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Continuous Monitoring of Extracellular Glucose Concentrations in the Striatum of Freely Moving Rats with an Implanted Glucose Biosensor

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Abstract: We have used a glucose oxidase-based sensor implanted in the striatum of freely moving rats to determine the concentration of extracellular glucose in two distinct ways. With a modification of the zero net flux method, in which different concentrations of glucose are infused through a dialysis probe glued to the biosensor, we calculated the concentration at which there was no change in glucose current by regression analysis; this gave a concentration of 0.351 ± 0.016 m*M*. Calculating the concentration from the basal current and the in vitro calibration of the biosensor was not significantly different from this. The basal extracellular glucose concentration determined by either method remained constant over a period of several days. Infusion of 50 μ*M* veratridine through the adjacent dialysis probe caused a steep decrease in glucose current as soon as the drug reached the brain in contrast to the delayed fall (7.5 min) seen with microdialysis in previous experiments from this laboratory. These results demonstrate that this biosensor provides a direct, real-time measure of the extracellular concentration of glucose. **Key Words:** Glucose biosensor—Brain glucose concentration—Veratridine—Rat striatum. *J. Neurochem.* **70,** 391–396 (1998).

The concentration of brain extracellular glucose has been calculated from whole-brain glucose and the volume of the extracellular compartment (Lund-Anderson, 1979); such measurements have estimated the extracellular glucose concentration as 2.0 m*M*. More recently, brain extracellular glucose has been monitored in vivo using microdialysis. The true extracellular concentration of glucose cannot be derived from dialysate concentration using the in vitro probe recovery (Morrison et al., 1991). Application of the zero net flux (ZNF) method (Lönnroth et al., 1987) has given values of 0.47 m*M* (Fellows et al., 1992) and 0.35 m*M* (Fray et al., 1997) in the striatum of unanaesthetised rats and 3.3 m*M* in the cortex of anaesthetised rats (Ronne-Engström et al., 1995). Another approach is to use an amperometric biosensor. Measurement of glucose with an implanted glucose oxidase (GOx) electrode has found a concentration of 2.5 m*M* in the hypothalamus of the sodium pentobarbitone-anaesthetised rat (Silver and Erecińska, 1994) and 1.5 m*M* in the cortex of the chloral hydrate-anaesthetised rat (Netchiporouk et al., 1996).

We have developed a glucose biosensor based on the immobilisation of GOx on Pt electrodes with the polymer poly(*o*-phenylenediamine) (PPD) (Lowry and O'Neill, 1994) and have used it to monitor continuously rapid changes in glucose in response to physiological stimulation (Lowry and Fillenz, 1997). An important stage in the development of this biosensor was the in vitro and in vivo characterisation of the sensor, which demonstrated that it has a fast response time, is linear over the relevant concentration range, is free of protein and lipid fouling, and has minimal interference from endogenous species (Lowry and O'Neill, 1994; Lowry et al., 1994*b*; Lowry and Fillenz, 1997).

There is some controversy concerning the immediate source of glucose in the extracellular compartment (Forsyth et al., 1996). An accurate determination of the true extracellular concentration is critical for the resolution of this controversy. The present study was designed to determine the true extracellular concentration of glucose using the implanted biosensor and to compare the two approaches to the measurement of extracellular glucose concentration (amperometry and microdialysis) in the same animal. We used a recent modification of the ZNF method of Lönnroth et al. (1987), in which an implanted electrode is combined with a dialysis probe (Miele and Fillenz, 1996) to

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Abbreviations used: aCSF, artificial cerebrospinal fluid; ECF, extracellular fluid; GOx, glucose oxidase; PPD, poly(*o*-phenylenediamine); ZNF, zero net flux.

determine the extracellular concentration of glucose in the striatum of freely moving rats. We then compared this value with that derived from using the in vitro calibration of the biosensor.

MATERIALS AND METHODS

Reagents and solutions

The enzyme GOx from *Aspergillus niger* (EC 1.1.3.4, grade I) was obtained from Genzyme (West Malling, Kent, U.K.). *o*-Phenylenediamine (free base) and veratridine (free base) were from Sigma Chemical Co. (Dorset, U.K.). D(+)-Glucose (AnalaR grade) was obtained from BDH Laboratory Supplies (Poole, U.K.). NaCl, KCl, NaH_2PO_4, NaOH, $MgCl_2$ (all BDH, AnalaR grade), and $CaCl_2$ (Sigma) were used as supplied.

Stock 1 *M* solutions of glucose for in vitro calibrations were prepared, left for 24 h at room temperature to allow equilibration of the anomers, and then stored at 4°C. Stock solutions of 100 m*M* ascorbic acid for in vitro calibrations were prepared just before use because of their gradual decomposition. All experiments in vitro were carried out in phosphate-buffered saline solution (pH 7.4): 150 m*M* NaCl, 40 m*M* NaH_2PO_4. Glucose solutions used in in vivo experiments (microdialysis) were prepared in artificial cerebrospinal fluid (aCSF): 147 m*M* NaCl, 4 m*M* KCl, 1.2 m*M* $CaCl_2$, and 1 m*M* $MgCl_2$ (de Boer et al., 1990). Veratridine solutions were prepared in aCSF and sonicated with heating to ensure complete dissolution. All solutions were prepared using deoxygenated, deionised water.

Instrumentation and software

A low-noise potentiostat (Biostat II; Electrochemical and Medical Systems, Newbury, U.K.) was used in all experiments. Data acquisition was performed using a Macintosh IIx computer and a National Instruments (National Instruments Corp., Austin, TX, U.S.A.) NB-MIO-16X multifunction data acquisition board. Further noise reduction was achieved by averaging 75 determinations to give 1 data point every 2 s. All experiments were performed using in-house developed software: LabVIEW (National Instruments Corp.). All analyses were performed using both in-house developed software and the commercial packages Igor Pro 2.0 (WaveMetrics, OR, U.S.A.) and Prism (GraphPad Software, CA, U.S.A.).

Surgery

Male Sprague–Dawley rats weighing 200–300 g were anaesthetised as described previously (Lowry and Fillenz, 1997). A mixture of 0.25 mg/kg fentanyl, 0.8 mg/kg fluanisone, and 0.4 mg/kg midazolam was injected intraperitoneally at a volume of 3.3 ml/kg. Surgery typically lasted 40 min, and anaesthesia was reversed by an intraperitoneal injection of naloxone (0.1 mg/kg; Sigma). Subsequently, animals were given Vetergesic (Reckitt and Colman Pharmaceuticals, Hull, U.K.) and narcotic buprenorphine (0.1 mg/kg s.c.) for pain relief. Occasionally, when surgery took longer, the animal was given a further intramuscular injection of 0.1 ml of Hypnorm every 10 min beyond 30 min.

Once surgical anaesthesia was established, animals were placed in a stereotaxic frame. Body temperature was maintained at 37°C throughout the surgery with an isothermal heating pad (Braintree Scientific, Braintree, MA, U.S.A.). The Pt/PPD/GOx electrode, glued to a microdialysis probe, was implanted in the right striatum [coordinates with the skull levelled between bregma and lambda were A/P +1.0 from bregma, M/L +2.5, and D/V −8.5 from dura (Paxinos and Watson, 1986)]. The reference electrode (8T silver wire, 200-μm bare diameter; Advent Research Materials) was placed in the cortex, the auxiliary electrode (8T silver wire) was placed between the skull and dura, and an earth wire (8T silver wire) was attached to one of the support screws. The electrodes and probe were attached to the socket fixed to the skull with dental screws and dental acrylate (Associated Dental Products, Swindon, U.K.). Animals were allowed 24 h to recover after surgery.

Animals were assessed for discomfort using the guidelines of Morton and Griffiths (1985). All animals used in this study had a score of ≤ 2, as defined by Morton and Griffiths. In cases where the score was >2, the experiment was terminated. All procedures were specifically licensed under the Animals (Scientific Procedures) Act, 1986.

Glucose biosensor

The Pt/PPD/GOx electrodes were made from 5T (125-μm bare diameter, 160-μm coated diameter) Teflon-coated platinum/iridium (Pt/Ir 90/10%) wire (Advent Research Materials). Approximately 4 mm of the Teflon was cut from one end to produce a 4-mm active length of bare wire. GOx was immobilised using PPD by potentiostatic electropolymerisation of the *o*-phenylenediamine monomer (300 mmol/L) following a modified form of a previously reported procedure (Lowry and O'Neill, 1994; Lowry and Fillenz, 1997).

In all experiments constant potential amperometry at +700 mV was used to detect glucose by oxidising H_2O_2 formed from the following enzyme reaction:

$$\text{D-glucose} + \text{GOx/FAD} \rightarrow \text{D-gluconolactone} + \text{GOx/FADH}_2$$

$$\text{GOx/FADH}_2 + O_2 \rightarrow \text{GOx/FAD} + H_2O_2$$

$$H_2O_2 \rightarrow O_2 + 2H^+ + 2e^-$$

Prior to implantation all Pt/PPD/GOx electrodes were preconditioned in vitro by continuous recording in 5 m*M* glucose over a 10-h period, which was found to improve the stability. Electrodes were then calibrated in vitro for both glucose (0–100 mmol/L) and ascorbic acid (0–1 mmol/L) to ensure selectivity and linearity of response of the sensor to glucose (Lowry and O'Neill, 1994). A characterised Pt/PPD/GOx electrode was glued with epoxy to a microdialysis probe ~1 h prior to implantation so that the 4-mm active lengths of both probe and electrode were directly aligned. Care was taken to ensure that the distance between the probe and electrode was within 1 mm.

Microdialysis probe construction

Concentric microdialysis probes were constructed as described previously (Demestre et al., 1997). The length of the probe was 4 mm, and the external diameter of the sampling region of the probe was 300 μm.

Experimental procedures

The rats were housed in large plastic bowls (diameter 20 in), in a windowless room (12-h light, 12-h dark cycle, lights coming on at 8 a.m.), with free access to food and water. Experiments were carried out with the animal in its home bowl. Implanted electrodes were connected to the potentiostat through a six-pin Teflon socket and a flexible screened six-core cable that was mounted through a swivel above the rat's head (Semat Technical, Herts, U.K.) at least 60 min prior to the start of each experiment. This arrange-

ment allowed free movement of the animal. Once the background current for the Pt/PPD/GOx electrode had stabilised (typically 30–45 min), experiments began.

For microdialysis experiments involving the ZNF method and the combined sensor/probe, the experimental procedure was similar to that used recently for the quantitative determination of brain extracellular ascorbic acid (Miele and Fillenz, 1996). In brief, glucose solutions ranging from 100 to 1,000 μM were perfused in random order through the dialysis probe at a flow rate of 2 μl/min. The current from the adjacent Pt/PPD/GOx electrode was recorded continuously during the perfusion. Changes in current were allowed to reach a steady state (typically between 10 and 20 min) before perfusion was stopped and the current allowed to return to baseline values. These changes were then plotted against the glucose inflow concentration; the point at which no change in current occurred, calculated by linear regression, was the point of ZNF.

Presentation of results

Data are presented as means ± SEM with n equalling the number of rats. Statistics (StatView; Abacus Concepts, CA, U.S.A.) were performed using the Student t test for paired observations (two tailed), with $p < 0.05$ considered to be significant.

RESULTS

ZNF experiments

Combined Pt/PPD/GOx sensors and microdialysis probes were implanted in the right striatum of freely moving rats and the modified Lönnroth ZNF method used to determine the true extracellular concentration of glucose in situ. Perfusion of glucose concentrations lower or higher than that in the extracellular fluid (ECF) resulted in a net loss or gain from the tissue into the dialysis probe and was characterised by a decrease or increase in the Pt/PPD/GOx current. A typical example of the effects of perfusing 1,000 μM glucose and aCSF is shown in Fig. 1. Random perfusion of concentrations above and below the point of ZNF resulted in quite rapid changes in the glucose current, which reached a steady state usually within 10–15 min. However, once perfusion was stopped, the return to baseline or control values was longer for the higher concentrations: 26 ± 3 min for 1,000 μM glucose (n = 4, six determinations) and 6 ± 1 min for aCSF (n = 4, six determinations). With certain concentrations there was little or no change in the glucose current, indicating that these concentrations were close or equal to the ECF concentration. As the animals were freely moving, there were often periods during the perfusion of glucose when the animals were quite active (e.g., feeding) and the glucose current required longer periods of time to reach steady-state conditions. Linear regression analysis of a plot of the observed change in current (I) versus perfused concentration yielded a straight line ($r^2 = 0.98 \pm 0.01$) and a mean ZNF point of 0.351 ± 0.016 mM (n = 4, 11 determinations) (Fig. 2A), which represents the extracellular concentration of glucose. In ZNF experiments carried out on 2 consecutive days (1 and 2) in four animals, the calculated extracellular concentrations were 0.373 ± 0.012 mM for day 1 and 0.358 ± 0.019 mM for day 2, a difference that was not statistically significant ($p > 0.57$). This is in contrast to our early microdialysis data in which dialysate glucose levels showed a progressive decrease (Fellows et al., 1992).

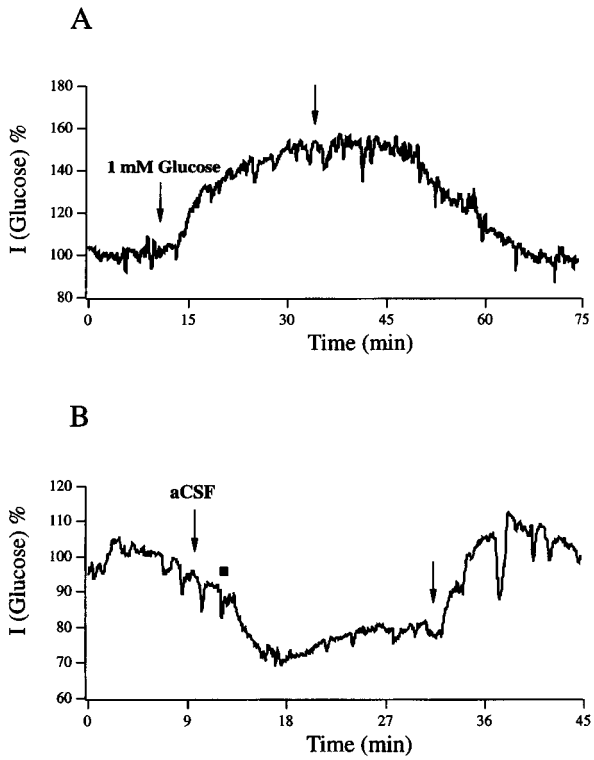

FIG. 1. The effect on the glucose current at the biosensor, shown as a percentage of basal current in the absence of perfusion, of the infusion of 1 mM glucose (**A**) and the infusion of aCSF (**B**) through the adjacent dialysis probe.

In vitro calibrations

In the alternative approach, we calculated basal glucose levels from the baseline currents recorded in vivo and in vitro calibration data, and compared these values with those obtained using the ZNF method. The in vitro sensitivity of the Pt/PPD/GOx electrodes for glucose was 61 ± 25 nA/mM (n = 4). The difference in glucose concentration calculated from the in vitro calibration of the four biosensors and the value calculated from ZNF experiments carried out 24 h after implantation was 48 ± 86 μM, a difference not different from 0 (n = 4, four determinations, $p = 0.61$, one-tailed t test). When the comparison was made between basal concentrations determined by these two methods on days 1 and 2 after implantation, the difference was 19 ± 49 μM (n = 4, eight determinations, $p = 0.7$). Similarly, there was no significant difference ($p > 0.38$) in the extracellular concentration calculated from in vitro calibrations for day 1 (0.422 ± 0.081 mM; n = 4) and day 2 (0.398 ± 0.069 mM; n = 4).

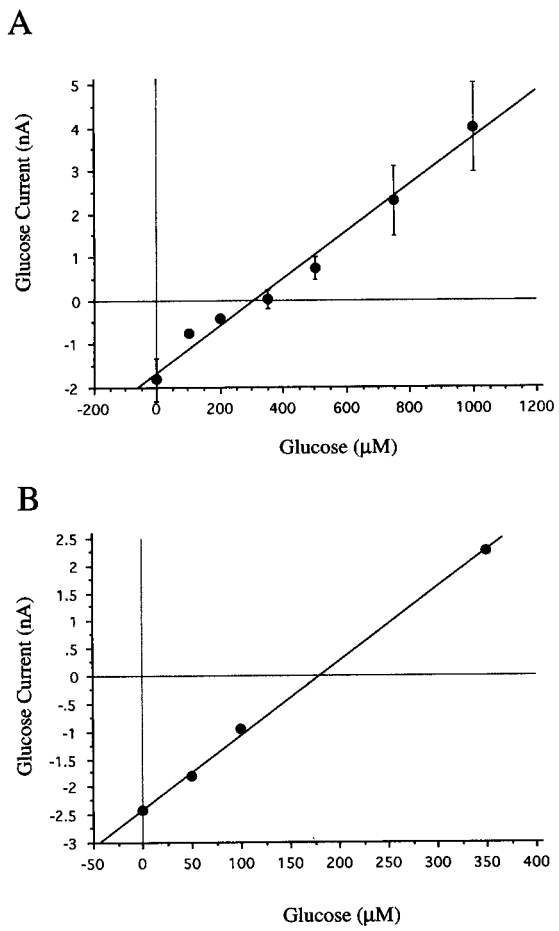

FIG. 2. Regression curves constructed from ZNF experiments during infusion of normal perfusion medium (**A**) and during perfusion with medium containing 50 μM veratridine (**B**). The curve in A is a mean of 11 determinations; the curve in B is from a single representative experiment.

Effect of veratridine

To see how accurately the biosensor can follow changes in the extracellular concentration of glucose, we carried out experiments using veratridine, which, by opening voltage-gated sodium channels, stimulates the Na^+,K^+-ATPase and so increases energy requirements and glucose utilisation (Whitesell et al., 1995). The basal extracellular current in the absence of perfusion was 24 ± 3 nA. Perfusion with aCSF reduced the basal value to 20 ± 3 nA. We next added veratridine to the perfusion medium to a final concentration of 50 μM. This caused a steep decrease in the glucose current, which reached a new steady value at 10 ± 3 nA (n = 2, four determinations). After allowing for the dead volume, the delay between veratridine reaching the brain and the onset of the decrease of the glucose current was <1 min (Fig. 3). With use of in vitro calibration of the biosensor, the concentrations of glucose calculated from the currents were 0.285 ± 0.024 mM in the absence of perfusion, 0.240 ± 0.010 mM during perfusion with aCSF, and 0.156 ± 0.025 mM during perfusion with veratridine (Fig. 1B). This represents a reduction of 15 ± 4% due to perfusion alone and a further reduction of 46 ± 6% due to the action of veratridine.

We next examined the effect of veratridine on the extracellular glucose concentration using the ZNF technique. In the absence of drug, the extracellular concentration calculated by regression analysis was 0.380 ± 0.010 mM. This was followed immediately by a ZNF experiment in the presence of 50 μM veratridine. Regression analysis (see Fig. 2B) gave a concentration of 0.181 ± 0.030 mM for the extracellular concentration of glucose in the presence of veratridine, which represents a reduction of 52 ± 9% (n = 4).

Calculation of fluxes

A valuable method for the comparison of a biosensor and microdialysis is to calculate the flux of glucose removed using the equation $J = i/nFA$, where J is the flux, i is the baseline current, n is the number of electrons per molecule of H_2O_2 oxidised, F is the Faraday constant, and A is the area of the biosensor. Taking 15 nA as the value for the baseline current, 2 for the value of n, 9.65×10^4 C/mol for the Faraday constant, and 1.57×10^{-2} cm^2 for the area of the biosensor yields a value of 5 pmol H_2O_2 (glucose) oxidised/s/cm^2.

For the microdialysis probe, the equation is $J = f/cA$, where J is the flux, f is the flow rate, c is the concentration in the dialysate, and A is the area of the probe. Taking 2 μl/min as the flow rate, 200 μM as the concentration in the dialysate, and 0.3×4 mm^2 as the area of the probe (Fray et al., 1996) gives a value of 186 pmol/s/cm^2.

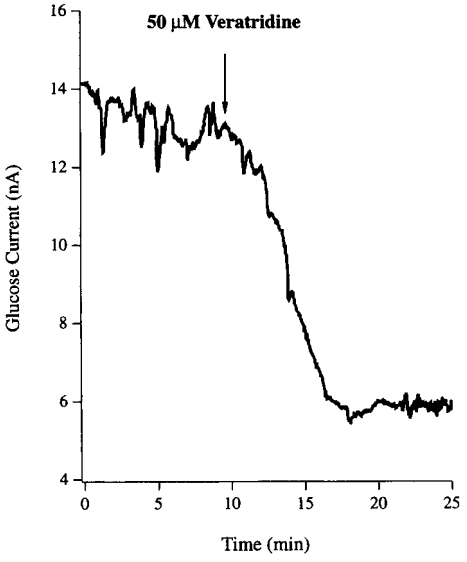

FIG. 3. The effect on glucose current of the infusion of 50 μM veratridine through the adjacent dialysis probe.

DISCUSSION

By testing the Pt/PPD/GOx biosensor in vivo, we have established that it provides a true measure of glucose concentration that is independent of changes in oxygen concentration and free of interference by ascorbate, the principal electroactive interferent present in the ECF. These sensors also exhibit no significant loss of sensitivity for several days following implantation (Lowry et al., 1997).

The present study was undertaken to determine the relation between the current recorded at the biosensor and the true extracellular concentration of glucose. We have shown previously, using microdialysis, that the calculation of the concentration of glucose in the extracellular compartment from the concentration in the dialysate requires a knowledge of the in vivo recovery, which cannot be derived from in vitro recovery of the probe (Fray et al., 1997). The ZNF method of Lönnroth et al. (1987) provides a value for both the extracellular concentration and the in vivo recovery.

In the present study, we have used a modification of the ZNF method, where instead of plotting infused concentration against (in–out) concentration, changes in the current at the implanted biosensor are used. The point of zero current change, arrived at by regression analysis, represents the point at which the infused concentration of glucose is the same as that throughout the ECF. This value was 0.351 ± 0.016 m*M*, which is the same as the value of 0.350 ± 0.020 m*M* derived from use of the ZNF method with microdialysis (Fray et al., 1997). It is clear (Fig. 1A) that infusion of glucose concentrations above this value increases the current and hence the local glucose concentration. We have discussed the possible causes and implications of this level of ECF glucose elsewhere (Forsyth et al., 1996; Fray et al., 1996).

We also calculated the basal extracellular glucose concentration from the basal current and the preimplantation in vitro calibration of the biosensor. There was no significant difference between this value and that determined by the ZNF method in individual rats and no difference between basal glucose determined by either of these methods 24 and 48 h after implantation.

This finding has several implications. The first is that there is little or no change in the sensitivity of the biosensor following implantation, supporting previous results (Lowry et al., 1994*a*). The second is that the close agreement indicates that in vivo the biosensor current represents only glucose. The third is that in contrast to microdialysis, measurements with the implanted biosensor provide a direct, real-time measure of the extracellular concentration of glucose.

In microdialysis the delivery of glucose to the dialysate is by diffusion along a concentration gradient. In addition to the dialysis membrane, the narrow and tortuous clefts of the extracellular compartment offer barriers to free diffusion and hence restrict the flux to the probe. Processes of supply and utilisation increase the concentration gradient and hence the flux from the brain (Boutelle and Fillenz, 1996).

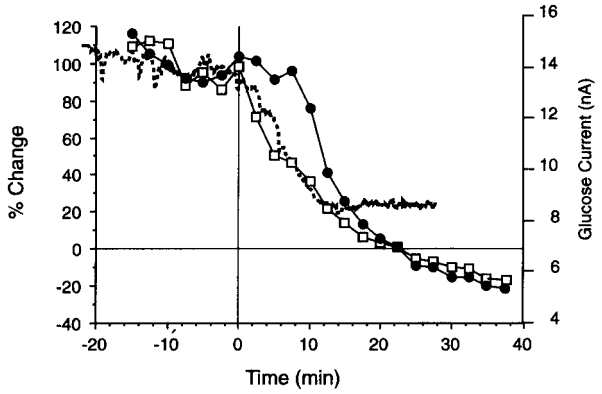

FIG. 4. A comparison of the time course of the effect of veratridine on the glucose current at the biosensor (from Fig. 3, dotted line) with the results from microdialysis experiments showing the difference between changes in dialysate (filled circles) and the calculated extracellular glucose concentration (open squares, from Fray et al., 1997).

The removal of glucose by microdialysis varies with flow rate and even at 2 μl/min is 41 times greater than the removal by the enzyme-based biosensor. An example of the effect of microdialysis is illustrated in Fig. 1B. The much smaller removal flux of the biosensor is much more easily met by the tissue processes surrounding the probe. Consequently, the local extracellular concentration is not perturbed by the electrode, which records the true extracellular concentration.

We have shown that with microdialysis the in vivo recovery increases with an increase in the rate of utilisation. With the local administration of veratridine, the steep decrease in extracellular concentration together with an increase in in vivo recovery meant that there was a delay of 7.5 min in the decrease in dialysate concentration (Fray et al., 1997). No such delay was seen in the present experiments when glucose was measured with the implanted biosensor (Fig. 4).

These findings all suggest that the biosensor provides a direct measure of the extracellular concentration of glucose, which can be calculated from the in vitro calibration.

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