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BIOMEDICAL APPLICATIONS OF LIQUID CHROMATOGRAPHY–ELECTROCHEMISTRY

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SUMMARY

Liquid chromatography–electrochemistry has proven to be a viable tool for solving a wide variety of practical analytical problems, primarily in biomedical research. Over 2000 papers have appeared and over 20 manufacturers participate in this area. In spite of these obvious measures of acceptance, there continues to be a general lack of understanding of how these systems work. This naturally stems from the unfamiliarity of chromatographers with electrochemistry and electrochemists with chromatography. Furthermore, there are aspects of both which are not yet understood in molecular detail by anyone. While there is more work to do to optimize the technique, liquid chromatography–electrochemistry clearly is very useful at its present state of development. This article presents an overview of the technology and more recently developed applications. Emphasis is placed on the chromatograph as a whole, dual-channel detectors, microdialysis sampling for in vivo determinations, derivatization and post-column reactions, and studies of xenobiotic metabolism.

INTRODUCTION

Electrochemistry has a number of advantages as an analytical methodology. Nevertheless, it perhaps remains best known in industry only for its deficiencies in relation to spectroscopy and chromatography. It is now clear that the unique difficulties of electrochemistry can often be overcome so that its strength can be applied to solving important problems [1,2]. It is a good mechanism and kinetics tool. It is a direct way to acquire thermodynamic values. It is ideal for study of transition metals and their complexes. It can be a remarkable means of looking at trace amounts of unstable substances in the liquid phase, such as free radicals and unusual oxidation states. And, if nothing else, electrochemistry can be used to examine extremely small amounts of ions or molecules in very tiny volumes. Electrochemical experiments often involve the reaction of 10^{-14} mol, or even less.

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Doing electrochemistry in microliter volumes is easy and useful. Doing electrochemistry in rat brains is not unusual.

The use of conventional electroanalytical chemistry in batch cells to determine individual substances in complex mixtures has significant limitations. In general, such methodology has been very poorly accepted because (1) the selectivity of voltammetry-based techniques is inadequate, (2) automation is difficult and awkward, and (3) electrodes tend to foul and often are irreproducible. All three of these problems are largely overcome by coupling electrochemical (amperometric) flow cells to liquid chromatography (LC) columns. Liquid chromatography–electrochemistry has therefore been one of the fastest growing analytical techniques in the last fifteen years.

The first practical thin-layer hydrodynamic experiments were carried out with detection cells at the University of Kansas in early 1972. The technological developments followed the need to solve an important problem in neuropharmacology: determination of neurotransmitters and metabolites in brain tissue and body fluids. At that time, diverse techniques such as fluorescence, gas chromatography–mass spectrometry, and various radiochemical techniques left much to be desired. In recent years, a number of new applications (environmental, industrial, as well as biomedical) have been developed. The liquid chromatography–electrochemistry technology has been extended to reducible substances. Derivatizing agents and various post-column reactions have been very successful for applications to amino acids, carbohydrates, carbonyl compounds, acetylcholine, etc. Electrochemical detection is ideal for small LC columns (I.D., 1 mm or less) as well as for more conventional 4–5 mm columns. In fact, application to column diameters of under 20 μm have been demonstrated [3]. The use of liquid chromatography–electrochemistry now includes the determination of less than 100 000 molecules in complex biological samples.

Multi-channel liquid chromatography–electrochemistry systems are now available which permit significantly improved performance. In particular, dual-working electrode transducers have been developed in both parallel and series configurations. The potential of each electrode can be controlled independently. This permits oxidizable and reducible substances to be detected simultaneously. It affords peak identification via current ratio comparisons. Both selectivity and detection limits can often be improved.

The aim of this report is to present an overview of some recent technology and biomedical applications. There have been many comprehensive reviews of liquid chromatography–electrochemistry, but I particularly recommend the chapter by Shoup [4]. Neurochemical applications continue to be predominant and two recent books cover the literature well with respect to a variety of competing analytical techniques [5,6]. A key to success with liquid chromatography–electrochemistry is a 'systems attitude' toward the technique. Those who consider an 'electrochemical detector' as an isolated device will often not meet their experimental goals.

GENERAL EXPERIMENTAL CONCERNS

Temperature control

Both liquid chromatography and electrochemistry require transport of molecules or ions in solution. They are therefore quite temperature-dependent. Even UV absorbance detectors have a strong temperature dependence when measurements at 0.001 A.U. or less are contemplated. It is therefore quite surprising that the majority of liquid chromatographs have no temperature control whatsoever and that columns often 'dangle in the air'. Many complaints about baseline drift, gradient imprecision, detector noise, retention time shifts, outgassing in pumps and detectors, and so forth can be attributed to neglect of temperature control. Often chromatographers ignore this and focus on mechanical, electronic, and chemical causes, which may (or may not) be at fault. For the best LC results, there is little argument that the injection valve, column, and detector should be isothermal. This is not always possible; however, all of our instruments incorporate at least column temperature control. Most often we use a BAS 200 liquid chromatograph (Bioanalytical Systems, West Lafayette, IN, U.S.A.) with the entire sample pathway as well as the mobile phase reservoirs under active temperature control. For liquid chromatography–electrochemistry with low-wavelength UV detection, this lead to a marked improvement in system reliability and precision.

As far as both UV and electrochemistry are concerned, it is most critical that temperature be constant. There is no optimum target temperature. This is fortunate in the sense that controlling temperature can provide optimization of chromatographic resolution in the difficult cases often encountered in biomedical situations. Establishing the temperature based on column performance comes first. If it is then tightly controlled, detectors will cooperate.

Deoxygenation

With liquid chromatography–electrochemistry, oxygen can be very critical. Depending on conditions, in a protonated environment oxygen is reduced to hydrogen peroxide or water.

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

$$O_2 + 4e^- + 4H^+ \longrightarrow 2H_2O$$

This can cause an enormous background current density even at slightly negative potentials (vs. Ag/AgCl) when trace work is contemplated. Oxygen has a secondary effect in the case of analytes which are easily oxidized electrochemically (often the most popular candidates for a liquid chromatography–electrochemistry assay). Such analytes can be reacted by dissolved oxygen prior to reaching the electrochemical detector. This is obviously very dependent on pH (in most cases) and the oxidation potential for the analyte.

Finally, when low-wavelength UV is used, solvated oxygen (e.g., in acetonitrile) can have a significant absorbance at much lower wavelengths than gas phase oxygen. This is very well documented in LC [7–9]. While oxygen removal is sen-

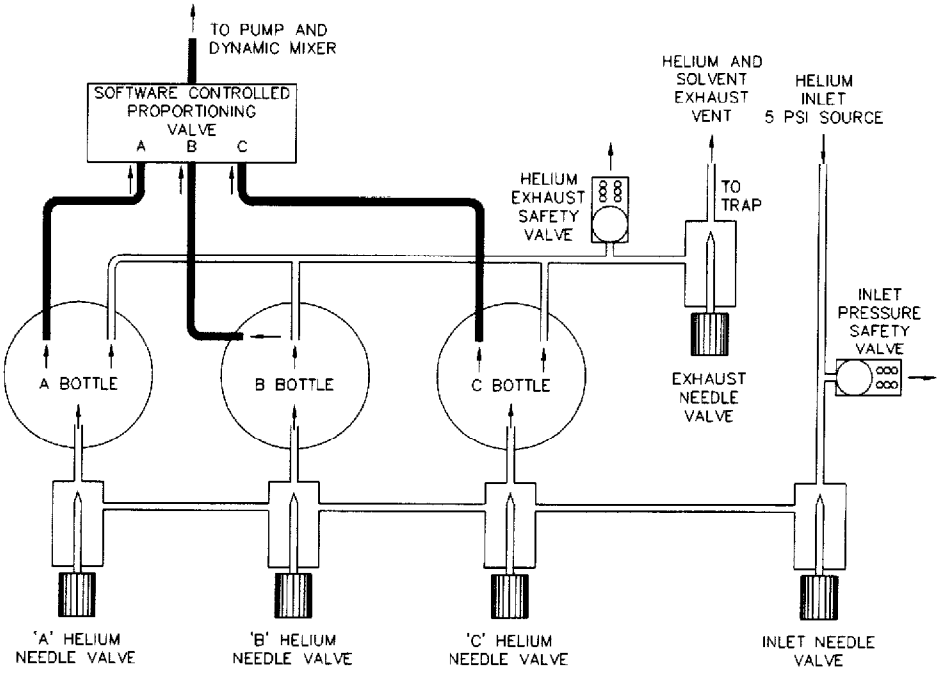

Fig. 1. Deoxygenation manifold for ternary-gradient elution features sealed reservoirs, safety relief valves, and a single-point exhaust.

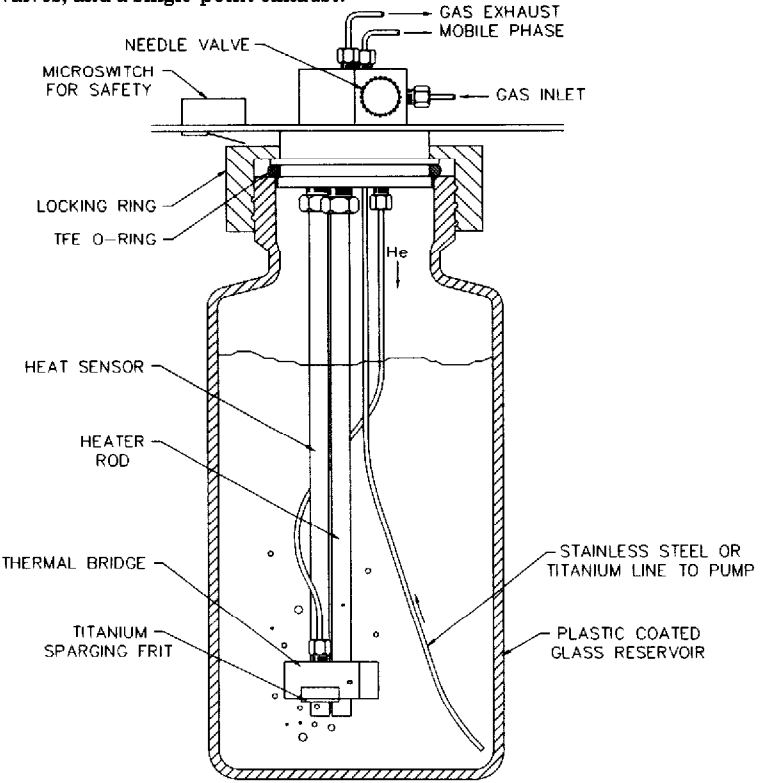

Fig. 2. Mobile phase reservoir specifically designed for liquid chromatography–electrochemistry and low-wavelength UV.

sible, there can be an opposite effect. Corrosion of stainless steel in oxygenated solutions results in release of Fe^{3+} whereas in rigorously deoxygenated mobile phases, Fe^{2+} can result. This observation by Shoup [10] demonstrated a frequent cause of high background currents in oxidative liquid chromatography–electrochemistry caused by oxidation of Fe^{2+}. This corrosion chemistry is complex. The influence is very dependent on mobile phase composition, materials (alloys) used in the LC system, and the electrode material chosen.

How do we remove oxygen? This has received limited attention in the literature [7–9,11], however, the general consensus is that helium sparging at elevated temperature is the fastest and most efficient approach that can be used conveniently. This requires a rigorously sealed mobile phase reservoir and *total absence* of Teflon (or related plastics) tubing from the gas tank, from the reservoir(s) to the pump, and from the column to the detector. Oxygen passes through Teflon with extreme ease, yet most chromatographs are replete with centimeters or even meters of Teflon tubing! Thus most chromatographs are not capable of performing a number of important liquid chromatography–electrochemistry applications. Teflon is inert and easy to work with, but it can be a source of problems in electrochemistry, UV absorbance, and fluorescence (O_2 quenching) as well. For trace biomedical work, this can mean the difference between success and failure.

Safety seems to be widely neglected by liquid chromatographers. Sparging with helium is frequently done in vessels without a good seal, allowing solvent vapors to escape into the laboratory. This is bad practice. Oxygen will back diffuse into the mobile phase and operators will be exposed to potentially toxic solvents.

The BAS 200 chromatograph developed in our laboratory provides a single exhaust port from the deoxygenation manifold (Fig. 1). It is therefore possible to trap vapors or vent them to a hood. In addition, back diffusion is virtually eliminated since solvent reservoirs (Fig. 2) are sealed and can be pressurized (typically 0.34 bar).

MacCrehan et al. [12] presented an interesting twist on deoxygenation by using a platinum catalyst in a cartridge prior to the injection valve. Mobile phase methanol is used as the reductant and the products are formaldehyde and formic acid. We have tried it and this works well, but there is the possibility of chemical contamination anytime reactive material is added to an LC flow stream.

Sample injection

This is often not a problem with liquid chromatography–electrochemistry, however, there are exceptions involving oxygen, temperature, and extremely small-volume biological samples. While removing oxygen from mobile phases is problem enough, removing it from samples is not at all straightforward. One method which works well is to sparge the sample in a syringe while it is placed on a seven-port valve (e.g. Rheodyne Models 7125, 8125, and 9125). This approach was first introduced by Lloyd [13], a forensic chemist interested in determining explosives. The idea is illustrated in Fig. 3 [14].

Dual series electrodes (see below) have also been used to minimize the influence of sample oxygen on liquid chromatography–electrochemistry. This ap-

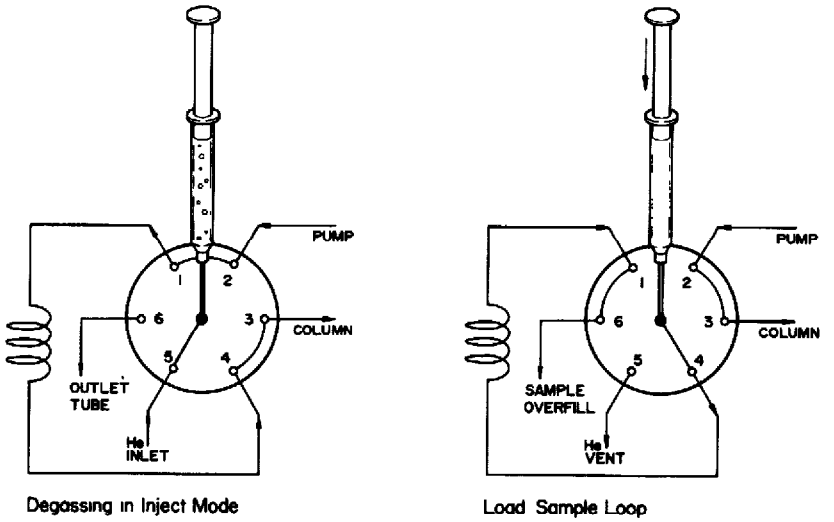

Fig. 3. Sample deoxygenation procedure. Left: deoxygenation in 'inject position'. Right: filling sample loop in 'load' position. A modified 1-ml syringe is used so this is not a procedure for microsamples.

proach has been extensively studied by Jacobs [15] with regard to nitro compounds. The process depends on the fact that oxygen reduction to peroxide

(upstream electrode) $O_2 + 2H^+ + 2e^- \longrightarrow H_2O_2$

(downstream electrode) $H_2O_2 \longrightarrow O_2 + 2H^+ + 2e^-$

cannot be reversed on a carbon electrode until quite high positive potentials. On the other hand, a nitro compound is reduced to a hydroxylamine which is very easily reoxidized.

(upstream electrode) $RNO_2 + 4e^- + 4H^+ \longrightarrow RNHOH + 2H^+$

(downstream electrode) $RNHOH \longrightarrow RNO + 2H^+ + 2e^-$

Hydrogen peroxide does not react at the downstream electrode used to detect the hydroxylamines (and thus indirectly the nitro compound analytes). Therefore oxygen gives no signal.

RESULTS AND DISCUSSION

Thin-layer amperometric detector

Chemical sensors based on hydrodynamic amperometry in thin-layer flow cells have been popular since we first developed them in 1970 and commercialized them in 1974. These simple devices have a number of practical virtues: (1) compatibility with a wide variety of electrode materials, (2) very rapid response time, (3) easily adjusted dead volume down to 100 nl or less, (4) ease of assembly and surface cleaning, (5) compatibility with optical devices, (6) ease of working with multiple electrodes simultaneously, (7) routine quantitation of 1 pmol or less for many analytes, and (8) relatively low cost. The latest detector cell we use is

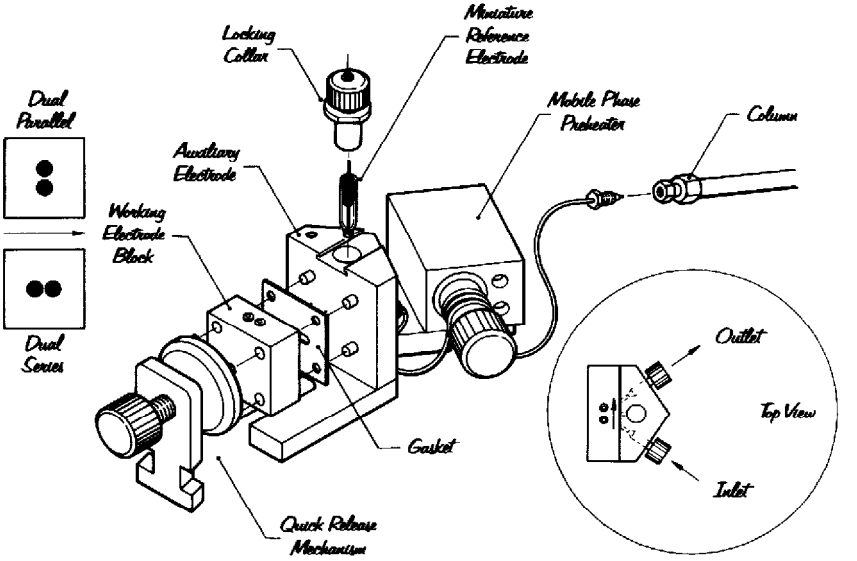

Fig. 4. Thin-layer amperometric detector for liquid chromatography–electrochemistry. The 'sandwich' is assembled without screws making it possible to quickly accommodate a variety of working electrode configurations. Dual parallel and dual series electrodes are illustrated.

shown in Fig. 4. It is designed for use with LC column diameters down to 0.5 mm. The electronics used with this cell provides programmable gain, autozero of baseline, and automated adjustment of applied potential and filtering throughout a chromatogram.

Electrode materials

There have been many misconceptions about electrodes for liquid chromatography–electrochemistry. Some workers believe (incorrectly) that the bigger the electrode, the better will be its performance. Others recommend using the smallest possible surface area. Some workers advocate polishing electrodes with toothpaste, while others oxidize the surface chemically (e.g. with chromate) or electrochemically (e.g. at +1.5 V). Some people believe that you simply cannot use electrodes with proteinaceous samples. A discussion of several of these issues was published elsewhere [16].

Part of the problem is that it is difficult to generalize about electrode performance without reference to a specific analyte (or series of analytes) detected under given conditions (e.g. mobile phase pH, choice of solvent, applied potential, electrode material). What may be reasonable for a glassy carbon electrode at −0.5 V vs. Ag/AgCl in pH 2, 50% methanol, may be totally inappropriate for a mercury film electrode at −0.5 V in pH 7, 20% acetonitrile. A safe answer to most questions about such issues is that "it depends on the experimental goal".

It is clear that better electrode materials are needed for particular applications. One of the reasons liquid chromatography–electrochemistry became popular is the difficulty in controlling selectivity at an electrode per se (Fig. 5). While it is difficult to build selectivity features into or onto an electrode, it is relatively easy

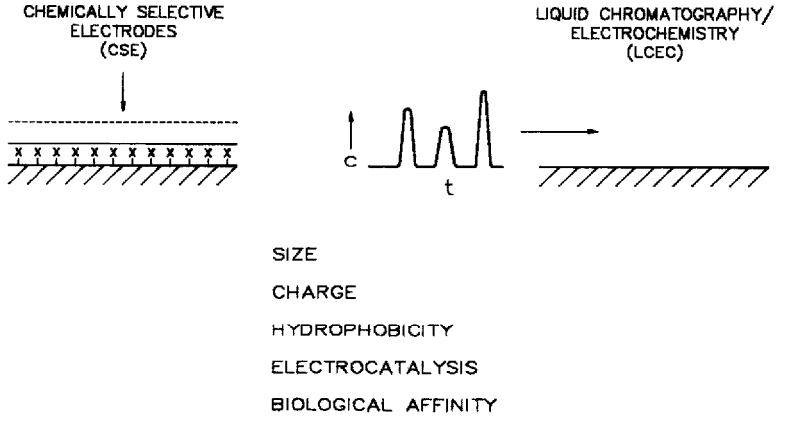

Fig. 5. Selectivity controlling features (listed) can be built onto an electrode surface, onto an LC stationary phase, or both. Control of selectivity via LC is currently more feasible and more reliable.

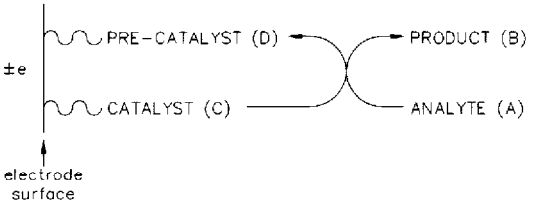

Fig. 6. A redox catalyst bound to an electrode can improve detection of analytes with slow electrode kinetics.

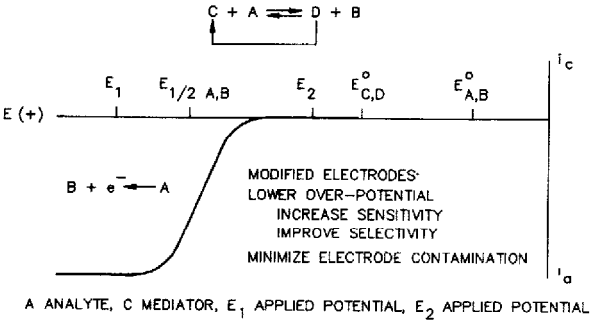

Fig. 7. The redox cross-reaction shown in Fig. 6 requires careful selection of catalyst $E^{\circ\prime}$ and the applied detector potential.

to use these molecular features to control differential migration rates in LC. This solves many of an electrochemist's problems. The trend lately has been to do both. Electrodes can be modified with surfaces which incorporate electrochemical catalysts (Fig. 6) making it possible to detect some chromatographic peaks more selectively. This well known process in electrochemistry takes advantage of a redox cross-reaction between the catalyst couple (fast electron exchange with both electrode and analytes) and the analyte couple (slow electron exchange with an unmodified surface). The principle is illustrated in Fig. 7. To date, such elec-

trodes have not been rugged enough for commercialization. The concept, however, has been proven in several academic laboratories and its future seems bright. There are many reviews on chemically modified electrodes [17,18].

A more mundane application of layered electrode surfaces is to build in size exclusion protection from higher-molecular-mass materials [19] or to add charge to a layer to discriminate against ions of like charge [20]. For the typical biomedical application it is often desirable to attain selectivity from (1) the sample preparation, (2) the LC column, and (3) the electrode surface. The best results are obtained when all three are used.

Electrode potential

Taking advantage of the potential dependence of electrode reactions has always been a feature of liquid chromatography–electrochemistry. Hydrodynamic voltammograms (as shown in Fig. 7) are typicaly used to confirm peak identity just as spectra are used in optical absorbance detection. Can this be achieved 'on the fly'? The answer is yes. There are two approaches as shown in Fig. 8. The spatial approach uses an array of electrodes poised at different potentials parallel to the flow stream. The temporal approach involves rapidly scanning a single electrode [3]. Both methods work, but both have problems. Developing arrays is problematic. It is hard to ensure that each element will be chemically equivalent and will stay that way throughout an experiment. Rapid scanning, including pulse waveforms, also is problematic. At speeds sufficient for LC, capacitance current (physical) will usually exceed the faradaic current (chemical) of interest. As a result, detection limits are often many times higher than for a fixed-potential experiment. As with UV absorbance, the ultimate problem might well be the relatively low information content of these 'electrochemical spectra'. In spite of these

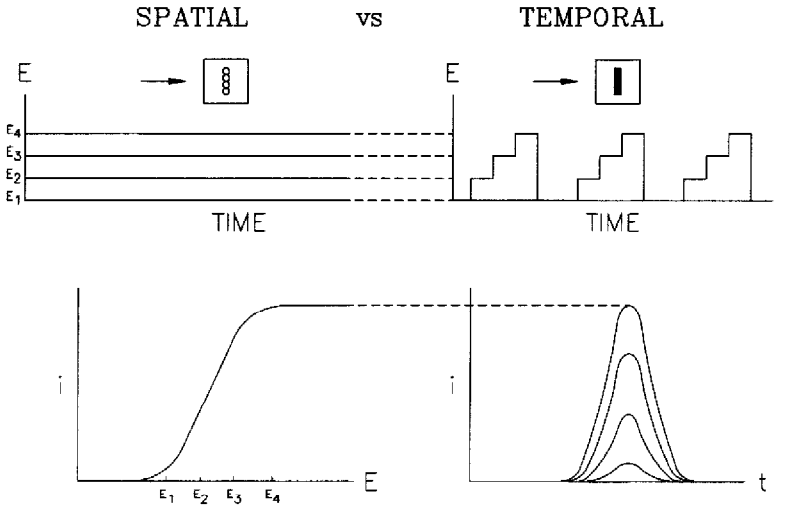

Fig. 8. Using electrode potential to gain qualitative information. Using four potentials in this example, the chromatographic peak heights provide voltammetric information for the analyte of interest.

reservations, good things can be accomplished with scanning, with multi-channel detectors, and with both combined.

Parallel dual-channel detection

In the 'parallel mode' the compounds eluting from the column pass over each electrode at the same time. The following applications are quite useful:

(1) The ratio of currents monitored at each electrode can provide confirmation of peak identity and purity.
(2) Oxidations and reductions can be carried out simultaneously. This saves time and enhances selectivity. This can be ideal for compounds present in several different redox states.
(3) Signals from low- and high-potential reactions can be recorded simultaneously, providing both greater selectivity and wider applicability in a single experiment.
(4) A difference signal can be plotted to subtract out 'common mode' information while enhancing detection of the desired compound [21].

Fig. 9 illustrates one example of a dual-parallel application. The identity of neurotransmitters and related metabolites is commonly confirmed using response ratios at two potentials [22].

Series dual-channel detection

In the 'series mode' the working electrode block is rotated 90° in relation to the flow stream as shown in Fig. 4. Products of the upstream electrode reaction can be detected downstream. If an oxidation is carried out upstream, a reduction is accomplished downstream and vice versa. The following applications are popular.

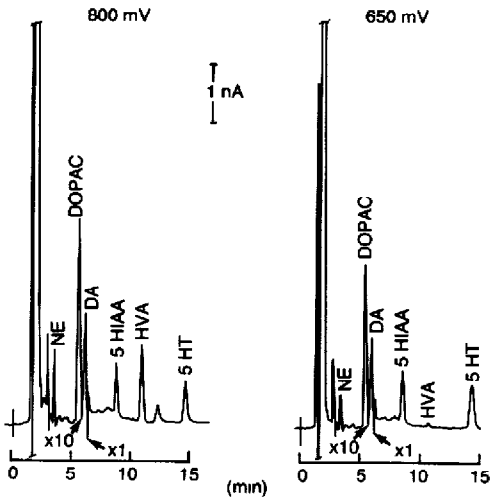

Fig. 9. Determination of neurochemicals in rat brain homogenate using dual-parallel liquid chromatography–electrochemistry to confirm the identity of individual peaks.

(1) The ratio of currents monitored at each electrode can provide confirmation of peak identity and purity.
(2) Selectivity is enhanced at the downstream electrode because compounds with chemically irreversible reactions upstream are discriminated against.
(3) The upstream electrode can 'derivatize' compounds to enhance detectability at the downstream electrode. Overall selectivity and detection limits can be greatly improved.
(4) Dissolved oxygen can be discriminated against, simplifying study of compounds that ordinarily would require mobile phase deoxygenation (e.g. nitro compounds, see above [11,15]).
(5) 'Common mode' currents can be discriminated against by taking the difference between the two signals [21].
(6) Scanning detection can be facilitated when the upstream electrode is swept across the potential axis, while the product is monitored at a fixed-potential downstream electrode. This approach has been pioneered by Lunte et al. [23]. The advantage here is that the capacitance current (always a problem with voltammetry) is not a problem at the measurement electrode itself.

One application of the dual series technique, disulfide detection, will be described in a subsequent section.

Both series and parallel dual-channel liquid chromatography–electrochemistry already provide many opportunities for study of neurochemistry and xenobiotic metabolism because of the wide range of redox properties involved. Industrial applications include determination of sulfur compounds in petroleum and antioxidants in food packaging. Multiple working electrodes (two or more) have many desirable features, however, the vast majority of applications are quite adequately carried out using a single (less expensive) working electrode. The possibilities for arrays of many electrodes on an electrode block is intriguing. At the present time, there are several practical limitations from the 'materials science' point of view. Arrays of gold are easy to achieve, but rugged arrays of platinum, carbon, and mercury remain elusive.

In vivo determinations using microdialysis sampling

Accuracy requires selectivity for complex biological samples. Selectivity requires care in sampling as well as separation and quantitation. Achieving the desired result in living animals is one of the more challenging aspects of analytical chemistry. In order to advance biomedical frontiers such as neuroscience, it has been traditional to monitor substances in biological fluids or to sacrifice large numbers of animals and dissect the organ of interest.

Microdialysis is a new bioanalytical sampling technique [24–26]. It is an attempt to imitate the function of blood vessels by introducing an exogenous 'blood vessel' — a microdialysis probe. This concept was first put forth in the early 1970s by two prominent neuroscientists, Delgado (Spain) and Ungerstedt (Sweden). Until liquid chromatography–electrochemistry for neurotransmitters was improved, the dialysis technique remained dormant. It now is rapidly growing in popularity and is a key sample preparation tool for LC as well as other instrumentation.

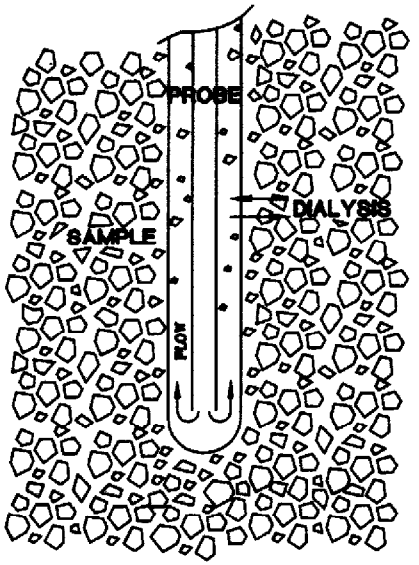

Fig. 10. Schematic diagram of the microdialysis process using a probe of concentric geometry to both add and remove substances from the extracellular space.

The probe may take several forms, however, it most commonly consists of two concentric steel cannulas covered at the tip by a dialysis membrane (Fig. 10). A physiological perfusion fluid is introduced through the inner cannula, flushes the inside of the membrane, and leaves by way of the outer cannula. Microdialysis is used to recover substances such as neurotransmitters and their metabolites, drugs and other xenobiotics, amino acids, purines, glucose, and peptides. The time scale of sampling is typically 10 min or less. The dialysis probe avoids selectivity problems and can be used without the extensive development costs of individual 'biosensors'. The great advantage of microdialysis is its relative simplicity in relation to the complex mechanisms that can be studied. The membrane protects the surrounding tissue from excessive damage and irritation during perfusion. The probe can be sterilized and the membrane which separates the tissue from the perfusing fluid is a further guarantee of sterility. Fig. 11 illustrates a typical experimental set-up for studies in rat brain. The rat is held in position by the 'ear bars' for surgery and acute experiments. After recovery from anesthesia and surgery, the animal can be permitted to freely behave while dialysis samples are collected [27]. A refrigerated microfraction collector–autosampler (CMA/200 Carnegie Medicin/Bioanalytical Systems) has been developed to interface the dialysis experiment to the liquid chromatograph.

The advantages of microdialysis generally outweigh the fact that probes typically are 200–500 μm in diameter and cannot operate on a time scale below a few minutes. Most known biosensors cannot give better spatial or time resolution for organic compounds. There are, of course, serious concerns for in vivo sampling.

(1) Changes in the biological system will accompany the measurement process. Will these changes be important to a given experimental objective?

(2) The region sampled is not always clearly defined. The probe samples from a

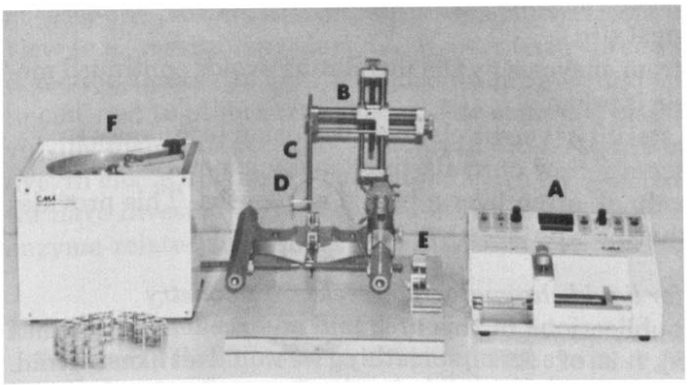

Fig. 11. Basic experimental arrangement for micodialysis sampling from the living rat brain. (A) CMA/100 micro injection pump, (B) stereotaxic instrument, (C) dialysis probe clamp, (D) probe, (E) liquid switch to select perfusion fluid, and (F) CMA/200 refrigerated microfraction collector.

three-dimensional space, the size of which depends on diffusion coefficients and diffusion barriers (e.g., cells, membranes) which may exist.

(3) Time resolution depends on detection limits. If the analysis provides exceptionally good detection limits and/or the analyte is in relatively high concentration, the temporal resolution can be good.

(4) Recovery can change with long-term use. This can result from coating of the membrane as the tissue reacts to it. For typical experiments of 4–6 h duration this has not been a serious concern.

(5) Protein bound material may not be recoverable.

(6) Very small volume samples must be processed. This eliminates some analytical techniques from consideration.

(7) Oxygen will be recovered from tissue and across Teflon tubing used to connect the dialysis system. This can lead to decomposition of reducing agents (e.g., catechols, some pterins, hydroxyindoles).

The following are several of the opportunities available from microdialysis sampling.

(1) Living animal.

(2) The spatial resolution is good and obviously far superior to sampling circulating fluids or urine.

(3) Sampling rates are *much* faster than can be achieved by sacrificing animals.

(4) Multiple analytes can be examined in the same location at the same time. This is a major deficiency of other so-called biosensors.

(5) A wide range of analytical techniques are already available (radioimmunoassay, mass spectrometry, liquid chromatography, gas chromatography, ion-selective electrodes, capillary zone electrophoresis, chemiluminescence, electrochemistry, etc.). Microdialysis can be used today.

(6) An animal can be its own control. Control experiments carried out on the same animal are often better controlled than experiments between different animals.

(7) Compounds (ions) can be added as well as removed, making it possible to

measure a response to pharmacological stimulus with the same apparatus in the same physiological site.

(8) Isolation of analytes from enzymes by the membrane avoids continued metabolism after a sample is taken.

(9) It is easy to maintain sterility (versus classical perfusion techniques).

(10) There is a good conservation of animals since many experiments can be done with the same animal, often over a period of months. This provides both economic and ethical advantages.

Pre-column derivatization for liquid chromatography–electrochemistry

There have been many publications in this area and an excellent review has appeared by Krull et al. [28]. It is, of course, something we would all like to avoid. Nevertheless, there are many examples where derivatization presents real advantages. A very recent example by Munns et al. [29] showed derivatization of carboxylic acids with 1-(2,5-dihydroxyphenyl)-2-bromoethanone. The resulting hydroquinones are, of course, oxidizable at a very low positive potential. Most attention has been given to amino acids. While they can be detected directly by electrochemistry using triple-pulse techniques [30] low detection limits require derivatization just as for UV and fluorescence.

Amino acids

Derivatization of amines with aromatic nitro reagents is by now an ancient practice of biochemists. Many such reagents have been considered for UV detection and several are quite popular. This is convenient for liquid chromatography–electrochemistry because all aromatic nitro groups can be easily reduced electrochemically [28].

The reaction between primary alkyl amines and *o*-phthalaldehyde (OPA) in the presence of an alkyl thiol is a widely used derivatization chemistry for determination of amines and amino acids by LC. The normal products of this reaction, 1-alkylthiol-N-alkylisoindoles, are formed rapidly in high yield and can be detected with good sensitivity using either fluorescence or electrochemical oxidation. A considerable problem associated with this chemistry stems from the poor stability of the isoindole derivatives, due to further reaction with excess OPA in the derivatization mixture. This problem has complicated many precolumn derivatization schemes and has resulted in numerous publications describing attempts to optimize reaction conditions. It has been shown that alteration in the thiol structure (e.g., from mercaptoethanol to *tert.*-butylthiol) [31] or substituting sulfite [32] for the thiol can result in markedly improved derivative stability. While such changes in derivative structure have relatively little impact on electrochemical reactivity, they have been shown to often degrade the quantum yield of fluorescence. Liquid chromatography–electrochemistry thus provides an attractive alternative to fluorescence for some applications of OPA or OPA-type reagents.

Pre-column use of enzymes and antibodies

One of the recent trends in LC is utilizing (and/or studying) enzymes for analytical purposes. Oxido-reductase enzymes are obviously good candidates. Often

at least one participant in the reaction is ideal from an electrochemical point of view (e.g., reactant, product, O_2, H_2O_2, pterin species, NADH, etc.) and the good detection limits can shorten incubation times and simplify sample preparation in contrast to other methodology. For example, classical colorimetric assays involving hydrogen peroxide generation often require an additional enzyme and a dye. In electrochemistry, the peroxide can be detected directly. In our laboratory we have invested a great deal of effort on pterins [33] to give one example of enzyme-related substances with interesting redox chemistry.

Electrochemical immunoassay

In recent years, non-isotopic immunoassays have gained an important share of the market for diagnostic tests in clinical laboratories. Among these, enzyme immunoassays (e.g., so-called EMIT or ELISA systems) have been quite popular. It is not surprising to find that electrochemistry is being considered as a viable candidate for such assays, many of which have already been developed commercially for absorbance or fluorescence apparatus. Often NADH is the monitored species (e.g., with glucose-6-phosphate dehydrogenase used as the enzyme in a homogeneous or EMIT, assay kit). In other cases a product, rather than a cofactor, can be monitored electrochemically (e.g., using alkaline phosphatase as the enzyme label in a heterogeneous, or ELISA-type, assay permits electrochemical detection of phenol released from phenolphosphate). W. Heineman and his group at the University of Cincinatti [34,35] have pioneered in the electrochemical immunoassay area as has Wilson and his team at the University of Kansas [36]. Two schemes (well known in immunoassay circles) are shown in Fig. 12.

A heterogeneous approach for electrochemical immunoassay is perhaps most satisfactory. The antigen (i.e., the analyte) and an enzyme-labeled antigen compete for the surface bound antibody (typically on the wall of a tube or on macroscopic particles). After equilibrium is achieved, the solution phase is removed and the activity of the bound enzyme is determined by incubation with a solution of appropriate substrate. The amount of enzyme bound to the surface (and thus its activity) is inversely related to the unlabeled antigen present initially. An advantage of this approach is that the final incubation solution, subject to electrochemical analysis, is very clean in contrast to the original sample. It is an ideal fit to liquid chromatography–electrochemistry using short ('high speed') LC columns and full automation of the assay procedure. The enzyme amplification makes it possible to determine only a few thousand molecules in an original sample.

It is quite clear that enzymes can be studied by liquid chromatography–electrochemistry and can also be put to work to assist in 'preparing samples' for a number of routine methods.

Post-column reactions

Post-column chemical reactions coupled to electrode reactions are also expanding the range of applications. Fig. 13 illustrates five configurations. In Fig. 13A a reagent is added, mixed, and reacted in a delay line followed by electrochemical detection. A superb example of this is the determination of acetylcho-

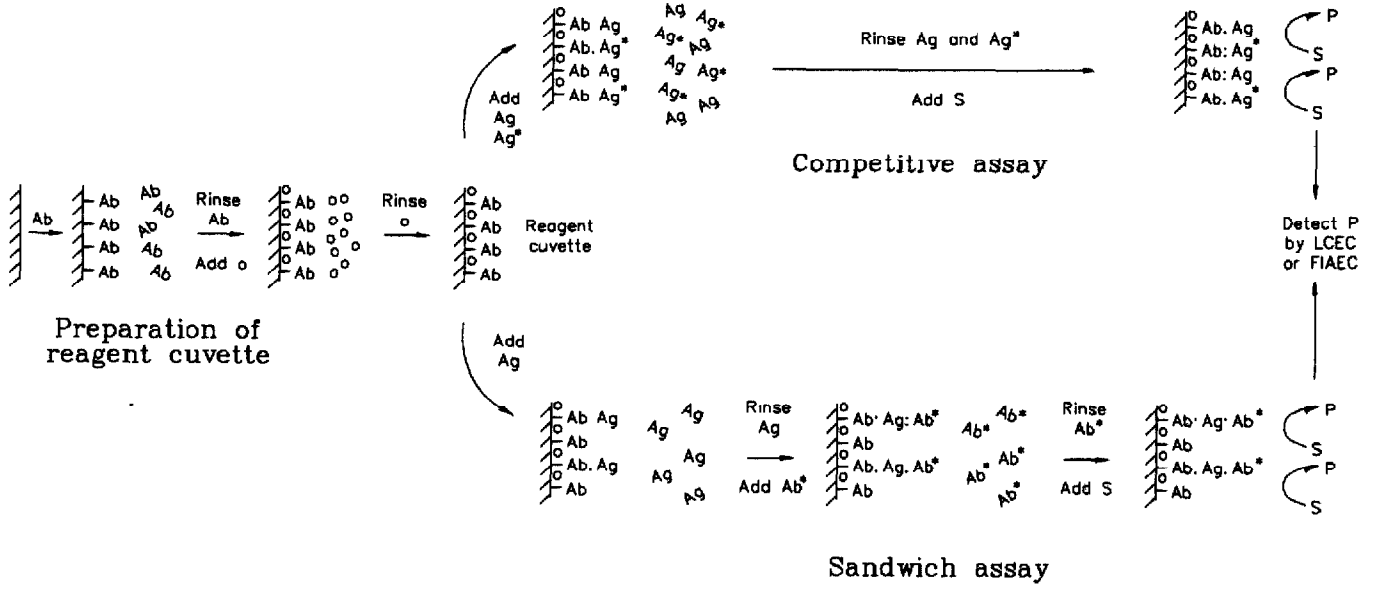

Fig. 12. Solid-phase electrochemical enzyme immunoassay.

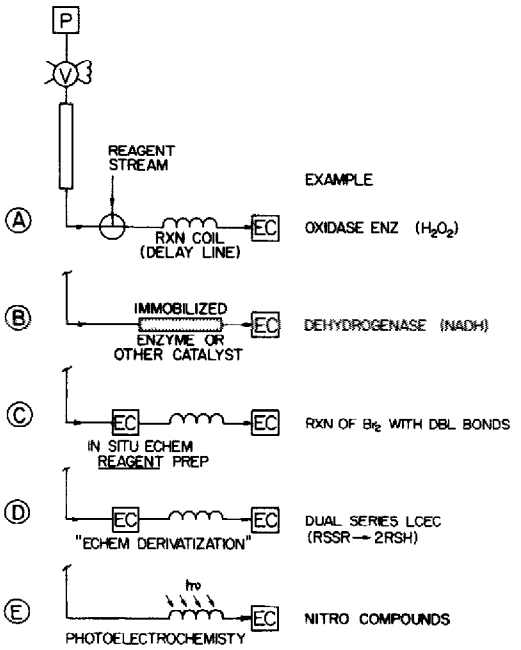

Fig. 13. Post-column reaction schemes for liquid chromatography–electrochemistry.

line in brain tissue by reversed-phase LC [37,38]. Acetylcholine esterase and choline oxidase are mixed in and the detection process proceeds as follows:

acetylcholine $\longrightarrow$ choline + acetic acid

choline $\longrightarrow$ betaine + H_2O + $2H_2O_2$

The peroxide is detected electrochemically at a platinum electrode. There are many pairs of esterases and oxidases which have been used to develop classical assays. Now many of these can be directly coupled to liquid chromatography–electrochemistry. In the future, it is likely that the same basic idea will become practical for certain phospholipids which are now very difficult to handle in small amounts. In addition, a possibility now being explored involves the use of enzymes to hydrolyze drug conjugates (e.g., sulfate esters) prior to electrochemical detection of the free phenol.

In Fig. 13B, a catalyst is immobilized and a cofactor is detected. For example, using a dehydrogenase enzyme, an alcohol can be detected indirectly by monitoring the turnover of NAD to NADH, the latter being ideal for electrochemical detection. Recent choline and glucose methods have also used the immobilized enzyme approach. This conserves enzyme and avoids the need for a reagent pump and post-column mixer. We have recently developed a glucose analyzer for microdialysates using this liquid chromatography–electrochemistry approach [39]. Samples of a microliter or less can be studied quite easily.

In Fig. 13C, an upstream electrode generates a reagent (e.g., Br_2 from Br^- in the mobile phase) which reacts with a non-electroactive compound (e.g., an un-

saturated fatty acid) and the *decrease* in reagent concentration is monitored downstream [40]. This method has been used subsequently for a number of substances where direct electrochemical detection is not feasible or lacks adequate selectivity [41,42].

In Fig. 13D, the analytes of interest are converted at an upstream electrode into a product which is more selectively detected (at a lower energy) downstream. Examples include reduction of a nitro compound to a hydroxylamine and reduction of a disulfide to a thiol. The latter is one of the more important applications and includes the determination of disulfide bridges in peptides and protein tryptic digests. This application has recently been reviewed [43]. Fig. 14 illustrates a chromatogram for a tryptic digest of a growth hormone. Low-wavelength UV (215 nm) as well as dual-channel electrochemistry was used. Fig. 15 shows the principle of disulfide detection (lower chromatogram) originally developed by Shoup and Allison. In this situation the electrode material itself (mercury) participates in achieving high selectivity.

Post-column photochemical reactions (Fig. 13E) have also been demonstrated to greatly extend the scope of applications. A large volume of work in this area has been published by Krull and co-workers of Northeastern University (see, for example, ref. 45).

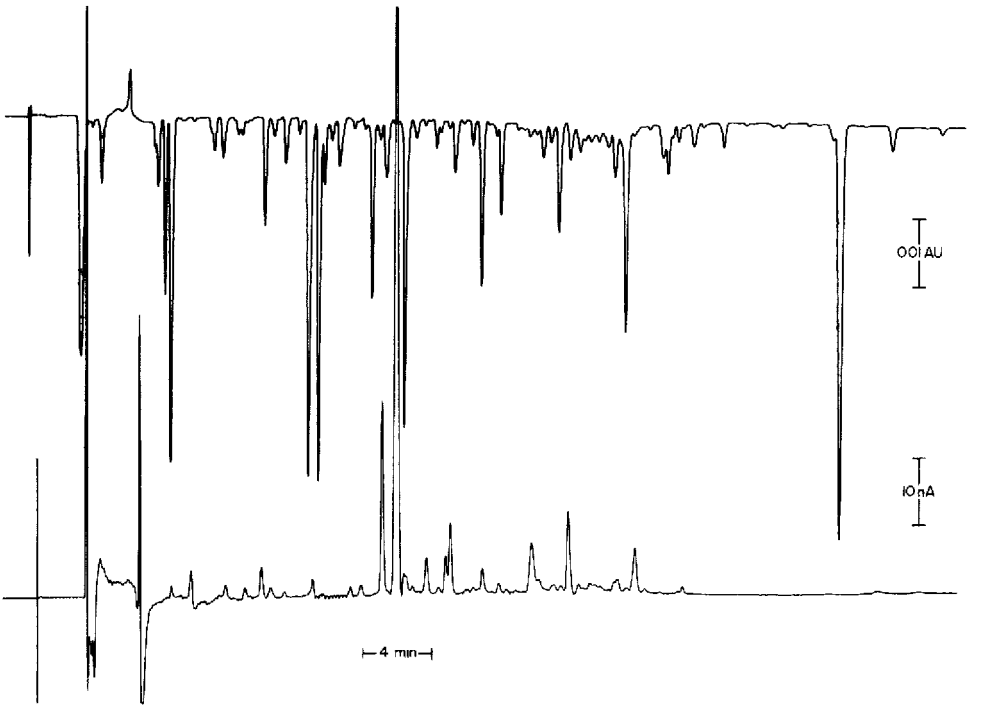

Fig. 14. Tryptic map of a bovine growth hormone with ternary-gradient reversed-phase LC. Upper chromatogram: UV detection of peptides at 215 nm. Lower chromatogram: electrochemical detection of sulfur-containing peptides. (See ref. 44 for details.)

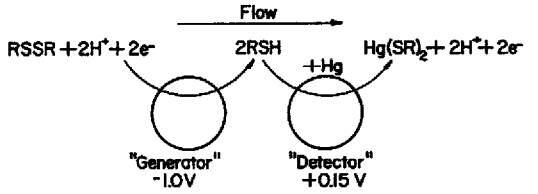

Fig. 15. Dual-electrode cell for determination of both thiols and disulfides using mercury film disk electrodes.

Metabolism of aromatic xenobiotics including benzene, phenol and aniline

Liquid chromatography–electrochemistry offers a distinct advantage over other methods of analysis for the detection and quantitation of aromatic xenobiotic metabolites in complex samples such as microsomal incubations. Many of the metabolites are electrochemically active at favorable potentials and are easily detectable in the picomole range without derivatization. Few, if any, endogenous electroactive compounds interfere in the analysis. For example, hydroquinone has been isolated and identified in microsomal incubations of benzene using liquid chromatography–electrochemistry [46]. The glutathione and cysteine conjugates of *p*-benzoquinone have been detected and conclusively identified in microsomal incubations of benzene and a sulfhydryl. Analogous reactions have been examined for anilines, phenylenediamines, aminophenols, and benzidines. Many of the metabolites can be identified in nanogram amounts to a high degree of certainty based on retention time and voltammetric properties. This can be achieved without elaborate methods of preconcentration or the use of ^{14}C-labelled compounds.

In addition to determination of these substances, electrochemistry is well suited to studies of the basic chemistry involved. The thermodynamics of various components can be revealed. The rates of addition reactions of nucleophiles can be determined and hydrolysis of reactive intermediates followed precisely. Cyclic voltammetry, chronoamperometry, and various forms of spectroelectrochemistry are quite useful in this regard. High-efficiency flow through electrochemical reactors can, in some cases, provide a means of obtaining metabolites for examination by infrared, nuclear magnetic resonance, mass spectrometry and other conventional techniques.

As described above, the use of series mercury film dual electrodes allows for the detection of both oxidized and reduced glutathione in a single chromatographic run. This makes it possible to readily explore redox cross-reactions between xenobiotic intermediates and this important liver component. Both reduced and oxidized forms of a variety of metabolites can all be determined in a similar manner by using a dual glassy carbon electrode in the parallel configuration. By utilizing these two methods of detection, the biological mechanism of oxidation of aromatic xenobiotics can be studied as well as the role of glutathione in the detoxification of the reactive intermediate.

Fig. 16 illustrates one series of compounds we have investigated in liver microsomes and biological fluids over the last decade [47]. Enzymatic hydroxylation followed by loss of electrons appears to be a general process leading to interme-

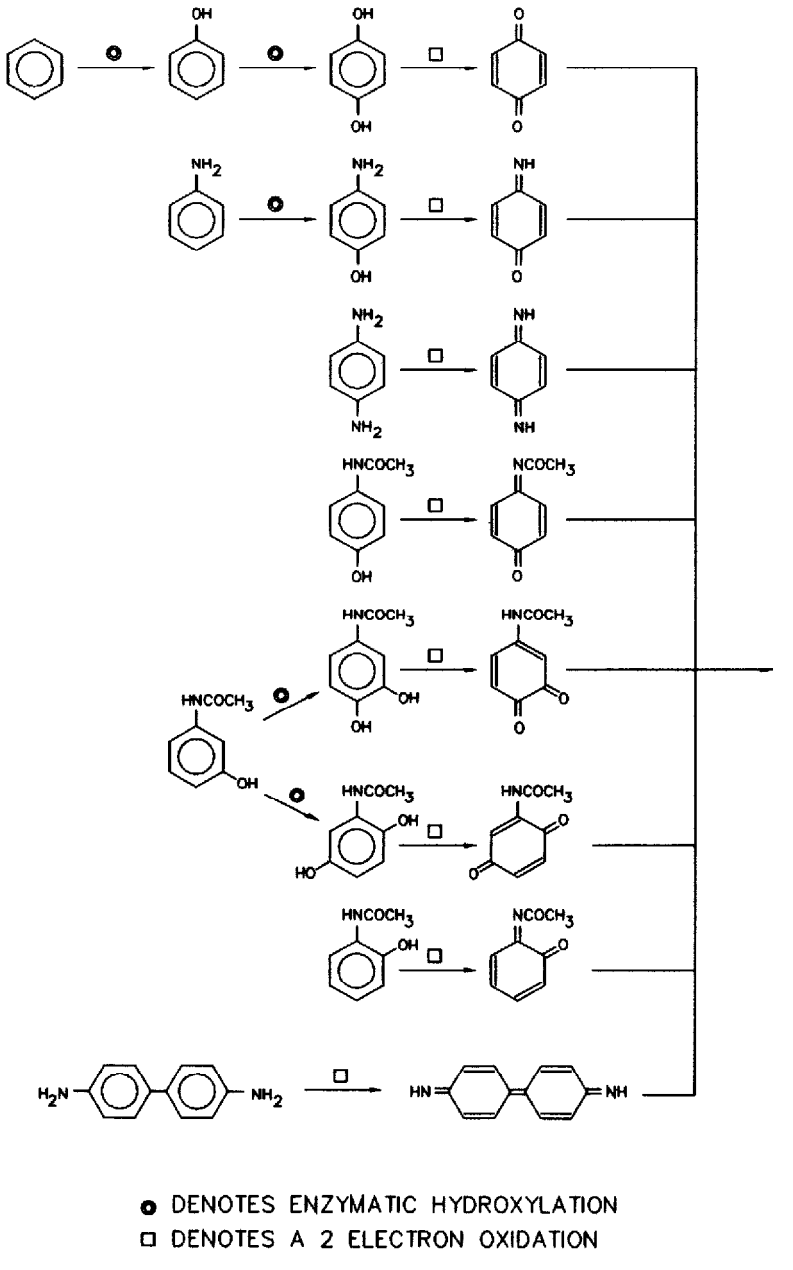

Fig. 16. One common metabolic pathway for aromatic xenobiotics involving hydroxylation and then two-electron oxidation to electrophilic intermediates which bind to endogenous biochemicals.

diates which can bind to macromolecules as well as upset the normal balance of redox components. The loss of electrons may in some cases involve formation of superoxide anion, which itself is thought to be a potent toxicant.

CONCLUSION

Electrochemistry has some of the characteristics of fluorescence. It is great when you know how and where to use it. If you do not, you can get into trouble and become discouraged very quickly. There are a lot of us who want to help! All you have to do is ask.

ACKNOWLEDGEMENTS

This article has been a quick look at over fifteen years of work. I admit to having done very little of it. It has been my very good fortune over the years to work with an extraordinary team of excellent graduate students, postdoctorals, visiting scientists, engineers, and friends. Craig Bruntlett, Don Evans, William Heineman (University of Cincinatti), Wesley Jacobs, Candice Kissinger, Craig and Susan Lunte, Donna Radzik, Ron Shoup, and Urban Ungerstedt (Karolinska Institut) contributed to the concepts developed in this article. There are a hundred others as well. We all wish Karel Macek the best on his birthday. The Journal of Chromatography has been a key participant in our research. Karel has contributed much to its quality.

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