

ELECTROCHEMICAL TECHNIQUES APPLIED TO BRAIN CHEMISTRY

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Ralph N. Adams is Professor of Chemistry at the University of Kansas. His training in electroanalytical chemistry was with the late N. Howell Furman at Princeton in the early 1950s. He and his coworkers studied the electro-oxidation of aromatic systems, radical ion intermediates in anodic electrochemistry, and solid electrode voltammetry for some 15 years before switching to the area of neurochemistry and biological psychiatry. Recently he completed three years of training in clinical psychiatry in an Interdisciplinary Studies program at the Menninger Foundation in Topeka, Kansas. He now holds a joint appointment in Neurobehavioral Sciences at the Menninger School of Psychiatry.

In 1970 I spent a sabbatical leave at the University of California, Irvine, essentially as a graduate student in their Department of Psychobiology, to get some fundamental training in the neurobehavioral sciences. I had become convinced that some 15 years of experience in electroanalytical chemistry could be applied to neurochemical systems and might provide some useful new methodology. As a result of this, the direction of our research shifted completely, and the laboratory might now be characterized as an electrochemically oriented psychobiology group. Actually, our work is not as far-out as that description might imply. We have applied some straightforward electrochemical methods to problems in neuropharmacology and neurophysiology. This article presents these approaches in the hope that they will interest workers in these fields and make them aware of their potentialities.

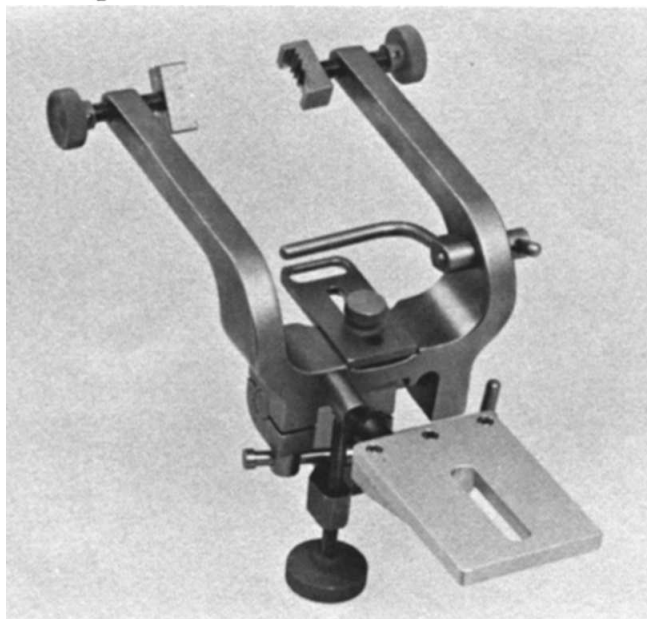
The basic electrochemical experiment involves a very simple circuit shown in Figure 1. A small source of voltage (e.g., 1.5 V dry cell) powers a low resistance voltage divider from which various potentials (E_{app}) between 0-1.5 V may be applied to a pair of electrodes. The reference electrode (which serves only as a bench-



mark and takes no part in the solution electrochemistry) is typically a Ag/AgCl electrode familiar in electro-physiology. The working electrode (so called because electrochemical reactions of interest "work" at its surface) is a highly inert material like platinum, gold or graphite. As E_{app} varies, electroactive species near the electrode surface can either gain electrons (reduction) or lose them (oxidation) to the electrode and a current flow results.

The circuit of Figure 1 is easily modified to automatically record E_{app} -current curves called voltammo-grams. In actual practice the simple circuit is usually replaced by an all electronic or computer-controlled potentiostat. The potentiostat maintains dynamic control of E_{app} at the working electrode with respect to the reference via a third auxiliary electrode. The potentiostat is similar in nature to, and serves the same purpose as, the classical voltage-clamp circuitry of electrophysiology. It "clamps" the potential of the working electrode and maintains it under instantaneous control. The clamped value can be varied in any wave form the experimenter desires. The potentiostat contains the control or clamp functions as well as high sensitivity current measuring capabilities and outputs to recording devices. The PAR 174 is a commercial unit well suited to many of the studies mentioned herein (1). Laboratory mini-computers routinely function as potentiostats with exceptional data handling capabilities.

(Continued on page 2)



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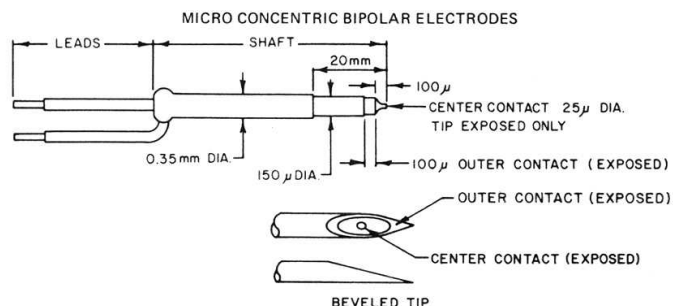
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A new metal electrode, the MCE-100, is available with or without beveled tip. It is a concentric bipolar electrode with an outer contact diameter of 150 microns and 25 microns inner contact diameter.

Continued

Whether we use the simple or potentiostat circuit, the experiment is the same. To study oxidations, in which we are primarily interested, the E_{app} of the working electrode is varied, say, from 0.0 V to +1.5 V, and the resulting voltammogram recorded. Two basic types of voltammograms are obtained, depending on whether the solution in which the working electrode is immersed is quiet or stirred.



NEW MCE-100

If the solution is stirred or rapidly flowed past the electrode, the voltammogram is like that in Figure 2A. As E_{app} is swept to positive values (more oxidizing), molecules near the electrode surface begin oxidizing (at characteristic potentials) and the electrons given up to the electrode constitute increasing current flow. The current reaches a steady-state, limiting value, i_{L} , because fresh electroactive material is constantly being supplied to the electrode by the stirring or forced flow. This is directly proportional to the concentration of electroactive material in solution.

Figure 2B shows the result in quiet solution, commonly called a peak voltammogram. In this case, fresh electrooxidizable species can only get to the electrode surface by diffusion. The current, which initially increases with E_{app} , begins to fall off as diffusion transport fails to "keep pace," and a maximum or peak in the current voltage trace results. The potential, E_p , is characteristic of the species undergoing oxidation and the peak current, i_p , is directly proportional to its concentration in solution. Much more information can be obtained in the quiet solution case if E_{app} is swept in a cyclic fashion (cyclic voltammetry). For example, suppose the initial (forward) potential sweep oxidizes a species A to B, then B, if stable, stays close to the electrode surface long enough to be re-reduced when the potential sweeps backward. Thus a peak voltammogram is seen on the reverse sweep for the reduction of B just produced at the electrode surface. But, if B rapidly decomposes or undergoes some fast chemical reaction, the reduction current may be missing or some entirely new peak current will be seen. Cyclic voltammetry is particularly useful in certain in vivo experiments mentioned later. (Cycling E_{app} in stirred solutions gives no extra information since anything formed at the electrode surface is quickly swept away.)

One other type of electrochemical measurement is of special interest in the present discussion. Rather than vary E_{app} , the potential of the working electrode is stepped or fixed at a value where the full extent of the reaction will occur (e.g., at a value corresponding to i_L in Figure 2A or past E_p in Figure 2B). Now, with E_{app} fixed, one monitors the electrolysis current. Obviously, in the stirred or flowing solution just a steady, time-dependent current is observed. If, however, the

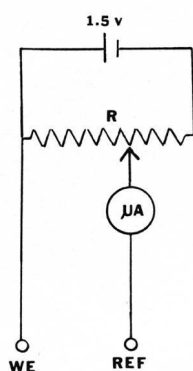


FIGURE 1

concentration of the electroactive species is changed by some external source, the electrolysis current will follow these changes. This is classical amperometry and neurochemists may be familiar with one style of it amperometric titrations of sulfhydryl groups in peptides with silver ion or iodine. An example will be given later where the current will rise and fall with the concentration of species eluting from a chromatographic column as they flow past an electrode in separated sequence.

In quiet solution if the potential is stepped and held fixed, the current will continuously decay. The current falls inversely with t^* . These measurements are termed chronoamperometry. Although not a steady-state measurement, the product it is directly proportional to concentration of electroactive species in solution. Chronoamperometry is highly analogous to voltage clamp measurements on squid axons. Rather than watch ionic current flow at a membrane following a potential step: one observes oxidation-reduction current (electron transfer) at an electrode surface.

All the characteristics of stirred or quiet solutions voltammograms are precisely described by electrochemical theory and apply to either oxidations or reductions. A more thorough introduction to voltammetry written especially for those interested in pharmacology is given in the literature (2) and a detailed monograph on organic oxidations is available (3).

The above discussions would appear to have little, if any, relationship to brain chemistry. However, it turns out that a variety of endogenous brain constituents are easily oxidized at inert electrodes like platinum and graphite. The curves of Figure 2 are experimental voltammograms for the oxidation of dopamine (DA) in pH 7.4 buffer at a graphite electrode. All the catecholamines and their metabolites are electro-oxidizable. Similarly, serotonin and its metabolite 5-hydroxyindole acetic acid (5-HIAA) are electroactive. Using ordinary size electrodes which have been commonplace for years in electrochemical practice one can develop extremely useful new analytical procedures as well as study a variety of in vitro interactions of these neurotransmitters with drugs, etc. Or, one can miniaturize the electrodes and use them for in vivo electrochemical measurements. A brief summary of these two approaches follows.

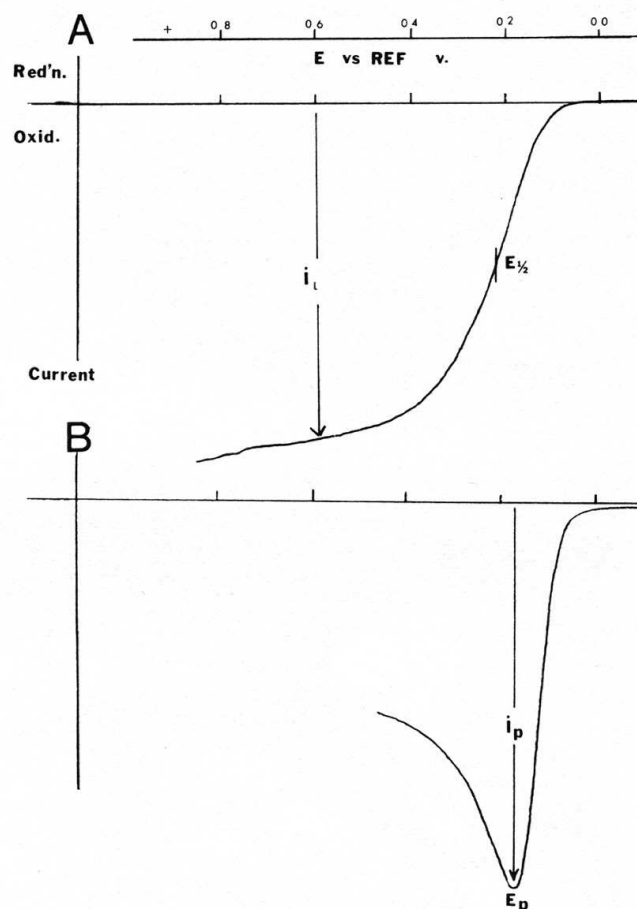


FIGURE 2

PICOMOLE LEVEL ANALYSES FOR ADRENERGIC TRANSMITTERS AND METABOLITES

The measurement of current in a flowing solution as described above can be made an exquisitely sensitive measure of concentration. This sensitivity, by itself, is often of little value in dealing with the complex mixtures in neurochemical analyses. We combined the ultrasensitivity of electrochemical detection with a technique of high separation capabilities by adding it to high performance liquid chromatography (HPLC). The latter is basically conventional column chromatography using very small particle size supports and hence has excellent resolution capabilities. The detector is just an amperometric electrode in the flowing solution at the end of the chromatographic column (4). The usual potentiostat fixes the detector electrode potential and measures current as each electro-oxidizable component elutes from the column.

With such simple equipment it is possible to determine ca. 20 picograms to 2 nanograms of NE and DA in brain tissue punches as small as 0.5mg on a routine basis. From the sensitivity perspective this method is directly comparable to the gas chromatography-mass spectrometry assay. A detailed report of this high sensitivity method, especially applicable to catecholamine brain mapping is available (5).

Probably of more interest to this readership is that the LC method is ideally suited to operate in conjunction with acute in vivo experiments. The high sensitivity

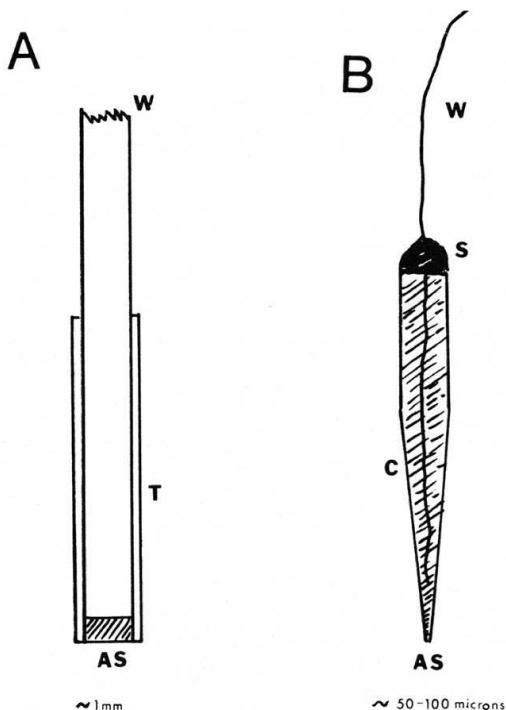


FIGURE 3

requires only 2-10 microliter samples. Thus rat or other small animal CSF can be sampled several times via a microsyringe and ventricular cannula without serious disturbance to the animal. These CSF samples can be analysed by direct injection into the liquid chromatograph (no sample pretreatment). For example, one can follow homovanillic acid (HVA) and 5-HIAA release into CSF this way after electrical stimulation of sub-stantia nigra and raphe, respectively, or HVA release following L-DOPA treatment (6). The sensitivity requirements of such experiments have, in the past, usually been met by using radiotracers. Even then considerable integration occurred because several milli-liters of perfusate were ordinarily collected for counting. The LC method has ample sensitivity for discrete, micro .sampling of CSF.

A most promising recent application is the monitoring of transmitter release from brain slices following drug or electrical stimulation. By bathing a brain slice in a minimal volume of solution (ca. 250 microliters), the concentrations of released transmitters are easily analysed by direct LC sampling. Without radioactive precursor treatments or perfusion, Plotsky (7) has studied various adrenergic neurotransmitter release processes. A particular advantage of the new method is that more than one released transmitter or metabolite can be determined simultaneously with the proper chromatographic analysis. With refinements it may be possible to combine LC sampling with the brain slice techniques practiced by Lynch and coworkers (8) and provide simultaneous chemical and electrophysiological monitoring of restricted neuronal populations.

In Vivo VOLTAMMETRY

While it may seem heretical to neurophysiologists, when electrochemical techniques are applied in the CNS we encourage current flow. In fact, as described earlier, its variation with applied potential is the very

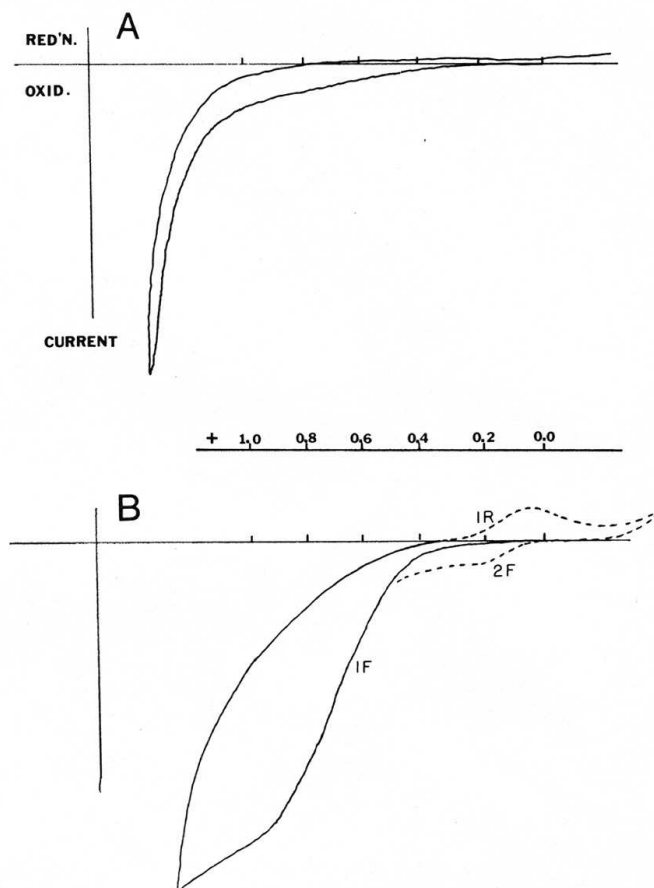


FIGURE 4

signal we want to record as a voltammogram. This is no contradiction of the working principles of electrophysiology which dictate minimal current drain from the signal source. With *in vivo* voltammetry one is measuring in a different substrate and signal domain the chemical domain. One is detecting not the electro-physiological potentials, but the chemicals in the CNS milieu which evoke and modify them. The dc current (always in the nanoampere range) is a measure of these chemical concentrations.

Since CNS tissue and CSF are highly conducting media, one can insert a small electrode almost anywhere in the brain and measure voltammograms. Graphite is a highly satisfactory electrode material. Mixing graphite into a dry paste with an inert organic liquid like Nujol (mineral oil) makes it an excellent inert surface for electrochemical oxidations. Two styles of miniature graphite electrodes we have used most are seen in Figure 3. These electrodes may be stereotactically positioned in tissue or in CSF via cannulae. The location of the reference and auxiliary electrodes is not particularly critical.

Suppose we insert the electrode of Figure 3A through a previously implanted cannula in the lateral ventricle of a rat or rabbit. The electrode depth is adjusted so its graphite surface is just immersed in the CSF. The cannula can serve as auxiliary electrode and the reference is placed out of the way in the contra-lateral cortex. The anesthetized animal is normally kept in a head holder so inadvertant movements will not

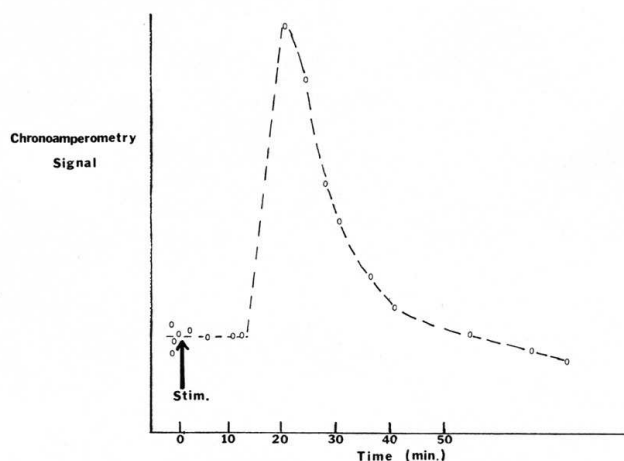


FIGURE 5

break electrical connections. Figure 4A shows the cyclic voltammogram obtained by starting the sweep of E_{app} from 0.0 V to +1.2 V and back. Not much of interest appears only a small, slightly increasing "residual" current is seen between 0.0 V and about +1.0 V and then the current rapidly increases and would go off scale if continued. Electrochemists call this a background voltammogram in the potential range examined and with the current sensitivity used, no major oxidation current signals are seen. Finally as E_{app} increases, the background constituents (here presumably the CSF electrolytes) oxidize and limit any further detection.

Actually there are oxidizable species in the CSF, but they are quite low in concentration in this resting or baseline state where the animal has not been subjected to any special stimulation. Thus LC sampling of the CSF reveals that the sum of all catecholamine and 5-HT metabolites (all of which are oxidizable at the graphite electrode) is ca. 20-30 μ molar. There is about 100 μ molar ascorbate present which oxidizes at about +0.4 V. All of these components make up the sloping residual current line between 0.0 V to +1.0 V. (If current sensitivity is increased one can see discrete peaks for some of these components. It should be noted that CSF movement is very slow by electrochemical standards. It can be considered a quiet solution; therefore, peak voltammograms are obtained. These are sometimes distorted by large molecular weight constituents which tend to film the electrode surface.)

Now assume this same animal had bipolar stimulating electrodes implanted in the substantia nigra. We excite the nigro-striatal DA pathway by electrical stimulation of substantia nigra and monitor the CSF voltammetry every minute or so. Figure 4B is a cyclic voltammogram recorded some 15-20 minutes after such stimulation which shows a startling change has occurred. On the first oxidizing sweep of E_{app} (IF), there is now a large flattened peak current beginning at ca. +0.8 V. This peak is identical with that obtained from authentic HVA in CSF. LC sampling of the CSF proves without question that there has been a large increase in HVA concentration but no significant changes in any other

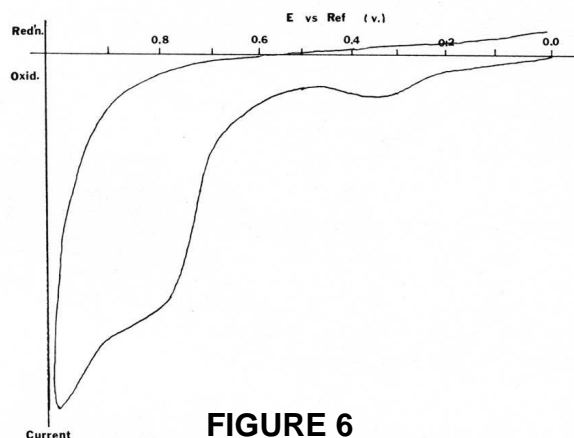


FIGURE 6

catecholamine metabolites. The voltammetry is detecting the expected release of caudate DA via its metabolite HVA seen in the ventricular CSF.

To continuously monitor the time course of HVA release, instead of cycling the E_{app} one steps the potential suddenly to, say, +0.9 V and measures the current for 200 milliseconds the chronoamperometry experiment. This pulsing is repeated at any desirable interval (every 60 seconds, for example) and the $i_{t < 4}$ values are directly proportional to HVA concentration. A typical plot of the time course of release and wash-out of HVA following stimulation is seen in Figure 5. After the CSF has returned to baseline conditions, repeated stimulations can be monitored.

Even without LC confirmation an electrochemist would know HVA had been detected from the pair of peaks at ca. +0.1 V in Figure 4B. These, seen only in the reverse sweep and subsequent cycles (indicated by dotted lines 1R and 2F), come from the oxidation and reduction of dihydroxyphenylacetic acid, DOPAC. This is formed at the electrode surface when HVA, after oxidation, splits off its methyl group a well established electrode reaction.

In similar fashion, other electrical and chemical stimulations of neural pathways may be monitored, e.g., midline raphe in the rabbit releasing 5-HIAA into CSF. All the electrochemical results are confirmed by LC analyses. A brief note on these results has appeared (9) and a full description will soon be available.

Measurements directly in CNS tissue are a bit less clear cut at present. Figure 6 shows a typical cyclic voltammogram at a micro capillary electrode in rat caudate nucleus. The same peak at +0.8 V for HVA is seen, but it may be a composite including some glutathione oxidation. There is an additional peak at +0.3 V clearly due to the oxidation of ascorbate (known to be present in about millimolar concentration in brain tissue). Unfortunately, this ascorbate peak obscures any DA or NE one might hope to see since all three compounds are oxidized at about the same potential. In this regard, a very significant achievement has recently been reported by Lane and Hubbard. Using an iodine-treated platinum electrode, they are able to differentiate electrochemically between ascorbate and DA and have been able to detect endogenous DA in rat caudate nucleus (10, 11).

There are many unanswered questions about in vivo tissue voltammetry at present. Much remains to be learned about quantitative responses, smaller electrodes

need to be designed, and theoretical questions about the electrode-solution-tissue interface need examining. While continuing to study these fundamental problems, we have gone ahead and surveyed some potential applications. We have chronically implanted the graphite-epoxy capillary electrodes (70-100 μ tip diameter) in rat caudate. Voltammetric signals have been recorded continuously for periods of days (intermittently over a 12 day period so far in one animal). The signals are uniformly like those of Figure 6. The quantitative chronoamperometry measurements are relatively constant in a given animal and undergo slow rhythmic changes. Electrodes in the rat caudate nucleus seem to respond in expected fashion to drugs known to stimulate catecholamine release, e.g., amphetamine treatment. These results, while very exploratory at present, are very exciting because they represent continuous in vivo monitoring of chemical changes in the brains of unanesthetized, unrestrained animals (12).

One other style of tissue voltammetry is of particular interest to neuropharmacology. A graphite electrode can be attached to the barrel of a Hamilton micro syringe and the pair placed in brain tissue. Now electro-active drugs can be injected near the electrode tip and detected. With species like the powerful neurotoxin 6-hydroxydopamine one can watch its rapid interactions with neural tissue. A variety of quantitative manipulations are possible with this technique which is fully described in the literature (13, 14).

Many questions have been asked about the effect of the voltammetric current flow. As mentioned previously, it is usually in the low nanoampere range and rarely greater than 600-700 nanoamperes. Thus, it is far below most physiological stimulating current levels. Indeed, it can couple with on-going electrophysiological events. However, it is likely that its influence is of the same order of magnitude as the chemical changes induced by electrode tissue injury. The latter can also affect physiological events. At present, the current flow is a second order perturbation which can be set aside to worry about when much more refined voltammetry in CNS can be accomplished.

It is hoped that the above paragraphs give a satisfactory introduction and feeling for the potentialities of the electrochemical techniques. The LC with electrochemical detection is a reliable and well-tested technique by now and many applications are possible in small animal neuropharmacology. The in vivo voltammetry is far more exploratory, but the results to date are very encouraging and offer exciting possibilities. All of the work is limited to the adrenergic and serotonergic systems. Unfortunately, acetylcholine, GABA and most of the other suspected neurotransmitters are not electrochemically active. It may be noted that the style of electrochemistry used so far is quite unsophisticated there is a whole bag of electrochemical tricks to be used as required. If the proper refinements can be made, in vivo voltammetry might help to unravel some of the ever-shifting chemical changes which orchestrate the electrical patterning of Sherrington's "enchanted loom." Given the present capabilities, this is a grossly over-enthusiastic evaluation. But without such enthusiasm no one in his right mind would even be trying electrochemistry in the brain.

ACKNOWLEDGMENTS

I am especially indebted to James McGaugh, Richard Thompson, Carl Cotman, Gary Lynch and the staff and graduate students at UCI for their initial training and encouragement. Any success the work may have had is due to my chemistry students and coworkers as well as the experimental psychologists working with us who have had the courage to pursue this borderline area.

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LEGENDS FOR FIGURES

FIGURE 1. SIMPLE CIRCUIT FOR VOLTAM-METRY

R, 10 turn Helipot or similar linear slide-wire, typically 50-100 ohm. uA, microammeter or other current measuring device. WE, working electrode REF, reference electrode For recording, the ammeter is replaced by a standard series resistor and the IR drop fed to the pen axis of an X-time recorder. The slidewire can be operated by a reversible synchronous motor correlated with the chart speed of the recorder.

FIGURE 2. VOLTAMMOGRAMS IN STIRRED vs. QUIET SOLUTION

- A. Typical Oxidation Voltammogram at Graphite Rotated Disk Electrode $E_{1/2}$, the potential where $i=0.5i_L$.
- b. Typical Oxidation Voltammogram at Graphite Miniature Electrode in Quiet Solution E_p , the potential at $i=i_p$.

FIGURE 3. TYPICAL MINIATURE GRAPHITE ELECTRODES

- A. Teflon Sleeve Type
 - W, 27 gauge stainless steel wire for electrical connection.
 - T, pressure-fitted Teflon tubing sleeve cut ca. 1 mm past end of wire.
 - AS, active surface of graphite paste electrode, ca. 1 mm dia.
- B. Micro Glass Capillary
 - W, stainless steel wire for electrical connection, extends about halfway down capillary. S, seal or cap to hold wire connector
 - C, glass capillary filled with graphite-Nujol-epoxy resin, hardened in place.
 - AS, active surface of graphite resin electrode, ca. 50 - 100 u tip dia.

FIGURE 4. CSF VOLTAMMETRY BEFORE AND AFTER STIMULATION OF SUBSTANTIA NIGRA

- A. Cyclic Voltammogram of Ventricular CSF baseline condition, before stimulation
- B. Cyclic Voltammogram of Ventricular CSF after electrical stimulation of substantia nigra IF, first forward (oxidizing direction; voltage sweep IR, first reverse (reducing direction; voltage sweep 2F, second forward voltage sweep

FIGURE 5. CONTINUOUS ELECTROCHEMICAL MEASUREMENT OF HVA RELEASE

Stim. Stimulation initiated (via bipolar electrodes in substantia nigra)

FIGURE 6. TYPICAL TISSUE VOLTAMMOGRAM WITH CHRONIC: GRAPHITE-EPOXY ELECTRODE

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