On-line, Real Time Measurement Of Extracellular Brain Glucose Using Microdialysis And Electrochemical Detection

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Extracellular glucose in the perfusate of in vitro and in vivo microdialysis samples was continuously measured by a method which utilized glucose oxidase immobilized onto the surface of a Os(II)polyvinylpyrridine wired HRP (Os-gel-HRP) glassy carbon electrode. Interference from ascorbic acid was eliminated by overcoating this "bilayer" (Glucose oxidase/Os-gel-HRP) electrode with a cellulose acetate film and by using a platinum tube generator. The microdialysis perfusate was mixed with a low pH phosphate buffer to minimize the oxidation of ascorbic acid, buffer the sensor from variations of pH and maximize enzyme efficiency. The high sensitivity (detection limit 5 µM of glucose in the dialysate) and linearity of current response, coupled with the technically simple requirements of the detection system should prove a useful simplification for the measurement of extracellular glucose. The suitability of this technique for neurophysiological investigation of changes in extracellular levels of striatal glucose in response to anesthesia, glucose loading and restraint is demonstrated.

Glucose, lactate and glutamate are fundamentally important to the brain in physiology and clinical pathologies, such as ischemia and hypoxia. The degree of interrelation of these compounds extends beyond physiology. This is illustrated by the fact that all three compounds are regularly assayed by the electrochemical measurement of H_20_2, the product of the reaction of each of these compounds with their respective oxidase enzyme (as per the following equation).

$$\text{Substrate} + O_2 \xrightarrow{\text{Enzyme}} \text{Oxidized substrate} + H_2O_2$$

The development of the microdialysis diffusion sampling technique by Ungerstedt in the early 1980s provided a means of easily obtaining protein-free dialysates of the extracellular fluid (ECF). At present, the concentrations of glucose, lactate and glutamate in the dialysates are most frequently assayed by LC systems which contain a column to temporally separate the analyte(s) of interest, a reactor column containing immobilized substrate specific enzyme(s) to generate H_20_2 and an electrochemical detector to quantify the product of the oxidation of H_20_2 at 500-700 mV. Although this technique is extremely sensitive and reliable, it requires technical expertise. The temporal resolution of the technique is dependent upon the retention time of the analyte in the column. Alternative strategies are based on attempts to produce an "on-line assay" that combines the collection procedure of the dialysate directly with a simplified form of the enzymatic assay. Ideally, "on-line real time assays" are the golden fleece of the neurochemist. The journey towards this goal has resulted in the development of three types of assays: 1) solutions of the enzyme are mixed with the dialysate and the H_20_2 is generated in the mixed solution (1); 2) the enzyme is immobilized on inert particles contained in a reactor that is positioned upstream of the electrochemical detector (2); and 3) enzymes are electrochemically immobilized onto the surface of the sensor itself (3,4,5). Each method has proved useful but each is subject to limitations.

Enzyme solutions provide great simplicity, but eventually they

foul the detector and the cost of less common enzymes can become a major consideration. Enzyme columns provide great sensitivity, but unless a valve is incorporated into the system, they induce back pressure that adversely affects the diffusion across the microdialysis membrane.

Enzymes immobilized directly onto the detector surface are economical and provide for rapid response time; however, the demands upon the detector for stable, interference-free performance in the face of ever changing dialysate concentrations is a Herculean task. In practice, the result is often drifting baselines and interference from oxidation of undesirable components in the dialysate, such as ascorbic acid. The latter can be reduced by coating the detector with selectively permeable membranes which protect both the detector and the enzyme, but this also reduces the signal. For substances with high ECF concentrations, such as glucose, lactate and glutamate, the reduction in sensitivity is not a problem and may in fact enhance longevity of the enzyme coated detector. For substances with low ECF concentrations, the use of a generator electrode positioned upstream of the detector provides for protection without reducing sensitivity. Regardless of the ECF concentration, the use of a "wired peroxidase electrode", which enables the detector potential to be reduced to 0 mV (6), provides significant advantages.

The aim of this study was to develop an on-line real time assay for ECF cerebral glucose with sufficient sensitivity to be coupled with the microdialysis sampling technique. A principal concern was the development of a technically simple procedure that did not require the use of specially modified equipment. We took advantage of the Bioanalytical Systems, Inc. (West Lafayette, IN, USA) "wired Os-gel-HRP electrode" which enabled detection of the enzymatically derived H_20_2 at 0 mV (*vs.* Ag/AgCl-re) (7). The enzyme L-glucose oxidase (1.5% by weight) from *Aspergillus niger* (Type X-S, EC 1.1.3.4 purchased from Sigma) was immobilized onto the surface of the Os-gel-HRP casted, glassy carbon electrode with a 2.5% solution of glutaraldehyde in 0.15 M phosphate buffer (pH = 5.7). This "bilayer" electrode, once dry, was overcoated with a 0.75% solution of cellulose acetate in butanone. A low volume platinum generator maintained at 250 mV (*vs.* Ag/AgCl) was positioned upstream of the detector to oxidize ascorbic acid and provide diagnostic information. The dialysate was mixed immediately prior to the detector with 10 volumes of oxygenated 150 mM phosphate buffer (pH 5.7). The buffer served to stabilize the sensor's environment, inhibit the oxidation of ascorbic acid at the sensor and maximize the kinetics of the glucose oxidase. This system proved an extremely sensitive and durable method for the on-line real time measurement of ECF cerebral glucose. The high sensitivity of the assay enabled the in vivo measurement of cerebral glucose using a small, commercially available, microdialysis probe. In vitro tests, which simulated hypoxia, demonstrated that the enzymatic reactions were not decreased by reducing the partial pressure of oxygen in the solution being perfused.

Reagents and Methods

A schematic of the system is presented in ***F1***. Ringer's solution (Na^+ = 150 mM, K^+ = 3 mM, Mg^{++} = 0.8 mM, Ca^{++} = 1.2 mM) was pumped by syringe pump 2 at the rate of 1.5 µL/min through the microdialysis probe and a platinum tube (0.1 mm x 60 mm, ID, internal volume less than 1 µL) (Nihonbashi Tokuriki, Japan). The speed of the perfusion is chosen by the need to facilitate the desired recovery across the microdialysis membrane. The perfusate (from channel A) was mixed with 150 mM phosphate buffer (pH = 5.7) pumped by pump 1 at 15 µL/min (channel B), thus the total flow rate of the mixture passing through the sensor was 16.5 µL/min. The Os-gel-HRP/Glucose oxidase/Cellulose acetate, 6 mm glass carbon electrode (overcoated bilayer electrode) was coupled to a radial flow cell from BAS (West Lafayette, IN, USA). A 50 µm gasket was used.

The current generated from the reduction of H_20_2 was measured with an LC-4C amperometric controller from BAS (West Lafayette, IN, USA) and stored on a hard disk using a ChromGraph DA-5 data acquisition interface (BAS, West Lafayette, USA). The platinum generator electrode was maintained at 250 mV (*vs.* Ag/AgCl) using the generator electrode control feature of the LC-4C. The current output from the generator electrode was also stored on ChromGraph. The tubing used in channel B was PE-10. All other components were connected by low internal volume FEP tubing and plastic connectors (CMA or BAS). Solutions were pumped in 0.25 mL or 2.5 mL glass syringes from BAS (West Lafayette, IN, USA) in order to reduce pumping noise (3). Two liquid switches (LS 1 and LS 2) (CMA 110, CMA/Microdialysis, Sweden) were incorporated into the circuit. Liquid switch 1 was used to apply standards or drugs. Liquid switch 2 was used to include or exclude the dialysate from the animal from entering the analytical circuit. Using this protocol, 1 µM of glucose in the dialysate generated between 0.5 - 1.0 nA current.

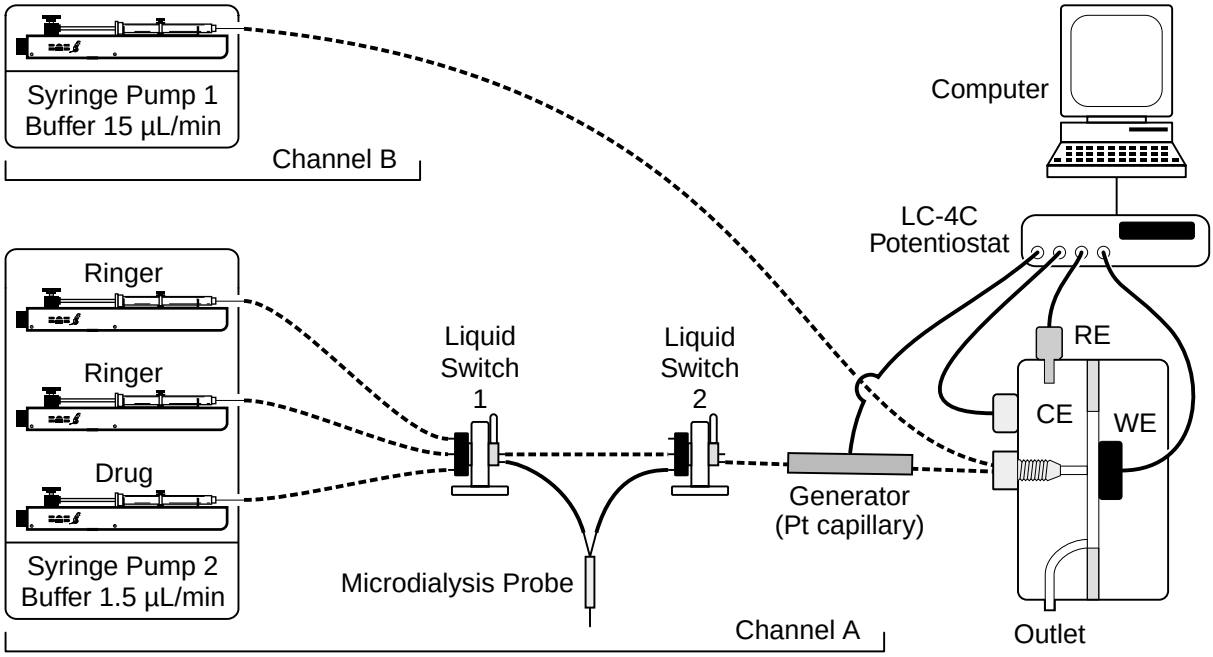
Calibration

Fast Calibration

A solution of buffer and Ringer's was combined in the ratio of the flow rates employed in the experimental conditions and was pumped, at the total flow rate utilized in the experimental measurement of glucose, through channel

F1

Schematic of on-line system. Pump 1 (Syringe pump with 3 syringes at same flow rate or 3 independent syringe pumps); Pump 2 (Separate syringe pump at a higher flow rate); GEN - Platinum tube (60 mm x 0.1 mm) poised at +250 mV vs. Ag/AgCl reference (RE), WE - 6 mm Glassy carbon "bilayer" working electrode casted in Osmium-gel-HRP/1.6% Glucose Oxidase in 2.5% Glutaladehyde and overcoated with 0.75% Cellulose Acetate poised at 0 mV (vs. Ag/AgCl) positioned in a radial flow cell. CE - counter electrode.



A. Channel B was blocked. Standard solutions of glucose and/or other drugs or samples were included/removed from the system by liquid switch 1 (***F1***). A three-point calibration curve was determined for the concentration of glucose *vs.* the current generated. Using a fast calibration method, a three-point standard curve could be performed in less than 30 minutes and pump noise was minimal.

Slow Calibration

The standard solution was perfused through Channel A at the speed employed in the experiment. Buffer was pumped through Channel B at the speed employed during the experiment. Using the slow calibration method, a three-point standard curve could be performed in 60 minutes. Using both methods, the actual concentration of glucose in the dialysate was calculated by adjusting for the factor by which the sample was diluted by the buffer (in this instance approximately x10). ***F2*** shows the reduction of sensitivity of an electrode used for approximately 3 hours a day for 5 days.

Buffer was pumped through Channel B. Ringer's solution was pumped through Channel A. Two syringes containing Ringer's solution were loaded. One syringe perfused the microdialysis probe, the other syringe connected directly to Channel B through liquid switch 2. Initially, liquid switch 2 excluded the dialysate from the analytical circuit. Once a stable current had been obtained, and the rat had been perfused for at least 90 min, the dialysate was switched into the analytical circuit. The basal level of glucose was calculated by comparing the current generated from baseline with the calibration curve. The time delay between the dialysate leaving the brain and reaching the detector was dependent upon flow rate and the length of tubing, but was typically less than 10 minutes. ***F3*** shows the stability of signal over 30 min perfusion with dialysate concentration of 400 µM glucose (approximately 4-5 times the brain dialysate concentration measured in vivo using a CMA 11 probe, 1 mm dialysing membrane).

Results and Discussion

In Vitro Experiments

Sensitivity to Interferents

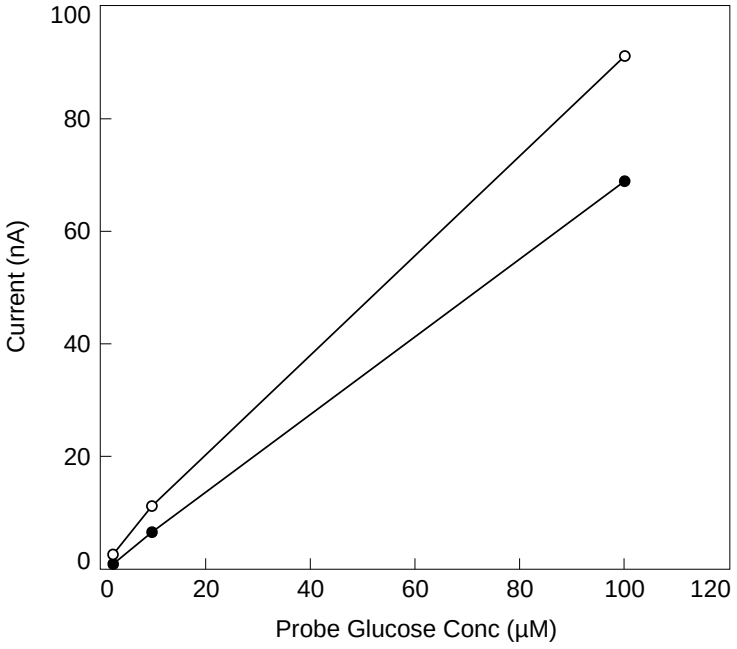
In a dialysate sample from a rat brain, the principal substance which could be anticipated to compromise the current generated by the enzymatic reduction of H_20_2 at 0 mV is the oxidation of ascorbic acid (AA). In the rat brain, the concentration of AA in the extracellular compartment is regionally dependent and varies between 100 and 500 µM as determined by cyclic voltammetric analysis (7). The effect of increasing the AA concentration in the perfusate from 0 to 100 µM on the baseline current generated for 10 µM glucose was determined using "bilayer" electrodes coated with CA both with and without the inclusion of a platinum generator electrode to oxidize AA. In both instances, 100 µM AA had no effect upon the current generated by glucose.

Oxygen Dependency

The production of H_20_2 by glucose oxidase occurs from an oxygen dependent reaction. Under experimental conditions, such as

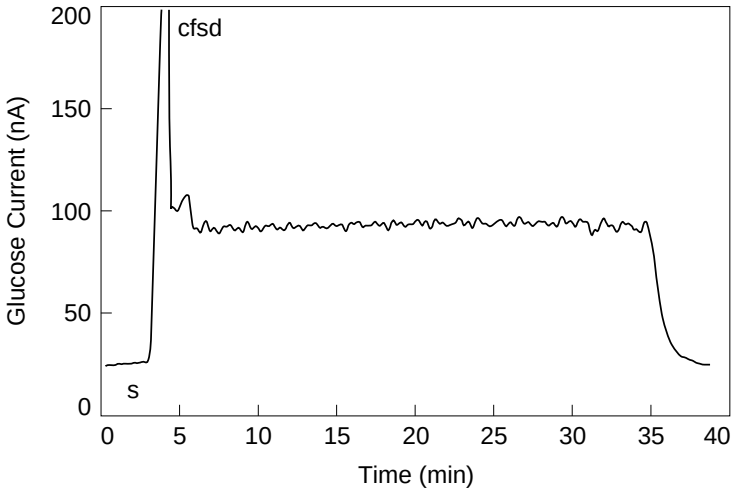
F2

Effect of time on linearity and sensitivity of current responses to glucose concentrations on the same compound electrode on Day 1 (open circles) and Day 5 (filled circles). Dialysate concentration using a 1 mm probe (CMA 11) was between 50-100 µM.



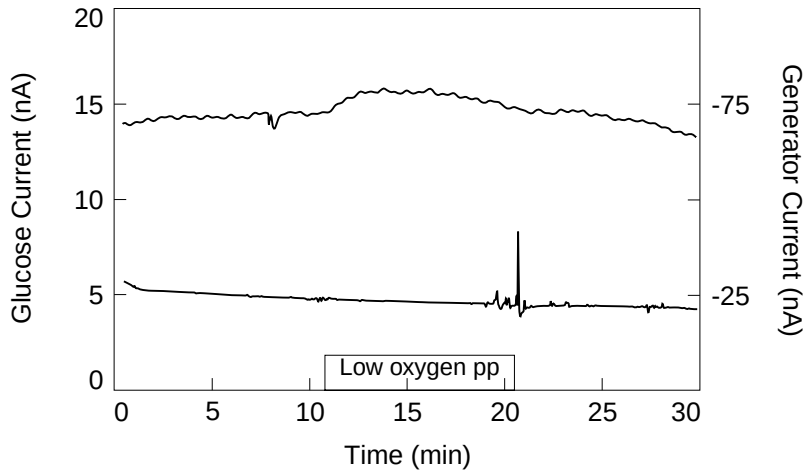
F3

Current response time of "bilayer" glucose electrode to perfusion with 400 µM glucose for 30 minutes. "s" - switch glucose in/out of analytical circuit. "cfsd" - change full scale deflection.



F4

The effect of changing the microdialysis probe from Ringer's solution at room temperature containing 470 µM glucose to a second Ringer's solution containing 470 µM glucose which had been bubbled for 1 hour with N_2 for 10 minutes. Then the microdialysis probe was returned to the initial solution. Top trace - glucose current. Bottom trace - generator current.



nonventilated anesthesia, experimentally induced anoxia or ischemia, the partial pressure of oxygen in brain ECF and hence the perfusate, may be reduced. The effect of reducing the oxygen partial pressure of the solution being perfused was determined by perfusing a microdialysis probe (CMA 11, 0.28 mm diameter, 2 mm membrane length) with Ringer's solution. The probe was immersed in solution containing 470 µM glucose (approximate concentration of ECF glucose (2) that had been (a.) allowed to stand in air at room temperature, or (b.) bubbled with pure nitrogen for 1 hour). ***F4*** demonstrates that reducing the partial pressure of oxygen in the solution being perfused did not significantly reduce the current generated and demonstrates the suitability of this assay technique for in vivo studies of ischemia or hypoxia.

In Vivo Experiments

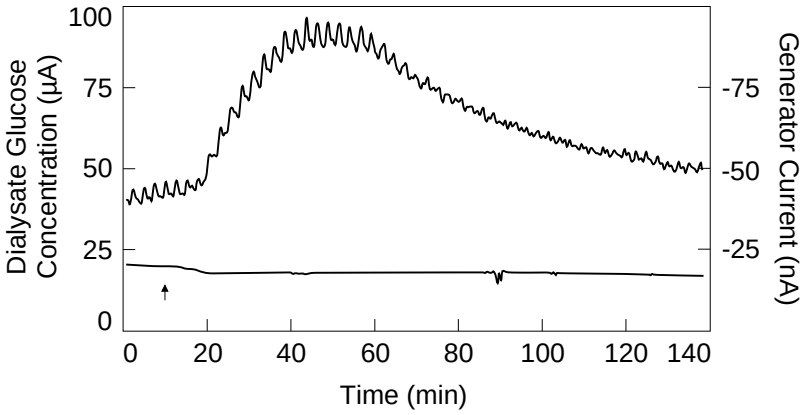
On-line Real Time Microdialysis

Under pentobarbital sodium anesthesia (50 mg/kg IP), a microdialysis probe with a concentric design (CMA 11) and a 1 mm dialysing membrane at its tip was permanently implanted into the striatum (AP = 0, L = 3, D = 7) using established procedures (8). Post surgically procaine (1%) was applied to the surface of the wound. Experiments were performed on the second to the fifth day after surgery on awake, unrestrained rats. In all experiments, the probe was perfused with Ringer's solution for 90 min before the perfusate was switched into the analytical circuit.

F5 shows that basal ECF glucose rapidly increases with anesthesia (40 mg/kg pentobarbital sodium IP). Hindlimb reflex was mildly apparent 80 min after injection, although, the rat remained anesthetized. The magnitude and profile of the increase in striatal ECF glucose is consistent with previously published data (2). ***F6*** shows an increase in ECF striatal glucose to IP injection of 1.5 mL of 10% glucose solution in 150 mM saline at t = 42 min. This result is consistent with increases in ECF hippocampal glucose measured in response to intravenous infusion (1). In another experiment, the rat was immobilized for 9 minutes by being restrained by hand and wrapped in a piece of cloth with the head protruding (9). ***F7*** shows that basal ECF glucose

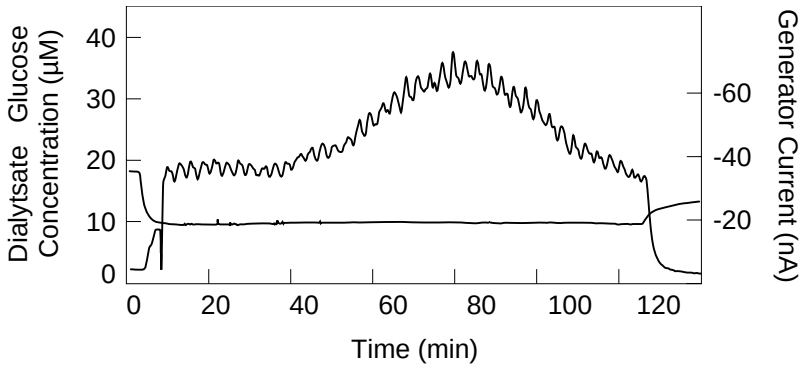
F5

The effect of anesthesia (40 mg/kg IP Nembutal sodium) on striatal ECF glucose. The rat was anesthetized at t = 11 minutes (arrow) and remained anesthetized for the duration of the experiment. Top trace - glucose current. Bottom trace - generator current.



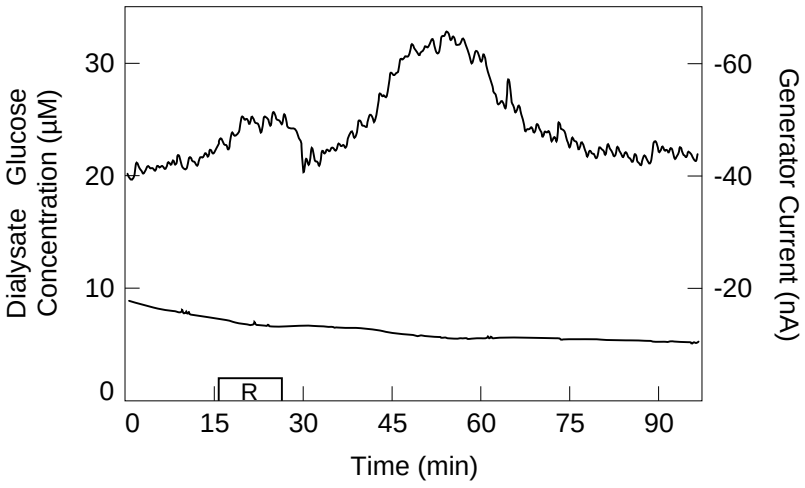
F6

The effect of 1.5 mL intaperitoneal injection of 0.25 g of glucose in 150 mM NaCl on striatal ECF glucose. Top trace - glucose current. Bottom trace - generator current. T = 5 min, dialysate switched into analytical circuit. T = 127 min, dialysate switched out of analytical circuit.



F7

The effect of a 9 min immobilization stressor of striatal ECF glucose. Top trace - glucose current. Bottom trace - generator current. The rat was restrained from T = 16-25 min.



increased in response to the immobilization. The increase in striatal ECF glucose observed in response to immobilization is consistent with previously published data (1).

Conclusions

This system proved a sensitive, specific and technically undemanding means of measuring extracellular glucose in solutions and dialysates. The substrate specificity is provided by the enzyme. Interference current, generated at the detector by oxidation of easily oxidizable compounds in the dialysate, is minimized by the combination of the following three factors: 1) the dilution of sample in 150 mM PBS, pH 5.7 buffer; 2) the use of high sensitivity osmium-gel-HRP polymer facilitated the reduction of H_20_2 at an applied potential of 0 mV (6); and 3) the use of cellulose acetate to repel AA coupled with a generator electrode, positioned "upstream" of the detector, poised at 250 mV which irreversibly oxidized AA (7). In addition, the buffer serves to stabilize the detector from fluctuations induced by changes in the pH of the perfusate. The buffer also ensures that enzyme reaction kinetics were maintained when the partial pressure of oxygen in the solution being perfused is reduced. The overcoated bilayer electrode retained usable sensitivity for about 4-5 days as sensitivity was reduced by 3% with each 1 hour of use. The high sensitivity of the assay enabled commercially available microdialysis probes to be used in in vivo experiments which demonstrated appropriate increases of ECF glucose in response to anesthesia, glucose loading and immobilization stressor. The high sensitivity and stability of this assay will enable region specific changes in brain ECF glucose to be investigated.

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