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Residual effects of tracer in sequential double label deoxyglucose studies*

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The validity of sequential double label deoxyglucose (DG) determinations of local metabolic rate for glucose (IMR_{glc}) was examined by quantifying the degree of trapping of residual first DG tracer during the second experimental period. One sciatic nerve was repetitively stimulated for 25 min, beginning either at the time of the DG injection or 25 min later. IMR_{glc} in the ipsilateral dorsal horn of the lumbar spinal cord was found to be 105% and 56%, respectively, greater than that of the contralateral unstimulated side. Attempts to lower the body burden of radioactive DG by exchange blood transfusion failed to reduce this delayed effect. These data indicate that residual effects of the first tracer could obscure possible differences in IMR_{glc} between two sequential experimental states.

The deoxyglucose (DG) method for determination of local rates of glucose utilization (IMR_{glc}) is used extensively to study regional brain responses to physiological, pharmacological and behavioral perturbations [14]. Because it was designed for use with quantitative autoradiography [15], the technique can measure IMR_{glc} only once in each animal. The ability to measure IMR_{glc} twice sequentially in the same animal [1] would allow a single animal to serve as its own control; experimental variance and the number of animals needed to obtain statistical significance could then be reduced. Furthermore, metabolic maps of the two conditions in the same brain sections would facilitate analysis of more subtle differences in anatomical localization of activated areas. Several groups [4, 7, 12] have proposed the use of double label autoradiographic DG studies with ³H- and ¹⁴C-labeled DG administered sequentially to the same animal with each injection temporally associated with a different experimental condition. The assumption underlying these double label measurements is that the IMR_{glc} measured with each species of labeled DG reflects the condition of the animal during the experimental period immediately following the injection [4, 7]. Only in the studies of Re-

dies et al. [12] was there consideration of the effect of the second condition on the residue of the first tracer in the tissue; in these studies application of a mathematical correction based on tracer kinetic modeling was proposed.

The purpose of the present study was to determine quantitatively and by direct experiment the effects of the second experimental condition on the metabolic trapping of the first tracer still remaining in the animal while keeping the total duration of the experiment close to the established optimum of 45 min [9]. We used the reproducible effects of unilateral, repetitive, electrical stimulation of the sciatic nerve on IMR_{glc} in the dorsal horn of the ipsilateral lumbar spinal cord of the rat for these determinations [6]. Stimulation during the 25 min beginning either at the time of DG injection or 25 min later resulted in rates of glucose utilization in the ipsilateral dorsal horn of the lumbar spinal cord that were 105% and 56%, respectively, greater than those of the contralateral unstimulated control side. Attempts to decrease the residual DG in the animal by exchange blood transfusion resulted in only small (~10%) reductions in the relative effect; metabolic trapping of the first tracer during the second experimental condition remained significant.

Male, Sprague–Dawley rats (300–430 g) (Taconic Farms, Germantown, NY, USA), fasted for 14–16 h, were anesthetized with sodium pentobarbital (45 mg/kg, i.p.) and polyethylene catheters were inserted bilaterally into femoral arteries and veins. Anesthesia was maintained at the level of extinction of the corneal reflex with

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sodium pentobarbital (i.v.) as needed. Body temperature was maintained at 37.5°C by means of a heat lamp. Arterial blood pressure, arterial blood pH, pO_2 , pCO_2 , and hematocrit were monitored during the experiment; the mean values \pm standard deviations for these variables in all experimental animals were 100 ± 12 mm Hg, $51 \pm 2\%$, 7.44 ± 0.05 , 82 ± 6 mm Hg and 45 ± 5 mm Hg, respectively. There were no statistically significant differences among experimental groups in any of the physiological variables.

Sciatic nerves were exposed bilaterally and transected near the gluteus muscles; paraffin oil was pooled around the nerves to prevent desiccation. The proximal portion of the right sciatic nerve was placed on a bipolar electrode (cathode proximal) and stimulated at 15 Hz, 200–400 μ A with pulses 2 ms in duration. Stimulation lasted for 25 min beginning either at 0 time (the time of tracer injection) or 25 min after tracer injection. Five groups were studied: (1) no stimulation, $n=4$; (2) stimulation from 0 to 25 min, $n=4$; (3) stimulation from 25 to 50 min, $n=4$; (4) stimulation from 0 to 25 min with exchange blood transfusion, $n=2$; (5) stimulation from 25 to 50 min with exchange blood transfusion, $n=4$. The purpose of the exchange transfusion was to decrease residual DG available during the second stimulation period. Donor blood containing heparin (30 U/ml) was pumped at 4 ml/min into the right femoral vein while radioactive blood was pumped out at the same rate from the right femoral artery into a waste container. Transfusions were started 10 min after tracer injection and lasted for 10 min resulting in at least one full exchange of total blood volume.

The period of measurement was initiated by an i.v. pulse injection of 2-deoxy-D-[1- 14 C]glucose (52.7 mCi/mole, DuPont-NEN, Wilmington DE, USA) (125 μ Ci/kg b.wt.). Timed arterial blood samples (50 μ l) were taken throughout the 50-min experimental time as follows: at 0 time (the time of tracer injection) blood was sampled continuously for 30 s followed by samples at 0.75, 1, 1.5, 3, 5, 7.5, 10, 15, 20 and 25 min; at 25 min blood was again sampled continuously for 30 s followed by samples at 25.75, 26, 26.5, 28, 30, 32.5, 35, 40, 45 and 50 min. This sampling schedule was designed to simulate a sequential double label procedure. All rats were given 2 ml of donor blood at 20 min. At 50 min rats were killed with sodium pentobarbital (i.v.), and the lower thoracic to upper sacral spinal cord was quickly removed and frozen in isopentane (-45°C). Serial cryostat sections, 20 μ m thick, were prepared for autoradiography [15] on OMC-1 film (Eastman Kodak Co., Rochester, NY, USA). Concentrations of ^{14}C in the medial portions of lamina 2–6 of the dorsal horn and lamina 7–9 of the ventral horn in alternate sections through the lumbar

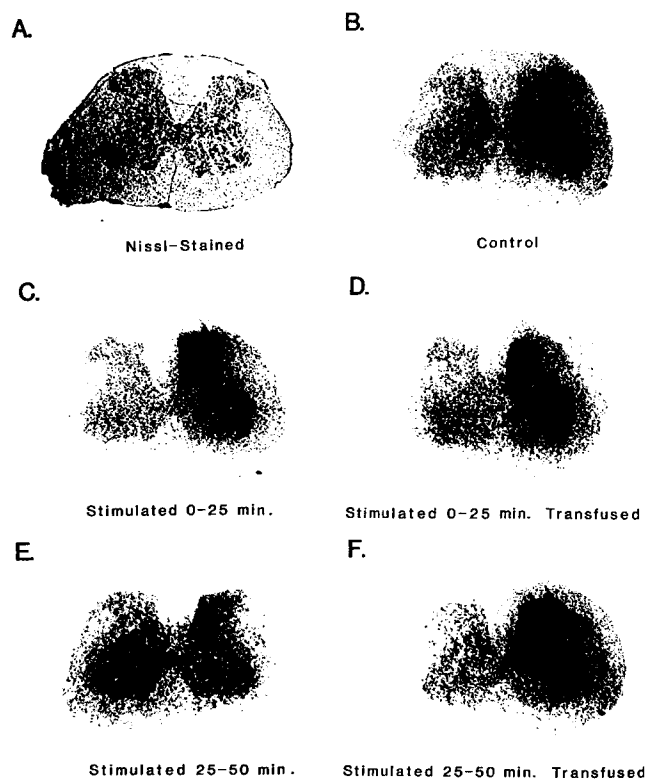


Fig. 1. Typical [^{14}C]deoxyglucose autoradiograms of sections of lumbar spinal cord illustrating each experimental condition (B–F) as indicated. A: a thionin-stained section of the lumbar spinal cord. Right side is on the right.

spinal cord were determined by image analysis of the autoradiograms (M1, Imaging Research Inc., St. Catharines, Ontario, Canada). Values for IMR_{glc} were calculated by the operational equation of the DG method [15]. Each regional IMR_{glc} is the mean value for 18–24 sections of lower lumbar cord. Side-to-side differences were analyzed by paired t -tests. Comparisons of side-to-side differences between groups were statistically analyzed by analysis of variance. With each data set subjected to three comparisons, effects were considered statistically significant with $P < 0.017$.

Electrical stimulation of one sciatic nerve, regardless of the period of stimulation, increased IMR_{glc} in the ipsilateral dorsal but not in the ventral horn of the lumbar spinal cord (Fig. 1, Table I). Stimulation of the sciatic nerve beginning at the time of DG injection (0 time) and continuing for 25 min resulted in a 105% ($P < 0.001$, paired t -test) increase in IMR_{glc} in the ipsilateral dorsal horn above that of the contralateral control side (Table I). When stimulation was during the second half of the experimental interval (i.e. from 25–50 min), IMR_{glc} in the ipsilateral dorsal horn was still statistically significantly increased over the contralateral control side by 56% ($P < 0.001$, paired t -test). Comparison of the side-to-side differences in IMR_{glc} in the dorsal horn between animals

TABLE I

EFFECTS OF UNILATERAL ELECTRICAL STIMULATION OF SCIATIC NERVE ON IMR_{glc} IN LUMBAR SPINAL CORD

Stimulation period and conditions	n	IMR_{glc} ($\mu\text{mol}/100 \text{ g}/\text{min}$)		Mean difference (stimulated-control)	Mean percent difference [†]
		Control side	Experimental side		
Dorsal horn					
No stimulation	4	35.8 ± 3.2	36.3 ± 3.5	-0.5 ± 0.6	-1.3 ± 1.6
0-25 min	4	31.5 ± 2.3	64.3 ± 3.6*	32.8 ± 1.8	105.2 ± 6.7
25-50 min	4	30.5 ± 2.4	47.2 ± 2.4*	16.7 ± 0.5**	55.9 ± 5.2
0-25 min with blood transfusion	2	31.5 ± 5.2	64.5 ± 4.7*	33.0 ± 0.5	108.0 ± 19.4
25-50 min with blood transfusion	4	33.4 ± 3.2	48.3 ± 4.9*	14.9 ± 1.9	44.2 ± 2.9 ^{††}
Ventral horn					
No stimulation	4	38.6 ± 2.9	38.7 ± 3.0	0.1 ± 0.2	0.2 ± 0.6
0-25 min	4	33.3 ± 2.7	39.5 ± 3.2	6.2 ± 2.4	19.1 ± 7.3
25-50 min	4	34.5 ± 2.0	36.7 ± 2.5	2.2 ± 0.5	6.2 ± 1.2
0-25 min with blood transfusion	2	37.6 ± 6.6	43.2 ± 6.8	5.5 ± 0.2	15.0 ± 2.1
25-50 min with blood transfusion	4	38.3 ± 3.7	39.4 ± 3.9	1.0 ± 0.4	2.6 ± 1.2

Values are the means ± S.E.M. for the number of animals indicated.

*Statistically significantly different from the control side, $P < 0.01$; paired *t*-test.

**Statistically significantly different from the difference between control and stimulated from 0-25 min, $P < 0.001$.

[†]Percent differences are means of individual percent differences and not percent differences between the means.

^{††}Statistically significantly less than that of the rats stimulated from 25-50 min but not transfused, $P < 0.05$ (one-tailed *t*-test).

stimulated from 0-25 min and 25-50 min (Table I) shows that while both periods of stimulation resulted in significant effects, the effects of stimulation during the first 25 min were statistically significantly greater than those of the stimulation during 25-50 min ($P < 0.001$, analysis of variance).

In an attempt to reduce residual blood and tissue levels of free DG during the second period (i.e. from 25-50 min), rats were subjected to exchange blood transfusions between 10 and 20 min after the pulse of [¹⁴C]DG, well after the peak of DG in plasma. Plasma [¹⁴C]DG levels were reduced by the transfusions, and the percent effect of stimulation from 25-50 min on IMR_{glc} in the dorsal horn was statistically significantly smaller in the rats which received the exchange blood transfusion as compared to those which were not transfused (Table I).

The design of a sequential double label autoradiographic DG study depends on adequate separation of the signals from the two tracers and separation of the metabolic effects of the two conditions. Separation of the tracers in the autoradiographic process can be achieved by the use of optimal ratios of ¹⁴C to ³H activity and mylar film, which absorbs the lower energy β -particles of ³H with minor attenuation of the ¹⁴C signal [5, 7]. Separation of the two conditions, however, is complicated by restrictions of the DG method and the slow clearance of free DG from tissues and plasma. The method requires that the product, DG-6-P, remains trapped in the tissue for

the duration of the experiment, but by 60 min after a pulse some loss of product occurs in the rat [10]. We tried, therefore, to restrict the total experimental interval to 50 min which is close to the established optimum of 45 min [9]. There were, therefore, 25 min allowed for each of the two experimental conditions, i.e. injection of the first tracer at time 0 and the second tracer at 25 min. A potential source of error is in the calculation of the integrated specific activity of tissue DG at 25 min after injection of the second DG tracer. This calculation depends on the estimated half-life ($T_{1/2}$ of tissue DG which is normally about 2.4 min [15]. If, however, the $T_{1/2}$ of DG in the tissue under the experimental conditions is reduced ($T_{1/2} \rightarrow 0$ min) or prolonged ($T_{1/2} \rightarrow 5$ min), then the error in the calculated integrated specific activity of DG in grey matter at 25 min would range from -7% to +10%, respectively [14].

In the present studies we used only a single label with the [¹⁴C]DG injected at 0 time because our goal was to examine the effect of the second experimental condition from 25-50 min on the metabolic response produced with the residual first tracer. In the case of stimulation from 0-25 min, the mean percent difference in IMR_{glc} in the dorsal horn between the stimulated and control sides was 105%, while in the case of stimulation during the second 25 min it was 56%, approximately half the effect produced by stimulation during the first 25 min (Fig. 1, Table I). The difference in the magnitude of the effects of

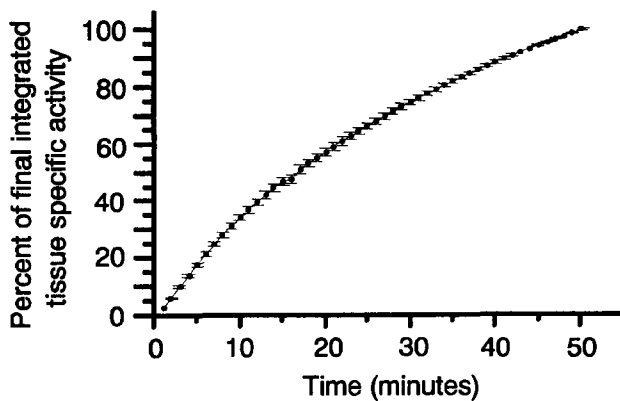


Fig. 2. Time course of the integrated specific activity of DG in grey matter as a percent of the final value at 50 min. Each point is the mean \pm S.E.M. of 4 experiments.

stimulation during the first and second periods on IMR_{glc} can be related to the differences in the magnitudes of the integrated tissue DG specific activities to which the brain is exposed during these two time intervals, as predicted by the model for the behavior of DG in brain. Based on this model, the values of the fitted rate constants for grey matter [15], and the time courses of the measured plasma levels of glucose and DG, the integrated tissue specific activity of DG can be calculated over the entire time course for each experiment. At 25 min, the integrated tissue specific activity for DG is 67% of the final value, whereas the tissue DG specific activity integrated from 25–50 min is approximately 33% of the final value (Fig. 2). The model, therefore, predicts that during the interval from 25–50 min following a pulse injection, the amount of DG available for phosphorylation is about 50% of the amount available during the interval from 0–25 min; this prediction is consistent with our results (Table I) in which the effect of stimulation from 25–50 min on IMR_{glc} in the dorsal horn is approximately 50% of the effect of stimulation from 0–25 min.

Effects of residual DG have been observed in studies of auditory stimulation in guinea pigs [16], visual stimulation in monkeys [3], and somatosensory stimulation in rats [2], but the magnitude of these effects were not quantified. Solutions to this problem have been proposed. Redies et al. [11, 13] used a model-dependent mathematical approach in their sequential 45-min intervals for the two tracers, i.e. total experimental period of 90 min. They used a model which included loss of product and an operational equation for IMR_{glc} that corrects the results obtained with the first tracer for both loss of labeled product and superimposed effects of the second condition on the estimated IMR_{glc} . The limitation of this approach is that its accuracy depends entirely on the valid-

ity of the model and the estimated values for the rate constants. Friedman et al. [5] have attempted to optimize the experimental intervals by allowing 35 min for clearance of the first tracer followed by a 10-min interval for the second tracer and second condition. Although this approach stays within the optimal 45-min total experiment time and, therefore, circumvents problems of loss of product, the accuracy of determinations of IMR_{glc} with a 10-min experimental interval is heavily dependent on the accuracy of the values of the rate constants [14].

In the present study we tried to reduce the level of first tracer available for phosphorylation during the second experimental period by replacing the experimental animal's blood with donor blood. The exchange of 40 ml of blood (equivalent to the entire blood volume) did increase the clearance of DG from the plasma; at 50 min plasma levels were about 33% below those of matched controls. The mean percent increase in IMR_{glc} in the dorsal horn during stimulation from 25–50 min (Table I) was reduced in the transfused animals, i.e. 44% as compared with 56%, but the effects of the transfusion were insufficient to eliminate the effect of the second condition in a double label experiment. The most likely explanation for the relative ineffectiveness of the blood exchange is that it is the tissues, e.g. liver, muscle, fat etc., that serve as a reservoir from which free DG is delivered to the blood and subsequently to the brain. It is possible that more extensive exchanges of blood might have more significant effects. The results of this study indicate, however, that the effect of the second experimental interval on IMR_{glc} determined with the first tracer is substantial and that exchange blood transfusion does not resolve this problem.

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