An enzymatic microassay method for deoxyglucose and deoxyglucose 6-phosphate

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ABSTRACT

An enzymatic microassay method was developed to determine 2-deoxyglucose (DG) and 2-deoxyglucose 6-phosphate (DG6P) in the nanogram to microgram amount of tissue samples. Glucose and glucose 6-phosphate (G6P) in the sample were first removed because these substrates caused a high blank in the present assay system. In the assay for *DG plus DG6P*, elimination of glucose and G6P was accomplished by converting them to fructose 1,6-diphosphate (FDP) in the reaction mixture containing hexokinase, glucose phosphate isomerase and phosphofructokinase; DG in the sample was simultaneously phosphorylated to form DG6P during this incubation. DG6P, originally present in the sample and newly formed in the above reaction, was then oxidized by adding excess NADP⁺, and glucose 6-phosphate dehydrogenase (G6PDH) at a high concentration. The resultant NADPH was amplified 3,000–60,000-fold by using NADP cycling, and fluorometrically determined. In the assay for *DG6P alone*, glucose in the sample was first converted to D-glucono- δ -lactone, and G6P to FDP. NADP⁺ and G6PDH were then added, and NADPH was determined as above. The amount of DG was obtained as the difference between *DG plus DG6P* and *DG6P alone*. With this method, DG and DG6P were measured in single mouse morulae and in microgram tissue samples from various parts of the rat central nervous system.

2-Deoxy-D-glucose (DG) is actively incorporated into cells, especially into neurons, by the same carrier as for glucose, and phosphorylated to 2-deoxy-D-glucose 6-phosphate (DG6P) by hexokinase (HK). DG6P is hardly metabolized further and accumulated in the cells. Sokoloff and his colleagues (5) developed the deoxyglucose method, in which [¹⁴C]DG or [³H]DG was administered to animal and DG6P accumulated in small cerebral regions was semiquantitatively determined by autoradiography. The degree of DG6P accumulation is thought to reflect the functional activity of the cerebral regions, since glucose is the main source of energy in neurons. Recently, Chi *et*

al. (2) found that glucose 6-phosphate dehydrogenase (EC 1.1.1.49, G6PDH; for other abbreviations, see Fig. 1) oxidized DG6P at a rate 1,000-fold slower than for G6P and they utilized G6PDH at a high concentration in enzymatic assays for DG and DG6P. In these enzymatic assays, glucose and G6P in samples need to be eliminated prior to the G6PDH reaction, since the endogenous glucose and G6P provide a high blank. When applied to small cerebral regions or single cells, their method is troublesome in practice and insufficient to eliminate the high blank (see Comments). We developed an enzymatic method that efficiently eliminated glucose and G6P,

Fig. 1 Enzymatic reactions and substrates in the microassay methods for DG and DG6P. G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; FDP, fructose-1,6-diphosphate; DG, 2-deoxyglucose; DG6P, 2-deoxyglucose-6-phosphate; 6PDG, 6-phosphodeoxygluconate; AE, aldose epimerase; GO, glucose oxidase; HK, hexokinase; G6PDH, G6P dehydrogenase; GPI, glucosephosphate isomerase; PFK, phosphofructokinase

and thus enabled us to determine DG and DG6P in single preimplantation embryos of mice and in small regions of the rat central nervous system.

Samples Male Sprague-Dawley rats (6 weeks of age, 180–200 g of body weight) were injected in the tail vein with 0.25–0.5 mmol of DG/kg of body weight in 0.2 ml of physiological saline and sacrificed 1–4 min later by immersion in liquid nitrogen. Freeze-dried sections (14–20 μ m thick) were prepared from the cerebellum, lumbar spinal cord and eyeball. Pieces of cerebellar layers, anterior horn of the spinal cord, white matter adjacent to the anterior horn, and neuroretina (0.7–2.3 μ g dry weight) were dissected out from the sections and weighed on a quartz fiber balance (4). Six female mice (Crj:B6C3F₁ strain, 6 weeks of age, 16–20 g of body weight) were injected intraperitoneally with 5 IU of pregnant-mare serum, followed 48 h later by intraperitoneal injection of 5 IU of human chorionic gonadotropin to induce superovulation. After the second injection, the females were placed with males, and 82–85 h later morulae were obtained by flushing the reproductive tract with culture medium (modified Briggs-Whitten-Whittingham medium containing 5.55 mM glucose). The isolated morulae were cultured for 30 min at 38°C in air. The medium contained 2.13 mM DG. The morulae were then cleaned in phosphate-buffered physiological saline, freeze-dried, weighed (32–35 ng dry

Fig. 2 Linearity of the assay for DG plus DG6P in the freeze-dried samples of anterior horn from rat spinal cord. A rat was injected intravenously with 0.25 mmol of DG/kg of body weight and sacrificed 2 min later. One microgram/assay on the abscissa corresponds to 0.52 mg dry weight/ml in 1.92 μ l of G6PDH reaction mixture; and 100 fluorometric divisions on the ordinate to 2.79 pmol of DG/assay. Closed circles represent the assay values. Open circles indicate the values for the anterior horn samples, which were prepared from a rat that did not receive DG. The concentration of endogenous glucose plus G6P was 4.54 μ M when 1 μ g dry weight of sample/assay was added in the elimination reaction mixture (1.44 μ l). Note that endogenous glucose and G6P were almost completely removed.

weight each), and used as a sample. In order to test the blank elimination reaction, the freeze-dried sample was prepared from the rat which did not receive DG (see Fig. 2).

Blank elimination reactions In the assay for DG plus DG6P, glucose and G6P were first converted to FDP (Fig. 1). The reaction mixture A consisted of 100 mM Tris-HCl (pH 8.1), 0.02% bovine serum albumin (BSA), 1 mM MgCl₂, 50 mM KCl, 0.5 mM phosphoenolpyruvate (PEP), 0.3 mM ATP, 15 μ g of HK/ml, 10 μ g of GPI/ml, 18 μ g of PFK/ml, 5 μ g of pyruvate kinase (PK)/ml and the sample. (All the enzyme preparations were purchased from Boehringer, Mannheim, F.R.G.) In this reaction mixture, DG was converted to DG6P, but not further since DG6P was not a substrate for GPI. As a result, the mixture contained the DG6P formed *in vitro* plus the DG6P originally present in the sample. To assay DG6P alone, glucose was converted to D-glucono- δ -lactone by two successive enzyme reactions catalyzed by AE and

GO (Fig. 1). Unless removed in this way, glucose was oxidized by G6PDH at a high concentration, and caused a high blank (2). Simultaneously, G6P was removed by conversion to fructose 1,6-diphosphate (FDP). The reaction mixture B was the same as the mixture A except for HK, which was replaced by 0.5 μ g of AE/ml and 60 μ g of GO/ml (Fig. 1). In the reaction mixtures A and B, ATP was produced by PK from PEP and ADP derived from added ATP; therefore the HK and PFK reactions were shifted to form FDP as much as possible.

Microassays for DG and DG6P The reaction mixture contained 100 mM Tris-HCl (pH 8.1), 0.02% BSA, 100 μ M NADP⁺, 50 μ g of G6PDH/ml and the sample. Volume of the reaction mixture was determined according to the size of samples. As an example, the assay procedure for the microgram tissue from the rat central nervous system will be described below. A weighed sample was added to 480 nl droplet of 0.1 M HCl placed at the bottom of a small well (3×3 mm), which was made in a Teflon plate (28×130×3 mm, oil-well rack) and filled with paraffin oil to prevent evaporation of the droplet (4). After heating the rack at 95°C for 15 min to destroy enzymes in the sample, the droplet was neutralized by adding 480 nl of 0.1 M NaOH. Subsequently, the same volume of 3-fold concentrated reaction mixture A was added to the neutralized droplet, and the endogenous glucose and G6P were eliminated as described above. Then, 480 nl of 4-fold concentrated G6PDH reaction mixture was added to the droplet and DG plus

DG6P were converted to NADPH. After adding 480 nl of 1 M NaOH, the oil-well rack was heated at 70°C for 30 min to destroy the unreacted NADP⁺, keeping the NADPH formed almost intact. The whole droplet was added to 50 μ l of NADP cycling mixture to amplify the NADPH from 3,000 to 12,000-fold for fluorometry (1). In the assay for DG6P, the elimination reaction mixture B was used in place of the mixture A. The procedure was the same as above. The assay mixture was used as a blank, since the endogenous glucose and G6P in samples were completely removed by the elimination reactions and did not contribute to the blank for assay (see Fig. 2). The blank and the standards, containing DG or DG6P at several appropriate concentrations in the assay mixture, were run in parallel with the assay. The difference in values between the *DG plus DG6P assay* and the *DG6P assay* was calculated to obtain the *DG value* (see Table 1).

For the assay of a single morula, the volume of HCl and NaOH and of the concentrated reaction mixtures was reduced from 480 to 20 nl. The procedure for the DG plus DG6P assay was the same as for microgram samples and the amplification by NADP cycling was 10,000-fold. In the DG6P assay for a single morula, the whole droplet, after destroying the unreacted NADP⁺, was added to 5.44 μ l of the NADP cycling mixture in an oil well and the 10,000-fold amplification reaction was carried out. The cycling mixture, heated to stop the reaction, was added to 100.5 μ l of the indicator reaction mixture (1) in a Pyrex tube, and

Table 1 *Contents of Glucose, G6P, DG and DG6P in Mouse Morulae*

	Glucose plus G6P (A)	G6P (B)	Glucose (A-B)
fmol/morula	779 $\pm$ 90*	46.3 $\pm$ 3.2	733
mmol/kg dry wt.	24.1 $\pm$ 1.2	1.35 $\pm$ 0.09	22.8
Number of samples	13	10	
	DG plus DG6P (A)	DG6P (B)	DG (A-B)
fmol/morula	398 $\pm$ 25	126 $\pm$ 7	272
mmol/kg dry wt.	12.1 $\pm$ 0.7	3.77 $\pm$ 0.18	8.3
Number of samples	10	14	

For the conditions under which morulae were incubated with DG, see the text. The dry weight of the morula was 33.9 $\pm$ 0.5 ng* for 24 samples. *Mean $\pm$ SEM

then NADPH formed, in an amount 10,000-fold that of DG6P, was converted to highly fluorescent substance by adding 1 ml of 6M NaOH, containing 10 mM imidazole and 3 mM H₂O₂ (strong alkali treatment; see reference 4). The overall amplification was 50,000–60,000-fold.

Microassays for glucose and G6P The assay procedures were the same as for DG and DG6P except that the elimination reactions were omitted. In the G6PDH reaction mixture, the concentration of G6PDH was lowered from 50 to 0.2 μ g/ml. *Glucose plus G6P* and *G6P* were measured separately, and the difference between the two assay values was calculated to obtain the *glucose value* (see Table 1).

Characteristics of the present assays

When glucose lower than 200 μ M was added to the mixture A, more than 98% of glucose were eliminated by HK, GPI, PFK and PK (Fig. 1). In the mixture B, the blank due to 1 mM glucose was almost completely eliminated by AE and GO. The fluorometric reading was proportional to the concentration of DG up to at least 25 μ M in the G6PDH reaction mixture containing 100 μ M NADP⁺. In the assay of freeze-dried anterior horn of rat spinal cord, the reading was linear up to 1.8 mg of dried tissue/ml of G6PDH reaction mixture (Fig. 2).

DG and DG6P contents in single morulae

When the morula was incubated with 2.13 mM DG, the content of DG increased but gradually leveled off during 1 h incubation. In contrast, the content of DG6P increased linearly with time during the incubation period (data not shown). The dry weight, and the concentrations of glucose and G6P did not alter during this incubation. The contents and concentrations of glucose, G6P, DG and DG6P in the single morula cultured with DG for 30 min are shown in Table 1.

DG and DG6P concentrations in the small regions of the nervous system As shown in Fig. 3, the neuroretina consisting of a dense population of small neurons showed a markedly high concentration of glucose. The gray matter also showed a higher concentration of glucose than did the white matter in the cerebellum and spinal cord. The concentration of G6P was lower than one-tenth of the glucose concentration in all the regions, similarly as in

Fig. 3 The concentrations of glucose, G6P, DG and DG6P in the small regions of the rat central nervous system. Two rats were injected intravenously with 0.5 mmol of DG/kg of body weight and sacrificed 4 min later. To minimize the individual variations, 5 samples from each rat were assayed and the SEM for 10 samples is indicated by the symbol T on the top of the open bar. A: Open bars indicate average G6P concentrations; solid bars, the concentrations of glucose calculated from the assay values of glucose plus G6P and G6P. B: The average concentrations of DG and DG6P are expressed in the same way as in A. Open bars represent DG6P; solid bars, DG. ML, molecular layer; GL, granular layer; WM, white matter; and AH, anterior horn

single morulae (Table 1). The higher activity of DG6P formation was found in the neuroretina than in the gray matter of the cerebellum and spinal cord. Both DG uptake and DG6P formation were less active in the white matter compared with the regions containing neurons, in accord with the previous reports based on the autoradiography using [¹⁴C]glucose (3) and [¹⁴C]DG (5).

Comments In the enzymatic assay method reported by Chi *et al.* (2), the endogenous glucose is converted to G6P by HK, and this G6P and the endogenous G6P are subsequently converted to NADPH by G6PDH at a low concentration, which negligibly oxidizes coexisting DG6P. The NADPH

is then destroyed in the acidified reaction mixture (4) to eliminate the blank from the endogenous glucose and G6P. The mixture is neutralized by adding alkali, and a high concentration of G6PDH is added to the mixture for determination of DG6P. Compared with our blank elimination reaction, the above procedure needs two more steps, *i.e.* acidification and neutralization steps, to eliminate the blank from the endogenous glucose and G6P. In the microassay of single morulae using 20 nl of HCl and NaOH solutions, these extra steps are very troublesome in practice. In addition, much NADP⁺ is needed to eliminate the endogenous glucose, whose concentration is more than several-fold higher than those of DG and DG6P in the tissue samples (Fig. 3). Prior to the cycling reaction, the unreacted NADP⁺ is destroyed by heating in the alkaline mixture as described in the Microassays for DG and DG6P. Consequently, the destruction product of excess NADP⁺ results in a high concentration in the cycling mixture and lowers the cycling reaction rate. Furthermore, a minute amount of NADP⁺, remaining after the destruction by alkali, is amplified by the cycling reaction with more than 10,000-fold amplification and causes a high blank in the assay of minute samples, including single morulae and single neurons. These disadvantages lead to a blank 6 times higher than that with our present method and make it practically impossible to assay a low level of DG6P, *e.g.* in a single morula cultured with DG for 5 min. The present assay method was successfully applied to a single morula, whose size is nearly equal to large neurons. The analysis of

glucose utilization in single neurons is now in progress.

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REFERENCES

1. CHI M. M.-Y., LOWRY C. V. and LOWRY O. H. (1978) An improved enzymatic cycle for nicotinamide-adenine dinucleotide phosphate. *Anal. Biochem.* **89**, 119-129
2. CHI M. M.-Y., PUSATERI M. E., CARTER J. G., NORRIS B. J., McDUGAL D. B. and LOWRY O. H. (1987) Enzymatic assays for 2-deoxyglucose and 2-deoxyglucose 6-phosphate. *Anal. Biochem.* **161**, 508-513
3. HAWKINS R. A., MANS A. M., DAVIS D. W., VIÑA J. R. and HIBBARD L. S. (1985) Cerebral glucose use measured with [¹⁴C]glucose labeled in the 1, 2, or 6 position. *Amer. J. Physiol.* **248**, C170-C176
4. LOWRY O. H. and PASSONNEAU J. V. (1972) *A Flexible System of Enzymatic Analysis*, Academic Press, New York/London, pp. 8-16 and pp. 221-260
5. SOKOLOFF L., REIVICH M., KENNEDY C., DES ROSIERS M. H., PATLAK C. S., PETTIGREW K. D., SAKURADA O. and SHINOHARA M. (1977) The [¹⁴C]deoxyglucose method for the measurement of local cerebral glucose utilization: Theory, procedure, and normal values in the conscious and anesthetized albino rat. *J. Neurochem.* **28**, 897-916