Enzyme Packed Bed System for the On-Line Measurement of Glucose, Glutamate, and Lactate in Brain Microdialysate

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Rapid measurement of glucose, glutamate, and lactate is important in understanding the dynamics of the energy balance of the brain. Glutamate is also the main excitatory neurotransmitter. A general immobilized enzyme-based flow injection assay system is described which uses oxidase and peroxidase enzymes to convert the analyte into an oxidized ferrocene species which is detected electrochemically by reduction. The enzymes glucose oxidase, glutamate oxidase, lactate oxidase, and horseradish peroxidase are immobilized with near 100% efficiency onto 10-μm tresyl-activated silica beads (1000- and 500-Å pore size). The beads are slurry-packed into 2- × 20-mm columns to give beds for glucose, glutamate, or lactate which are stable for >40 days. The flow injection assays described have detection limits from 1.8 to <20 pmol and have been configured to have linear calibration responses over the range of basal and stimulated levels of the three compounds found in 5-μL microdialysate samples from the rat striatum. The assays are used for automated on-line measurement of glucose, glutamate, and lactate in striatal microdialysate at 2.5-min intervals.

INTRODUCTION

Microdialysis is a powerful sampling technique[1,2] originally developed to study the release of neurochemicals in the brain, but with great potential for use in other envionments. A short length of hydrophilic tubular membrane connected to a flow system is placed in the area to be sampled, and a perfusing solution is slowly pumped through. The resulting dialysate is then collected and assayed, usually by HPLC with oxidative electrochemical detection. The perfusing solution is chosen to match the tissue being sampled but can also be used to deliberately perturb the sampled area, allowing the dynamic properties of the systems to be studied in addition to basal conditions. In the brain this corresponds to local application of a drug, perhaps a neurotransmitter receptor agonist, to the sampled brain area. The molecular weight cutoff of the membrane is chosen to select for the smaller analyte molecules at the expense of any larger interferent molecules. This is particularly important in biological tissues such as the brain where, for example, neurotransmitter molecules released by neurones close to the membrane can be removed from the degradative enzymes and reuptake processes.

Since its first description by Ungerstedt[3] the main limitation to the application of microdialysis has been the sensitivity and selectivity of the available assays. This has particularly applied to the sampling interval, that is the time over which the dialysate is collected to provide sufficient analyte to detect. For catecholamine neurotransmitters in the rat brain (detected by electrochemical oxidation) this started at 30 min[4] and, over a period of 5 years, has reduced to 5 min.[5] Although the assays have now become even more sensitive, 10-min sampling is still normally used because of the time taken for the peaks to elute from the HPLC column. Analytes not detectable by oxidation have proved more difficult. Pyruvate and lactate have been detected in brain dialysate by absorbance[6] following separation on an HPLC column, but this has succeeded because of their high extracellular concentration rather than a sizeable molar extinction coefficient. The on-line sampling rate is limited by a 5-min elution time from the column. Precolumn derivatization with the fluorophore *o*-phthaldialdehyde[7] has proved successful for amino acids including the neurotransmitters glutamate and GABA.[8] The method is sensitive and with column switching and careful optimization can give elution times of 2.5 min for glutamate and 4 min for GABA. However, both assays are complicated and require an autosampler and multiple HPLC pumps, so in practice sampling intervals of 10 and 5 min are used.

Enzymes can be used very effectively to react with a target analyte. Regeneration of the enzyme then occurs, producing a mediator molecule which can be detected electrochemically or fluorometrically. Korf et al. have mixed enzyme solution with the microdialysate stream and detected lactate in real time[9] or glucose at 2-min intervals.[10] A simpler method is to immobilize the enzymes in an enzyme bed. We have shown that a packed enzyme bed electrode is inherently more sensitive than an electrode with enzyme localized by a membrane,[11] and have used enzyme beds in assays of microdialysate for glucose[12] and acetylcholine.[13] Other enzymatic assays for acetylcholine have also been reported.[14,15]

In this paper we describe enzyme-based assay systems for glucose, lactate, and glutamate—three important neurochemicals not detectable by direct electrochemical oxidation. Rapid measurement of these compounds permits the study of the dynamics of the energy balance of the brain. Glutamate is also the main excitatory neurotransmitter. We wanted assays

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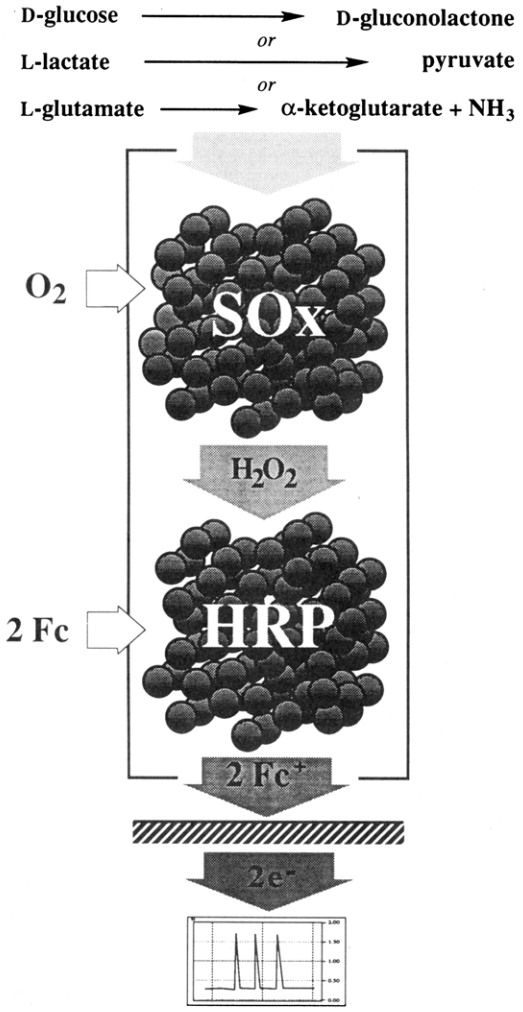

Figure 1. Schematic reaction sequence occurring within the immobilized enzyme packed bed. The sequence is shown for a generalized substrate oxidase (SOx) and horseradish peroxidase (HRP). In use, glucose oxidase (GOx) is used for glucose, lactate oxidase (LOx) for lactate, and glutamate oxidase (GLOx) for glutamate.

which were sensitive enough to allow rapid on-line sampling and which did not require chromatographic separation and hence slower sampling because of column elution times. We also wished the assays to be stable, share common components, and be simple to use. We chose to use the flavinadenosine dinucleotide-containing oxidase enzymes glucose oxidase (E.C.1.1.3.4, GOx), lactate oxidase (LOx), and the recently available glutamate oxidase (EC 1.4.3.11, GLOx[16]) rather than the equivalent dehydrogenase enzymes. As a group these enzymes are more stable (being of extracellular origin) and faster and have stable covalently bound cofactors. All the enzymes produce hydrogen peroxide (H_2O_2). This could be detected directly by oxidation; however the electrochemistry is easily poisoned and the high electrode potentials required would also detect other compounds present in the dialysate. We have used instead a combination first suggested by Frew et al.[17] of the enzyme horseradish peroxidase (EC 1.11.1.7, HRP) and a modified ferrocene (Fc) as a solution mediator. The complete method is shown schematically in Figure 1.

The enzymes are immobilized on solid supports and packed into a bed. H_2O_2 produced by an oxidase enzyme (generalized substrate oxidase SOx) from an injected sample is converted to water, and the HRP is regenerated by the oxidation of two Fc species present in the buffer pumped through the bed. The Fc^+ species produced are electrochemically reversible (with an $E_{1/2}$ = 0.225 V) and are detected highly efficiently by reduction at a downstream electrode. The low potential of the electrode (0 mV vs $Ag^+/AgCl$) prevents oxidation of other species in the sample, and a single peak is produced. The assay can consequently run in the flow injection analysis (FIA) mode without a chromatographic separation step.

EXPERIMENTAL SECTION

Materials. The LiChrospher Diol 1000 and 500 beads were from Merck, U.K. Tresyl chloride (2,2,2-trifluoroethanesulfonyl chloride) was from Fluka Chemicals. Glucose oxidase (grade II) and horseradish peroxidase (grade I) were from Boehringer Mannheim, FRG. L-Glutamate oxidase (from *Streptomyces*) was from Yamasa Shoyu, Tokyo, Japan, L-Lactate oxidase (from *Pediococcus*) was from Genzyme Diagnostics, Maidstone, U.K. Ferrocenemonocarboxylic acid was from Sigma Chemicals. All other chemicals were of at least AnalaR grade. Microdialysis probes were of a concentric design, constructed by inserting a plastic-coated silica tube (VS170/110; Scientific Glass Engineering) into a polyacrylonitrile dialysis fiber (o.d. 320 μm; Hospal, France). The fiber was glued into a stainless steel cannula, leaving an active length of 4 mm, and the tip was sealed with epoxy. A second silica tube inserted into the cannula served as the outlet. The probes were perfused with an artificial cerebrospinal fluid (aCSF) containing NaCl (147 mM), KCl (4.0 mM), $CaCl_2$ (1.2 mM), and $MgCl_2$ (1.0 mM) in sterile water. Anaesthesia was obtained using Hypnorm (Janssen, Oxford) and Hypnovel (Roche Products).

Activation of Diol–Silica with Tresyl Chloride. Tresyl chloride cannot react directly with the –OH groups on a silica surface; therefore silica coated with a hydrophilic layer of glycerylpropyl groups was used.[18] This also minimizes the adsorption of analyte.[19] Whilst any silica support can readily be coated with a glycerylpropyl layer,[18] we used the commercially available 10-μm spherical particle diol–silica Lichospher DIOL from Merck, U.K. To facilitate access by the enzymes, 1000- and 500-Å pore sizes were chosen.

Activation of the beads was performed using a method similar to that developed by Nilsson, Larsson, and Mosbach.[19,20] The dry 1000-Å pore beads were washed with dry acetone (50 mL, dried over 4-Å molecular sieves) and placed in a dry flask containing dry acetone (2 mL). Dry pyridine (600 μL) was added and the flask cooled to 0 °C with magnetic stirring. Tresyl chloride (600 μL) was added dropwise to the stirred suspension. After 15 min, during which the beads turned the yellow/cream color typically found in sulfonylations, the beads were washed with 50 mL each of water, 50% water/acetone, and dry acetone and dried in vacuo overnight. The beads were stored frozen with a dessicant.

The 500-Å pore beads were activated using the same method but using 900 μL of dry pyridine and 810 μL of tresyl chloride. These conditions gave an estimated coverage of tresyl sites of 60 and 400 μmol of tresyl/g of dry beads for the 1000-Å and 500-Å beads, respectively.

Coupling of the Enzymes to the Tresyl-Activated Beads. Dry beads (40 mg of each of two enzymes in 2- × 20-mm packed bed) were added to 1 mL of coupling buffer (0.2 M NaH_2PO_4 adjusted to pH 8.5) and degassed under vacuum and by sonication. A known weight of the chosen enzyme (typically 4 mg) was dissolved in 1 mL of the phosphate buffer and the resulting solution added to the bead suspension. The mixture was gently rolled for 24 h at 4 °C using a Spiramix 5 mixer (Jencons, U.K.). The beads were gently spun down (170 g for 5 min), and the supernatant was drawn off. The moist beads were then washed twice. Washing was by resuspension in 2 mL of coupling buffer followed by centrifugation and removal of the supernatant. The beads were then resuspended in 2 mL of Tris–HCl (0.2 M, pH 8.0) and rolled at room temperature for 2 h in order to remove

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any interference from unreacted tresyl groups. After washing, the beads were either packed immediately or stored at 4 °C in phosphate buffer (0.2 M pH 7.0) containing 0.05% of the biocide Kathon CG (Rohm and Haas).

Measurement of Immobilized Enzyme Activity. The effectiveness of each immobilization of the oxidase enzymes was assayed by measuring the rate of oxygen uptake by a known weight of beads upon addition of excess substrate. This was performed using a Clark O_2 electrode in a closed chamber thermostatically maintained at 37 °C. A comparison with a known concentration of free enzyme was then made to give micrograms of enzyme per milligram of beads and milliunits of activity per milligram of beads. All assays were in phosphate buffer (0.05 M, pH 7.0) containing 0.05% Kathon CG.

The effectiveness of the immobilization of HRP was checked in two ways. Firstly, by monitoring spectrophotometrically at 403 nm ($\epsilon = 9.1 \times 10^4\ M^{-1}\ cm^{-1}$) the disappearance of the enzyme's heme absorbance from the coupling buffer. Secondly, by monitoring spectrophotometrically the consumption of the chromogen ABTS at 340 nm ($\epsilon = 3.6 \times 10^4\ M^{-1}\ cm^{-1}$) according to the method of Childs and Bardsley.[21]

Packing the Enzyme Packed Bed. The enzyme beds were HPLC guard columns (2-mm diameter × 20 mm) fitted with 10-μm porosity nonmetallic frits. To fill a bed, 80 mg of enzyme-loaded beads were required. This weight was either split equally between the oxidase enzyme beads and the HRP beads, or the ratio was determined by the found immobilized activities of the two enzymes (taking account of the two Fc^+ species produced for each H_2O_2 shown in Figure 1). The beds were slurry-packed using the FIA mobile phase at 4 mL min^{-1} (to a maximum pressure of 10 MPa) for 2 h.

Storage and Lifetime of the Enzyme Packed Beds. The beds were generally in use 5 h/day (40–50 samples), 4–5 days a week. When not in use, they were filled with the FIA mobile phase containing Kathon CG, sealed, and stored at 4 °C. The daily calibrations provided a measure of the bed lifetimes under these conditions. We have successfully stored beds in this way for 1–2 months before use.

Flow Injection Analysis System. The enzyme packed bed for the desired analyte was connected to an HPLC pump (LKB 2248, Pharmacia, U.K.) and a high-pressure injection valve with a 20-μL loop. The mobile phase used was Na_2HPO_4 (0.05 M), ferrocenemonocarboxylic acid (0.5 mM), and EDTA (1 mM) adjusted to pH 7.0 with hydrochloric acid, with Kathon CG (0.05% by volume) added to inhibit bacterial growth. The flow buffer was vacuum-filtered before use. The bed outflow was connected via low dead volume tubing to the downstream detection electrode. The electrode was of the thin-layer cell design (BAS LC 4B Bioanalytical Systems), with a glassy-carbon working electrode held at 0.0 mV relative to an $Ag^+/AgCl$ reference electrode. The potentiostat used was of our own design and allowed currents from 50 μA to 0.2 pA to be measured without the need for filter stages. This is important given the rapid nature of the FIA response.

Assay Calibration. The height of a given FIA peak reflects the number of moles injected onto the column. At a given analyte concentration, the peak height varies with sample volume, which at a constant flow rate is determined by the sampling interval. The calibration curves for each assay were expressed in terms of the injected molar content in order to determine the range of sampling intervals over which the assays were most sensitive.

On-Line Microdialysis. The apparatus used is shown schematically in Figure 2. Male Sprague-Dawley rats (230–270 g) were anaesthetized with a mixture of Hypnorm and Hypnovel and a microdialysis probe implanted into the anterior right striatum according to published procedures.[22] The rats were allowed to recover for 24 h in large plastic bowls with free access to food and water. On experimental days, the probes were connected to a microinfusion pump (CMA/100, CMA Microdialysis, Sweden) through a liquid swivel, allowing the animals free movement, and perfused with aCSF at 2 μL min^{-1}. The outflow of the microdialysis probe was connected via low dead volume FEP tubing (0.1 μm cm^{-1}, CMA Microdialysis) to a 22-gauge square-ended injection syringe needle (Hamilton, Switzerland). After checking that the correct volume flow rate through the probe had been achieved, the needle was inserted into the VISF-1 injection port of an HPLC valve (Valco C6W) mounted as part of a CMA/160 on-line injector (CMA Microdialysis). The sampling volume of the valve, controlled by the microinfusion pump, was usually set at 5 μL, giving a 2.5-min sampling time, although it functioned well at 2 μL (1 min). Standards could be injected at any time with an injection syringe via the VISF-1 injection port. A three-point calibration was performed over the range of the dialysate content of the analyte. Typically, this calibration was linear; exceptionally, it was necessary to perform a nonlinear regression.

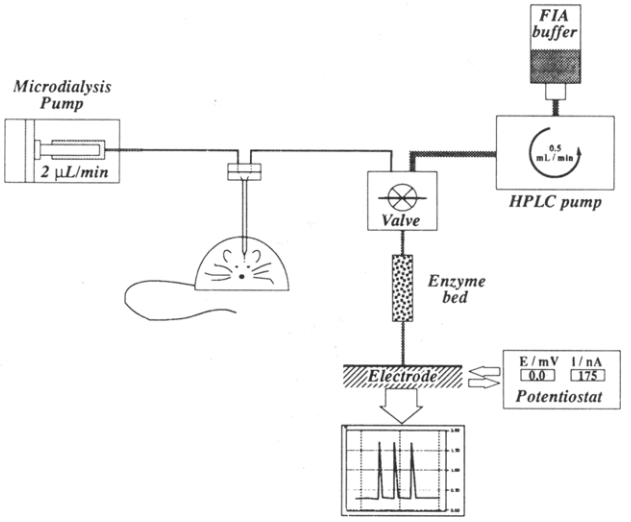

Figure 2. Apparatus for on-line microdialysis in the freely moving rat.

Table I. Enzyme Immobilization onto Tresyl Chloride Activated Silica Beads

enzyme	rel mass	enzyme coupled μg/mg dry beads	bead activity, mU/mg dry beads	bead sites coupled, %
GOx	160 000	3.85	886	40
GLOx	140 000	7.97	62.6	94
LOx	80 000	7.82	263	>100
HRP	40 000	13.14[a]	3286[a]	83[a]
		13.2[b]	3300[b]	83[b]

[a] Values based on loss of heme adsorbance at 403 nm from coupling solution. [b] Values based on assay of beads using ABTS (at 340 nm) as redox chromogen.

RESULTS AND DISCUSSION

Enzyme Immobilization. For a sensitive FIA packed bed we required high enzyme loading compared to the dead volume of the bed. For a practical assay the immobilization support had to be rigid (to prevent back-pressure buildup) and have mild, but very stable, covalent immobilization chemistry. Potential supports and immobilization chemistries have been extensively reviewed.[23,24] We experimented widely for each of the enzymes[25] before deciding on a variant of the Nilsson, Larsson, and Mosbach[19,20] tresyl-activated silica method as the method of choice. Details of the beads produced are summarized in Table I.

The oxidase enzymes showed similar properties and were immobilized most efficiently on 1000-Å pore size beads. The

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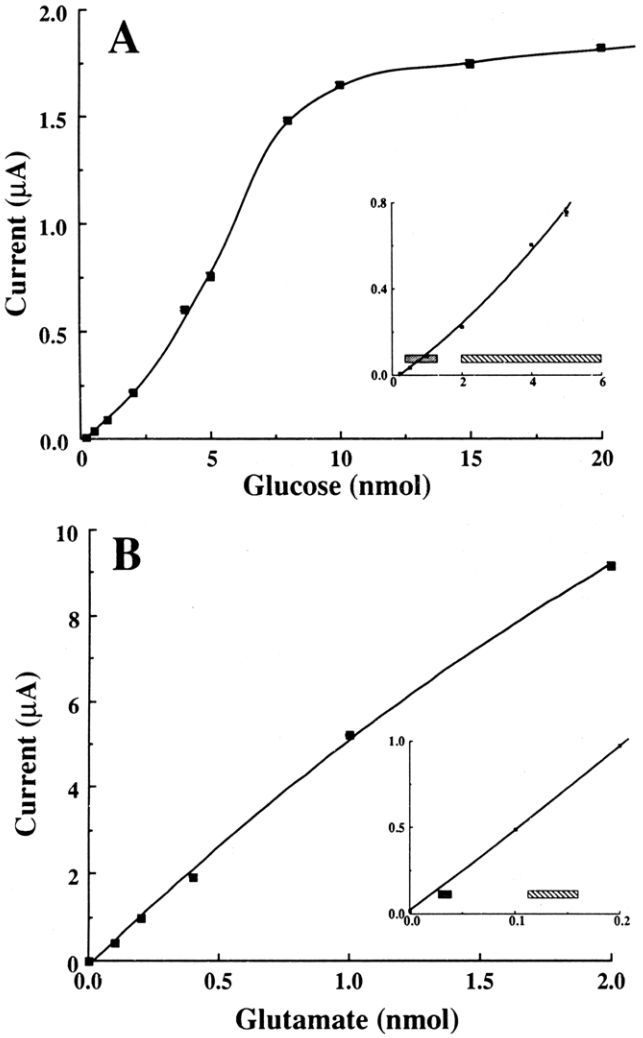

Figure 3. (A) Calibration of the glucose assay. The values given are mean ± standard error ($n = 5$). The inset shows the low level response to the assay. The dark bar indicates the range of stimulated and basal glucose molar contents found in 2.5-min (5-μL) microdialysis samples from the rat striatum; the light bar indicates the range found in 10-min (20-μL) samples. (B) Calibration of the glutamate assay. The values given are mean ± standard error ($n = 5$). The inset shows the low level response of the assay. The dark bar indicates the range of stimulated and basal glutamate molar contents found in 2.5-min (5-μL) microdialysis samples from the rat striatum; the light bar indicates the range found in 10-min (20-μL) samples.

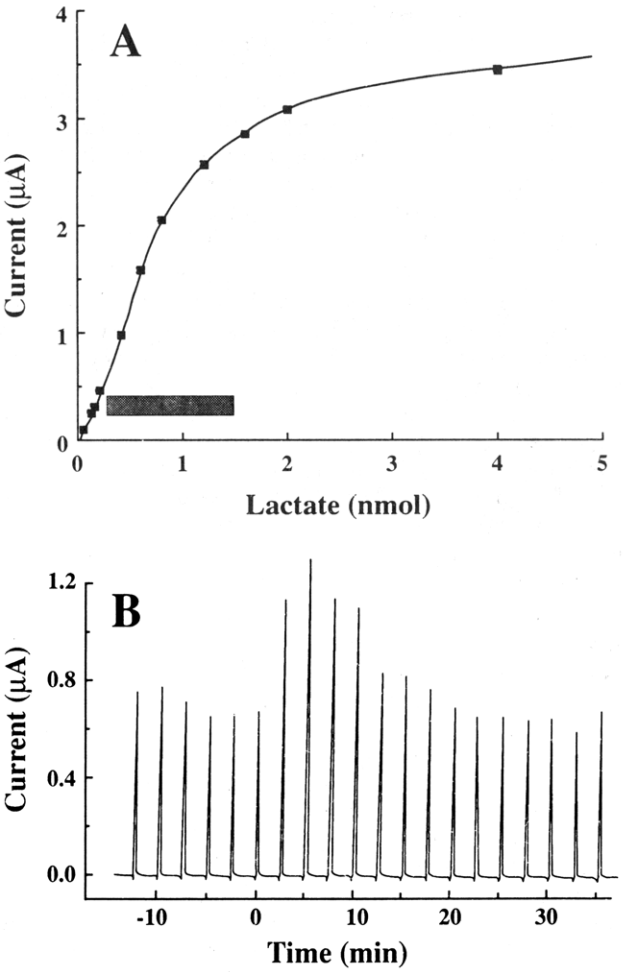

Figure 4. (A) Calibration of the lactate assay. The values are mean ± standard error ($n = 5$). The dark bar indicates the range of stimulated and basal lactate molar contents found in 2.5-min (5-μL) microdialysis samples from the rat striatum. (B) On-line assay of microdialysate from the striatum of a freely moving rat. Each peak reflects the lactate content of a 5-μL sample collected in the sample loop over a 2.5-min period. At time zero a 5-min period of behavioral stimulation (mild tail pinch) was begun.

lower immobilization efficiency obtained for GOx probably reflects a combination of protein impurities in the enzyme and the inaccessibility of some binding sites due to the larger size of the enzyme. However, this loading is much greater than in our original glucose assay,[12] allowing smaller beds to be used. The smaller HRP gave highest loadings on 500-Å pore beads, in agreement with the trend described by Olsson and Ögren.[26] Two values for HRP loading are given. The first is calculated from the loss of HRP heme absorbance in the coupling solution; the second is calculated from the activity of enzyme found on the beads. The close agreement between these two values shows that no significant denaturing of the enzyme occurs in the coupling solution or upon immobilization.

It was found in early experiments that the enzyme packed beds could suffer a sudden apparent loss of activity after 1–2 months of use. Enzyme denaturation or hydrolysis of the covalent link to the beads would be expected to result in a gradual decline in bed activity. The most likely cause of this rapid deterioration was massive growth of bacteria (known to be efficient scavengers of H_2O_2). Addition of EDTA to complex out trace metal ions and the biocide Kathon CG prevented this problem, and neither compound affected the activity of the immobilized enzymes (data not shown).

Glucose Assay. All three assays gave a single peak within 6 s of sample injection. A mean calibration curve for the glucose assay is shown in Figure 3A. The assay was sensitive, with a detection limit of <20 pmol. No loss of activity occurred within 30 days, and only a 20% loss of activity was found after 60 days. An on-line assay at 2.5-min intervals of microdialysate from a microdialysis probe implanted into the striatal region of the brain of a freely moving rat gave stable basal glucose levels of 0.7–1.2 nmol/5 μL of sample. We have used the assay to examine the effects on basal glucose in the rat striatum of systemic insulin[27] and local application of the ion channel modulators veratridine and tetrodotoxin.[28] The improvement over the 10-min sampling period of our earlier assay[12] has recently allowed us to detect transient changes in striatal glucose following the mild behavioral stimulation of

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tail pinch for the first time.[29] The range of glucose molar contents found under basal and stimulated conditions with 2.5-min sampling is shown by the dark bar, and with 10 min sampling by the light bar, in the inset in Figure 3A. We have also used the assay together with the variation of concentration method of Lönnroth et al.[30] in which the concentration of glucose applied through the probe is varied. The point of no net flux estimates the unperturbed extracellular glucose concentration in the rat striatum to be 0.47 ± 0.18 mM (n = 5).[28]

Glutamate Assay. A mean calibration curve for the glutamate assay is shown in Figure 3B. The assay was very sensitive, with a detection limit of 1.8 pmol, and very stable with no loss in response over 40 days. An on-line assay at 2.5-min intervals of microdialysate from a microdialysis probe implanted into the striatal region of the brain of a freely moving rat gave stable basal levels for glutamate of 30–40 pmol/5 μL of sample. The range of values for glutamate molar content found under basal and stimulated conditions is shown for 2.5-min samples by the dark bar and for 10-min samples by the light bar in the inset in Figure 3B.

Lactate Assay. A mean calibration curve for the lactate assay is shown in Figure 4A. The assay was sensitive, with a detection limit of <7.7 pmol. The response of the assay fell in the first two days to become linear to 4 nmol. After this there was a slow fall in response with time, but the assay was still sensitive enough to assay microdialysis samples after 40 days. An on-line assay at 2.5-min intervals of microdialysate from a microdialysis probe implanted into the striatal region of the brain of a freely moving rat is shown in Figure 4B. The average molar content of lactate in the first 10 min of this record is 0.64 ± 0.03 nmol/5 μL of sample. At time zero the animal was given a behavioral stimulation for 5 min (mild tail pinch[22]). An increase in the lactate response occurs very quickly, with a return to basal values 5 min after the stimulation period. The speed of this response is such that it would be missed by slower sampling. The range of values for lactate content found under basal and stimulated conditions is shown by the bar in Figure 4A.

Interference. The oxidase enzymes used have high specificity for their substrates. The electrode potential however is a potent promoter of electron-transfer reactions. The use of HRP followed by reduction of the electrochemically reversible oxidzed ferrocene species allowed the electrode to be held at a mild potential of 0 mV ($Ag^+/AgCl$), but it was important to rule out any contributions to the FIA peak from electroactive species present in the dialysate. Injection of dialysate in the absence of an enzyme packed bed gave no response.

A second form of interference is also possible. Species present in the sample could alter enzyme activities or interfere with the electron-transfer sequence at any point between the oxidase reaction and the detection of the oxidized ferrocene. This possibility was tested by adding known amounts of the substrate to samples of dialysate. The resulting peak height was found to be the sum of the original dialysate content and the added standard. This demonstrates that the assay is not subject to this sort of interference from any species present in the dialysate.

We have used a wide range of drugs in our neurophysiological studies including tetrodotoxin (1 μM), veratridine (50 μM), *N*-methyl-D-aspartate (10 mM), MK 801 (100 μM), and apomorphine (100 μM). None of these gave significant peaks themselves or had any effect on the size of the substrate peak.

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Registry No. Glucose, 50-99-7; glutamate, 56-86-0; lactate, 50-21-5.

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