The voltammetry of NADH has been characterized at carbon-fiber microelectrodes at scan rates up to 100 V/s. Electrochemical pretreatment of the electrode dramatically changed the properties of the modified electrode. Anodic pretreatment of the surface resulted in an adsorptive wave for NADH oxidation, while less adsorption was evident under more moderate conditions. The pH of the buffer used for the anodization played a critical role in determining the voltammetric peak shape. Oxidation of NADH at slow scan rates (<10 V/s) fouled the electrode. In contrast, consistent and reproducible voltammetry of NADH was observed at faster scan rates (100 V/s). This voltammetric measurement was used to monitor NADH generated during the oxidative deamination of glutamate catalyzed by glutamate dehydrogenase. A 150-μm-i.d. microdiode fiber was used to entrap the enzyme near the microelectrode tip, forming the dehydrogenase-modified carbon-fiber microelectrode.

INTRODUCTION

Fast-scan voltammetry (>100 V/s) at carbon-fiber microelectrodes has been used extensively to monitor the release and uptake of the easily-oxidized neurotransmitters (i.e. dopamine) in vivo. Unlike the catecholamines, most other neurotransmitters and metabolic intermediates are not electroactive at analytically useful potentials. In fact, most of these molecules have very few physical properties which can be utilized for chemical analysis (i.e. visible or ultraviolet absorption, fluorescence, or electrochemical activity). Chemical derivatization of these molecules requires extensive sample handling, and this requires the expenditure of time to carry out the physical handling and derivatization reactions. Thus, the small quantities and low concentrations of analyte limit the utility of derivatization methods for in vivo measurements, especially when the analysis must be performed on a second or millisecond time frame.

One possible solution to this problem is the use of an enzyme-modified microelectrode. Even though there are many "biosensors" reported in the literature, relatively few have the requisite size, sensitivity, selectivity, and temporal resolution to make such measurements. Additionally, most of these biosensors use oxidases, which liberate or consume oxygen. Since oxygen levels vary dramatically during neurotransmission, it can be difficult to dissociate physiological fluctuations from the oxidase-generated changes in oxygen concentration. This problem might be alleviated through the use of other enzymes coupled to different redox equilibria. Very few biosensor reports involve the use of dehydrogenase-catalyzed reactions, which are linked to the NAD+/NADH redox couple. This is because of the difficulty in the electrochemical measurement of NADH.

The electrochemical oxidation of NADH shows a large overpotential at most electrode materials (the $E_{1/2}$ for the electrochemical oxidation is typically 0.50 to 0.80 V vs SCE compared to the $E^0$ calculated for the homogeneous reaction, which is -0.56 V vs SCE). Additionally, the products of NADH oxidation have been shown to adsorb strongly on these electrodes and passivate the electrode surface. This problem is exacerbated when NAD$^+$ is present; the electrode surface is fouled very quickly, albeit consistently. Many attempts have been made to reduce this large overpotential for NADH oxidation through use of immobilized redox mediators. The problem with this approach is one of selectivity—can its oxidation potential be decreased selectively, so that other easily-oxidized species are not measured? This is difficult, since most of the protocols which reduce the overpotential for NADH oxidation will do the same for other interferents (e.g., ascorbic acid, uric acid, DOPAC, etc.).

Voltammetry at ultramicroelectrodes has several advantages over that performed at larger electrodes. Electrodes of micrometer dimensions have extremely low charging currents and fast response times. This facilitates the use of faster voltammetric scan rates (>100 V/s) which allows one to take analytical data with better temporal resolution. Additionally, fast scan techniques can differentiate species on the basis of their electron-transfer kinetics as well as their redox potentials. Species which have fast electron-transfer kinetics show reversible voltammetry at fast sweep rates, while less reversible species exhibit larger and larger overpotentials and, consequently, are easily distinguished from the more reversible species. This strategy has been very effective in minimizing interferences for the measurement of dopamine in vivo, where nanomolar levels can be measured selectively in the presence of a 10 000-fold excess of ascorbic acid. Since the rate of heterogeneous electron transfer for the oxidation of NADH is fast, the same strategy should be useful for the measurement of NADH. Another advantage of these methods...
fast electrochemical measurements is that electrode passivation is minimized.

Several electrochemical pretreatments were examined to optimize the quality and reproducibility of the voltammetric measurement. Anodic pretreatments at neutral or basic pH were found to increase adsorption of NADH/NAD+. This adsorption was short-lived, in that continuous application of the 100 V/s waveform resulted in diminution and eventual disappearance of the adsorbed wave. The same anodic pretreatment in acidic solution yielded a more reproducible, diffusion-controlled profile for the oxidation of NADH at 100 V/s. The voltammetric response was linear from 1 mM NADH to the detection limit of 7 μM. The response time for this measurement was determined with flow injection analysis to be 184 ± 65 ms, which is as fast as could be measured with this system. A microdialysis electrode was constructed where the enzyme, glutamate dehydrogenase, was entrapped within a 150-μm-i.d. hollow microdialysis fiber. This served as a prototype biosensor for the measurement of enzyme-generated NADH.

EXPERIMENTAL SECTION

**Chemicals.** Glutamate dehydrogenase (GDH, 40 units/mg, (EC 1.4.1.3), β-nicotinamide adenine dinucleotide (NAD+), β-nicotinamide adenine dinucleotide, reduced form (NADH), L-glutamic acid (Sigma); Epon 828 Resin (Shell Oil) and 1,4-phenylenediamine (Aldrich) were used without further purification. The phosphate buffer consisted of 150 mM sodium chloride and 100 mM sodium phosphate heptahydrate, adjusted to pH 7.4 unless otherwise indicated. All solutions were prepared in water degassed with nitrogen and purified by a Milli-Q water purification system (Millipore).

**Carbon-Fiber Microelectrodes.** Fabrication of carbon-fiber microelectrodes (Thornel P-55S, Amoco Performance Products) has been described previously. Briefly, a single 10-μm-diameter carbon fiber is aspirated into a glass capillary tube (1.2-mm o.d., 0.68-mm i.d., A-M Systems No. 60201, and a tapered end is produced after the capillary tube is pulled by a Narishige Model PE-2 microelectrode puller. A seal between the fiber and the capillary tube is achieved by utilizing Epon 828 epoxy with 12% by weight 1,4-phenylenediamine as hardener. Initial activation of the carbon surface was achieved by polishing for 10 min with diamond paste (1 μm, Buehler). The polished electrode was sonicated in hot toluene for 10 s and deionized water for 10 s to remove residual polishing material. Electrochemical pretreatments consisted of a 5-s application of a 50-Hz cyclic potential waveform (initial potential, ~0.2 V), with a switching potential of either 1.8 or 2.0 V. All potentials were referenced versus a Ag/AgCl reference electrode. The pretreatment was performed in either pH 12 phosphate, pH 7.4 phosphate buffer, or 1.0 M HCl.

**Instrumentation.** Cyclic staircase voltammetry was performed with an EI-400 potentiostat (Ensman Instruments, Bloomington, IN) where all waveforms were generated and currents acquired via an 80386 personal computer using an A/D-D/A interface (Labmaster DMA, Scientific Solutions, Solon, OH). A 100-MHz digital oscilloscope (Hewlett-Packard 54501-A) was used to observe all transients, and a Hewlett-Packard ColorPro was used to plot data. The flow injection analysis system consisted of a pneumatic actuator (Rheodyne, Model 5701) controlled via a solenoid valve (Rheodyne kit, Model 7163). A locally-built solenoid driver circuit allowed computer control of the compressed air-driven valves which switched flow from the buffer loop to the sample loop of the FIA. The electrochemical

![Scheme I](image-url)
cell was constructed from Plexiglas in such a way as to allow the microelectrode to be positioned ca. 60 mm from the output of the FIA sample loop. This cell was designed to match the internal diameter of the FIA tubing (0.75 mm) in order to minimize diffusional broadening of the analyte as it was transported to the microelectrode (specifications critical for the measurement of a subsecond response time). Finally, the flow of buffer (0.81 mL/min) was controlled by a syringe pump (Sage Instruments Model 341-B) or by gravity flow.

**Construction of a Microdialysis Electrode.** Two procedures were used to secure a dialysis fiber to the end of a carbon-fiber microelectrode (Scheme 1). A 2-cm length of 150-μm-i.d. hollow dialysis membrane (9000 MWCO, Spectrum Medical Industries, Los Angeles, CA) was attached to an empty glass capillary pipet with 5-min epoxy. The dialysis fibers were then flushed with ethanol for 10 min and with deionized water for 30 min. Next, a suspension of glutamate dehydrogenase was transferred into the membrane. The free end of the enzyme-filled membrane was then carefully guided onto the polished carbon-fiber microelectrode tip and attached with epoxy. When dry, the membrane was cut with a scalpel approximately 500 μm from the end of the electrode tip and sealed with epoxy.

Alternatively, the dialysis fiber (length ca. 1 cm) was cleaned with ethanol for 10 min, followed by deionized water for 30 min. The fiber was placed onto a microscope slide under a stereo microscope (Bausch and Lomb) and allowed to dry. A suspension of glutamate dehydrogenase was dialyzed for 24 h (12 000–14 000 MWCO, Spectrum) versus phosphate buffer to remove excess salts. One drop of the dialyzed solution was placed at one end of the fiber; capillary action filled the fiber with the enzyme solution. The fiber was then allowed to dry, and the technique was repeated to increase the enzyme concentration inside the fiber. A polished carbon-fiber microelectrode was mounted onto a three-dimensional stereotaxic manipulator (Kopf Instruments, Tujunga, CA) and carefully positioned into one end of the dialysis fiber. The fiber was cut approximately 0.2 mm beyond the electrode tip, and epoxy was applied to both the electrode/fiber junction and the open end of the attached dialysis membrane to prevent outward diffusion of the enzyme once placed in solution.

The distance between the epoxy at the fiber end and the electrode tip was minimized to reduce longitudinal diffusion of NADH to the electrode surface. The final assembly was allowed to cure in air for 45 min and then was immersed in a 12% v/v EtOH/phosphate buffer solution to facilitate rehydration of the dialysis fiber. Once hydrated, the electrode was transferred to a buffer solution containing 1 mM NAD⁺ for 30 min, then immersed in the FIA flow cell and flushed with fresh buffer (containing 1 mM NAD⁺) for 10 min. Both methods of construction were equally successful, although the latter was considerably more efficient. All sensors were stored in phosphate buffer at 0–5 °C to prevent the dialysis membrane from drying.

**RESULTS AND DISCUSSION**

Voltammetry of NADH at Carbon-Fiber Microelectrodes. The half-wave potential (E₁/₂) for the oxidation of NADH at a polished carbon-fiber microelectrode is approximately 500 mV vs Ag/AgCl (Figure 1, top), which is very similar to that observed at macroscopic glassy carbon electrodes.5-7 This represents at least a 1-V overpotential as compared to the E° calculated for the homogeneous reaction (−0.56 V vs SCE).6,7 A large overpotential can indicate slow electron-transfer kinetics, but this is certainly not the case for the oxidation of NADH at carbon fibers. As shown in Figure 1 (bottom), the voltammetry is quite electrochemically reversible at scan rates up to 100 V/s. This is interesting to note, since many previous investigators have ascribed this large overpotential to slow electron-transfer kinetics. From these data, it is easy to see that electron-transfer kinetics are not responsible for the observed overpotential.

There is considerable variability in the reversibility of NADH oxidation at different carbon-fiber electrodes as demonstrated by voltammetry at 100 V/s (Figures 1 and 2). A clean, polished carbon-fiber surface can produce an
oxidative wave with a diffusive peak potential \( (E_p) \) anywhere between 800 and 1000 mV vs Ag/AgCl (Figure 2A). Though there can be great variation from one electrode to another, the voltammetry of NADH at the same electrode is very consistent. A mild electrochemical pretreatment was employed to improve the reversibility of the voltammetry, since several investigators have reported that the presence of oxygen functionalities on carbon facilitates electron transfer with NADH.\(^{14-16} \) Oxidation in a neutral or basic pH buffer decreases the overpotential for the diffusion-controlled wave but also introduces a very sharp, symmetrical prepeak at roughly 500 mV, which is presumably due to product adsorption (Figure 2B).\(^6 \) This pretreatment decreases the reproducibility of NADH voltammetry from electrode to electrode, in that the magnitude of the peak current and potential of both diffusion-controlled and absorptive waves are extremely variable (Figure 2B,C).

Adsorption of an analyte onto an electrode surface has often been used to increase the sensitivity of an electrochemical measurement.\(^2 \) This has been especially true for the measurement of the catecholamine neurotransmitters, where fast adsorption kinetics allow preconcentration of dopamine onto the electrode surface, resulting in greatly enhanced detection limits without loss of temporal response.\(^1,2 \) In the case of NADH, adsorption could serve the same function, i.e., to improve detection limits. However, the magnitude of NADH adsorption was found to diminish as a function of the number of voltammetric scans applied to an electrode. As shown in Figure 3 (top), the diffusion-controlled component for the oxidation of 100 \textmu M NADH was virtually unchanged over 2850 scans, while the absorptive wave at 525 mV gradually disappeared as the electrode was continuously scanned in pH 8.5 buffer. Diffusion-controlled behavior could be obtained at any of these surfaces simply by cycling the electrode in buffer for a few thousand scans at 100 V/s. Once this was done, very reproducible voltammetry could be obtained at the electrode. This is demonstrated by the concentration independence of NADH voltammetry (Figure 3, bottom). As shown, the voltammetry at 100 V/s is independent of concentration in the diffusion-controlled region (>750 mV), but some adsorption of NADH is apparent at lower concentrations. The time course for the appearance of both diffusion-controlled and absorptive waves was identical (<200 ms; the limit for our FIA cell), giving additional support to the idea that the prepeak was a result of the adsorption of an electrochemically-generated product. When the current is averaged between 750 and 1000 mV in each scan, the result is linear with concentration from 1 mM NADH to a detection limit of 7 \textmu M \((r^2 = 0.9995, n = 6)\).

Fortunately, an oxidative pretreatment which produced the most consistent NADH voltammetry generally did not produce an absorptive wave. Application of a -0.2- to 2.0-V cyclic waveform (50 Hz for 3 s) in 1 M HCl produced an ideal surface for the diffusion-controlled oxidation of NADH (Figure 2D). The \( E_p \) for NADH oxidation (719 ± 20 mV vs Ag/AgCl, \( n = 3 \)) and the peak current \((i_p = 9.89 ± 0.26 \text{nA}, n = 3)\) were extremely reproducible from electrode to electrode. Anodic treatments in highly acidic media have been reported to produce the greatest increase in carbon–oxygen bonds (particularly OH and C=O).\(^{17} \) Since there is a great deal of evidence to suggest that electron transfer to NADH is mediated via the quinone/hydroquinone redox couple on the carbon surface,\(^{14-16} \) it is not surprising to see faster electron-transfer rates at surfaces oxidized under acidic conditions. In contrast, the same electrochemical conditions used in neutral or basic pH buffers produce far less extensive oxidation of the surface, and what oxidation does occur takes place at alcohol/ketone groups already present at edge sites on the carbon surface.\(^{17} \) Thus, electrochemical oxidation of the carbon surface at high pH may even reduce the density of hydroxyls and carbonyls, while increasing the density of carboxylates. This, in turn, may reduce the number of electron-transfer sites for NADH oxidation, effecting a less reproducible surface for electron transfer.

The oxidation of NADH is chemically irreversible due to the rapid protonation kinetics occurring in the NAD\(^+\)/NADH redox couple.\(^6 \) As demonstrated recently for synthetic analogs of NADH, the electrochemical oxidation of NADH follows an ECE mechanism where the initial electron-transfer step is not rate-limiting. The subsequent proton transfer is rate-limiting, but is so rapid that it can only be outrun by voltammetry at very fast scan rates (>10 000 V/s) in very basic solutions.\(^{18} \) The products of NADH oxidation have been shown to adsorb strongly on these electrodes and

\( \text{Figure 3. Stability of NADH adsorption on pretreated carbon-fiber microelectrodes. Top: Voltammetry (100 V/s) of NADH at an electrochemically-treated micro electrode (application of a cyclic potential waveform between -2.0 and 1.8 V vs Ag/AgCl at 50 Hz for 3 s in pH 7.4 phosphate buffer) as a function of the total number of scans at the electrode. NADH (100 \mu M) was introduced briefly (during 12 scans) into the FIA system (pH 8.5 phosphate buffer) after 100 (A), 950 (B), 1900 (C), and 2850 (D) scans. Bottom: The concentration dependence of NADH voltammetry at a preconditioned electrode (2000 conditioning scans were applied in phosphate buffer). The current was normalized to the concentration of NADH to show the relative contribution of adsorbed NADH to the voltammetric signal. The concentration of NADH was 1 mM, 250 \mu M, 100 \mu M, 75 \mu M, 50 \mu M, and 25 \mu M. } \)
passivate the electrode surface. This is particularly true at oxidized carbon electrodes or where catechols have been specifically introduced to the electrode surface. There is considerable evidence suggesting a chemical reaction between the oxidation products and catechol moieties on the surface forming a stable semiquinone. Indeed, several investigators have shown that the voltammetry for NADH oxidation observed at glassy carbon electrodes is strongly dependent on electrode history. This type of behavior is also observed at carbon-fiber microelectrodes at slow scan rates (1 V/s), where the high current density produces a large amount of oxidation products (Figure 1, top). The half-wave potential shifts out to higher positive potentials and the faradaic current decreases slowly with each successive scan, both indicative of surface fouling.

Voltammetry at faster scan rates (100 V/s, 15 mV step height, 150 μs per step) is minimally affected by exposure to NADH (Figure 1, bottom). The background-subtracted voltammogram obtained after thousands of scans is virtually identical to the very first scan acquired. This is true even in the presence of an excess of the cofactor NAD⁺ (Figure 4, top), which is necessary for optimal activity of the dehydrogenase. Thus, another advantage of the fast scan technique is that electrode passivation is minimized under these conditions. This may be due to the small amount of oxidation products generated with extremely short electrolysis times, as well as the rapid transport of these materials away from the electrode due to divergent diffusion.

Fast-scan voltammetric methods allow time course data to be acquired on the millisecond time scale. Since a single voltammetric scan takes less than 20 ms to complete at 100 V/s, the voltammetry can be repeated with a time interval of a few milliseconds. The current is then averaged in the potential region of interest (a digital sample and hold), and this average current is plotted as a function of time after a 2-s injection of 100 μM NADH.

![Figure 4](image)

**Figure 4.** Voltammetry of NADH in the presence of a 100-fold excess of NAD⁺. Top: Background-subtracted voltammograms of 50 μM NADH at 100 V/s were obtained in the presence of 5 mM NAD⁺ (+++ or in phosphate buffer (—)). Bottom: The time course of the appearance of NADH in a FIA system was monitored at the same electrode using repetitive fast scan cyclic staircase voltammetry (100 V/s; 2-a interval between scans). The current (averaged between 750 and 1000 mV on each scan) is plotted as a function of time after a 2-s injection of 100 μM NADH.

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Fast-scan voltammetric methods allow time course data to be acquired on the millisecond time scale. Since a single voltammetric scan takes less than 20 ms to complete at 100 V/s, the voltammetry can be repeated with a time interval of a few milliseconds. The current is then averaged in the potential region of interest (a digital sample and hold), and this average current is plotted as a function of time. Flow injection analysis is ideal for the characterization of the response time of such a measurement since it is possible to position a probe of micrometer dimensions directly at the outlet of the injection system. This allows for a rapid and brief exposure of the microelectrode to each sample, thereby facilitating subtraction of background currents. Additionally, the response time of the electrode can be evaluated by monitoring the time course of the appearance of the sample following injection. The response observed to this "square-wave" input will be characteristic of the system response of the electrochemical measurement. As shown in Figure 4 (bottom), the response of the voltammetric measurement to the injection of NADH in a flow injection analysis experiment is quite rapid; the response time of the entire instrument is 184 ± 65 ms (n = 10). This permits the use of NADH as the diffusive redox mediator for immobilized dehydrogenases without the need for continuous regeneration of the electrode surface. Additionally, it indicates that the voltammetric measurement of the enzyme-generated NADH will not be the rate-limiting step.

**Glutamate Dehydrogenase Microdialysis Electrode.** Since the objective of this paper was to demonstrate that dehydrogenase-generated NADH can be monitored at a carbon-fiber microelectrode, the simplest approach for electrode modification was used (i.e., physical entrainment of the glutamate dehydrogenase within a 150-μm-i.d. hollow...
fiber microdialysis membrane mounted at the microelectrode tip). Glutamate dehydrogenase catalyzes the oxidative deamination of glutamate and simultaneously reduces NAD⁺ to NADH. The NADH generated in this manner acts as an electron-transfer mediator and can be monitored with fast scan cyclic voltammetry at the carbon-fiber microelectrode surface. This allows the indirect detection of glutamate. Microdialysis electrodes were placed on-line in the FIA system, such that the response of the integrated sensor could be characterized. An injection of 10 mM glutamate generated a response at the microdialysis electrode with a response time of 17 ± 6 s (n = 6, Figure 5). The response time of the probe is limited by the diffusion of glutamate through the 150-µm dialysis fiber since the fast response was restored after removal of the membrane (data not shown). As shown in the inset, a background-subtracted scan of the NADH produced by the action of glutamate dehydrogenase was voltammetrically identical to authentic NADH. In this design, the voltammetry of NADH was often severely degraded after exposure of the electrode to the GDH enzyme. The peak potential shifted to much higher overpotentials, and the current response dropped by over an order of magnitude, presumably due to the adsorption of GDH onto the carbon surface. While these are significant problems, this work does demonstrate the feasibility of dehydrogenase-modified ultramicroelectrodes for the indirect measurement of amino acid neurotransmitters. Optimization of this design will ultimately allow its utilization for the analysis of neurotransmitter dynamics in vivo.

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