previous experiments of theirs in which they observed during constant intravenous infusions of [^{14}C]glucose-6-phosphate positive cerebral arteriovenous differences for [^{14}C]glucose-6-phosphate and negative differences for [^{14}C]glucose (Sacks and Sacks, 1968). They interpreted these observations as evidence for significant glucose-6-phosphatase activity in brain, which would therefore invalidate the DG method.

The results of the present studies demonstrate that the experimental data presented and cited by Sacks et al. (1983) bear no relationship to the conclusions they drew from them and have no relevance to the presence of glucose-6-phosphatase activity in brain or to the validity of the DG method.

MATERIALS AND METHODS

Chemicals

Hexokinase (EC 2.7.1.1) and glucose-6-phosphate dehydrogenase (EC 3.2.1.3) from yeast were purchased from Boehringer Mannheim, Indianapolis, IN, U.S.A. ATP, NADP⁺, imidazole, acid-washed activated char-

coal, and isobutyric acid were purchased from Sigma Chemical (St. Louis, MO, U.S.A.). The ion-exchange resins, AG 1-X 8 formate (200–400 mesh) and AG 50-X 8 H⁺ form (200–400 mesh), were obtained from BioRad Laboratories, Richmond, CA, U.S.A. All other chemicals were of the highest purity commercially available. Flexible 20 × 20 cm cellulose chromatography sheets (no. 13255), used in TLC analyses, were obtained from Eastman Kodak, Rochester, NY, U.S.A. [^3H]Sucrose (sp act 10.1 Ci/mmol), 2-[1- ^{14}C]DG (sp act 51.1 mCi/mmol), [γ - ^{32}P]ATP (sp act 27–34 mCi/mmol), and disodium D-[1- ^{14}C]glucose-6-phosphate (sp act 54.4 mCi/mmol) were purchased from New England Nuclear, Boston, MA, U.S.A.

[^{32}P]Glucose-6-phosphate was synthesized enzymatically by incubation of D-glucose with [γ - ^{32}P]ATP, hexokinase, and magnesium chloride. Unreacted ATP was adsorbed onto freshly activated charcoal (Norit A), and the radiochemical purity of the [^{32}P]glucose-6-phosphate was verified by TLC which demonstrated a single spot with the same R_f as authentic [^{14}C]glucose-6-phosphate. The solvent used to develop the chromatogram was 66% isobutyric acid, 33% water, and 1% concentrated ammonium hydroxide (by vol).

Scintillation counting

^{14}C , ^3H , and ^{32}P were counted with appropriate energy windows in either a Beckman Model No. LS 255 (Beckman Instruments, Fullerton, CA, U.S.A.) or a Packard Tri-carb, Model No. 3375 (Packard Instruments, Downers Grove, IL, U.S.A.) liquid scintillation counter. Efficiencies for counting ^3H , ^{14}C , and ^{32}P were calibrated for each sample by recounting with appropriate internal standards, e.g., [^3H]toluene, [^{14}C]toluene, and [^{32}P]ATP, respectively.

Time course of [^{14}C]DG-6-P

Under light N₂O halothane anesthesia polyethylene catheters (PE 50) were inserted into a femoral vein in 17 rats and into both a femoral artery and vein in 12 rats. The free ends of the catheters were tunneled beneath the subcutaneous tissue to exit at the nape of the neck. After at least 2 h of recovery from anesthesia both groups of rats were administered intravenous pulses of 120 $\mu\text{Ci}/\text{kg}$ of [^{14}C]DG. Timed arterial plasma samples were withdrawn from the femoral arterial catheter in the group of rats with arterial catheters until the time of killing. Arterial plasma concentrations of glucose and [^{14}C]DG were measured by means of a Beckman Glucose Analyzer II and liquid scintillation counting, respectively. Brains were obtained from both groups at various times after the pulse ranging from 2 to 45 min by means of the freeze-blowing technique of Veech et al. (1972). The frozen brains were crushed and ground to a fine powder under liquid N₂ in a cryostat maintained at -35°C . The powder was divided into weighed portions of approximately 100 mg each which were stored at -65°C until processed as follows. Perchloric acid, 3 M, cooled to -10°C was added to the frozen brain powders, and the tissue was allowed to thaw at this temperature (Lowry and Passonneau, 1972). The resulting suspensions were diluted with water to a final concentration of 0.64 M perchloric acid and vortexed vigorously. The pellet was removed by centrifugation at 4,300 g for 10 min, and the supernatant fluid was adjusted to pH 7.0–7.5 with 2 M KOH-0.4 M KCl-

0.4 M imidazole within 20–40 min after acidification. The KClO_4 precipitate was separated by centrifugation and discarded.

Free [^{14}C]DG and [^{14}C]DG-6-P in the neutralized extract were separated by passage through an anion-exchange column (AG-1 formate, 0.8×4 cm), and the free [^{14}C]DG was assayed in a portion of the effluent by liquid scintillation counting. The acidic products retained on the anion-exchange column were then displaced from the resin with 30 ml of 4 M formic acid, and the concentration of ^{14}C contained in the acidic metabolites of deoxyglucose (mainly [^{14}C]DG-6-P) was similarly measured by liquid scintillation counting.

Comparison of time courses of cerebral arteriovenous differences for [^{14}C]DG and [^{14}C]glucose

The model of the DG method leads to specific predictions about the time courses of the cerebral arteriovenous differences for tracer doses of [^{14}C]DG and [^{14}C]glucose. The full details of the model are provided in the original report of the method (Sokoloff et al., 1977). A predictive equation was derived from the model as follows.

The DG method is ultimately based on the Fick principle which can be expressed mathematically by the following equation:

$$F(C_a - C_v) = dC/dt \quad (1)$$

where F equals the rate of blood flow per unit mass of tissue; C_a and C_v are the arterial and cerebral venous concentrations of the labeled tracer, respectively; C_i is the concentration of label in the tissue; and t is the variable time.

The rate of change of concentration of the label in the tissue, dC/dt , is in turn equal to the difference in the rates of influx of the labeled hexose from plasma to tissue and efflux back from tissue to plasma. Therefore,

$$dC/dt = k_1 C_p - k_2 C_E \quad (2)$$

where C_p is the arterial plasma concentration, C_E is the concentration of the free labeled hexose in the tissue, and k_1 and k_2 are the rate constants for inward and outward transport across the blood-brain barrier, respectively.

Combining Eqs. 1 and 2:

$$F(C_a - C_v) = k_1 C_p - k_2 C_E \quad (3)$$

But according to the model [see Eq. 8 in the publication of Sokoloff et al., 1977], C_E is a function of the history of the arterial plasma concentration, the rate constants for inward and outward transport, and k_3 , the rate constant for the metabolism of the free hexose in the tissue.

$$C_E = k_1 e^{-(k_2+k_3)T} \int_0^T C_p e^{(k_2+k_3)t} dt \quad (4)$$

Combining Eqs. 3 and 4:

$$F(C_a - C_v)(T) = k_1 C_p(T) - k_2 k_1 e^{-(k_2+k_3)T} \int_0^T C_p e^{(k_2+k_3)t} dt \quad (5)$$

If, as is reasonable, it is assumed that in the very short transit time of the blood within the cerebral vascular bed, the hexose is exchanged only between the plasma and

brain tissue with negligible loss of hexose from plasma to red cells, then

$$F_p (C_p - C_{vp}) = F(C_a - C_v) \quad (6)$$

where F_p is the rate of cerebral plasma flow, and C_{vp} is the cerebral venous plasma concentration of tracer.

Substituting Eq. 6 into Eq. 5, rearranging, and solving for C_{vp} ,

$$C_{vp}(T) = C_p(T) - k_1 C_p(T)/F_p + k_1 k_2 e^{-(k_2+k_3)T} \int_0^T C_p e^{(k_2+k_3)t} dt / F_p \quad (7)$$

Eq. 7 was used to compute and compare the time courses of the cerebral venous plasma [^{14}C]DG and [^{14}C]glucose concentrations predicted by the model during identical arterial plasma inputs for both hexoses. The arterial plasma curves were designed to simulate closely the time course of the arterial input function that would be obtained in a rat during and following a constant intravenous infusion of the tracers for 1 min. A value of 0.5 ml/g/min was used for F_p , the cerebral plasma flow; this is a reasonable average value for an animal with a normal hematocrit of 50% (Sakurada et al., 1978). The values for k_1 , k_2 , and k_3 that were used for the computation of the [^{14}C]DG cerebral venous plasma curve were 0.189, 0.245, and 0.052, respectively; these are the values that were experimentally determined in normal, normoglycemic, conscious rats (Sokoloff et al., 1977). Reported values for the transfer rate constants, k_1 and k_2 , for glucose have varied, but all reports agree that they are about two-thirds those for DG because of the lower affinity of the carrier in the blood-brain barrier for glucose (Pardridge and Oldendorf, 1975; Pardridge et al., 1982; Gjedde, 1982; Pardridge, 1983). There is, however, unanimity that k_3 , the rate constant for the phosphorylation of glucose, is three times that of the equivalent rate constant for DG. This is self-evident from the known Michaelis-Menten kinetic constants of hexokinase for the two hexoses. It can be readily derived from the Michaelis-Menten rate equations that

$$k_3^*/k_3 = V_m^* K_m / V_m K_m^*$$

where k_3^* and k_3 are the rate constants for phosphorylation of DG and glucose, respectively; V_m^* and V_m are the maximal velocities of hexokinase with DG and glucose, respectively; and K_m^* and K_m are the Michaelis-Menten constants for DG and glucose, respectively.

Grossbard and Schimke (1966) have shown that rat brain hexokinase exhibits equal maximal velocities for both hexoses but has a K_m for DG approximately three times that for glucose. From the above relationship k_3 must, therefore, be three times that of k_3^* .

In view of the uncertainty about the exact values of the transport constants for glucose to be used, the time course of the glucose concentration in the cerebral venous plasma was computed twice by Eq. 7 with two sets of values for k_1 and k_2 . In one set k_1 and k_2 for glucose were set at 0.126 and 0.163, respectively, approximately two-thirds those of the equivalent values for DG. These values were considered to represent reasonable estimates of these parameters, at least relative to those for DG, on the basis of the information in the literature. In the second set, k_1 and k_2 were set equal to the equivalent values of

DG, the worst case possible to demonstrate differences in the kinetics of the exchange of DG and glucose between plasma and brain. In both computations k_3 for glucose was set at 0.156, three times the value used for deoxyglucose.

Cerebral arteriovenous difference and cerebral uptake of [^{32}P]glucose-6-phosphate

To determine whether glucose-6-phosphate is taken up by brain from the blood and metabolized, cerebral arteriovenous differences for [^{32}P]glucose-6-phosphate and cerebral ^{32}P uptake were measured during continuous intravenous infusions of the labeled compound. Preliminary experiments had shown that within the first few minutes after the onset of an intravenous infusion of [^{14}C]glucose-6-phosphate a large fraction of the radioactivity in arterial blood was contained in free [^{14}C]glucose, probably because of recirculation from tissues, such as liver and kidney, with high levels of glucose-6-phosphatase activity. It was, therefore, impossible to discriminate from radioactivity found in brain whether it was derived from [^{14}C]glucose-6-phosphate or [^{14}C]glucose uptake. [^{32}P]Glucose-6-phosphate was, therefore, selected as the tracer compound because free glucose derived from its hydrolysis would not be labeled, and inorganic phosphate is known not to cross the blood-brain barrier (Bakay, 1956).

In nine rats under pentobarbital anesthesia polyethylene catheters (PE 50) were inserted into one femoral artery and vein, and a metal cannula was inserted into the confluence of the sinuses through a trephined hole in the skull and held in place by a threaded metal holder screwed into the skull. The animals were then allowed to recover from the anesthesia for at least 2 h. During continuous intravenous infusions of approximately 25–55 μCi of [^{32}P]glucose-6-phosphate over a 10-min period, timed arterial and cerebral venous blood samples were drawn and stored on ice until subsequently analyzed for their [^{32}P]glucose-6-phosphate concentrations. At the end of the infusion the animals were decapitated, and their brains were removed by dissection. The brains were stripped of pineal gland, meninges, and choroid plexus to remove at least some of the blood and other than neural tissues, and then weighed and homogenized in water.

The brain homogenates were diluted to 10 ml with water, and 200- μl portions containing approximately 35 mg of brain tissue were added to 10 ml of Aquasol (New England Nuclear, Boston, MA, U.S.A.) and assayed for ^{32}P content in a liquid scintillation counter. Counting efficiency for each sample was calibrated by recounting again after addition of internal standards.

The blood samples were assayed for their [^{32}P]glucose-6-phosphate and $^{32}\text{P}_i$ contents by dilution of 25- μl portions with 1 ml of water, deproteinization with 50 μl of 10 M perchloric acid, neutralization with 2 M KOH/0.4 M KCl/0.4 M imidazole, removal of the precipitate by centrifugation, and separation of the organic ^{32}P and $^{32}\text{P}_i$ in the supernatant solution by the method of Martin and Doty (1949). The two phases obtained by this procedure, the aqueous phase containing the [^{14}C]glucose-6-phosphate and the isobutanol-benzene phase containing the $^{32}\text{P}_i$, were both assayed for their ^{32}P contents by liquid scintillation counting as described above.

In the first six of the nine animals studied the [^{32}P]glucose-6-phosphate was administered by a constant rate of intravenous infusion. Under these conditions a

continuously rising arterial curve was obtained with no semblance of steady-state conditions. The determination of arteriovenous differences in nonsteady-state conditions is fraught with artifacts, not the least of which is due to the difficulty in temporal matching of appropriately corresponding samples of arterial and venous blood when both are changing rapidly and sampled at a distance via catheters. That timing errors do occur was confirmed by the results of similar experiments with constant infusions of Evans Blue dye, a tracer that binds to albumin and is known not to cross the blood-brain barrier; apparent positive arteriovenous differences were sometimes observed under the nonsteady-state conditions. In the remaining three animals, therefore, the [^{32}P]glucose-6-phosphate was infused intravenously at an exponentially declining rate over the 10-min period to achieve a near constant arterial concentration and steady-state conditions.

Assay of blood-brain barrier permeability to glucose-6-phosphate by single-pass extraction technique

The permeability of the blood-brain barrier to glucose-6-phosphate was assayed directly by measurement of its single-pass extraction by means of the indicator diffusion technique of Crone (1963) with [^3H]sucrose used as the nondiffusible reference tracer. Three rats were anesthetized by the intraperitoneal injection of 30 mg/kg of sodium pentobarbital. A polyethylene catheter (PE 50) was inserted into one external carotid artery and threaded to the carotid bifurcation; the common carotid artery was ligated caudal to the tip of the catheter. A metal cannula was also inserted into the confluence of sinuses as described above for the sample of cerebral venous blood. The animals were then heparinized with 100 IU of heparin. A blood sample was drawn to which was added a mixture of [^3H]sucrose and [^{14}C]glucose-6-phosphate. At zero time 100 μl of the blood containing approximately 5 μCi of [^3H]sucrose and 20 μCi of [^{14}C]glucose-6-phosphate in one experiment and 125 μCi and 20 μCi of the two compounds, respectively, in the other two experiments were injected as a pulse into the carotid artery. Timed samples of cerebral venous blood were sequentially collected on filter paper discs at 1-s intervals. A sample of injectant was also collected on a filter paper disc. The discs were soaked in 1 ml of ice water for 1 h, 50 μl of perchloric acid were added to precipitate the hemoglobin, and the mixture was shaken at room temperature for 24 h. The precipitate and filter paper were removed by centrifugation, and the supernatant solution was assayed for ^3H and ^{14}C contents by liquid scintillation counting calibrated with internal standards. The ^3H and ^{14}C contents in the acid extracts of the cerebral venous blood samples were normalized to their respective concentrations in the injectant and used to calculate the cerebral single-pass extraction of [^{14}C]glucose-6-phosphate relative to that of the nondiffusible reference substance, [^3H]sucrose, as described by Crone (1963).

RESULTS

Time course of [^{14}C]DG-6-P accumulation in brain

Sacks et al. (1983) reported that following an intravenous pulse of [^{14}C]DG the brain concentrations

of [¹⁴C]DG and [¹⁴C]DG-6-P in brain remained constant with approximately 90% of the total radioactivity contained in the phosphorylated product throughout the 45-min experimental period of the DG method. Inasmuch as the model of the DG method predicts progressively falling [¹⁴C]DG and rising [¹⁴C]DG-6-P concentrations in brain during the experimental period (see Fig. 5 of report of Sokoloff et al., 1977), they interpreted their results as evidence of the invalidity of the model, due mainly to glucose-6-phosphatase activity. In their experiments, however, Sacks et al. (1983) removed the brains by dissection, allowing ample time for rapid phosphorylation of the free [¹⁴C]DG due to the well-known acceleration of glycolysis that occurs when the cerebral circulation is interrupted (Lowry et al., 1964). When the experiments were carried out in the present studies with rapid termination of cere-

bral metabolic activity by means of the freeze-blowing technique, the time courses of the [¹⁴C]DG and [¹⁴C]DG-6-P concentrations in brain followed the expected precursor-product relationships and conformed with those predicted by the model (Fig. 1). The results of Sacks et al. (1983) are a reflection, therefore, of a postmortem artifact.

In the group of animals in which arterial plasma samples were drawn and assayed for [¹⁴C]DG and glucose concentrations, there was a similar progressive accumulation of [¹⁴C]DG-6-P in brain (Fig. 2), but the slope of the accumulation was less steep and tended to decrease more with time than occurred in the experiments illustrated in Fig. 1. The reason for the difference between the two series of experiments is unclear, but in the animals from which arterial samples were drawn there were progressive rises in plasma glucose concentrations throughout the experimental period to two to three times the initial levels, probably in response to excessive blood withdrawal. Increased plasma and tissue glucose concentrations would by competitive

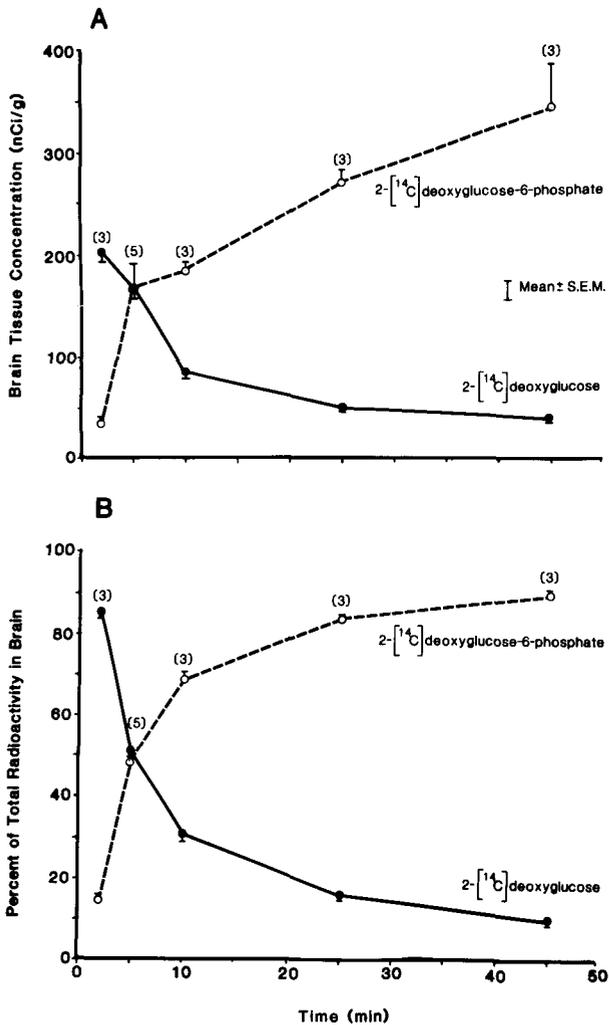


FIG. 1. Time courses of [¹⁴C]DG and [¹⁴C]DG-6-P contents in rat brains removed by freeze-blowing following an intravenous pulse of [¹⁴C]DG. The data in (A) and (B) are derived from the same experiments. The means ± SEM were obtained from the number of animals indicated in parentheses.

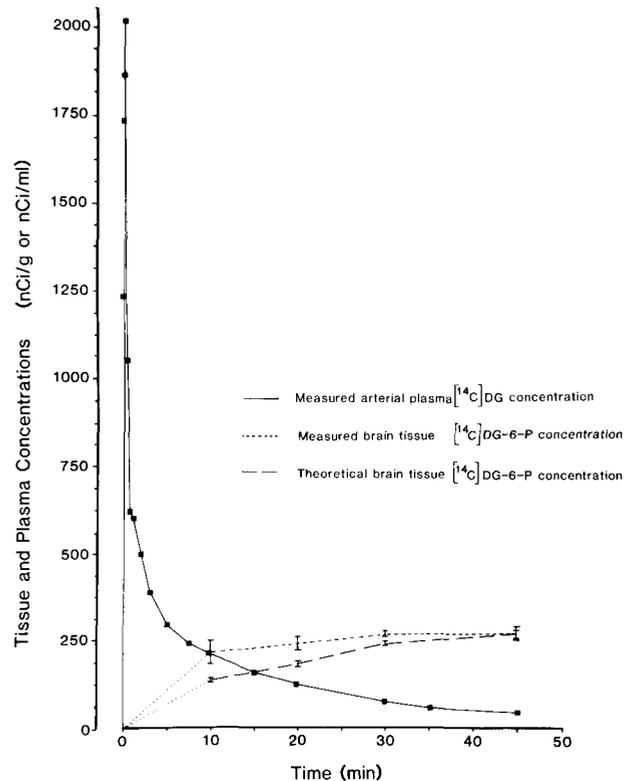


FIG. 2. Measured and theoretical time courses of [¹⁴C]DG-6-P concentrations in brain following an intravenous pulse of [¹⁴C]DG. The theoretical curve was computed by Eq. 8 which is derived from the model of the DG method (see Materials and Methods and Results). The values represent the means ± SEM of three animals for each time. In the same animals in which [¹⁴C]DG-6-P was measured, the theoretical concentrations were calculated by Eq. 8 on the basis of each animal's unique time courses of plasma [¹⁴C]DG and glucose concentrations up to the time of killing.

inhibition slow the rates of uptake and phosphorylation of [^{14}C]DG by brain and lead to flatter curves of [^{14}C]DG-6-P accumulation. The availability of arterial plasma [^{14}C]DG and glucose time courses made it possible, however, to use a transposed version of the operational equation of the DG method (Sokoloff et al., 1977), as Hawkins and Miller (1978) had done previously, to calculate the time course of the [^{14}C]DG-6-P concentration in brain predicted by the model and to compare it with the experimentally measured one. The transposed version of the equation is as follows:

$$[[^{14}\text{C}]\text{DG-6-P}](T) = R_i \times LC \left[\int_0^T (C_p^*/C_p) dt - e^{-(k_2^* + k_3^*)T} \int_0^T (C_p^*/C_p) e^{(k_2^* + k_3^*)t} dt \right] \quad (8)$$

where [^{14}C]DG-6-P(T) equals the brain concentration of [^{14}C]DG-6-P at the time of killing, T ; C_p^* and C_p are the arterial plasma concentrations of [^{14}C]DG and glucose, respectively; k_2^* and k_3^* are the rate constants for [^{14}C]DG transport from brain to plasma and for phosphorylation by hexokinase, respectively; LC equals the lumped constant; and R_i equals the average rate of glucose utilization of the brain as a whole.

The values for k_2^* and k_3^* used in the computations were 0.245 and 0.052, respectively; these are the arithmetic means of the values determined by Sokoloff et al. (1977) in some representative gray structures in normal conscious rats. For LC the value, 0.48, was used; this is the value for the lumped constant experimentally determined by Sokoloff et al. (1977) in rats. For R_i it was necessary to assume a single reasonable value for average rate of cerebral glucose utilization; the value assumed was 0.7 $\mu\text{mol/g/min}$, approximately equivalent to the rate of glucose utilization predicted on the basis of the measured rate of cerebral oxygen consumption in the rat and the usual stoichiometric relationship between cerebral oxygen and glucose consumptions (Nilsson and Siesjö, 1976; Siesjö, 1978).

Comparison between predicted and measured concentrations of [^{14}C]DG-6-P in brain provides no evidence for significant deoxyglucose-6-phosphatase activity during the 45-min period. The predicted values were calculated by an equation based on the model of the DG method, which assumes no deoxyglucose-6-phosphatase activity. Phosphatase activity, if present, would cause the measured values to be below those of the theoretical values, and the divergence would increase with time because the effects of phosphatase activity would be cumulative. The results show the opposite (Fig. 2). The theoretical values are below the measured values at early times, and the difference between

them diminishes with time until they become very similar at 45 min, the time recommended as optimal for the use of the DG method.

The discrepancy between measured and theoretical concentrations of [^{14}C]DG-6-P in brain at early times and the diminution in the difference with time almost certainly reflects inaccuracies in the values of the rate constants. The rate constants vary from structure to structure in the brain; the values used were arithmetic averages of some representative gray structures and could not possibly be accurate for the brain as a whole. Even the average of the rate constants for all the brain structures weighted for their relative sizes would not be appropriate in computations for the whole brain because of the many compartments in the brain. The terms in the equation that contain the rate constants diminish with time following a pulse of [^{14}C]DG until at 45 min their effect, and, therefore, also that of the rate constants, become very small (Sokoloff et al., 1977). It was, in fact, precisely to minimize the effects of inaccuracies in the rate constants that a 45-min period was selected for the duration of the experimental period in the use of the DG method (Sokoloff et al., 1977). If they were accurately known, then the method could be used with much shorter experimental periods.

If inaccuracies in the rate constants could cause falsely low predicted values for the brain [^{14}C]DG-6-P concentrations at early times, might they not also have caused the predicted values to fall artifactually below the measured values and thus obscure the effects of deoxyglucose-6-phosphatase activity? This possibility can readily be excluded. First of all, at 45 min, when the effects of the phosphatase would be maximum and the influence of the rate constants negligible, the theoretical and measured values agree very closely. Secondly, if the theoretical values for [^{14}C]DG-6-P concentration in brain are repeatedly recomputed with progressively higher values for $(k_2^* + k_3^*)$, they progressively increase most at early times, less so at intermediate times, and minimally at 45 min. Even when $(k_2^* + k_3^*)$ is arbitrarily set to infinity, which leads to the highest possible values for the predicted [^{14}C]DG-6-P concentrations, these concentrations come close to the experimentally measured values but never exceed them significantly at any time.

Another problem in the analysis of these data is the value of R_i , the rate of whole brain glucose utilization in the rat. There is a wide range of values reported in the literature reflecting mainly methodological differences and deficiencies. Siesjö (1978) reviewed this field and concluded that "since three different methods have given a cerebral metabolic rate for glucose (CMR_{gl}) of 0.6–0.8 $\mu\text{mol} \cdot \text{g}^{-1} \cdot \text{min}^{-1}$ for the rat brain, there is little doubt about its validity." We therefore chose 0.7 $\mu\text{mol/g/min}$ for the computation of the theoretical

curve in Fig. 2. It is obvious from Eq. 8 that the calculated $[^{14}\text{C}]\text{DG-6-P}$ is directly proportional to R_i . If we had used the upper limit of the range, $0.8 \mu\text{mol/g/min}$, the theoretical curve would have been raised approximately 14% above the one in Fig. 2. This would have brought the experimental and theoretical curves in closer agreement at earlier times and raised the theoretical value approximately 10% above the measured value at 45 min. Considering all the uncertainties in the values of the rate constants and R_i , it is rather remarkable that the theoretical and experimental curves are so close together, particularly at later times. With the spread of the experimental data in three rats a 10% difference between experimental and theoretical values at 45 min is hardly a sufficient basis on which to conclude that there are significant effects of DG-6-P within the prescribed 45 min of the DG method.

Comparison of cerebral arteriovenous differences for $[^{14}\text{C}]\text{DG}$ and $[^{14}\text{C}]\text{glucose}$

Sacks et al. (1983) observed that following an intravenous injection of $[^{14}\text{C}]\text{DG}$ or $[^{14}\text{C}]\text{glucose}$ there was initially a positive cerebral arteriovenous difference with either tracer. After termination of the injection the arterial concentration fell rapidly, the venous concentration more slowly, and the arteriovenous difference diminished and approached zero with increasing time. With $[^{14}\text{C}]\text{DG}$ the venous concentration curve eventually intersected and crossed the arterial curve resulting in a small but sustained negative arteriovenous difference. With $[^{14}\text{C}]\text{glucose}$ the arterial and venous curves converged but never intersected, and, therefore, no negative arteriovenous difference was ever ob-

served. Sacks et al. (1983) interpreted this difference in the time courses of the arteriovenous difference as evidence that the model of the DG method could not apply to glucose utilization because of hydrolysis of $[^{14}\text{C}]\text{DG-6-P}$ by phosphatase and the return of free $[^{14}\text{C}]\text{DG}$ for transport back from tissue to blood.

The results in Fig. 3 demonstrate that the model of the DG method fully predicts the difference in the time courses of the cerebral arteriovenous difference in the time courses of the cerebral arteriovenous difference for $[^{14}\text{C}]\text{DG}$ and $[^{14}\text{C}]\text{glucose}$ without the need to invoke glucose-6-phosphatase activity. With identical inputs for both tracers the time courses of the cerebral venous concentrations were computed by means of Eq. 7 (see Materials and Methods), which was derived from the model of the DG method. The computed arteriovenous differences reproduce almost exactly the phenomenon observed by Sacks et al. (1983). The arteriovenous difference for $[^{14}\text{C}]\text{DG}$ eventually reverses; that for glucose does not. The only differences in the computations of the venous curves for $[^{14}\text{C}]\text{DG}$ and $[^{14}\text{C}]\text{glucose}$ were in the values of the rate constants used. The rate constants used for $[^{14}\text{C}]\text{DG}$ were those previously determined for normal conscious rat brain (Sokoloff et al., 1977). In Fig. 3A the rate constants for inward and outward transport of glucose across the blood-brain barrier, k_1 and k_2 , respectively, and for its phosphorylation by hexokinase, k_3 , were adjusted from the rate constants for DG to take into account the known lower affinity of the transport carrier for glucose than for DG and the lower K_m of rat brain hexokinase for glucose than for DG (see Materials and Methods). Even

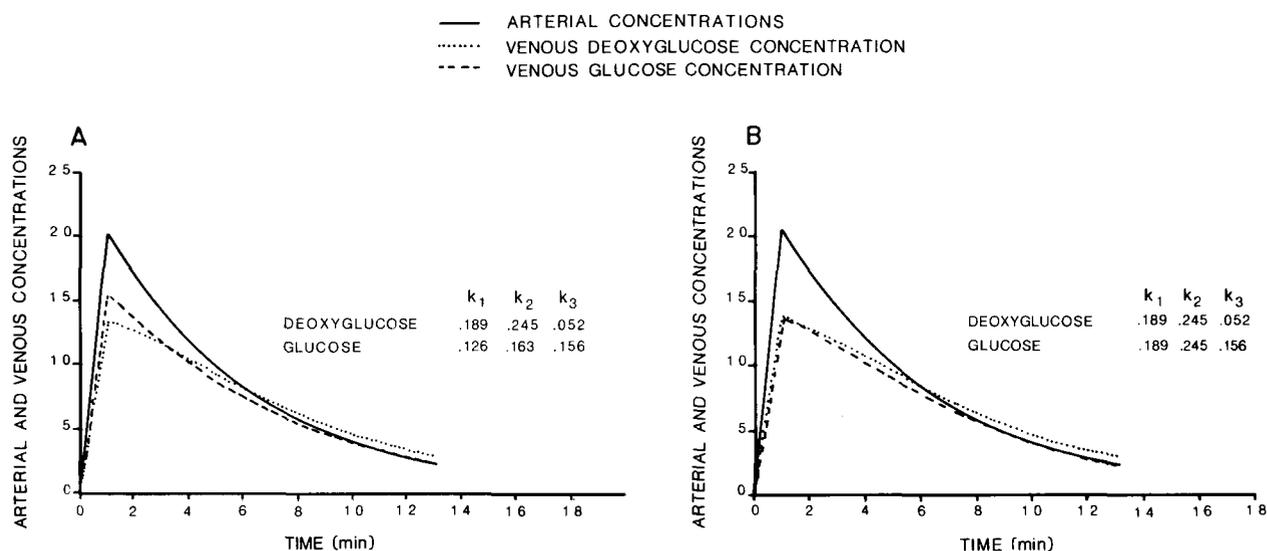


FIG. 3. Theoretical time courses of cerebral arteriovenous differences for $[^{14}\text{C}]\text{DG}$ and $[^{14}\text{C}]\text{glucose}$ during identical arterial concentrations of either tracer. The arterial curve was designed to simulate one obtained during and following a 1-min infusion of the tracer. The venous curves were computed by Eq. 7 (see Materials and Methods) with the rate constants used displayed in (A) and (B). The units of arbitrary inasmuch as no dose is specified.

when the same rate constants for transport, the values of k_1 and k_2 experimentally determined for DG, are used in the computations for [^{14}C]glucose and [^{14}C]DG and only k_3 is adjusted to accommodate the higher affinity of hexokinase for glucose, the difference in the time courses of the arteriovenous difference for the two hexoses is retained (Fig. 3B). The critical factor responsible for the different arteriovenous differences with the two hexoses is k_3 or, more precisely, the ratio of k_3 to k_2 . Because k_3 , the rate constant for phosphorylation is three times greater for glucose than for DG, glucose is more rapidly phosphorylated, thus lowering the free glucose concentration in the tissue. The steady-state distribution ratio for glucose between brain and plasma is no more than half that for DG (Hawkins and Miller, 1978). Inasmuch as k_2 , the rate constant for outward transport for glucose is no higher and probably lower than that of DG and the [^{14}C]glucose concentration is lower than that of [^{14}C]DG in the tissue, the transport of glucose from tissue to blood is less than that of DG and insufficient to raise the cerebral venous concentration above that of the falling arterial concentration. The model of the DG method and its operational equation takes into account the differences in the rate constants for DG and glucose. Contrary to the conclusions of Sacks et al. (1983), the difference in the time courses of the cerebral arteriovenous difference for [^{14}C]DG and [^{14}C]glucose does not invalidate the model of the DG method but, in fact, is predicted and explained by it (Fig. 3).

Transport of glucose-6-phosphate across blood-brain barrier

Sacks et al. (1968, 1983) reported that during constant intravenous infusions of [^{14}C]glucose-6-phosphate they observed positive cerebral arteriovenous differences for [^{14}C]glucose-6-phosphate and negative cerebral arteriovenous differences for [^{14}C]glucose. They interpreted these findings as evidence of cerebral glucose-6-phosphate activity that would invalidate the DG method which assumed that [^{14}C]DG-6-P was irreversibly trapped in the cerebral tissues for the 45 min of the experimental period.

Although it is generally believed that sugar-phosphates cannot cross the blood-brain barrier, we reexamined this question in view of the report of Sacks et al. (1968, 1983). The issue was investigated by three independent approaches. First, the cerebral arteriovenous difference for [^{32}P]glucose-6-phosphate was determined under conditions more closely approximating steady-state conditions than those existing during the studies of Sacks and Sacks (1968). Secondly, the labeling of brain tissue with ^{32}P during infusions of [^{32}P]glucose-6-phosphate was measured. Finally, the permeability of the blood-brain barrier to [^{14}C]glucose-6-phosphate

was directly examined by means of the single-pass capillary extraction technique of Crone (1963) with [^3H]sucrose used as the nondiffusible reference tracer.

In three animals cerebral arteriovenous differences for [^{32}P]glucose-6-phosphate were determined during intravenous infusions of the labeled glucose-6-phosphate which was infused at logarithmically declining rates to achieve near constant arterial concentrations and steady-state conditions. In one of these animals there was a small positive arteriovenous difference illustrated in Fig. 4A. In the other two experiments, one of which is illustrated in Fig. 4B, there was no evidence of any significant arteriovenous difference for [^{32}P]glucose-6-phosphate. When a paired t test was applied to all the paired arterial and venous samples of the three experiments, there was no statistically significant arteriovenous difference ($p \cong 0.3$).

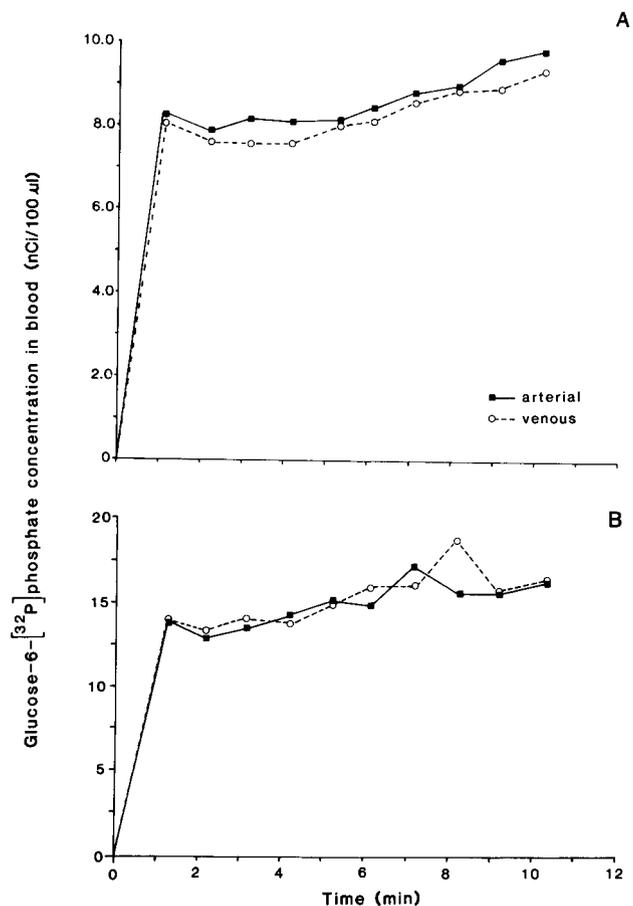


FIG. 4. Cerebral arteriovenous differences for [^{32}P]glucose-6-phosphate during continuous logarithmically declining rates of intravenous infusion of [^{32}P]glucose-6-phosphate. Of three experiments the only one with any evidence of an arteriovenous difference is illustrated in (A). The results of an experiment representative of the other two is illustrated in (B). The mean cerebral extraction of [^{32}P]glucose-6-phosphate in each animal was 2.2%, 4.3%, and -2.4%. The mean extraction for all three animals of 1.4% was not significantly different from zero.

In nine animals, including the three used for the determination of cerebral arteriovenous differences for [³²P]glucose-6-phosphate, the brains were dissected out after 10 min of intravenous infusion of [³²P]glucose-6-phosphate. The brains were assayed for their ³²P content which was compared to that of femoral arterial blood samples. Any inorganic ³²P released into the circulation by the hydrolysis of [³²P]glucose-6-phosphate in the peripheral tissues could not enter the brain because of the impermeability of the blood-brain barrier. Any labeling of the brain with ³²P would, therefore, have to come from the transport of intact [³²P]glucose-6-phosphate into the brain, and even if the hexose-phosphate were metabolized or hydrolyzed within the cerebral tissues, the ³²P would be expected to enter the various phosphates pools and be at least in part, retained within the brain. The results demonstrate that the ³²P concentration in brain was 2.2% ± 0.2 (mean ± SEM) of that of the blood, an amount that is fully accounted for by the ³²P contained in the blood content of the brain (Table 1). Even in the experiment illustrated in Fig. 4A, in which there appeared to be a positive cerebral arteriovenous difference for [³²P]glucose-6-phosphate, there was no cerebral uptake of ³²P (experiment 7, Table 1), indicating that the arteriovenous difference was more apparent than real. There was, therefore, no evidence of cerebral uptake of [³²P]glucose-6-phosphate.

Finally, the permeability of the blood-brain barrier to [¹⁴C]glucose-6-phosphate was assayed directly by the single-pass extraction technique of Crone (1963). A calibrated mixture of [¹⁴C]glucose-6-phosphate, the test substance, and [³H]sucrose, the nondiffusible reference substance, was injected as a pulse into a carotid artery, and the cerebral venous blood was monitored for the relative concentrations of the two tracers during the first 15 s after the pulse. Inasmuch as the nondiffusible ref-

erence substance cannot leave the circulation, its venous concentration reflects only the effects of dilution of the injected dose by the blood in the cerebral vascular bed. If the test substance can cross the blood-brain barrier, then some of it is lost from the blood into the brain, and its venous concentration reflects not only dilution by the blood but also the amount lost to the brain. The results of two of three such experiments, the worst case and best case, are illustrated in Fig. 5. The cerebral venous concentrations of [¹⁴C]glucose-6-phosphate and [³H]sucrose, normalized to their relative concentrations in the injectant, are essentially superimposable, confirming that the blood-brain barrier is impermeable to glucose-6-phosphate.

DISCUSSION

The results of the present studies demonstrate that the criticisms of the deoxyglucose method raised by Sacks et al. (1983) are based on misinterpretations of their experimental data.

1. Their findings of constant [¹⁴C]DG-6-P and [¹⁴C]DG concentrations in brain with the [¹⁴C]DG-6-P concentration maintained at approximately 90% of the total ¹⁴C content throughout the entire time

TABLE 1. Lack of uptake of [³²P]glucose-6-phosphate into brain

Rat	Dose (μCi/kg)	Blood [³² P] (nCi/ml)	Brain [³² P] (nCi/g)	Brain/blood ratio × 100
1	25.9	173	2.4	1.4
2	24.9	59	1.7	2.8
3	20.7	84	1.3	1.5
4	46.8	194	6.6	3.4
5	47.4	124	1.7	2.0
6	48.1	181	3.8	2.1
7	33.4	179	4.3	2.4
8	53.1	214	4.5	2.1
9	52.7	279	6.1	2.2
				Mean = 2.2 ± 0.2 (SEM)

Blood and brain samples obtained after infusing [³²P]glucose-6-phosphate for 10 min.

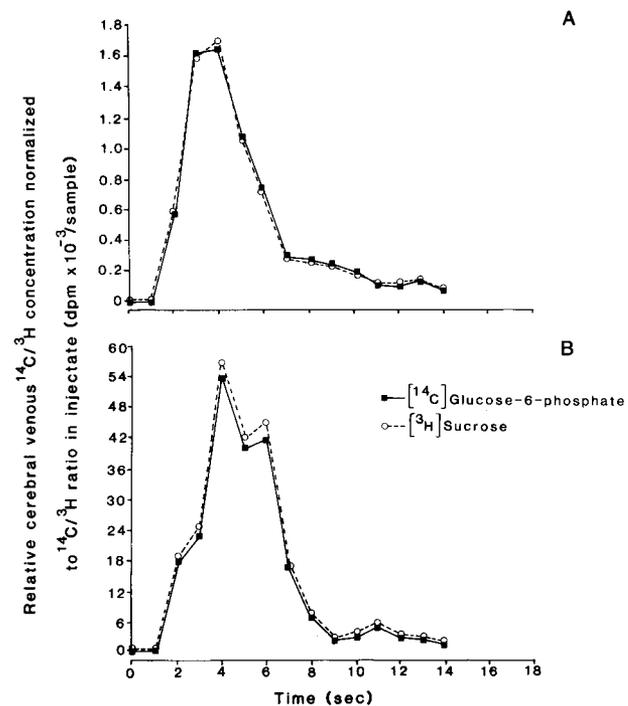


FIG. 5. Single-pass capillary extractions of [¹⁴C]glucose-6-phosphate in brain. [³H]Sucrose was used as the nondiffusible reference tracer (see Materials and Methods). Of three experiments the one with the least extraction is illustrated in (A) and the one with the greatest extraction is illustrated in (B). The calculated mean percent extraction of [¹⁴C]glucose-6-phosphate relative to that of [³H]sucrose was 2.8% (SEM = ±2.1, n = 3).

course following an intravenous pulse of [^{14}C]DG reflects the effects of the postmortem phosphorylation of the [^{14}C]DG during the accelerated rate of anaerobic glycolysis that occurs while the brain is being removed by dissection. The failure of Sacks et al. (1983) to recognize this postmortem artifact was recently pointed out by Gjedde (1984). When the experiments are repeated without postmortem dissection of the brain but with freeze-blowing, which avoids the consequences of the Pasteur effect by rapid freezing, the time courses of the [^{14}C]DG and [^{14}C]DG-6-P concentrations in brain following the pulse follow the normal precursor-product relationships predicted by the model of the DG method (Fig. 1).

2. The observation by Sacks et al. (1983) that following termination of an intravenous infusion the cerebral arteriovenous difference for [^{14}C]DG eventually reverses from positive to negative while that of [^{14}C]glucose does not is not, as they concluded, a reflection of [^{14}C]deoxyglucose-6-phosphatase activity in brain. It is predicted by the model of the deoxyglucose method which assumes no effects of [^{14}C]deoxyglucose-6-phosphatase activity during the duration of the 45-min experimental period. It is fully explained by the different proportions of the rate constants for phosphorylation and outward transport for the two hexoses in the brain (Fig. 3).

3. The observation by Sacks et al. (1983) of a positive cerebral arteriovenous difference for [^{14}C]glucose-6-phosphate and a negative arteriovenous difference for [^{14}C]glucose during a constant intravenous infusion of [^{14}C]glucose-6-phosphate, which they interpreted as evidence that [^{14}C]glucose-6-phosphate was taken up in brain and hydrolyzed by glucose-6-phosphatase, was an artifact due either to the non steady-state conditions during the measurement of the arteriovenous differences or inadequate separation of [^{14}C]glucose-6-phosphate and [^{14}C]glucose in the blood samples. When [^{32}P]glucose-6-phosphate is infused at a logarithmically declining rate to achieve near-constant arterial concentrations and steady-state conditions, there is no evidence of any significant cerebral arteriovenous difference for [^{32}P]glucose-6-phosphate (Fig. 4) or uptake of ^{32}P into brain tissue (Table 1). Furthermore, assay of the permeability of the blood-brain barrier to [^{14}C]glucose-6-phosphate by the single pass capillary extraction technique of Crone (1963) confirms the fact that the barrier is impermeable to it (Fig. 5). The observations of Sacks et al. (1983) have, therefore, no relevance to glucose-6-phosphatase activity in brain. Even if the activity were present in brain, it could not possibly hydrolyze a potential substrate in blood that cannot cross the blood-brain barrier and have access to it.

In their criticisms Sacks et al. (1983) relied heavily on previously published allegations that the

DG method is invalid because of glucose-6-phosphate activity. The first of these reports was by Hawkins and Miller (1978), who performed experiments similar to those carried out in the present studies and illustrated in Fig. 2. They also compared directly measured [^{14}C]DG-6-P concentrations in brain at various times following an intravenous pulse of [^{14}C]DG with the theoretically calculated values predicted by Eq. 8. Their predicted values, unlike those in Fig. 2, were two to three times the measured values, and Hawkins and Miller (1978) attributed the discrepancy to loss of [^{14}C]DG-6-P due to glucose-6-phosphatase activity. There were, however, errors in the calculation of the predicted values that could account for the discrepancy. The theoretical values were calculated by Eq. 8, just like those in Fig. 2. This calculation requires the insertion of values for R_i , the average rate of glucose utilization in rat brain, and LC , the lumped constant. Hawkins and Miller (1978) used the same value for R_i as in the calculations for Fig. 2 (i.e., $0.7 \mu\text{mol/g/min}$), but they used the value of 1.25 for the lumped constant, despite the fact that the lumped constant had been experimentally measured and found to be 0.48 (Sokoloff et al., 1977). Cunningham and Cremer (1981) and Gjedde (1982) have subsequently confirmed that the lumped constant for normal rat brain under normoglycemic conditions is in the range of 0.35–0.55. It is obvious from Eq. 8 that the use of a lumped constant more than two times the true value would lead to predicted values for [^{14}C]DG-6-P concentrations in brain more than twice the true values. Indeed, when the data of Hawkins and Miller (1978) are used to compute the theoretical [^{14}C]DG-6-P concentrations in brain with the correct value for the lumped constant, 0.48, then the predicted and measured concentrations of [^{14}C]DG-6-P in brain are in remarkably good agreement through most of the time course, proving, contrary to their conclusions, that there is no significant loss of [^{14}C]DG-6-P due to glucose-6-phosphatase or any other cause during the 45-min experimental period of the DG method (Sokoloff, 1980, 1982).

This refutation of Hawkins' and Miller's claim of significant effects of glucose-6-phosphatase during the use of the DG method has been previously published (Sokoloff, 1980, 1982), but strangely ignored by Sacks et al. (1983) and others who have considered the issue of the effect of glucose-6-phosphatase on the validity of the DG method (Huang and Veech, 1982; Cunningham and Cremer, 1985). Possibly, it was because of unawareness that Hawkins and Miller had used a value of 1.25 for the lumped constant or because of lack of conviction that this value was wrong. It is, therefore, important to examine just how they arrived at that value. Instead of accepting the experimentally determined and published value of 0.48 for the lumped constant (So-

koloff et al., 1977), they chose to calculate the lumped constant from their experimental data by a transposed version of Eq. 8.

$$LC = \frac{[^{14}\text{C}]\text{DG-6-P} (T)}{R_i \left[\int_0^T \left(\frac{C_p^*}{C_p} \right) dt - e^{-(k_2^* + k_3^*)T} \int_0^T \left(\frac{C_p^*}{C_p} \right) e^{(k_2^* + k_3^*)t} dt \right]} \quad (9)$$

where the symbols are the same as those described for Eq. 8.

They assumed the same value, 0.7 $\mu\text{mol/g/min}$ for R_i , and solved this equation for the lumped constant, LC , at various times by using their measured values for [^{14}C]DG-6-P concentration in brain at each of these times. The value for LC thus calculated was 1.25 at 5 min, their earliest time, and progressively declined with time until at 45 min it was 0.4–0.5. They reported, however, only the theoretical [^{14}C]DG-6-P concentrations in brain that were computed with the lumped constant calculated at 5 min. Had they recomputed the theoretical values with each of the lumped constants calculated at the various times to see which fit the measured values best, they would have found the close agreement that exists between theoretical and experimental values when the lumped constant determined at 45 min is used. They may have rejected the later values of the lumped constant on the presumption that they were lower because of the effects of glucose-6-phosphatase. In that case, prior conclusions already arrived at would have been circularly incorporated into the analysis of experimental data that were collected to examine the validity of those conclusions.

The lumped constant of 1.25 at 5 min was, however, the most erroneous one of all. Their calculation of the lumped constant reflected the consequences of at least two serious errors that are responsible both for the high value at 5 min and the apparent decline of the lumped constant with time. First of all, in their sampling of arterial blood they failed to draw any samples during the first minute after the pulse of [^{14}C]DG. Instead, they extrapolated the arterial curve between 1 and 2 min semi-logarithmically back to zero time. They, therefore, lost the peak of the arterial curve which occurs during the first minute. The function, $\int_0^T (C_p^*/C_p) dt$, in the denominator of Eq. 9 represents the area under the curve for the specific activity in the arterial plasma with respect to time. The area under the peak that was lost represents a substantial fraction of the area during the first 5 min. Therefore, its loss results in too low a denominator and, therefore, a falsely high calculated lumped constant. The lost area under the peak is a constant; as the area under the curve increases with increasing time, the contribution of the lost area to the total area diminishes until by 45 min it becomes negligible. The ap-

parent decline of the lumped constant reported by Hawkins and Miller (1978), therefore, represents not a true change in the lumped constant but the consequences of a diminishing effect of the lost peak with time, which results in falsely high values at early times declining toward the correct value at later times. Secondly, in the exponential term in the denominator of Eq. 9 Hawkins and Miller (1978) used values for k_2^* and k_3^* which are arithmetic averages for some representative gray structures determined by Sokoloff et al. (1977) in normal rats. For reasons explained in Results these values could not possibly be accurate for the whole brain which is made up of innumerable structures, each with different rate constants and different masses. The exponential term in the denominator containing the rate constants is extremely critical at early times following the pulse, and at such times inaccuracies in the rate constants can produce large errors (Sokoloff, 1982). The exponential term, and, therefore, also the impact of the rate constants diminishes with increasing time until they become negligible at 45 min. It is precisely to minimize errors due to inaccuracies in the rate constants that 45 min was prescribed as the experimental period for the DG method. Calculation of the lumped constant by Eq. 9 at 5 min is just as subject to error as the use of the DG method with a 5-min experimental period. The most accurate value of the lumped constant determined by Hawkins and Miller (1978) is, therefore, the one that they determined at 45 min and the one that leads to close agreement between their predicted and their measured values of [^{14}C]DG-6-P concentration, thus negating their claim of significant deoxyglucose-6-phosphatase activity in brain (Sokoloff, 1980, 1982).

There is also direct experimental evidence that the lumped constant does not change with time. The lumped constant is essentially the ratio of the cerebral extraction of DG from the blood to that of glucose when both are in steady states (Sokoloff et al., 1977). This is the basis of the method for measurement of the lumped constant (Sokoloff et al., 1977). In practice, a programmed infusion designed to achieve a constant arterial plasma [^{14}C]DG concentration is administered and maintained until a steady state of uptake of [^{14}C]DG by brain is reached as already exists for glucose. The steady state is manifested by a constant arteriovenous difference while the arterial concentration is also constant. If the arteriovenous differences for both hexoses remain constant with time, then the lumped constant must also remain constant because it is calculated from them. In Fig. 6 are illustrated the results of three such experiments in three different species of animals. It is clear that constant arteriovenous differences are achieved after a period of equilibration and then maintained constant up to at least 40–45 min. The lumped constant is, therefore,

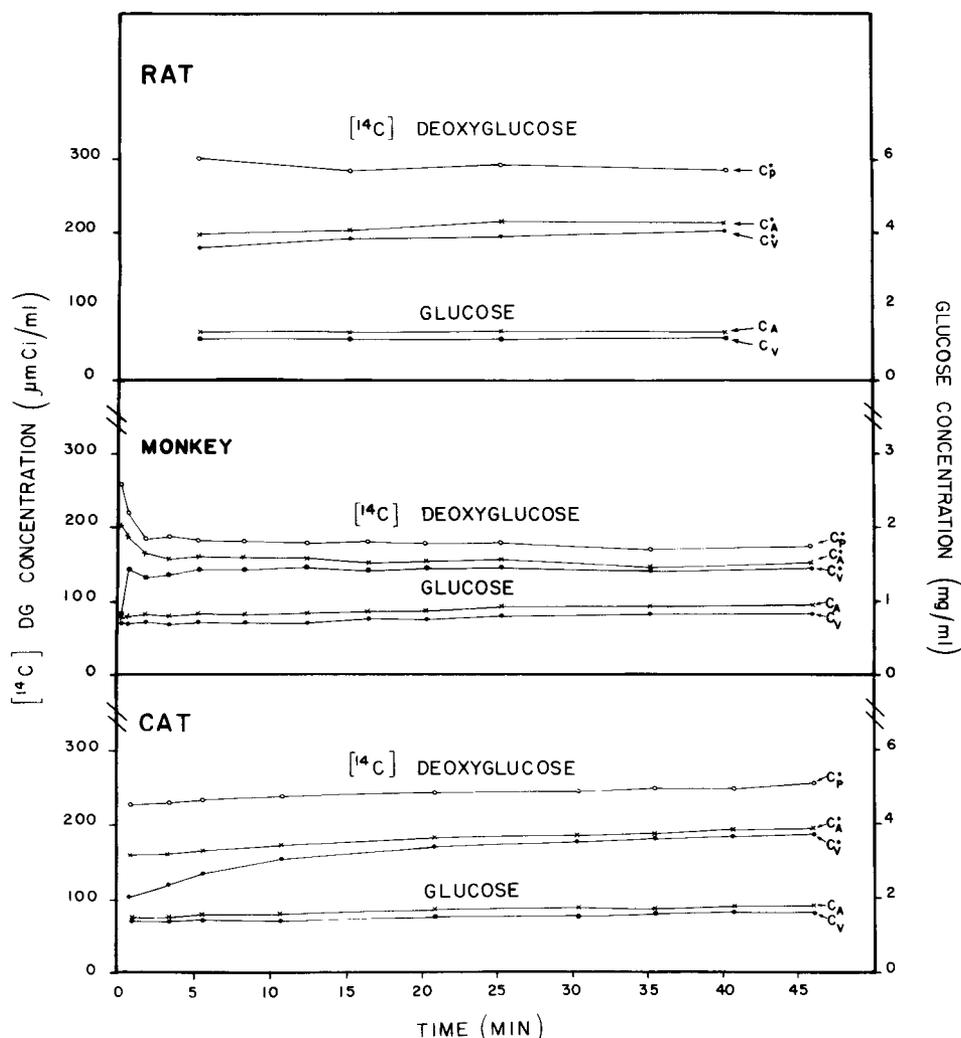


FIG. 6. Representative cerebral arteriovenous differences for [^{14}C]DG and glucose during programmed intravenous infusion of [^{14}C]DG to achieve constant arterial plasma concentrations and steady-state conditions in three species of animals. C_p^* represents arterial plasma [^{14}C]DG concentration. C_a^* and C_v^* are arterial and cerebral venous blood concentrations of [^{14}C]DG respectively, and C_a and C_v are the corresponding concentrations for glucose. Note that there is a period of equilibration following the onset of infusion at zero time, which varies in duration from animal to animal, but that eventually a steady state is achieved that is characterized by a sustained constant arteriovenous difference for [^{14}C]DG as it is for glucose (Kennedy et al., 1976; Sokoloff et al., 1977). It is inappropriate to average the above curves from all the animals because the actual concentrations in blood and plasma and the arteriovenous differences vary with various conditions, such as the dose of tracer, plasma glucose concentration, blood flow, rate of cerebral glucose utilization, etc. in each animal. The calculation of the lumped constant utilizes the same data but normalizes for all these extraneous factors by depending primarily on the ratio of the fractional extractions of DG and glucose from the blood. The data in the curves are used directly in the calculation of the lumped constant which is given by the following relationship: $LC = (E^*/E) (C_a^*/C_a) (C_p^*/C_p)$, where $E^* = (C_a^* - C_v^*)/C_a^*$ and $E = (C_a - C_v)/C_a$. The lumped constant for each animal is normally determined as the average of the values obtained by the above relationship at various time points throughout the steady-state period unique to that animal and not from the final value. Almost all animals achieved the steady state by 20 min. When we compute the correlation coefficient between the calculated lumped constant at individual time points obtained in all of the animals with time over the steady-state period of 20–45 min, no significant correlation with time is found ($r = -0.219$, $p = 0.676$). Any downward drift with time in the cerebral extraction of DG from the blood due to deoxyglucose-6-phosphatase activity would be reflected by a comparable downward drift in the value of the lumped constant. The lumped constant does not appear to change, therefore, with time between 20 and 45 min, the period of the steady state.

constant over the same period. Furthermore, the results in Fig. 6 provide additional evidence that there are no significant effects of [^{14}C]deoxyglucose-6-phosphatase activity in brain. For a steady state of uptake and a constant arteriovenous differ-

ence of [^{14}C]DG to be achieved, there must be an irreversible trap. Because deoxyglucose metabolism does not proceed substantially beyond DG-6-P, the trap must be at the phosphorylation step. If there were significant glucose-6-phosphatase ac-

tivity, then the back reaction, i.e., the release of free [^{14}C]DG by hydrolysis, would increase as the [^{14}C]DG-6-P accumulated. The efflux of [^{14}C]DG from brain would then gradually equilibrate with the influx, resulting in a progressive decline in the arteriovenous difference toward zero. There is no evidence from the results in Fig. 6 of any systematic decline in the cerebral arteriovenous differences for [^{14}C]DG with time, after the steady state has been achieved, indicating no significant effects of glucose-6-phosphatase activity over the duration of the experimental period.

Hawkins and Miller (1978) also claimed that their experimental data showed a constant rather than increasing level of [^{14}C]DG-6-P in brain between 10 and 45 min after the pulse and attributed this to glucose-6-phosphatase activity. They measured the brain [^{14}C]DG-6-P concentrations at six different times after the pulse. Their data show a fair degree of scatter. If one had complete faith in the absolute accuracy of the data, then one would have to conclude that the [^{14}C]DG-6-P concentration in brain oscillates with time, an unlikely possibility. The value of 10 min is considerably higher than those at 20 and 30 min and approximately equal to the values at 25 and 45 min. It also had the largest SEM despite the fact that it represented the average of the largest number of animals in the series, suggesting that there was considerably greater scatter in this group than in those of the other groups. Even with the scatter the other five points demonstrate a clear trend for progressive accumulation of [^{14}C]DG-6-P in brain somewhere between those seen in Fig. 1 and 2. If the value at 10 min is accepted as fully reliable, then it does appear to obscure the trend. The experimental data presented by Hawkins and Miller (1978) are, therefore, highly problematical on a statistical basis and hardly adequate evidence on which to conclude that there is deoxyglucose-6-phosphatase activity. In any case, the experimental data summarized in Figs. 1 and 2 do not support their conclusions.

More recently, Huang and Veech (1982) reported what they considered to be evidence of unexpectedly high levels of glucose-6-phosphatase activity in brain. They injected as a pulse into a carotid artery of rats a mixture of [$2\text{-}^3\text{H}$]glucose and [$\text{U-}^{14}\text{C}$]glucose, removed the brains by freeze-blowing at various times up to 5 min later, and assayed the tissue glucose content for the $^3\text{H}/^{14}\text{C}$ ratio in it. The principle is as follows. Any [$2\text{-}^3\text{H}$]glucose that reaches the fructose-6-phosphate step in glycolysis loses the ^3H label, but [^{14}C]fructose-6-phosphate does not lose the ^{14}C label. By reversal of the phosphohexose isomerase step some fructose-6-phosphate is converted back to glucose-6-phosphate, and if there is glucose-6-phosphatase activity, the glucose moiety enters the glucose pool without losing the ^{14}C label, but without the ^3H label. The

$^3\text{H}/^{14}\text{C}$ ratio in the glucose pool should then fall in case of phosphatase activity, and this is what they claim to have observed. The principle is sound.

A key issue, however, is the separation of glucose from all the other possible labeled products of glucose metabolism in brain tissue that, if present, could lead to the same results without the need to invoke phosphatase activity. In the purification procedure used by Huang and Veech (1982), the fraction designated by them as glucose is contaminated with significant amounts of other labeled substances that have lost the ^3H but not the ^{14}C (Nelson et al., 1985*a,b*). On the other hand, when a more extensive purification procedure is used to ensure purity of the glucose fraction, there is no change in the $^3\text{H}/^{14}\text{C}$ ratio of the glucose in brain with time from that of the glucose recirculating in the plasma or in the injectant (Nelson et al., 1985*a,b*). The result of Huang and Veech (1982) was an artifact due to contamination of the fraction that they designated as glucose with labeled products of glucose metabolism. There is, therefore, no evidence of significant glucose-6-phosphatase in brain on the basis of selective detritiation of [$2\text{-}^3\text{H}$]glucose in brain because it does not occur.

In a subsequent report Huang and Veech (1985) claimed to have demonstrated significant rates of dephosphorylation of [^{14}C]DG-6-P by fitting an equation containing rate constants for both phosphorylation of [^{14}C]DG and dephosphorylation of [^{14}C]DG-6-P to measured values of free [^{14}C]DG and [^{14}C]DG-6-P in rat brain obtained in experiments similar to those illustrated in Fig. 1A. The choice of the equation to be fitted is arbitrary and may include parameters that do not in reality exist. The fitting routine, however, assigns best-fit values to the parameters included in the equation regardless of the reality. In other words, the parameters to be fitted are already predetermined by the model that has been selected. With sufficiently scattered experimental data fits can be obtained with a variety of equations and models which cannot reliably discriminate between those that are valid and those that are not. The experimental data of Huang and Veech (1985) were extraordinarily scattered. Their fitted curve fit the measured values for [^{14}C]DG-6-P concentration in brain obtained early in the time course, when the effects of glucose-6-phosphatase are still minimal, fairly well, but clearly underestimates the [^{14}C]DG-6-P concentrations compared to the measured values late in the time course when the effects of glucose-6-phosphatase would be most apparent (see Fig. 4 of report of Huang and Veech, 1985). This study of Huang and Veech (1985) does not prove the existence of deoxyglucose-6-phosphatase activity in brain because fitting routines can only provide estimates of parameters in models already decided on and cannot prove the validity of a model, particularly with widely scattered data.

The lack of effects of glucose-6-phosphatase activity on the DG method, when it is used as prescribed, is not to be construed as evidence that the enzyme is absent in brain. Its presence in brain has been demonstrated by histochemical techniques (Stephens and Sanborn, 1976; Broadwell et al., 1983), and assays of the enzyme activity in whole or fractionated homogenates of brain *in vitro* have shown low levels of activity in the native state (Hers, 1957; Sokoloff et al., 1977) that are enhanced when detergents are added to the incubation medium (Anchors and Karnovsky, 1975; Hawkins and Miller, 1978). Furthermore, when the experimental period of the DG method is extended beyond the recommended limit of 45 min, effects of deoxyglucose-6-phosphatase activity begin to appear which become progressively more profound with increasing time (Sokoloff, 1982; Deuel et al., 1985). The kinetics of a long lag followed by progressively increasing activity of the enzyme indicates a rate-limiting step interposed between the formation of the [¹⁴C]DG-6-P in the cytosol and its hydrolysis by the phosphatase located on the inner surface of the membranes of the cisterns of the endoplasmic reticulum. Fishman and Karnovsky (1986) have found that the specific carrier that transports hexose-6-phosphates across the endoplasmic reticular membrane is absent in brain, thus making the transport step more rate-limiting and the lag longer. The effect of this lag in the expression of the glucose-6-phosphohydrolase activity provides, in effect, a window in time between the pulse of labeled DG and loss of its phosphorylated product. The procedure of the DG method is designed to take advantage of this window. The prescribed duration of the experimental period, 45 min, was selected because it was long enough to minimize errors that might accrue from inaccuracies in the values of the rate constants and yet short enough to avoid the consequences of the phosphatase activity. The present report demonstrates that all the allegations of errors introduced by glucose-6-phosphatase activity during that experimental period are without validity.

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