# Peroxidase Electrode Kit

October 1995

## MF-9079 INSTRUCTION MANUAL

Peroxidase Electrode Kit
for Cross-Flow Cell
or UniJet™ Cell

Bioanalytical
Systems, Inc

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West Lafayette
Indiana 47906

MANUFACTURER'S NOTE

This instrument, either wholly or in part, is manufactured for research purposes only. Use for medical diagnosis is not intended, implied or recommended by the manufacturer. Use for this purpose and accountability for the same rests entirely with the user.

UniJet technology is the property of Bioanalytical Systems, Inc.
US Patent 5,399,256

Wired Enzyme™ Sensor technology is licensed from E. Heller and Co.
US Patents 5,320,725 and 5,262,035

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# Section 1. Introduction

## Background

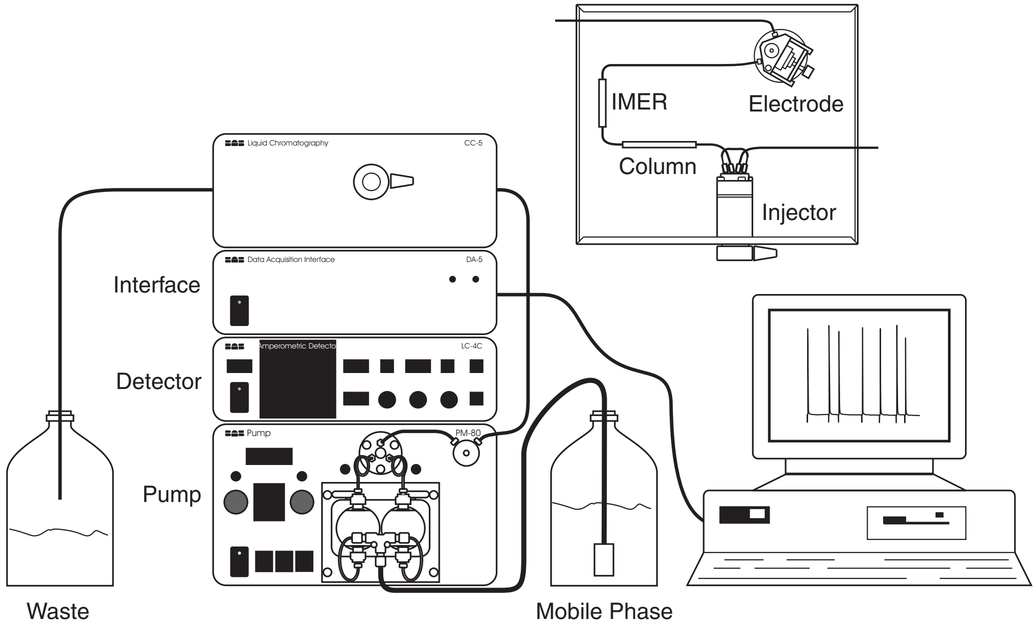
The electrochemical determination of hydrogen peroxide formed in the presence of various oxidase enzymes has been employed to measure a number of oxidase substrates.[1-4] Amperometric oxidative methods are most widely used to determine the hydrogen peroxide produced during enzymatic reactions as follows:

$$S(red) + O_2 + H_2O \xrightarrow{\text{Oxidase}} S(ox) + H_2O_2$$

$$H_2O_2 \rightarrow O_2 + 2H^+ + 2e$$

where S(red) and S(ox) are the reduced and oxidized forms of the substrate, respectively. The first reaction is catalyzed by an oxidase enzyme; in the second reaction, $H_2O_2$ is oxidized at the working electrode, which is often a platinum electrode operating at +500 to +700 mV (vs. Ag/AgCl).[3,5,6] This method has found wide application in liquid chromatography (LC) coupled with post-column immobilized enzyme-reactors (IMER), where $H_2O_2$, the product from the oxidase IMER, is electrochemically detected[7,8] (Figure 1). The combination of LC and IMER with electrochemical detection (EC) results in high overall selectivity and sensitivity. The detection of $H_2O_2$ with the Pt electrode has an inherent problem. The analyte solution usually contains other compounds which can be oxidized nonselectively at the applied potential, resulting in significant interference with the signal from the substrate under study. Nevertheless, this problem can be minimized by an LCEC system, in which the sample components are separated prior to the electrochemical step. The Pt electrode in the LCEC system, however, may suffer from the liabilities of baseline drift following a change in the system and then a re-equilibration time after the change. A freshly polished Pt electrode requires time for its initial equilibration, usually overnight.

**Figure 1.** An LCEC system coupled with an immobilized-enzyme-reactor (IMER).



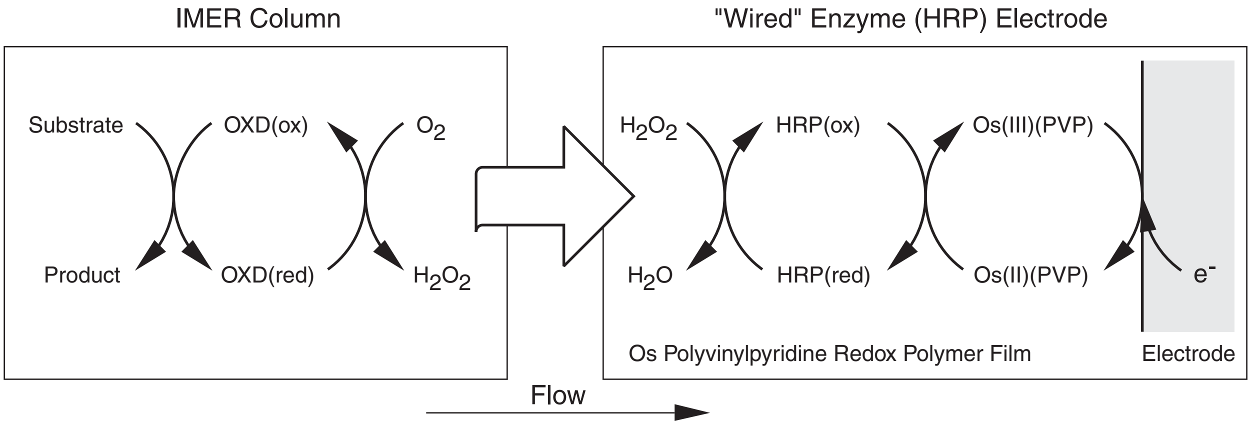
Recently, various amperometric electrodes with immobilized peroxidase have been developed for the detection of $H_2O_2$.[9-13] These enzyme electrodes usually have the advantages of high sensitivity and mild operating potential. There are two conventional types of peroxidase modified electrodes: “mediated” and “mediatorless.” Mediated enzyme electrodes are based on monitoring the electroreduction of the oxidized mediator generated by the peroxidase catalyzed reaction, while mediatorless enzyme electrodes are based on monitoring direct electroreduction of peroxidase-active centers oxidized by $H_2O_2$. Mediated enzyme electrodes employ the high efficiency of the electron transfer by effective mediators, while mediatorless enzyme electrodes have the apparent advantage of simplicity. In a recently developed “wired” enzyme electrode, the peroxidase is covalently linked to a redox hydrogel coated on the surface of an electrode, and its oxidized form can be electroreduced effectively through the redox centers of the polymer network.[9,10] Electrodes based on the wired peroxidase have shown increased sensitivity and speed, the absence of leachable components, low sensitivity to interferants, and high stability. The wired peroxidase has been coupled to oxidase enzymes in electrochemical sensors for flow injection analysis.[10,14] The wired peroxidase electrode, however, has not previously been used to detect $H_2O_2$ in LC systems with oxidase IMERs. The IMER approach has the potential advantage of a much greater loading of an active catalyst in a rugged format, and a single peroxidase electrode design can be used with a number of different oxidase IMERs.

This BAS kit provides a wired peroxidase electrode compatible to a flowing system for the determination of oxidase substrates with LC and an appropriate post-column oxidase IMER. Compared with a conventional platinum anode used in LCEC for the detection of $H_2O_2$, the peroxidase cathode exhibits improved sensitivity, detection limit, and operational stability. When the peroxidase enzyme electrode is coupled to LC-oxidase IMER systems, the initial stabilization of the background current is significantly faster than for the conventional Pt electrode.

**Kit Principle**

A redox polymer film containing horseradish peroxidase (HRP) is coated on the surface of a glassy carbon electrode. The redox polymer, derived of poly(vinylpyridine) partly complexed to $[Os(bpy)_2Cl]^{2+/3+}$ redox centers, electrically "wires" the peroxidase to the electrode.[9] The wired peroxidase electrode can be operated at +100 mV (vs. Ag/AgCl) for the reduction of $H_2O_2$ produced from post-column IMERs (Figure 2). Such an electrode is frequently referred to in the literature as a "biosensor." In this case the biosensor is used in a flow system. This kit provides a means of preparing the biosensor and regenerating it for a minimal cost.

**Figure 2.** Redox cycles for oxidase IMER and wired peroxidase electrode.



## Kit Inventory

Cross-Flow Cell Version of the BAS Peroxidase Electrode Kit (MF-2095)

| Quantity | BAS P/N | Description |
|---|---|---|
| 1 ea | MF-9079 | Peroxidase electrode kit manual |
| 1 vial | CF-1070 | Peroxidase/polymer coating solution, 0.3 mL |
| 1 vial | CF-1075 | Surfactant Solution, 0.5 mL |
| 2 ea | MF-1000 | 3 mm dual glassy carbon electrode |
| 1 ea | MD-00001 | 1 µL dispensing syringe |
| 1 ea | MF-1046 | 51 µm Teflon® gasket for regular bore LCEC |
| 1 ea | MF-1044 | 13 µm thin-layer gasket for microbore LCEC |
| 1 ea | A-1425 | Storage capsule |
| 1 ea | A-1803 | Insulated shipping container |
| 1 ea | A-1804 | Refrigerant gel pack |

Refill Kit (MF-2096)

| Quantity | BAS P/N | Description |
|---|---|---|
| 1 bottle | CF-1070 | Peroxidase/polymer coating solution, 0.3 mL |
| 1 vial | CF-1075 | Surfactant Solution, 0.5 mL |
| 1 ea | A-1803 | Insulated shipping container |
| 1 ea | A-1804 | Refrigerant gel pack |

UniJet™ Cell Version of the BAS Peroxidase Electrode Kit (MF-2098)

| Quantity | BAS P/N | Description |
|---|---|---|
| 1 ea | MF-9079 | Peroxidase electrode kit manual |
| 1 vial | CF-1070 | Peroxidase/polymer coating solution, 0.3 mL |
| 1 vial | CF-1075 | Surfactant Solution, 0.5 mL |
| 2 ea | MF-1003 | 3 mm glassy carbon UniJet electrode |
| 1 ea | MD-00001 | 1 µL dispensing syringe |
| 1 ea | MF-1055 | 13 µm thin-layer gasket for UniJet LCEC |
| 1 ea | A-1425 | Storage capsule |
| 1 ea | A-1803 | Insulated shipping container |
| 1 ea | A-1804 | Refrigerant gel pack |

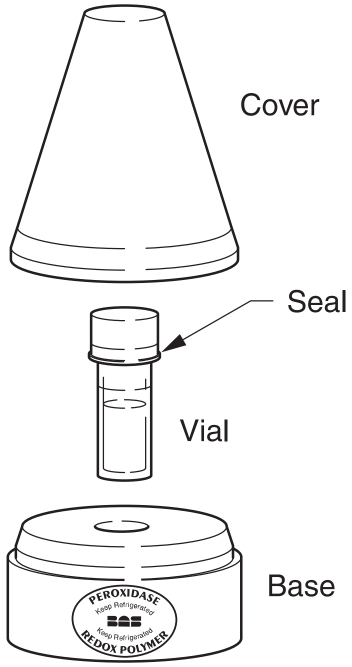
## Section 2. Operating Procedure

### Coating Solution Storage

Follow these steps to store the coating solution (see Figure 3).

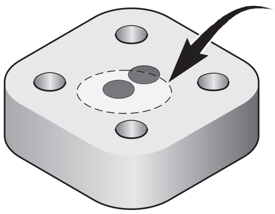
1. Remove vial seal.
2. Put vial into vial base.
3. Put the glass cover onto the base.
4. Mark glass with expiration date listed on the checklist shipped with the kit. It is normally 4 months after the shipping date.
5. Put the capsule into the refrigerator.

**Figure 3.** Steps for solution storage. Remove vial seal, put vial into base, cover base, mark expiration date on cover, refrigerate.

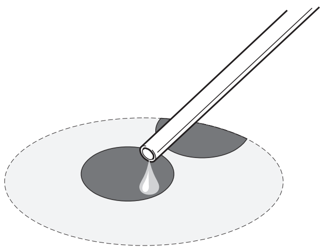


## Electrode Preparation

The electrode must be clean, dry, and free from particulates before it is coated. If you are using a new electrode, which has just been removed from the original package, make sure that the glassy carbon electrode surface is dust-free. If you have just finished repolishing the electrode, make sure that there are no residual polishing particles or film residue on the electrode surface. Clean the electrode before step 1 with a methanol-wetted wipe and allow it to dry.



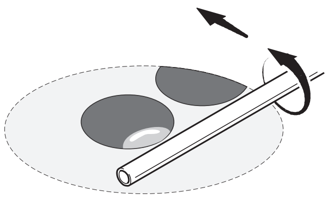
1. Locate the capped vial labeled “Surfactant” which was shipped as part of the Wired Enzyme kit. Open the vial and use the 1 µL syringe to remove 0.5 µL of surfactant solution.



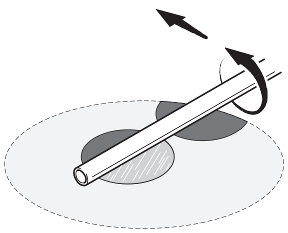
2. Position the syringe needle at the edge of the electrode surface and dispense the 0.5 µL of surfactant. Using the smooth edge of the syringe needle, roll the droplet of surfactant over the 3 mm electrode surface until it is completely coated.

3. Allow this surface to dry completely. This should require approx. 10 minutes.

4. Rinse the syringe with distilled water by filling and emptying the syringe at least 10 times. Thoroughly rinse the exterior of the needle with distilled water and wipe dry.

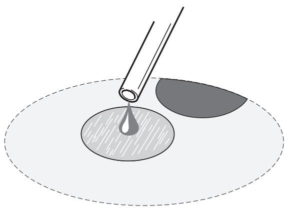


5. Using the 1 µL syringe, remove 0.5 µL of peroxidase/polymer solution from the vial. Carefully dispense the 0.5 µL droplet from the syringe in the center of the 3 mm working electrode. Do not gouge or scrape the surface while dispensing the solution. If necessary, use the side of the needle to push the fluid evenly across, but not out of, the electrode surface.

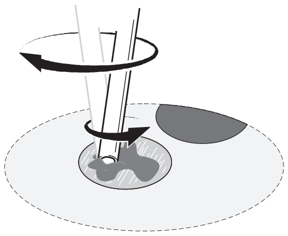


6. Return the vial of peroxidase/polymer solution to its container and refrigerate. Clean the syringe and the needle thoroughly with distilled water.

7. Allow the electrode to dry and cure overnight at room temperature. Cover the electrode with a clean, inverted beaker to keep the surface dust-free.



8. After the coated electrode is cured overnight at room temperature, it is ready to use. If it is not to be used immediately, it should be stored dry in the refrigerator at 4 °C. The shelf life of the coated electrode in the refrigerator is one week. Avoid warm and humid places for storage. Do not wipe, touch, or place the electrode surface in contact with other materials once it is coated with the peroxidase/polymer film.



For the cross-flow dual working electrode, although only one of the two electrodes on the same block will be used, it is recommended that both electrodes be coated. Use 0.5 µL of surfactant and polymer for each electrode. If the electrode initially connected is deactivated accidentally (e.g., the electrode is exposed to a wrong and extreme potential) during the first hour of its operation, the other one can be used as a backup. See Notes and Cautions (section 3, item 7).

The coated electrode can be continuously used in a flowing system for over two weeks. During this time, the electrode response may drop gradually, but the drop is usually less than 30% after two weeks of intensive use. When the response is too small for use, the electrode can be regenerated using the following procedure:

1. Remove the old peroxidase/polymer film by polishing the electrode with 0.5 μm alumina and then sonicating it in distilled water. After 2-3 minutes of sonicating, rinse the electrode well with distilled water and wipe dry. Refer to the polishing manual (A-1302) provided with your BAS electrochemical detector for more details.

2. Repeat the above electrode coating procedure (steps 1–4).

NOTE: Return the peroxidase/polymer solution to the storage capsule and refrigerate immediately after use. Keep the solution cold at all times.

## System Start-up

1. Flush out the pump and injection valve (both LOAD and INJECT positions) as required for LC systems.
    a. Pass through 50 mL of $H_2O$ (deionized $H_2O$ filtered with 0.2 µm filter).
    b. Pass through 50 mL of nitric acid solution (1:2 v/v concentrated $HNO_3$ to $H_2O$).
    c. Pass through 50-100 mL of filtered $H_2O$ until the pH is 3 or greater.
    d. Pass through 50 mL of mobile phase.

2. Install the analytical column and the immobilized enzyme-reactor (IMER), and flush them with mobile phase at the appropriate flow rate for 10 minutes or more.

3. Make sure the cell mode is at STBY, and set potential at +100 mV for cross-flow electrode and 0 mV for UniJet electrode.

4. Assemble the electrochemical transducer cell by installing the reference electrode and attaching the peroxidase working electrode to the auxiliary electrode.

5. Attach the effluent line of the enzyme reactor to the assembled cell.

6. Flush out the entire system with mobile phase at the required flow rate for **2-5 minutes** to wet the polymer film and to ensure that air has been removed from the cell.

7. Attach the cell leads and turn on the detector cell.

8. Equilibrate the system with mobile phase at the required flow rate for 1-2 hours until a stable baseline is achieved.

9. The system is now ready for injections.

10. If the system will be used every day or every few days, **do not turn the detector cell to STBY** when no injections are made. Leave the detector cell on and recycle the mobile phase when not making any injections.

11. After being used continuously for several weeks, the electrode may show some decrease in response and will need to be regenerated (see Electrode Preparation). The two working electrode blocks included in the kit can be used interchangeably to keep the system running. Turn the detector cell to STBY when changing the working electrode and follow steps 4 through 10. When the enzyme/polymer solution is depleted, a refill kit (MF-2096) is available. For the cross-flow dual working electrode, the two electrodes on the same electrode block cannot be used successively; the electrode not being used will lose its activity rather quickly in the flowing system, so only one of the two electrodes on the same block can be used for each operation.

**Note:**
Change the OXD/RDN Toggle Switch on back panel of LC-4C controller to RDN!

The electrochemical reaction that occurs at the “wired” enzyme electrode is a reduction of OS(III) to OS(II). This reaction creates a current of opposite polarity to that of an oxidation reaction. If you have been using your detector for oxidative LCEC, you will find that the chromatographic peak will go below baseline in a direction opposite of that you expect. This is easy to correct.

Look for the OXD/RDN toggle switch on the back panel of the LC-4C controller and change the position to RDN. **(The oxidation or reduction parameters are not affected by this switch.)** The peak will now rise above the baseline as usual. Do not change the position of the recorder or output leads. Always connect the green (+) banana jack to the (+) terminal of the data collection device and the black (-) banana jack to the (-) negative terminal.

## Shutdown and Storage

If the system will be used every day or every few days, leave the electrode in place and keep the system running. The mobile phase can be recycled when there is no injection, but fresh mobile phase should be prepared and used every week.

If the system will be unused for more than several days, it should be shut down and the electrode stored dry in the refrigerator. (A used and still active electrode can be stored dry in the refrigerator for over 2 months without losing any activity.) The following procedure is recommended for the system shutdown:

1. Turn the detector cell to STBY.
2. Take off the peroxidase working electrode and store it dry in a refrigerator in the plastic box used for shipping.
3. (This step is for the cross-flow cell only.) Take off the Ag/AgCl reference electrode and soak its porous tip in 3 M NaCl.
4. Remove the oxidase IMER and put it in the original shipping tube containing fresh mobile phase. Store in refrigerator.
5. Handle the analytical column as outlined in the individual kit. Flush the rest of the system with cleaning solvents as instructed.

**The cleaning solvents must not reach the peroxidase electrode or the oxidase IMER.**

## *Section 3. Applications of the Peroxidase Electrode*

The peroxidase enzyme electrode has been used to detect $H_2O_2$ for the determination of glucose, lactate, and acetylcholine/choline with LCEC and post-column IMERs. This section discusses examples that compare the peroxidase enzyme electrode and a conventional Pt electrode for these analyses.

All chromatography for these comparisons was performed at room temperature using a BAS 480 liquid chromatography system (PM-80 pump, LC-4C amperometric detector, CC-5 flow cell and column compartment, DA-5 data acquisition interface, and ChromGraph® software for data collection and analysis). The peroxidase electrode was operated at +100 mV vs. Ag/AgCl; the conventional Pt electrode (MF-6012, BAS) was operated at +500 mV vs. Ag/AgCl.

(Results obtained using the UniJet cell are not shown here. If you are using a UniJet cell, add 10 mM NaCl to the mobile phases used in the following examples. Both the baseline noise and the signal will be higher with the UniJet cell than with the cross-flow cell.)

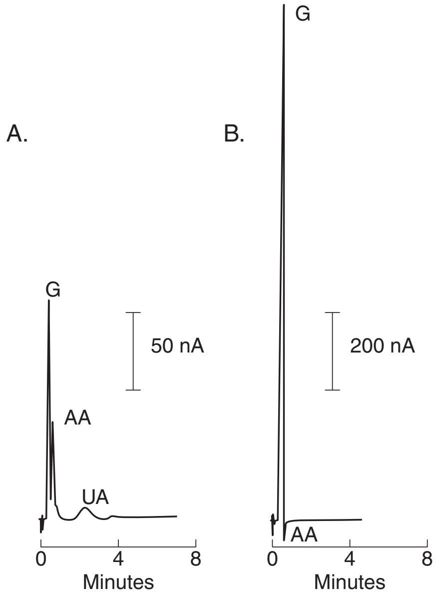
### Glucose Determination

A BAS Glucose Applications Kit (MF-8925), including an ODS column (3 µm particle diameter, 40 x 3.2 mm) and a glucose oxidase enzyme reactor, was used. The mobile phase was 20 mM $NaH_2PO_4$ and 0.05% dimethylhexylamine, pH 5.5, and the flow rate was 0.8 mL/min. Kathon® CG reagent (1% v:v) (CF-2150, BAS) was added to the mobile phases at 5 mL/L to retard bacterial growth.

The coupling of a glucose oxidase catalyzed reaction to electrochemical oxidation of the enzymatic reaction product, $H_2O_2$, has been the most common method for the detection of glucose in biological samples. A platinum working electrode is often used for the electrochemical measurement of $H_2O_2$ at a potential of +0.5 to +0.7 V vs. Ag/AgCl.[7] Under these conditions, reducing agents such as ascorbic acid and uric acid, which are fairly common in biological samples, can also be oxidized easily and therefore cause interference. This has been a problem in many glucose oxidase based amperometric sensors for glucose and also in the flow injection analysis of glucose employing the glucose oxidase catalyzed reaction.[15] In this analysis using the BAS Glucose Applications Kit, however, ascorbic acid and uric acid were separated from glucose by using an LC ODS column and an ion-pairing reagent.[7] As shown in Figure 4A, obtained from a glucose assay using the conventional Pt electrode at +500 mV vs. Ag/AgCl, glucose has almost no retention, while both ascorbic acid and uric acid show retention and elute after glucose.

When the Pt working electrode was replaced by the peroxidase enzyme electrode, the current for $H_2O_2$ reduction was measured. The new enzyme electrode coupled to the LC-IMER system required less than 1 hour for equilibration (the freshly polished Pt electrode required an overnight equilibration to achieve a stable baseline when it was coupled to the LC system). With the peroxidase electrode operating at +100 mV vs. Ag/AgCI, ascorbic acid showed an oxidation current response almost equal to that shown with the Pt electrode, and uric acid showed no significant response; on the other hand, the glucose response was nine times greater than with the Pt electrode (Figure 4B).

**Figure 4.** Chromatograms of a standard of 1 nmole glucose (G), 0.23 nmole ascorbic acid (AA), and 0.24 nmole uric acid (UA) with (A) the Pt electrode as an anode; (B) the enzyme electrode as a cathode.



The separation of ascorbic acid and uric acid from glucose can be changed by varying the content of the ion-pairing reagent in the mobile phase. Better separation, however, requires a longer operation time. In many cases when a large number of samples need to be analyzed, especially when the samples are facing degradation, fast assay is desired. Therefore, the ascorbic acid peak is usually set very close to the glucose peak, as shown in Figure 4, to save time. This could affect the accuracy of the glucose measurement when the ascorbic acid response is relatively high. Since the peroxidase electrode is much more sensitive to $H_2O_2$, but not to ascorbic acid, compared to a Pt electrode, it offers better selectivity, which is important in the case of a high ascorbic acid concentration.

Many researchers have applied various perm-selective membranes to the electrode surface to prevent ascorbic acid and uric acid from reaching the electrode.[15,16] In this experiment, as a comparison, Nafion coating was also applied to both Pt and peroxidase electrodes. It resulted in the elimination of the ascorbic acid and uric acid responses and also in a 60% drop in glucose response for both coated electrodes. Although much attention is given to this issue, glucose is 100-fold more concentrated than ascorbic acid in most biological samples, so the problem is actually minimal.

The dependencies of the peak currents on the amount of glucose injected, from the experiments using both Pt and enzyme electrodes, are shown in Figure 5. The currents from both electrodes initially increased linearly with the glucose amount, and then reached a plateau at the same glucose amount. The consistency of the upper limit of the linear range of the two electrodes indicates independence of the response linearity of the electrode under the experimental condition.

**Figure 5.** Dependence of absolute peak currents on the amount of glucose injected with ❍ the Pt electrode as an anode and ● the enzyme electrode as a cathode.

One of the applications of the glucose analysis is to determine the glucose concentration in rat subcutaneous microdialysate. The microdialysis perfusion medium, Ringer's solution (147 mM $Na^+$, 2.3 mM $Ca^{++}$, 4 mM $K^+$, 155 mM $Cl^-$, pH 6.0), can give a response at the same retention time as glucose, and this response is usually a limiting factor in the detection limit for glucose determination of undiluted dialysate.[15] The magnitude of this response from Ringer's solution was determined to be about the same with both the Pt electrode and the enzyme electrode, but because the enzyme electrode had a much higher glucose sensitivity, it could reliably detect a much smaller amount of glucose in the presence of Ringer's solution. Overall, the enzyme electrode offered a lower detection limit and better sensitivity for the determination of glucose than the Pt electrode (Table 1).

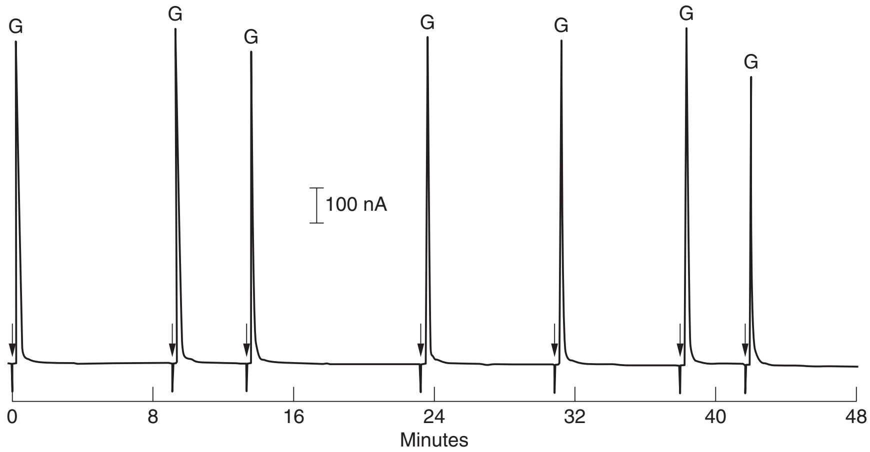
**Table 1.** Comparison of the "Wired" Peroxidase Electrode and the Pt Electrode in Glucose, Lactate, and Acetylcholine/Choline Determinations

| Assay | Electrode | Sensitivity (nA/nmole) | Detection Limit (pmole) | Linear Range |
|---|---|---|---|---|
| Glucose | Pt | 84.5 | 250[a] | 0.25 - 10 nmole |
| | Enzyme | 917 | 25[a] | 0.025 - 10 nmole |
| Lactate | Pt | 138 | 50[b] | 0.05 - 5 nmole |
| | Enzyme | 356 | 20[b] | 0.02 - 5 nmole |
| ACh/Ch | Pt | 421 | 0.05[a] | 0.05 - 2 pmole |
| | Enzyme | 1790 | 0.01[a] | 0.01 - 2 pmole |

[a] Measured with a signal-to-noise ratio of 3:1. [b] Calculated with a signal-to-noise ratio of 3:1.

Figure 6 shows the measurement of glucose in rat subcutaneous microdialysate (collected with a BAS DL-2 microdialysis probe perfused with Ringer's solution at 7 µL/min from an awake rat) using a peroxidase electrode. After 8 days of continuous injection of the dialysate (50 injections/day), there was only a 5% decrease in the analysis sensitivity from the calibration with standard glucose solutions. Previous experiments with Pt or Nafion-coated Pt electrodes had shown a much larger variation in sensitivity during the glucose assay of microdialysate.

**Figure 6.** Chromatograms of sequential injections of rat subcutaneous microdialysate samples with the enzyme electrode. Arrows indicate injections.



## Lactate Determination

For lactate analysis, an ion exchange column and a lactate oxidase enzyme reactor were used. The mobile phase was 50 mM $Na_2HPO_4$ and 5 mL/L Kathon CG reagent (CF-2150, BAS) at pH 8.0, and the flow rate was 1 mL/min.

Both the enzyme electrode and the Pt electrode were used and compared for the measurement of $H_2O_2$ in this analysis, and the enzyme electrode showed a higher sensitivity (Figure 7), similar to the results with glucose. When currents were measured for different amounts of lactate injected, the enzyme electrode and the Pt electrode showed the same linear range (Figure 8).

**Figure 7.** Chromatograms of (a) Ringer's solution; (b) standard of 0.5 nmole lactate with (A) the Pt electrode as an anode; (B) the enzyme electrode as a cathode.

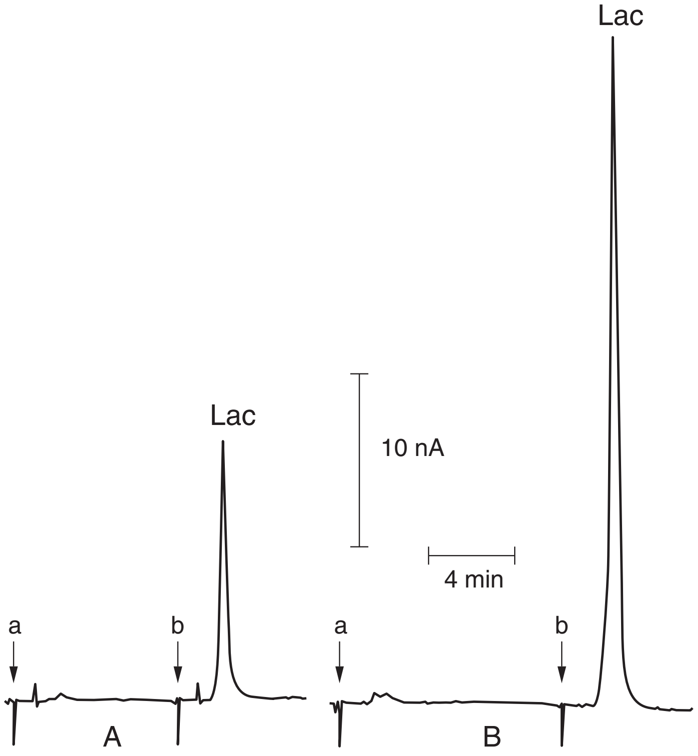
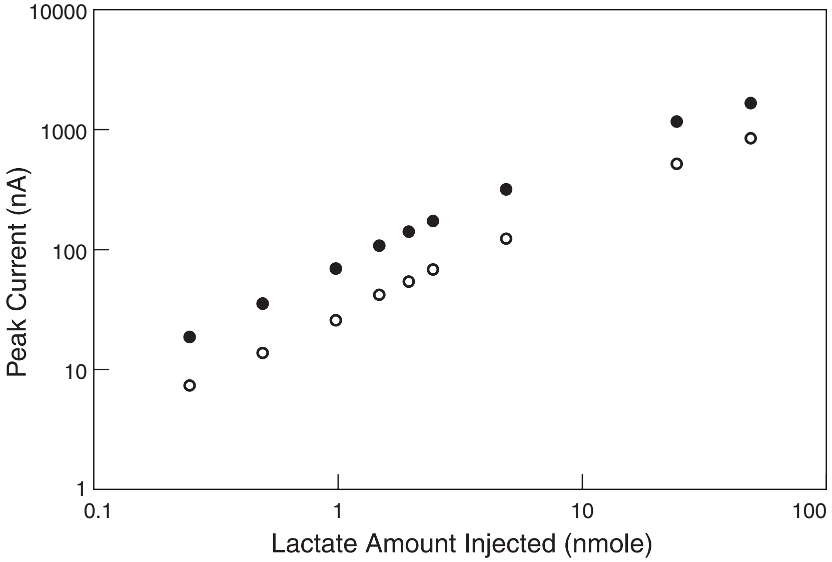


**Figure 8.** Dependence of absolute peak currents on the amount of lactate injected with ❍ the Pt electrode as an anode and ● the enzyme electrode as a cathode.



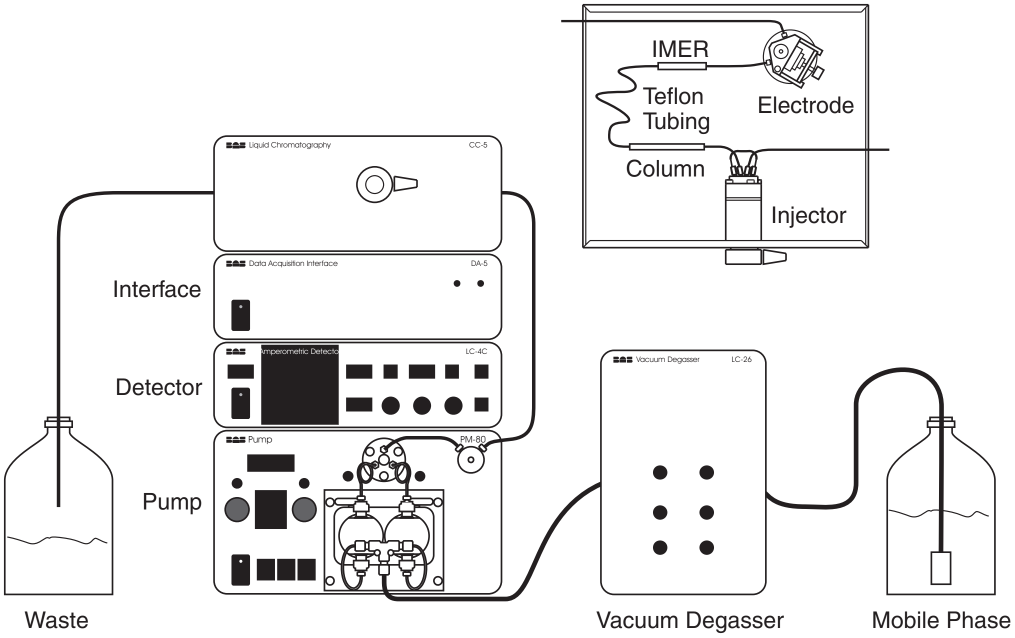
In this analysis, uric acid was very well separated from lactate, while the retention of ascorbic acid was quite close to that of lactate. As in the glucose assay, the ascorbic acid oxidation current response at the enzyme electrode and the Pt electrode were approximately the same. Therefore, the enzyme electrode was again more selective due to its higher sensitivity for $H_2O_2$. Separate injections of water, mobile phase, and Ringer's solution all gave small responses at the same retention as lactate, restricting the detection limit. Since the magnitudes of these blank solution responses were the same with the Pt and enzyme electrodes, the higher response of the enzyme electrode resulted in a lower detection limit for lactate (Table 1).

When the peroxidase enzyme electrode was used to determine the lactate concentration in rat subcutaneous microdialysate samples, no significant decrease in response was observed after 7 days of continuous operation.

## Acetylcholine/Choline Determination

A BAS Microbore Acetylcholine (ACh)-Choline (Ch) Kit (MF-8908) (including a microbore ACh/Ch analytical column (1 x 530 mm, 10 µm) (MF-8904) and an enzyme reactor column (1 x 50 mm, 10 µm) (PN MF-8903) containing acetylcholinesterase (AChE) and choline oxidase (ChO)) was used. The mobile phase contained 50 mM $Na_2HPO_4$ and 5 mL/L Kathon CG reagent (CF-2150, BAS), pH 8.0. The flow rate was 140 µL/min. A BAS vacuum degassing unit (MF-8500) was used to partially degas the mobile phase, and a piece of microbore Teflon tubing (I.D. = 0.009", 30 cm) was used to connect the analytical column and the IMER in order to regain some oxygen and maintain the activity of the IMER (Figure 9). The vacuum degasser and the microbore Teflon tubing were used together to reduce the background current and improve the detection limit.

**Figure 9.** The LC-EC system for acetylcholine/choline analysis.



Microdialysate samples were collected from the striata of freely moving animals. Sprague-Dawley rats were anesthetized with an intraperitoneal injection of 0.01 mL/kg of KX (10 mL Ketamine (100 mg/mL) + 1 mL Xylazine (100 mg/mL)), after which a guide cannula (MF-5429, BAS) was stereotaxically implanted. The animals were allowed to recover from surgery for approximately 4 days. On the day of the experiment, the guide cannula dummy stylette was removed and replaced with a microdialysis probe (CMA/12 4 mm, MF-5392, BAS). The probe was perfused with Ringer's solution at 2 µL/min via a syringe pump (MF-5102, BAS). After five hours of perfusion with Ringer's solution, as a comparison experiment, the probe in the same rat was perfused with Ringer's solution containing 10 µM neostigmine. In both cases, the dialysate fractions were automatically collected (CMA/142 fraction collector, MF-5202, BAS) into small plastic vials and the collection time for each sample was 10 minutes. Microdialysis samples (5 µL) were then injected directly onto the LC system. During microdialysis sampling, the awake unrestrained rat was housed in the BAS "Bee Keeper Rodent Residence" containment and swivel system (MD-1575).

The extracellular concentration of ACh in rat brain is typically very low due to its rapid conversion to Ch by the action of endogenous AChE. Many investigators have used esterase inhibitors to restrain this conversion so that the ACh concentration in the rat brain tissue could be measurable. These esterase inhibitors may affect the physiology of the system and thus the accuracy of data interpretation. A low detection limit for ACh is required to allow the investigator to determine basal ACh in the rat brain without using an esterase inhibitor.

Figure 10 shows the acetylcholine/choline responses at the Pt and peroxidase electrodes. As in the glucose and lactate assays, the enzyme electrode was more sensitive than the Pt electrode. The baseline noise of the enzyme and Pt electrodes were about the same. Therefore, the enzyme electrode offered a better detection limit than the Pt electrode (Table 1). The conventional Pt electrode had the disadvantages of long equilibration time and lack of stability at the high amplification required for basal ACh determinations. The peroxidase electrode, however, required less than 2 hours for initial equilibration and showed very good operational stability.

**Figure 10.** Chromatograms of a standard of 0.5 pmole acetylcholine/choline with (A) the Pt electrode as an anode; (B) the enzyme electrode as a cathode.

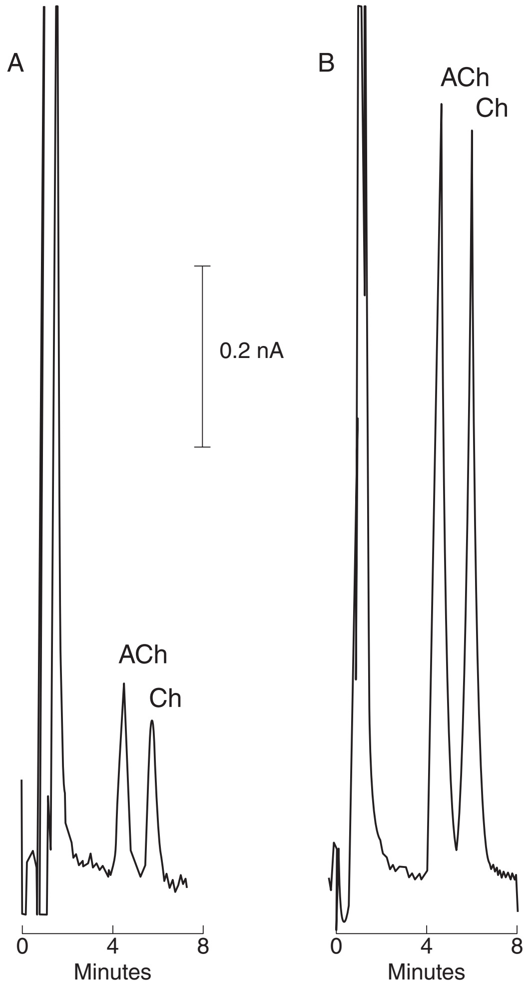


**Figure 11.** Chromatograms of (A) Ringer's solution; (B) 10 fmole ACh/Ch standard; (C) 50 fmole ACh/Ch standard; (D) 500 fmole Ch standard; (E) rat striatal microdialysate. The sample size was 5 µL. Arrows indicate the time of injections.

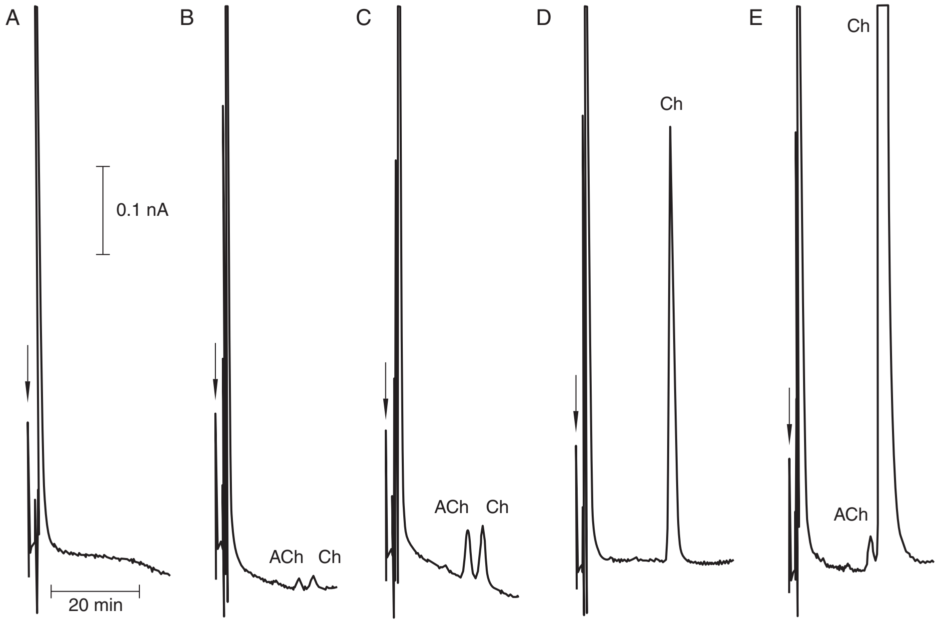


**Figure 12.** Dependence of absolute peak currents on the amount of standard ACh injected.

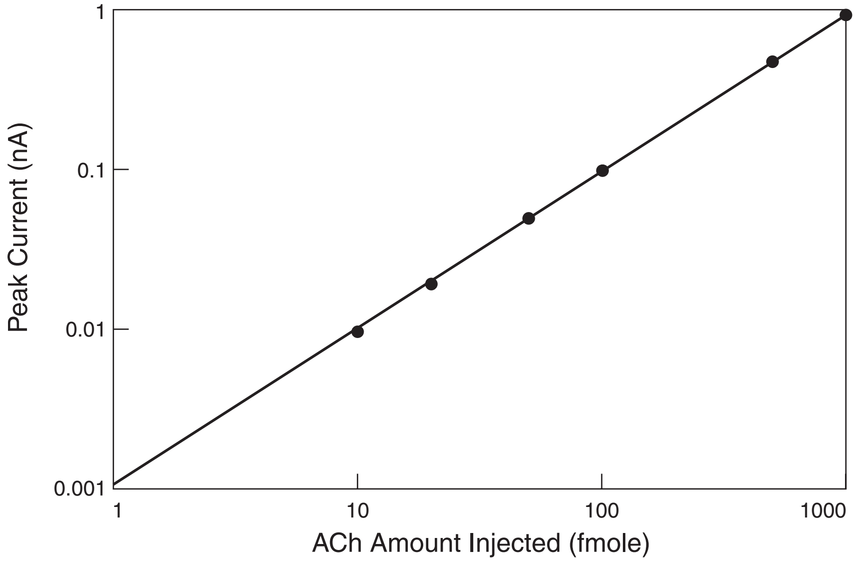


Figure 11 shows typical chromatograms using the peroxidase electrode for the determination of ACh/Ch in standards and rat brain microdialysates. Injections of 10 fmole ACh/Ch consistently gave signal-to-noise ratios above 3 as shown in Figure 11B. Therefore, 10 fmole was determined to be the practical detection limit for ACh in this experiment. Figure 12 shows that the peroxidase electrode response is linear with the amount of ACh injected over at least two orders of magnitude. The $r^2$ value from the linear regression of these data is 0.999, and the slope of the linear regression line is $9.01 \times 10^{-4}$ nA/fmole. Figure 11E shows the chromatogram of a rat brain microdialysate sample collected without the use of any esterase inhibitors. The basal striatal concentration of ACh in the dialysate was determined to be 31 ± 5 (mean ±SD, n=6) fmole/5 µL. The response of Ch can be seen to be much higher and off-scale in the chromatogram. During five hours of perfusion with Ringer's solution without any esterase inhibitors, the concentration of ACh in the rat dialysate samples remained relatively constant. After the five hours of perfusion with Ringer's solution, the perfusion solution was changed to Ringer's solution containing 10 µM neostigmine. The concentration of ACh in the dialysates increased 10-fold during the first hour of perfusion with the solution containing neostigmine. Longer perfusion times resulted in even higher concentrations of ACh in the dialysates. The rats appeared to become increasingly active during the neostigmine perfusion, indicating an effect of the esterase inhibitor on their physiology.

## Conclusion

Results indicate that the LCEC system used during these experiments provides a sufficiently low detection limit for basal ACh determination in a very small sample (5 µL). In addition to its high sensitivity and low detection limit for ACh, this LCEC system has also shown satisfactory operational stability. For this system, there are two sources of instability. These are the AChE/ChO IMER and the enzyme-modified electrode, both of which are limited by enzyme stability. We found that the AChE/ChO IMER did not exhibit any decrease in response over a six-month period, during which it had been used intensively and stored at 4 °C while not in use. As for the peroxidase electrode, no significant variation in electrode response was observed after one week of continuous operation. The peroxidase electrode operating at +100 mV vs. Ag/AgCl exhibited a background reduction current. It was found that the LC pump noise was proportional to the magnitude of this reduction current. In order to achieve a low detection limit for ACh, the noise (and thus the background current) needs to be minimized. The background reduction current was found to increase as the oxygen concentration in the mobile phase increased. Because oxygen is necessary for the ChO catalyzed reaction in the IMER, it cannot be simply eliminated from the system to reduce the background current. In this experiment, a vacuum degassing unit was used to reduce the oxygen concentration of the mobile phase (and thus the background current). Teflon tubing was used to connect the analytical column and the IMER in order to regain some oxygen and maintain the activity of the IMER (Figure 9). In our previous ACh analysis, with the conventional Pt electrode, ethylenediaminetetraacetic acid (EDTA) was added to the mobile phase to reduce the noise. In this assay, however, the presence of EDTA was seen to contribute to the background reduction current for some unknown reason, so EDTA was avoided in the mobile phase.

**Notes/Cautions**

1. A thick peroxidase/polymer film on the electrode surface may result in poor current sensitivity, peak tailing, and baseline noise. On the other hand, a peroxidase/polymer film that is too thin may result in short operational life and low sensitivity. Therefore, when coating the electrode, use 0.5 µL of the peroxidase/polymer solution for a 3 mm electrode.

   The syringe provided with the system is designed to deliver fluid volumes < 1 µL without wasting material. The syringe needle is filled with a plunger pin. As the plunger is withdrawn, the pin withdraws and fluid is drawn into the needle. Follow the flat metal scale on the exterior of the syringe to dispense 0.5 µL.

2. Remember to clean the syringe with distilled water after using it to coat the electrode. Dried polymer will clog the syringe.

3. A coated electrode after overnight-cure at room temperature should be stored dry in the refrigerator. Avoid warm and humid places for storage. Do not wipe, touch, or place the electrode surface in contact with other materials once it is coated with the peroxidase/polymer film. **Do not** immerse the electrode in any kind of buffer for storage.

4. In a flowing system, the peroxidase electrode will lose its activity faster in an open circuit than in a closed circuit, so **do not** turn the detector cell to STBY when no injections are being made. Leave the detector cell on and recycle the mobile phase when not making any injections.

5. However, ALWAYS make sure the detector cell is set to STBY when removing or replacing any of the electrodes.

6. Incorrect applied potentials will damage the redox polymer. Make sure the potential is set at +100 mV vs. Ag/AgCl (0 mV for UniJet cell) before the cell is turned on. Do not remove the reference electrode or change the potential when the cell is on.

7. For a cross-flow dual working electrode, use only one of the two electrodes on the same block. When the response is too low for use, regenerate the electrode by cleaning and recoating it following the instructions in the Electrode Preparation section. The second electrode on the same block can only be used as a backup during the first hour of operation, because the peroxidase electrode will significantly lose its activity after several hours in a flowing system with an open circuit.

8. During system shutdown, always take off the peroxidase electrode and the oxidase IMER (for some kits, also the analytical column) before flushing the system with cleaning solvents. The cleaning solvent should not reach the peroxidase electrode or the oxidase IMER.

9. When the detector is set to high gain (sensitive, low-current scale), a noisy and drifting baseline may be observed when:
    a. the analytical column is changed.
    b. the IMER is changed.
    c. the mobile phase is changed.
    d. the system has just started.

    Equilibrate the system overnight on these occasions. When fresh mobile phase is needed, prepare it as consistently as possible.

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