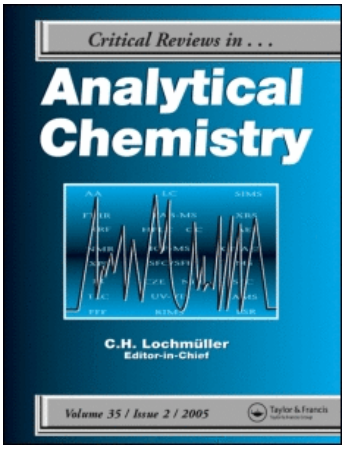

Critical Reviews in Analytical Chemistry
Publication details, including instructions for authors and subscription information:
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To cite this Article McCreery, Richard L. and Wilson, George S.(1978) 'Bioelectrochemistry: An Examination of Some Examples', Critical Reviews in Analytical Chemistry, 7: 2, 89 — 119
To link to this Article: DOI: 10.1080/10408347808542699
URL: http://dx.doi.org/10.1080/10408347808542699

BIOELECTROCHEMISTRY: AN EXAMINATION OF SOME EXAMPLES

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I. INTRODUCTION

During the last decade or so, the term bioelectrochemistry has been used to describe a variety of research endeavors involving electrochemical phenomena of biological systems. The phenomena grouped under this heading include such diverse areas as generation of potentials across membranes, the oxidation-reduction chemistry of macromolecules, and the application of electrochemistry to medical problems such as bone repair and tissue growth. Although this diversity has lead to fairly rapid growth, it has also resulted in difficulty in defining the field of bioelectrochemistry. The often quoted statement, "bioelectrochemistry is what bioelectrochemists do," does not allow one to identify the area as a viable scientific subdiscipline. It is partly the intent of this discussion to describe several areas of active research which, in the author's opinion, justifiably fall under the heading of bioelectrochemistry.

Anyone familiar with the diversity of electrochemical phenomena associated with biology will realize that a comprehensive review of such work would be exceedingly difficult, if not impossible. In keeping with the format of this publication, five areas of bioelectrochemistry will be examined with the primary objective being identification of areas of current interest rather than a review of all work undertaken. The first of five areas to be considered involves faradaic reactions of low-molecular weight materials, including vitamins and pharmaceuticals. Second, electrochemical investigations of charge-transfer processes of macromolecules will be examined with particular emphasis on the cytochrome systems of the electron transport chain. Third, the analytical applications of electrochemistry in biology are considered, with particular regard to those analyses which are not easily performed by other methods. The electrochemistry of biological membranes comprises the fourth area, the only one to be discussed which is known to involve primarily nonfaradaic processes. Finally, the sparse but important direct applications of electrochemistry in therapeutic situations will be discussed.

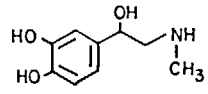
It is anticipated that this article will serve two purposes of which the reader should be aware. Primarily, an attempt is made to identify the merits of electrochemistry when it is applied to biological systems. What types of information have been obtained which are unique to this approach and not easily obtained otherwise? What types of electrochemical experiments appear most promising for learning more about biological systems? In addition, it is hoped that bioelectrochemistry will be better defined if its principal areas are discussed in one article.

II. FARADAIC REACTIONS INVOLVING SMALL MOLECULES

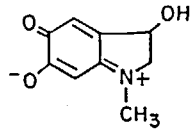
A. Techniques and Approaches

Probably the most active area of those mentioned above has involved the electrochemistry of low-molecular weight materials (< 1000 daltons). A large variety of biological molecules has been investigated electrochemically, usually by conventional methods existing at the time the studies were conducted. The history of this area is a long one, beginning with the polarography of reducible vitamins such as thiamin and riboflavin in the 1940s.[1]

A useful but somewhat limited approach used in the past was the investigation of systems under conditions where the chemistry was relatively simple. The reason was that techniques were not sophisticated enough nor fast enough to follow the complex mechanisms associated with many biological systems. A somewhat dated but nevertheless relevant example is the oxidation of adrenalin[2] (Structure 1). It has been known for several decades that adrenalin oxidation leads to adrenochrome (Structure 2) and eventually to a complex melanin-like pigment. If the electrochemical oxidation is examined below pH 2, only oxidation of the catechol nucleus is observed, and the reaction is easily studied by classical potentiometry.[3] However, at pH 3 and above, the reactions are much more complicated but at the same time more biologically important. It was not until



Structure 1



Structure 2

cyclic voltammetry was used that the complex electrochemistry was understood.

In 1 *M* H_2SO_4, the voltammogram of Figure 1 was observed, corresponding to the simple, quasi-reversible catechol-*ortho*quinone couple. At pH 3, the more complex voltammogram of Figure 2 was observed. The large anodic wave indicated generation of the *ortho*quinone, which at this pH, undergoes a Michael addition-type cyclization to form reduced adrenochrome (adrenolutin, Structure 3). After this initial oxidation, the appearance of the adrenolutin/adrenochrome couple (peaks 3 and 4) allowed identification of the products and proposal of a mechanism (Scheme 1).

The pseudo first-order rate constant for cyclization is strongly pH-dependent due to protonation of the nucleophilic nitrogen. Results for various electrochemical determinations of k are shown in Table 1. Notice that the biologically important production of adrenochrome would have been missed if the conditions were restricted to pH 2 or below. It was necessary to improve techniques to examine this reaction rather than to simplify the system until existing methods were usable. In this system, the situation becomes more difficult as one approaches physiological pH since k becomes much faster. It was necessary to use more

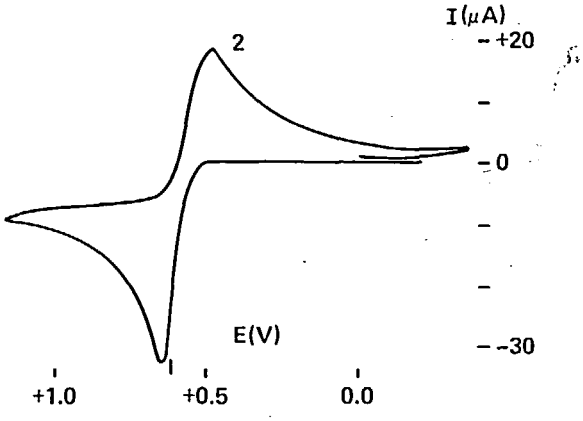


FIGURE 1. Cyclic voltammogram of adrenalin in 1.0 *M* H_2SO_4, scan rate = 0.04 V/sec. (Reprinted with permission from Hawley, M. D., Tatwawadi, S. V., Piekarski, S., and Adams, R. N., *J. Am. Chem. Soc.*, 89, 447 (1967). Copyright by the American Chemical Society.)

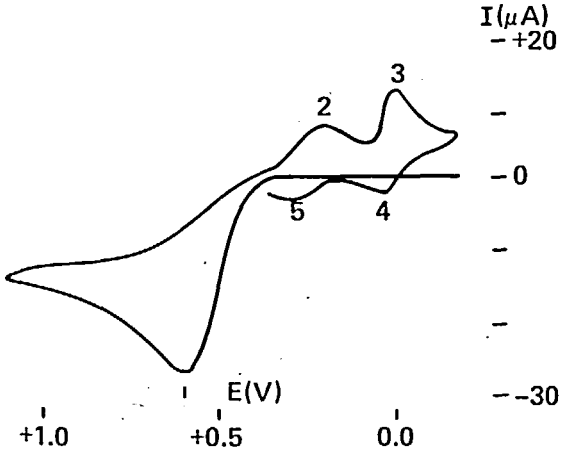
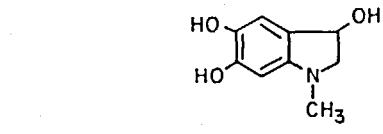
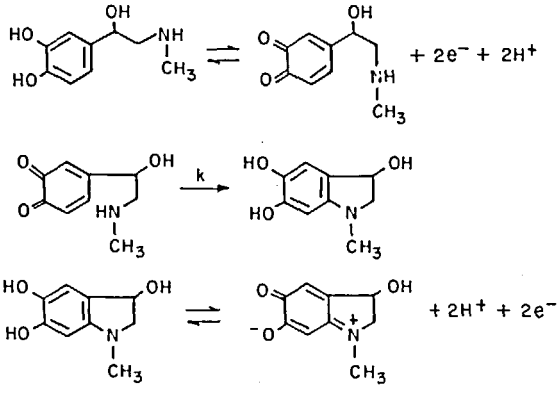


FIGURE 2. Cyclic voltammogram of adrenalin at pH 3.0, scan rate = 0.04 V/sec. (Reprinted with permission from Hawley, M. D. et al., *J. Am. Chem. Soc.*, 89, 447 (1967). Copyright by the American Chemical Society.)



Structure 3



Scheme 1

TABLE 1

Pseudo First-order Rate Constants for Cyclization of Adrenaline *o*-quinone

pH	k (sec^{-1})	Half-life (sec)
3.5	0.025[a,2]	28
4.0	0.098[a,2]	7.1
4.5	0.27[a,2]	2.6
5.0	0.99[a,2]	0.70
6.3	19[b,4]	0.036
7.15	20[b,4]	0.035

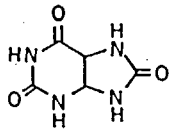
[a]Using chronoamperometry.
[b]Using rotating disc electrochemistry.

sophisticated rotating disc methods to monitor the reaction in the physiological pH region.[4] It is apparent that fairly powerful electrochemical techniques were necessary to cover the wide range of half-lives observed for the adrenalin cyclization.

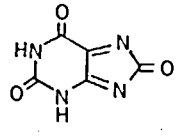
Another example of the necessity for the use of sophisticated techniques is provided by studies of the oxidation of purine bases. The electrochemical oxidation of adenine and the related compound uric acid (Structure 4) produced a variety of products, including alloxan, parabanic acid, alantoin, and urea.[6,7] Since these products are also found metabolically, the proposal was made that any intermediates discovered electrochemically may be involved in the biological degradation as well. Based mainly on product analysis and coulometry, a dication intermediate (Structure 5) was proposed, without observing it directly, either electrochemically or otherwise.[6] Although a complete mechanism was proposed at that point, some flaws were apparent when the system was examined using fast cyclic voltammetry. At scan rates above about 0.5 V/sec, a reversible wave could be observed corresponding to the oxidation of uric acid to the reactive intermediate. By examining the pH dependence of the wave's potential, it was found to be a 2e-2H$^+$ oxidation, contrary to predictions based on a dication intermediate which would be a pH-independent 2e$^-$ oxidation. Combining this and other evidence, the mechanism shown in Scheme 2 involving a bis-imine intermediate (Structure 6) was proposed.[8] More recent work on a model compound, diaminouracil (Structure 7), supports this mechanism.[9]

B. Nicotinamide Nucleotides

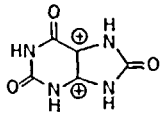
Since the nicotinamide nucleotides NAD and NADH comprise a primary redox system involved in a wide variety of metabolic reactions, their electrochemistry has been actively studied in recent years.[10] Most of the efforts have been directed toward the reduction of NAD$^+$ since the reaction is easily studied by conventional polarography. A series of papers describing the use of dc and ac polarography and cyclic voltammetry on NAD and related compounds[11,12] has resulted in a fairly clear picture of the electrochemical reduc-



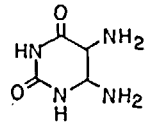
Structure 4



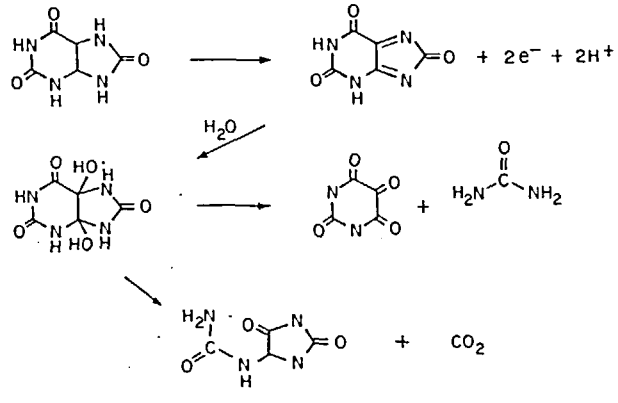
Structure 6



Structure 5



Structure 7



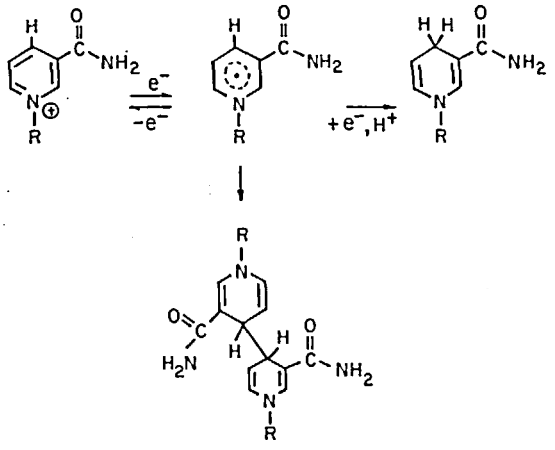
Scheme 2

tion scheme. Two reduction processes were described, one at -0.89 V vs. SCE and the other at about -1.6 V. Both potentials were pH independent, but the more negative wave was not observable below pH 9.3. After a thorough examination of NAD and model compounds, the authors proposed Scheme 3. The reduction wave at -0.89 V was attributed to the reduction of NAD (Structure 8)[13] to a free radical, which can couple to form a dimer (Structure 9). At sufficiently negative potentials (-1.6 V), NAD may be reduced via a two-electron process to NADH (Structure 10). It should be noted that the 1,6-isomer of NADH was also observed as a product, as well as the 4′,6′- and 6′6′-dimers. The electrochemical reduction was complicated by adsorption processes and other effects which distorted the observations.

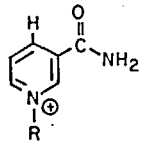
Although this study of NAD reduction provides a great deal of chemical information, it is unfortunate that the reactions do not correspond to the biologically observed mechanism. The reversible potential for NAD/NADH at pH 7 is -0.57 V vs. SCE, compared to the -0.89 V observed polarographically. Furthermore, the biological reduction product is totally 1,4-NADH; the dimer and 1,6-NADH are not believed to be biochemically important. These differences should be kept in mind when considering the biological significance of the electrochemical data.

A related effort by different investigators dealt with the electrochemical oxidation of NADH.[14] A platinum electrode was employed to examine the oxidation in phosphate or pyrophosphate buffers at pH 6 to 9. Compared to the reduction of NAD, the oxidation of NADH was well behaved. The voltammetric peak was linearly related to NADH concentration and to the square root of the scan rate, indicating a diffusion controlled oxidation without significant adsorption. Coulometric oxidation generated a 92% yield of enzymatically active NAD measured by comparing the spectrophotometrically determined concentration with the coulombs of charge passed. The authors comment that this electrochemical generation of oxidized material may be useful in driving enzymatically catalyzed reactions involving the NAD/NADH couple.

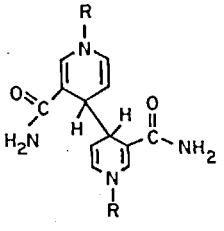
In order to avoid problems caused by electrode adsorption and nonspecific reduction of pyridine nucleotides, the reduction process has been coupled to the electrode via a charge-transfer mediator.[15] The details of this approach will be discussed later, but the example of interest here is the reduction of NADP, closely chemically related to NAD and differing only by a phosphate group.[13] NADP, methylviologen, and an enzyme catalyzing NADP reduction, spinach ferredoxin-NADP-reductase, were placed in an electrochemical cell utilizing a tin oxide-working electrode. The viologen was electrochemically reduced, and the homogeneous reduction of NADP to NADPH by reduced viologen was



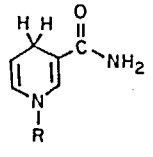
Scheme 3



Structure 8



Structure 9



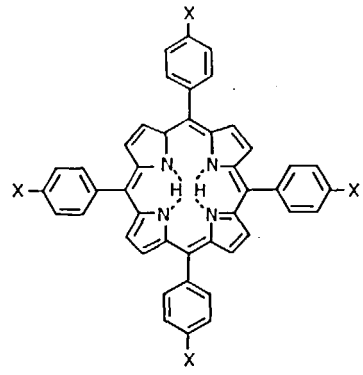
Structure 10

catalyzed by the enzyme. It was demonstrated that the reduced NADPH was fully active and had the expected spectral and electrochemical characteristics. Side reactions involving adsorption or dimerization were not observed as they were with unmediated NAD reduction. One can conclude that the mediated system is much "cleaner" and biochemically relevant although the presence of the mediator and enzyme make extraction of information more complex. The oxidation waves appear at the mediator potential, not that of NADP, and one must consider the rate of charge transfer not only between electrode and mediator, but also between mediator and biological component.

C. Porphyrin Models

Of the large amount of work performed on the reactions of metals in biological systems, the examples of interest here deal with the study and modeling of metalloenzymes. Applications of electrochemistry have been frequent in this area, since involvement in redox processes is the dominant characteristic of systems such as the cytochromes, dehydrogenases, and other electron-transport components. Although the intact enzymes themselves have been studied with electrochemical techniques, the model systems are more appropriately covered in this section. Several classes of metalloenzymes and their model systems are apparent, including the metal porphyrin entity of the electron- and oxygen-transport systems, the iron and molybdenum complexes of flavins associated with flavoproteins, and the enzymes incorporating several trace metals such as manganese and vanadium. Two aspects of the redox reactions of these materials are important to consider with regard to biological significance. First, the nature of the various oxidation states with regard to structure and electron delocalization will have a bearing on the stoichiometry and energetics of the redox processes. Second, the kinetics of charge transfer to the model system or intact enzyme will provide insight into the details of the associated biological events.

Research on the porphyrin system has been particularly active because of its importance to the cytochromes, hemoglobin, and photosynthetic processes. The redox chemistry is complicated by protonation equilibria and limited solubility in water, so many studies have been carried out in nonaqueous media on simplified systems. Commonly studied models include tetraphenyl porphyrin (H_2TPP, Structure 11), porphyrin IX, dihydroporphyrins, and various other substituted porphyrins.



Structure 11

The electrochemistry of metal/porphyrin complexes is complicated by the fact that both the ligand and the metal center may undergo redox reactions. Furthermore, an added electron may be delocalized over the entire complex rather than residing completely on the metal or the ligand. One approach to this problem is the study of porphyrin electrochemistry with and without the metal present. A cyclic voltammetric investigation of uncomplexed H_2TPP in methylene chloride revealed four distinct couples, corresponding to two oxidations and two reductions,[16] as shown in Figure 3. The observed couples were assigned to the reactions:

$$[H_2TPP]^{+2} + e^- \rightleftarrows [H_2TPP]^+$$

$$[H_2TPP]^+ + e^- \rightleftarrows [H_2TPP]^O$$

$$[H_2TPP]^O + e^- \rightleftarrows [H_2TPP]^-$$

$$[H_2TPP2]^- + e^- \rightleftarrows [H_2TPP]^{-2}$$

Substituent and solvent effects on the redox potentials for these reactions have been investigated.[16]

A similar approach was used to examine H_2TPP electrochemistry in dimethylformamide. H_2TPP and its isomeric dihydroporphyrins, phlorin and chlorin, were examined with the aid of the additional techniques of spectroelectrochemistry and electron spin resonance.[17-19] The authors point out that if the porphyrin redox reactions are carried out in protic media, the dihydroporphyrin

derivatives are likely to form and the total number of possible products increase.

Addition of a metal ion to the system complicates matters by introducing an additional redox center.[20] A cyclic voltammogram of Co(II)TPP is shown in Figure 4. The workers assigned the couples to reactions corresponding to electron transfer to both the ligand and the metal:

$$[Co(III)TPP]^{+3} + e^- \rightleftarrows [Co(II)TPP]^{+2} \quad (1)$$

$$[Co(III)TPP]^{+2} + e^- \rightleftarrows [Co(III)TPP]^{+} \quad (2)$$

$$[Co(III)TPP]^{+} + e^- \rightleftarrows [Co(II)TPP]^{O} \quad (3)$$

$$[Co(II)TPP]^{O} + e^- \rightleftarrows [Co(I)TPP]^{-1} \quad (4)$$

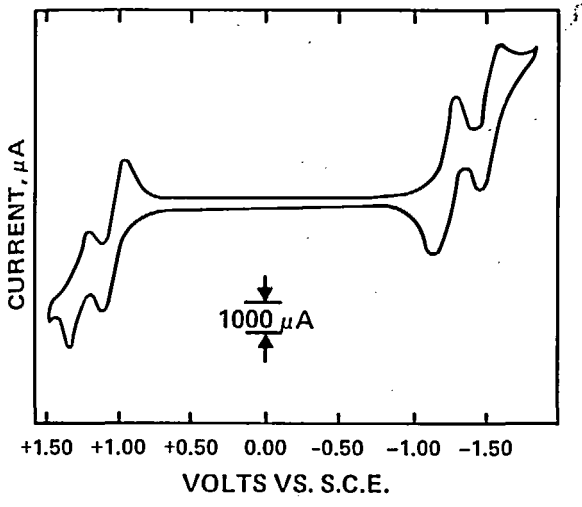


FIGURE 3. Cyclic voltammogram of tetraphenylporphyrin in CH_2Cl_2, scan rate = 0.05 V/sec. (Reprinted with permission from Kadish, K. and Morrison, M., *J. Am. Chem. Soc.*, 98, 3326 (1976). Copyright by the American Chemical Society.)

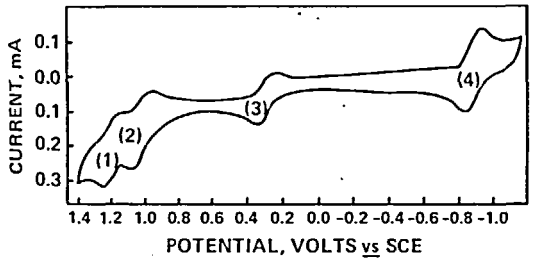
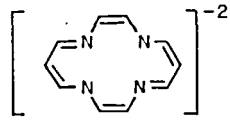


FIGURE 4. Cyclic voltammogram of Co(II) (p-OCH_3) TPP in butyronitrile, scan rate = 0.10 V/sec. Numbers in parentheses refer to reactions 1 to 4 in text. (Reprinted with permission from Walker, F., Beroiz, D., and Kadish, K., *J. Am. Chem. Soc.*, 98, 3484 (1976). Copyright by the American Chemical Society.)

A systematic study of substituent effects on reactions of other metal porphyrin complexes has been made for reactions occurring in nonaqueous media.[21-23] Interactions of iron tetraphenylporphyrin with the electrode have been studied.[24]

A fairly extensive examination of the effect of the metal center on redox behavior has been carried out for the case of octaethylporphyrin complexes in butyronitrile and dimethylsulfoxide. Depending upon the identity of metal center, the electron was transferred either to the porphyrin ring or to the metal itself. It is interesting to note that the porphyrin ring system is able to stabilize some uncommon metal oxidation states such as Ag(III) and Co(I).[25]

Although the equations corresponding to Figure 4 include assignments of oxidation states of the metal center and ligand, it is often difficult to discern the degree of delocalization of the added electrons. The assignments in the CoTPP system represent the extreme of isolation of oxidation-state changes on either metal or ligand without significant overlap. At the other extreme is the complete delocalization of electrons in molecular orbitals shared by both ligand and metal. This problem has been examined in detail using electron spin resonance (ESR) to identify the location of unpaired electron density for complexes of a different porphyrin model, the tetraazaannulene system (Structure 12). In this study,[26] the Ni^{+2} complex of Structure 12, $[Ni(II)L^{-2}]^{O}$, was oxidized by the removal of one electron and the ESR spectrum was obtained. A calculation of spin density placed the electron predominantly on the ligand, with minor contribution of the metal to the spectrum. Thus, it was concluded that at least in this case, the charge distribution is more accurately represented by $[Ni(II)L^{-}]^{+}$ rather than $[Ni(I)L^{-2}]^{+}$.



Structure 12

In cases where electrons are localized on the ligand or metal center, the possibility for intramolecular-electron transfer exists. Such a process has been documented for the NiTPP complex[27] and a variety of tetraazamacrocyclic[28] complexes

and can be represented by an equation of the form:

$$[M(II)L^{-}]^{+} \rightleftarrows [M(III)L^{-2}]^{+}$$

The biological importance of this exchange has been discussed.[21,29]

As mentioned above, there is particular interest in the redox potentials of porphyrin/metal model systems because of their obvious biological importance. In addition to the identity of the metal center, at least three major factors which affect the redox potential have been examined. With both the TPP and tetraazamacrocyclic series, ligand ring structure and substituent effects on potentials have been studied.[16,21] Since electron transfer to the complex may occur through an exposed ligand edge in the biological system, ligand substituents may play significant biological roles. The effect of the solvent environment on the redox potential has been investigated,[30,31] and it was found that the potential for the iron/porphyrin complex is 300 mV higher in nonaqueous solvents than in water. Since the biologically active heme system is enclosed in an essentially nonaqueous protein sheath, solvent effects on potentials cannot be ignored. The influence of the axial ligand on potentials reveals effects which emphasize the importance of the local environment on the chemistry of the porphyrin/metal complex.[32,33] Finally, comparisons of the potentials of a variety of tetraazamacrocyclic Fe^{+2}/Fe^{+3} complexes have been made with those of naturally occurring iron-based redox systems.[34,35]

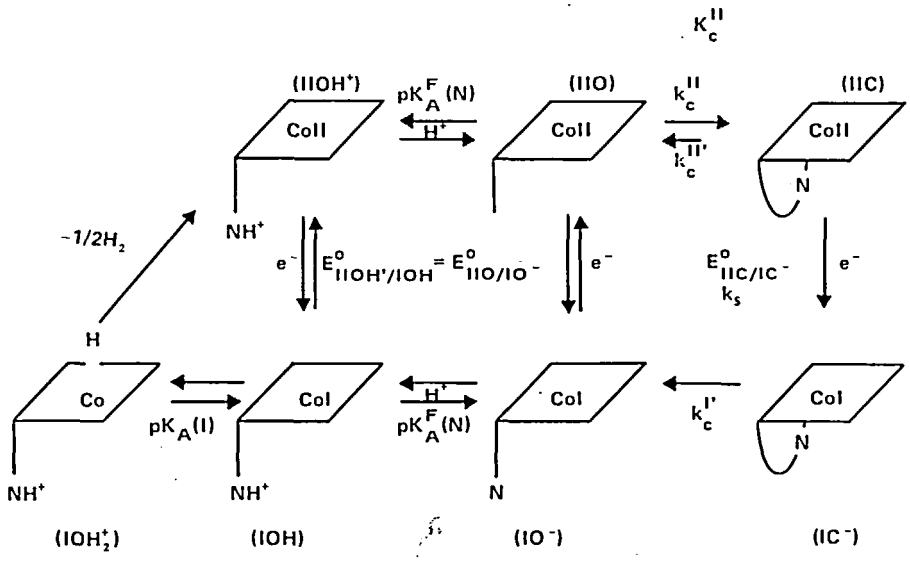
The electrochemistry of porphyrin complexes has been studied in aqueous environments, but only to a limited degree. The problems of solubility, protonation equilibria, and dimer formation complicate matters to the point where the results are difficult to interpret.[19] A broader effort has been expended in examining the metal/porphyrin center in the intact enzymes as discussed below.

D. Vitamin B_{12} and Related Cobalamins

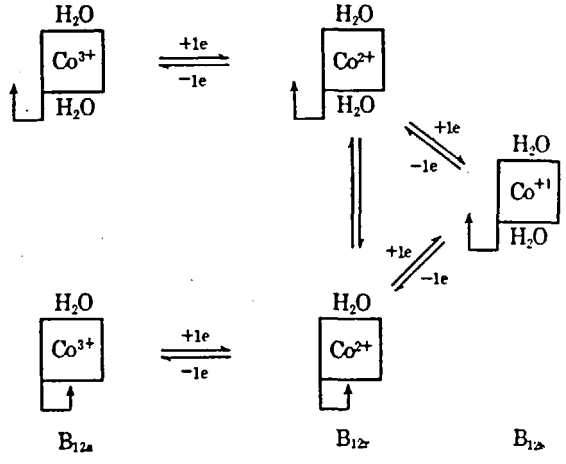
Vitamin B_{12} consists of a cobalt complex with a corrin ring, structurally similar to the porphyrin system.[36] In addition, B_{12} has a cyanide group as one axial ligand, and the corrin ring has an attached benzimidazole group which may be coordinated as the other axial ligand (so-called "base-on" configuration). The majority of electrochemical studies of B_{12} have been on the aquocobalamin form, where the cyano group is substituted with water. Early polarography and voltammetry indicated that B_{12} itself was not electroactive; therefore, study was limited to the aquocobalamin system. The Co(II) aquocobalamin complex has been labeled B_{12r}; its Co(I) and Co(III) states are B_{12s} and B_{12a}, respectively.

The complexity of the redox reactions of the B_{12a} system in aqueous solution is apparent from recent work on the B_{12r}/B_{12s} redox couple.[37] By examining pH effects and electron-transfer kinetics, the potentials and K_a values for all observed protonated and base-on/base-off species were determined. A mechanism for this couple was proposed which involved the B_{12r}/B_{12s} redox pair and species arising from deprotonation of the coordinated water and benzimidazole ring, as well as the base-on/base-off equilibrium (Scheme 4). It should be kept in mind that this complex mechanism involves only the one-electron B_{12r}/B_{12s} couple.

Much of the difficulty in using electrochemistry to examine the complex cobalamin system is caused by electrode adsorption and slow charge transfer kinetics. When electrochemical control of potential is combined with optical monitoring of the solution species, many of these problems are alleviated. Of particular value in the B_{12} problem was the use of an optically transparent thin-layer electrode.[38] Diffusional mixing in the cell was rapid enough so that the entire solution could be equilibrated with the electrode, the rate-limiting process being charge transfer rather than diffusion. Since solution species were optically monitored, there was no requirement for fast charge transfer, and adsorption did not interfere. By using this approach, it was found that not only B_{12a} but also B_{12} could be electrochemically oxidized, the B_{12} oxidation being missed previously because of very slow charge transfer. Furthermore, both the Co(I)/Co(II) and Co(II)/Co(III) charge transfer could be observed by stepping the potential by small increments and plotting the solution composition as a function of potential. Finally, the role of the base-on vs. base-off reaction was examined. For further detail, the original papers should be consulted, but the authors proposed a mechanism which is repeated in Scheme 5. The B_{12}/cobalamin system provides an important example of the utility of coupling optical probes with electro-



Scheme 4. (Reprinted with permission from Lexa, D. and Saveant, J. M., *J. Am. Chem. Soc.*, 98, 2652 (1976). Copyright by the American Chemical Society.)



Scheme 5. (Reprinted with permission from Kenyhercz, T. M., DeAngelis, T. P., Norris, B. J., Heineman, W. R., and Mark, H. B., Jr., *J. Am. Chem. Soc.*, 98, 2469 (1976). Copyright by the American Chemical Society.)

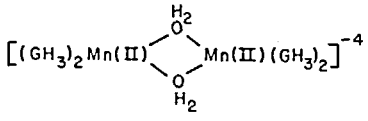
chemical methods. It is true that much was learned from an exclusively electrochemical approach, but the addition of an optical probe permitted more information to be obtained with increased clarity.

E. Metalloenzyme Models

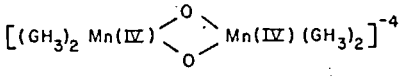
Significant effort has been expended in electrochemical studies of metalloenzyme systems which frequently involve the examination of low-molecular weight models. A variety of compounds of trace metals such as manganese and molybdenum have been investigated, often in non-aqueous solvents. Two examples are of particular interest here since they model two biologically important processes. It was found that manganese/gluconate complexes parallel many of the characteristics of photosystem II, the complex of metalloenzymes involved in oxygen evolution from green plants.[39] The photosystem is believed to utilize Mn(III) and Mn(IV) oxidation states, and the Mn/gluconate complex stabilizes the higher oxidation states in basic solution. Based on polarographic, spectroscopic, and magnetic susceptibility data, it was found that electrochemical oxidation

of [Mn(II) (gluconate)$_2$] $^{-2}$ (Structure 13) yielded an Mn(IV) dimer (Structure 14) which evolved oxygen, as shown in Scheme 6. Note that the process is catalytic in the Mn(II) complex and eventually oxidizes two molecules of water to molecular oxygen. It remains to be seen how closely this cycle parallels the photosynthetic oxygen evolution step, but a Mn(IV) complex appears to be a viable intermediate.

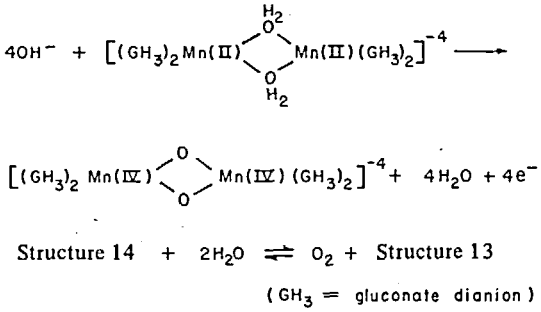
The second example relates to the nitrogenase enzyme which reduces molecular nitrogen to ammonia in plants and bacteria. A commonly studied model reaction for this process is the reduction of acetylene to ethylene and ethane by molybdenum/cysteine complexes. It has been known for some time that reduction of Mo(IV)/cysteine complexes with borohydride or dithionite in the presence of acetylene yields ethylene, but the catalytic species was uncertain. By careful bookkeeping of electrons, recent workers were able to show that the Mo(III)/cysteine complex was responsible.[40] Ethylene production could be affected by electrochemical reduction of Mo(V)/cysteine in the presence of acetylene or by reduction to Mo(III)/cysteine before addition of acetylene. The mechanistic importance of Mo(III) was discussed. Additional examples of model systems for metalloenzymes based on vanadium,[41,42] molybdenum,[43] and manganese[44] have been examined.



Structure 13



Structure 14

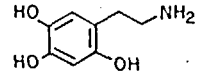


Scheme 6

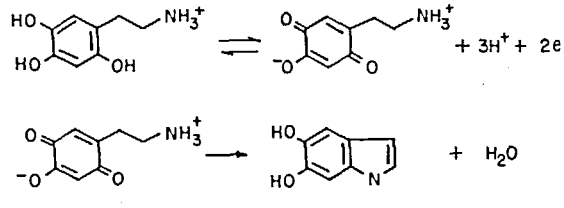
F. Pharmaceutical Compounds

An additional group of small molecules which can be classified as biologically important are pharmaceutical materials whose pharmacology involves redox reactions. A major reason for studying the electrochemistry of compounds is the importance of redox chemistry to their activity, metabolism, and stability as dosage forms. For example, electrochemistry has been used to monitor the stability of ascorbic acid,[45] vitamin A,[46] L-dopa,[47] diazepoxide tranquilizers,[48] and phenothiazine major tranquilizers.[49]

An interesting example of the importance of redox chemistry to drug activity is provided by the large amount of work on the neurotoxic compound, 6-hydroxydopamine (Structure 15). Because of its structural similarity to the adrenergic neurotransmitters, it is absorbed and concentrated in adrenergic nerve cells. After treatment with 6-hydroxydopamine, nerve cells irreversibly degenerate by a mechanism which remains a matter of controversy. Through an electrochemical study,[50,51] it was found that Structure 15 is easily oxidized to a reactive *para*quinone or 5,6-dihydroxyindole, which has the potential to convalently bond to nerve membranes (Scheme 7). Further work indicated that this oxidation does occur in the brain environment[52] and that the quinone does react with brain components fairly rapidly. The important conclusion is that the electrochemical information was very useful in examining the mechanism of action of the material. The biological mechanism of the drug's activity remains uncertain, but present theories depend upon redox reactions elucidated by the electrochemical studies.



Structure 15



Scheme 7

Correlations between electrochemical oxidation and metabolic degradation of drugs are quite clear in many cases, and the electrochemical evidence is useful in predicting metabolic routes. For example, electrochemical oxidation of the major tranquilizer chlorpromazine leads to the sulfoxide,[49] a major metabolic product. Furthermore, the electrochemical oxidation proceeds through a cation radical intermediate which is too short-lived to be observed metabolically, but is the initial product of all proposed chlorpromazine degradation schemes. The electrochemical oxidation of chlorpromazine metabolites also closely parallels the biological oxidation.[53]

By considering the examples above, one may ascertain the types of information available from electrochemistry. First, the basic chemical and physical information about reactants, products, and redox potentials is of value whether or not the data are directly applicable to biological processes. The complexity of physiological systems often precludes the determination of such information within the biological framework; electrochemistry provides a simplified alternative for examining basic processes. For example, merely the identification of the products of 6-hydroxydopamine oxidation by electrochemistry provided insight into the metabolic fate of the drug. Furthermore, information about its low-oxidation potential provided a major clue to the nature of its toxicity. A second type of information available from electrochemical experiments involves the analysis of the biological compounds of interest. If an electrochemical technique is used for analysis of a drug or naturally occurring material, obviously the electrochemical characteristics must be known. A third type of knowledge available is mechanistic in nature. Many biological redox systems involve short-lived intermediates or alternate pathways which are exceedingly difficult to sort out in vivo. The time frame of electrochemical methods can be extended down to the submillisecond region, making them powerful tools for generating and monitoring reactive species. Although the electrochemically determined mechanism may not be the same as the biological pathway, the likely intermediates are revealed. An example of the utility of such an approach is the adrenalin oxidation, where the quinone intermediate could only be observed by a dynamic technique such as cyclic voltammetry, and the time frame had to be shortened as the pH approached the physiological region.

A major consideration which arises when examining any of this work is the question of biological relevance. Since the true biological environment can never be precisely duplicated in an in vitro experiment, how can one be sure that information determined in a simplified environment is of any biochemical value? This issue has been discussed actively, and there are at least two answers. First, when the relationship between the electrochemical information and the biology is not known, the electrochemical experiments often provide insights into mechanisms and intermediates which can then be examined in the biochemical systems. In many cases, the electrochemically derived information would have been buried in the complexity of the biological system and virtually inaccessible. Second, when the biological process appears to closely parallel the electrochemically observed reactions, electrochemistry can provide a much more complete description of the process. Redox potentials, kinetics, and mechanisms from the simpler experiments can aid greatly in understanding the more complex biological process.

III. FARADAIC REACTIONS OF BIOLOGICAL MACROMOLECULES

A. Direct, Heterogeneous Charge Transfer

Examination of the redox chemistry of large molecules has been very active in recent years, particularly in the area of electron-transport enzymes. The applications of electrochemistry to the study of macromolecules are best considered in two sections, one involving the direct redox reactions of large molecules at an electrode surface and the other involving indirect study through the use of charge-transfer mediators.

Direct reduction of macromolecules at a mercury surface has been carried out in a variety of systems, including DNA,[54] bovine serum albumin (BSA),[55] and electron-transfer components,[56] by conventional polarography. In the polarographic reduction of BSA,[55] the principal reaction involves formation of a mercury bond with protein sulfhydryl groups. Severe adsorption and irreversibility accompany the process, making results somewhat complex to interpret. DNA is similarly complex with serious adsorption and irreversibility. Several hypotheses on the nature of the reduction have been critically examined,[57] and attempts to explain polarographic results at the molecular level have been made. Reduction waves

in the region of −1.3 V vs. SCE have been related to adenine and cytosine nucleosides of adsorbed DNA accompanied by helix uncoiling and fragmentation of the double helix backbone. It is possible that in time these types of experiments will yield additional physical information about the molecules, but at present they suffer from two major drawbacks. First, molecules such as DNA and BSA do not exhibit any obvious redox reactions in the physiological environment, so electrochemical studies cannot directly provide information about biological reactions. Second, the electrochemistry is complicated by adsorption and interfacial problems to such a degree that the information derived is of limited utility when answering biological questions.

Electrochemical investigations of large molecules with clear physiological redox reactions have proven more fruitful. A particularly active area of research has been the cytochrome series of the electron-transport chain, particularly cytochrome c. Before 1960, several studies had been made of the direct electrolytic reduction of oxidized cytochrome c.[58] More recent work includes a detailed electrochemical and spectroscopic examination of cytochrome c electrochemistry.[56] Unlike many macromolecules, cytochrome c exhibited a well-defined polarographic wave at potentials only slightly more negative than the $E^{0'}$ value. Although the wave was significantly distorted by adsorption and slow charge transfer, the limiting current was proportional to concentration. A coulometric reduction of oxidized cytochrome c yielded an n value between 0.83 and 0.94, consistently lower than the expected value of 1. To measure the formal potential for cytochrome c, the reduction was carried out potentiostatically by incrementing the applied potential in a coulometric cell and allowing the system to equilibrate between steps. The ox/red ratio was measured spectrophotometrically and its logarithm was plotted vs. the applied potential. The resulting line, shown in Figure 5, had a slope of 0.054 to 0.062 and an intercept of 0.249 to 0.266 V vs. SCE, comparing reasonably well with data from other methods discussed below. Additional experiments presented by the same workers indicated that the nonideal behavior of cytochrome c, evidenced by a nonintegral n value and variable Nernstian slopes, was caused by adsorption and slow charge transfer. The irreversibility of the reduction of cytochrome c was further indicated by the inability to oxidize the reduction product.

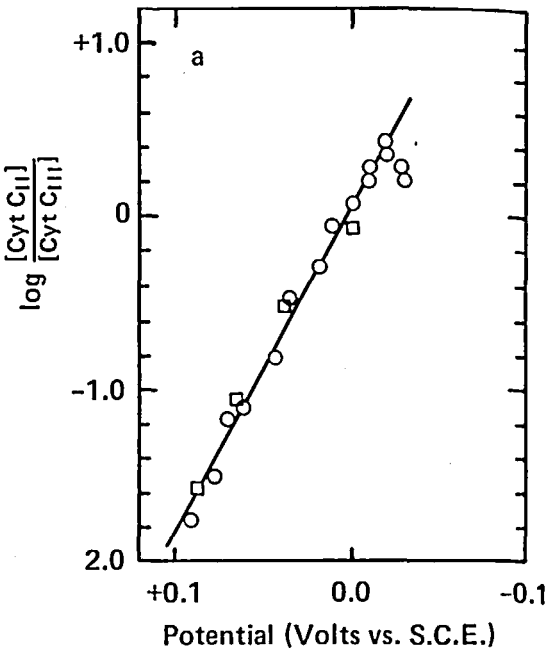


FIGURE 5. Equilibrium ratio of reduced to oxidized cytochrome *c* measured spectrophotometrically at various applied potentials using a platinum electrode. (Reprinted with permission from Betso, S., Klapper, M., and Anderson, L., *J. Am. Chem. Soc.*, 94, 8197 (1972). Copyright by the American Chemical Society.)

While this direct electrochemical study adequately established that the electrolytic process primarily involved freely diffusing material, the distortions caused by interfacial effects were unavoidable. In this instance, the experiments yielded valuable information despite these problems, but the cytochrome c system is probably a special case because of its relatively low molecular weight. For larger molecules, such as methaemoglobin, the results are more complex[59] and the relationship between electrochemical and physiological results more difficult to establish. It is reasonable to conclude that direct electrochemical studies of macromolecules are usually clouded by adsorption and charge-transfer problems.

B. Indirect, Electrochemically Controlled Titrations

Given that macromolecules are not commonly amenable to direct electrochemical study, several methods have been developed to circumvent the poor interaction between large molecules and potentiometric or voltammetric electrodes. A common technique used in biochemistry is the redox titration of macromolecules with a strong reducing

agent (e.g., dithionite) in the presence of an indicating redox dye. Knowing the formal potential for the dye, the Nernstian potential of the solution may be determined spectrophotometrically by monitoring the absorbance of the dye. Assuming equilibrium between the dye and the macromolecule, the formal potential for the macromolecule may be determined by monitoring its absorbance. Although this technique has yielded much useful data, there are a few problems. The dye must not only have a formal potential close to that of the macromolecule, but also have absorbance peaks which do not interfere with optical monitoring of the biocomponent. The electron-transfer rates between dye, reducing agent, and macromolecule should all be fast, yet complicating side reactions must be avoided. Finally, the reducing agents are often unstable and difficult to standardize.

Several methods for relieving these problems have been developed in a number of different laboratories. The more recent approaches are based on a combination of the dye/mediator titration methods with spectroelectrochemistry.[60] The macromolecule and a redox mediator are placed in a spectroelectrochemical cell utilizing an optically transparent electrode. Charge may be injected into the solution by normal electrochemical experiments, and the mediator is oxidized or reduced so that a new Nernstian potential is established. Provided the mediator interacts with both the macromolecule and the electrode, the Nernstian ratio for the biocomponent will equilibrate with the applied potential. The redox state of the biocomponent or mediator may be monitored optically.

As pointed out by workers in the field,[61] three types of information are usually desired when using this approach. First, the stoichiometry of the redox reaction embodied in the n-value for the macromolecule may be determined from the absorbance vs. charge plots of the electrochemical titration. Second, the thermodynamics of the reaction, reflected in the formal potential, may be determined from the absorbance vs. potential plots. Finally, the kinetics of charge transfer between mediator and macromolecule may be studied by observing absorbance changes immediately after charge injection and before equilibrium is attained.

The earliest experiments of this type were on the cytochrome c and cytochrome c oxidase systems.[62,63] In this application, a small volume (~2 ml) spectroelectrochemical cell was used which allowed maintenance of very low oxygen concentrations.[63] Both a tin-oxide optically transparent electrode (OTE) and a platinum potentiometric electrode were included in the design, so that both optical and potentiometric measurements could be made during the course of mediator-coupled oxidation or reduction. The determination of an n-value by this approach is perhaps best illustrated by the experiment on modified cytochrome c. Figure 6 is a plot of cytochrome absorbance vs. coulombs for reduction and oxidation of modified cytochrome coupled to the electrode by methylviologen for reductions or ferricyanide for oxidations. It can be shown that the n-value can be determined directly from these data without knowing the cytochrome concentration:

$$n = \frac{\Delta Q}{\Delta A}\epsilon$$

where ΔQ is the charge injected per milliliter of solution, ΔA is the change in absorbance per centimeter of path length resulting from ΔQ, and ϵ is the molar absorptivity of the cytochrome. These experiments yielded n-values within 2% of the expected one-electron value. A similar approach was used very successfully to examine the stoichi-

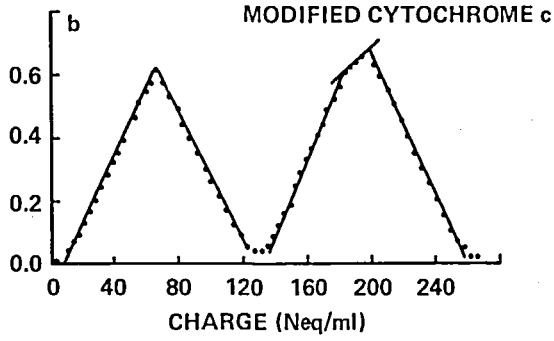


FIGURE 6. Electrochemically driven titration of modified cytochrome *c*. Absorbance monitored at 550 nm. Solution contains 53 μM cytochrome *c*, 0.5 mM methyl viologen dication, and 1.0 mM ferrocyanide. Rising portions of curve represent electrochemical reduction of cytochrome *c* mediated by the viologen: the falling portion is oxidation mediated by ferrocyanide. (Reprinted with permission from Hawkridge, F. and Kuwana, T., *Anal. Chem.*, 45, 1021 (1973). Copyright by the American Chemical Society.)

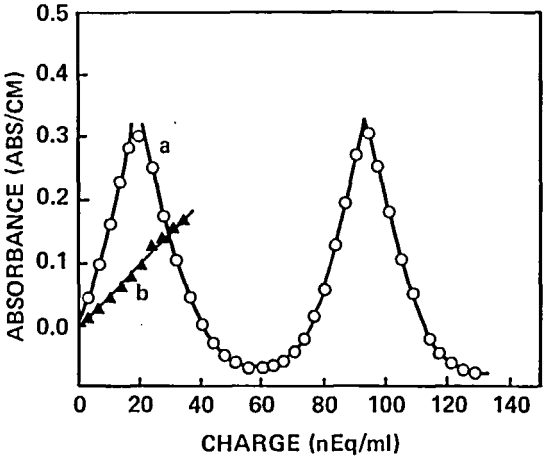


FIGURE 7. Curve a is the mediated titration of unmodified cytochrome *c*, conditions the same as Figure 6. Curve b is the result of the same experiment with O_2 present in solution. (Reprinted with permission from Hawkridge, F. and Kuwana, T., *Anal. Chem.*, 45, 1021 (1973). Copyright by the American Chemical Society.)

ometry of the more complex cytochrome c oxidase system.[63]

Two methods were used to evaluate the formal potentials for the cytochromes studied. First, the Pt indicator electrode was used to monitor solution potential during the titration process, an approach nearly identical to the older chemical titrations except for the use of electrochemical charge injection. Although the values of $E^{0'}$ determined from this method (0.262 V vs. NHE for cytochrome c) agree reasonably well with other determinations, the slope of the Nernstian plot is 0.039 V, yielding a nonintegral *n*-value (1.55). The reason for this discrepancy may be the lack of a well-poised electrode in this potential region. A second technique to determine $E^{0'}$ is by careful analysis of the absorbance vs. charge plots.[64] For the titration of unmodified cytochrome c, the curves do not have an ideal triangular shape, but rather the nonlinear shapes of Figure 7. The curvature is caused by the proximity of the cytochrome $E^{0'}$ to that of the titrant, ferricyanide. The reaction between the two compounds is not complete, and reduction by electrogenerated viologen will result in a mixture of reduced cytochrome and ferrocyanide. The theoretical shape of the absorbance/charge curve for such a case may be calculated by assuming an *n*-value and a difference, $\Delta E^{0'}$, between the cytochrome and ferrocyanide formal potentials. $\Delta E^{0'}$ may be adjusted until the best least squares fit is achieved between the experimental data and the calculated curve; then $E^{0'}$ for the cytochrome may be determined from $\Delta E^{0'}$. In this case, an assumed *n*-value of 1 and $\Delta E^{0'}$ of 0.166 V allowed a determination of $E^{0'}$ for cytochrome c (0.258 ± .005), which was very close to an average of literature values (0.257 ± .017). The same method was applied to cytochrome c oxidase[65] and cytochrome c oxidase in the presence of carbon monoxide.[66]

Although this method of electrochemically driven titrations has been valuable in investigating a variety of difficult problems, there are some drawbacks. The number of useful mediator/titrants is small and the values of their potentials are critical for the success of the method. The disagreement between *n*-values determined from absorbance/charge curves and those from the $E^{0'}$ determination is perplexing. A slightly different experiment partly alleviates these problems. When contained in an optically transparent thin-layer electrode, a solution will equilibrate fairly rapidly with the working electrode. The use of a mediator system in a solution containing a protein redox system allows the Nernstian ratio of the mediator, and therefore the enzyme, to be controlled potentiostatically.[67] The ox/red ratio of the enzyme, determined optically, may be compared with the applied potential to determine $E^{0'}$.

This technique has been applied to the cytochrome c system, mediated by 2,6-dichlorophenolindophenol.[67] The procedure involved incrementing the applied potential, waiting for equilibrium, then measuring absorbance of the cytochrome. The ox/red ratio was calculated from absorbance data and the applied potential was plotted vs. the logarithm of the Nernstian ratio. Figure 8 is a typical plot. Curve A is an experiment on the mediator itself, monitored at 600 nm; curve B is cytochrome c plus mediator, monitored at 550 nm, where only cytochrome c absorbs. Curve A has a slope of 31.3 mV, close to the expected value for a two-electron process. Curve B has a slope of 59.1 mV for cytochrome c, corresponding to a one-electron process, and an $E^{0'}$ of 0.262 V vs. NHE. The results for the cytochrome $E^{0'}$ and *n* determinations are summarized in Table 2. While this thin-layer technique yields good results in this case, it nevertheless is applicable only to systems with sufficiently high molar absorptivities, and where a suitable mediator is available.

As mentioned above, the third type of information desired about electron-transport components

TABLE 2

Redox Potentials and *n*-values for Cytochrome c Determined by Various Methods

Method	Nernstian slope (mV)	*n*	$E^{o'}$ (mV vs. NHE)
Direct coulometry[48]	–	0.83–0.94	
Direct potentiostatic[48]	54–62	1.1–0.95	249–256
Indirect electrochemical titration, Pt indicator electrode[54,55]	39	1.55	262
Indirect electrochemical titration, curve fittings[56]	59[a]	1.00[a]	258
Indirect thin layer spectroelectrochemistry[59]	59.1	1.00	262
Average of literature values[56]		1.00	257

[a]Assumed.

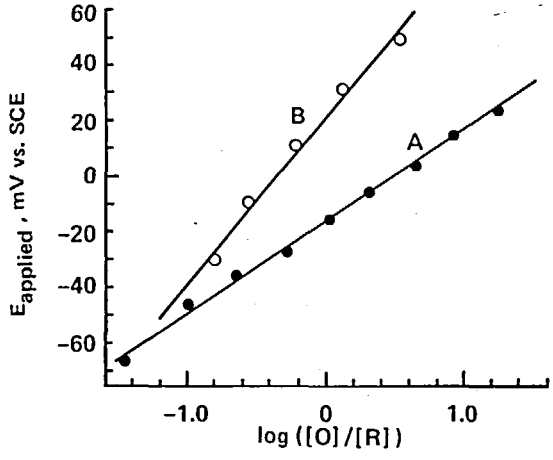


FIGURE 8. Results of potentiostatic experiments in a thin-layer optically transparent cell. The applied potential was incremented and the solution composition determined spectrophotometrically after equilibration. Curve A: 2,6-dichlorophenolindophenol monitored at 600 nm. Curve B: cytochrome c monitored at 550 nm. (From Heineman, W., Norris, B., and Goetz, J., *Anal. Chem.*, 47, 79[1975]. With permission.)

is the rate of electron transfer between the macromolecule and an oxidizing or reducing agent. This information is valuable for deducing the mechanism of charge transfer and providing insight into the conformation of the macromolecule during the redox reaction. Conventional approaches to determining this type of kinetic information have been reviewed.[68] The original spectroelectrochemical work on the reaction between viologen cation radicals and oxidized cytochrome c was performed in the cell using a tin oxide OTE described earlier.[69] By stepping the potential to a value where viologen dication is completely reduced to cation radical at the electrode surface, the radical will subsequently reduce oxidized cytochrome c to regenerate viologen dication. This sequence is usually dubbed the EC catalytic mechanism and can be written as:

$$B^{+2} + e^- \rightleftarrows B^{+} \quad (5)$$

$$B^{+1} + \text{cyt c}_{ox} \xrightarrow{k} B^{+2} + \text{cyt c}_{red} \quad (6)$$

where B is a viologen species. Provided the homogeneous reaction is thermodynamically strongly favored and cytochrome c is not itself reduced at the electrode, the absorbance of viologen is a function of both time and the homogeneous electron-transfer rate constant. Using this approach, values of 10^4 to 10^5 M^{-1} sec^{-1} were obtained for second-order rate constants for several viologens reducing cytochrome c. It was later observed[70] that these values were lower-than-pulse radiolysis results by several orders of magnitude,[71] and the electrochemical results were reexamined. The discrepancy was primarily attributed to spectral overlap and problems with repetitive time-averaged experiments. The experiment required averaging of a large number of runs, and the cytochrome became depleted near the electrode if sufficient time for reequilibration was not allowed between runs. After correcting this problem, rate constants greater than 10^8 were observed in agreement with radiolysis data.[70]

A thorough and detailed analysis of the effect of spectral overlap and diffusion coefficient values on rate constants has been carried out in a different laboratory.[72] Nevertheless, the rate constants determined by the two laboratories differ by a factor of 100, a discrepancy which remains to be resolved.

The electrochemically driven redox titration approach has been used to study a much more complicated system, that of chloroplasts from green plants.[73] The fluorescence yield of photosystem II is related to the redox state of the environment since quenchers of that fluorescence are believed to be quinones which will quench effectively only in the oxidized state. The chloroplasts were placed in a conventional spectroelectrochemical cell, along with several mediator titrants, to cover a fairly wide potential range. As the solution was electrochemically reduced, both potential and fluorescence yield were monitored. The results yielded support for the premise that the quenching molecule is membrane-bound plastoquinone. In a related study, the chlorophyll-hydroquinone interactions were used to attempt photogalvanic generation of current.[74]

As was the case with smaller biological molecules, the important consideration at this point is the question of the advantages of electrochemical experiments over the more conventional approaches in the study of macromolecules. It is obvious that nonideal electrode behavior will often be a problem in direct electrolysis of large molecules. In a few cases, such as those discussed above, direct electrochemistry can yield valuable information, but unfortunately these cases appear to be the exceptions rather than the rule. On the other hand, indirect methods using a charge transfer mediator are much more promising since interaction between the macromolecule and the electrode is not required. The spectroelectrochemical approach to redox titrations allows much better control than the conventional chemical titrations since the "titrant," namely charge, is much more accurately dispensed. Provided care is taken, the stoichiometry and energetics (redox potentials) may be accurately determined from spectroelectrochemical measurements. The virtue of these two measurements arises from the fact that the solution is homogeneous and diffusion processes are not important to the measurements.

For the kinetic investigations, however, the solution is not homogeneous, and one must accurately describe the diffusion profiles near the electrode in order to evaluate kinetic constants. These complications, combined with the possibility of spectral overlap, have led to serious disparity in electrochemically determined rate constants. The complexity of a spectroelectrochemical measurement is probably its most serious drawback. The technique has proven extremely valuable for examining redox reactions of macromolecules, but the user must be conscious of the complexities.

IV. ELECTROCHEMICAL ANALYSIS OF BIOLOGICAL SYSTEMS

A. Direct and Enzyme-coupled Electrochemical Analysis

There has been a significant resurgence of interest in the use of electrochemical methods for biochemical analysis caused primarily by two fairly recent developments. First, the selectivity and sensitivity of voltammetric and amperometric methods have been greatly increased by the development of pulse methods and by utilization of an electrochemical detector for chromatographic effluents. Second, significant miniaturization of electrochemical probes has permitted the performance of electrochemical experiments directly in living tissue, thus allowing constant monitoring of electroactive materials in vivo. As will be apparent from the discussion below, electrochemistry has some significant advantages over other techniques for certain applications.

Conventional polarographic and voltammetric methods have been available for decades for the analysis of a variety of biological and pharmaceutical materials. The poor detection limits ($\sim 10^{-5}$ *M*) and selectivity of standard dc methods limit their use, however, so recent efforts have been directed toward relatively pure or concentrated samples such as pharmaceutical dosage forms. Ascorbic acid has been analyzed by simple polarography[45] and amperometry at a tubular carbon electrode.[75] L-dopa (dihydroxyphenylalanine) can be determined using similar methods.[76] Other examples include a coulometric analysis of nucleotides,[77] polarographic analysis of sodium pentothal,[78] and a polarographic determination of pyrithioxine.[79] It is apparent that most successful voltammetric analyses have been applied to relatively pure samples, simply because of poor selectivity. The situations where direct

voltammetry is useful for biological samples occur when the species of interest is the only significant electroactive species at a particular potential. For example, uric acid may be determined in serum with a carbon paste electrode because possible interferences are very low in concentration.[80,81] Similarly, the ability of the liver to hydroxylate aniline may be electrochemically monitored by spiking the liver homogenate with a relatively concentrated portion of aniline, then observing the metabolite, *para*-aminophenol, with direct amperometry.[82] Except in special cases such as these, direct voltammetry will rarely be adequate for complex biological samples.

An important improvement in selectivity can be achieved by coupling an electrochemical analysis to an enzymatic system. There are numerous examples of ion-selective electrodes coated with immobilized enzymes, such as an AMP-sensitive electrode consisting of an NH_3-selective electrode coated with AMP deaminase.[83] Although NH_3 is the species actually measured, selectivity is provided by the highly specific degradation of AMP to form NH_3. The Clark oxygen electrode has been used extensively in a similar configuration, an example being the determination of glucose oxidase.[84] Electrochemistry may be used to monitor homogeneous enzymatic reactions when an electroactive species is coupled to the reaction of interest. The oxidation of tyrosine to L-dopa by tyrosinase was used to determine tyrosine by monitoring the consumption of oxygen with a Clark electrode.[85] The selectivity of the enzymes eliminated the cleanup steps for even a complex blood serum sample. A flowing system similar to that used in clinical autoanalyzers was used to amperometrically detect alcohol in blood and urine.[86] Alcohol dehydrogenase (ADH) was used to oxidize alcohol to acetaldehyde; then the NADH generated was coupled to an electroactive dye, dichlorophenolindophenol (DCPIP):

$$CH_3CH_2OH + NAD^+ \overset{ADH}{\rightleftharpoons} NADH + CH_3CHO$$

$$NADH + (DCPIP)_{ox} \overset{diaphorase}{\rightleftharpoons} NAD^+ + (DCPIP)_{red}$$

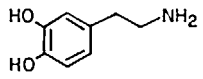
The $(DCPIP)_{red}$ was oxidized at a tubular carbon electrode. The change in steady state current in the presence of alcohol was used to determine the alcohol concentration. The technique is highly specific and sensitive enough to yield accurate results on 10 to 20 μl of blood. The application of similar methods for enzyme rather than substrate analysis is obvious and has been well documented in the literature.[87,88] Additional examples of enzyme-coupled electrochemical probes include a potentiometric immunoelectrode[89] and an alcohol oxidase electrode.[90]

The application of differential pulse techniques to biological samples has greatly improved sensitivity, particularly when the sample is comparatively pure. Pulse polarographic methods were used to analyze the urinary metabolites of a common tranquilizer, flurazepam.[91] The differential pulse technique was sufficient to measure total metabolites, but to determine individual species, a TLC separation preceded polarography. Detection limits for urinary metabolites were in the region of 0.2 to 0.5 μg/ml (10^{-6} *M*), referenced to the original sample and taking recovery of the TLC step into account. A similar procedure was developed for chlordiazepoxide (Librium®) and its metabolites using a TLC separation followed by differential pulse polarography.[92] Sensitivity was comparable with detection limits in the region of 0.05 μg/ml (10^{-7} *M*). The reduction of a nitro group has been used in several drug analyses, both for systems containing a nitro group, such as the nitroimidizoles,[93] and for nitro-containing derivatives of electroinactive drugs. Phenobarbital in blood was determined by differential pulse polarography after extraction and nitration.[94] Finally, the detection limit of pulse polarographic techniques was improved to compete with electron capture GC by careful cell miniaturization and concentration during an extraction step. Levels of various diazepines in blood were accurately monitored at the 20-ng/ml (2×10^{-8} *M*) level.[95] The absolute limit of the technique was the 10 ng contained in the cell.

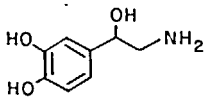
B. Electrochemistry Coupled to Liquid Chromatography

Although the sensitivity of differential pulse polarography has been adequately demonstrated, its poor resolution usually necessitates a chromatographic or extraction "cleanup" step. The examples above involving a TLC separation preceding polarography are somewhat tedious, with problems often resulting from irreproducible recovery from the TLC plate. A significant improvement of such an approach results when an amperometric detector is combined with a liquid chromatograph. The original motivation for the

development of such a device was provided by the need to determine the concentration of adrenergic neurotransmitters in animal and human nervous tissue. The compounds are metabolic precursors of adrenaline and are believed to be related to a variety of types of mental illness. The compounds of interest here are dopamine (Structure 16) and norepinephrine (Structure 17). The electrochemical oxidations of Structures 16 and 17 are similar to that of adrenalin, leading to the *ortho*quinones at a pH below 4.[96] The amounts of dopamine and norepinephrine in a mouse brain are about 800 and 400 ng, respectively, corresponding to a range of 1 to 5 μM. Except with specialized electrodes, voltammetric detection of these low-level constituents is precluded by interference from relatively large amounts of ascorbic acid.



Structure 16



Structure 17

By preceding an amperometric detector with a chromatographic column, the dopamine and norepinephrine could be separated from each other and from the surrounding interferences.[97,98] The experimental apparatus for such an approach is shown in Figure 9. The chromatographic separation was accomplished by a conventional high-performance ion exchange column, followed by a carbon paste amperometric detector which is maintained at a constant potential sufficient to oxidize dopamine and norepinephrine A chromatographic run using amperometric detection is shown in Figure 10. The sensitivity is very high with easily measurable peaks resulting from 10 to 50 pg of electroactive material, corresponding to a flow velocity of approximately 10^{-15} mol/sec. The high sensitivity results from several aspects, the most important being the high purity of the sample reaching the detector. The chromatographic separation removes any background materials, except those present in the mobile phase. In addition, the constant-applied potential eliminates charging current, and one actually detects the difference in current over the steady state background as an electroactive material passes the electrode.

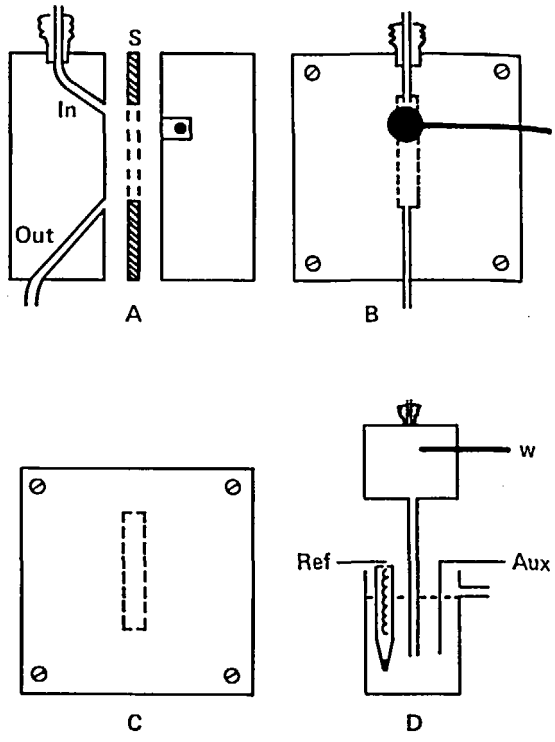


FIGURE 9. Construction of amperometric detector for liquid chromatography based on carbon paste electrode. S is a 5-mil teflon gasket which forms the edges of the electrolytic chamber. In detail D, the positions of the auxiliary and reference electrodes downstream are indicated, the chromatographic column is attached at the fitting shown at the top of details A, B, and D. (Reprinted with permission from Kissinger, P. T., Refshauge, C., Dreiling, R., and Adams, R. N., *Anal. Lett.*, 6, 465 (1973). Copyright by Marcel Dekker, Inc.)

Since its development in 1974, this HPLC/electrochemical approach has been applied to a variety of biological analyses. Extensions to other phenolic metabolites include homovanillic acid[99] and homogentisic acid.[100] Animal cerebrospinal fluid may be directly injected into the LC to analyze for the dopamine metabolite, homovanillic acid.[101] Several enzymes involving these compounds can be determined by monitoring the reactants or products of the enzymatic reactions.[102] Alterations to the method itself include a dual-working electrode permitting electrolysis at two different potentials[103] and several alternative working electrodes.[104-106]

The analytical methods for biological samples discussed above are useful for analysis of fluids

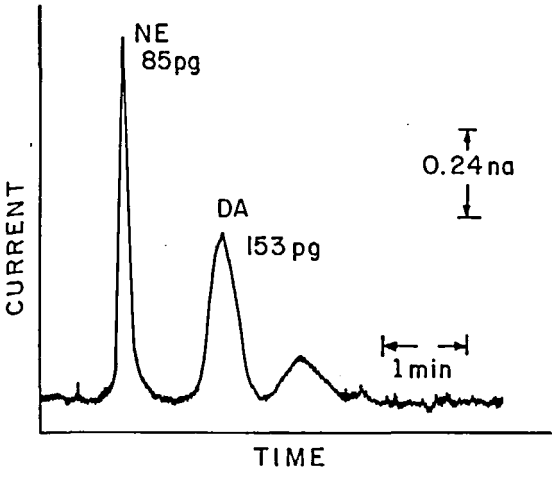


FIGURE 10. Chromatogram of subnanogram quantities of norepinephrine and dopamine obtained using amperometric detection. (Reprinted with permission from Kissinger, P. T., Refshauge, C., Dreiling, R., and Adams, R. N., *Anal. Lett.*, 6, 465, (1973). Copyright by Marcel Dekker, Inc.)

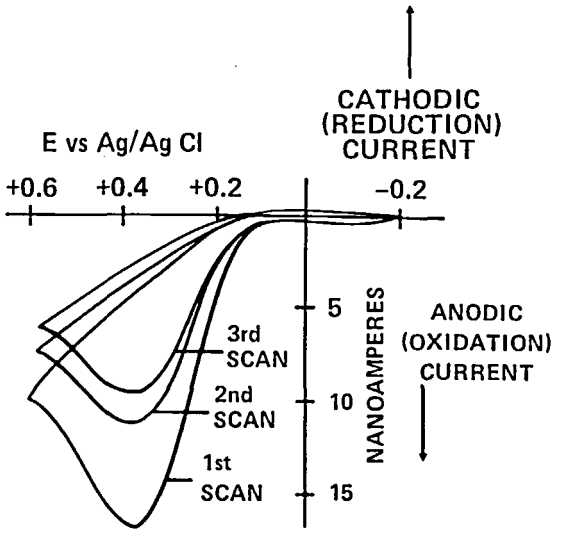


FIGURE 11. Cyclic voltammogram from a 0.75 mm diameter carbon paste electrode implanted in the brain of an anesthetized rat, scan rate = 0.1 V/sec. (From Kissinger, P. T., Hart, J. B., and Adams, R. N., *Brain Res.*, 55, 209 (1973). With permission.)

such as blood, urine, and cerebrospinal fluid, or for homogenized tissue samples (the so-called "grind and find" approach). These methods are of unquestionable value in studying the biochemistry of the organisms and the pharmacology of drugs, but they are often limited in informational content. Many biological molecules, particularly neurotransmitters, are highly localized in certain cells or regions; this localization is missed by whole brain analysis. Microdissection followed by liquid chromatographic analysis can provide more information about local concentration, but resolution is limited to a few cubic millimeters of tissue. Furthermore, levels of neurotransmitters and other metabolic materials change drastically as a function of nervous activity, pharmacological manipulation, and trauma such as death. Post-mortem analysis, or even urinary or blood analysis, often reveals little about concentration changes in the living animal.

C. Electrochemical Probes for in vivo Analysis

Ideally, one would desire a miniature analytical probe which could be implanted in living tissue while causing a minimum of disturbance to the organism. Electrochemical probes have been used in the past to attempt to solve this problem with varying degrees of success. A potentiometric microelectrode was implanted in a rat brain, and potentials were monitored as a function of respiration.[107] Voltammetry at a platinum electrode was carried out in liver where currents related to endogenous and injected glutathione and ascorbic acid were obtained.[108-110] In both cases, signals were far from ideal but nevertheless responded to manipulation in predictable fashions. Miniaturized Clark oxygen electrodes have been used in several applications for continuously monitoring oxygen in the vascular system.[111,112] Concurrent with these developments has been significant progress toward monitoring the neurotransmitters dopamine and norepinephrine and their metabolites in brain tissue in living animals.

The first attempt to monitor dopamine in living tissue involved implanting a small (0.75 mm diameter) carbon paste working electrode in the caudate nucleus of an anesthetized rat.[113] The caudate nucleus is known to be high in dopamine (approximately 5×10^{-5} *M*) by bulk analysis and is physically large enough to make implantation easy. Reference and auxiliary electrodes were placed near by on the surface of the brain, and a conventional cyclic voltammogram was obtained. The result, shown in Figure 11, is a reasonably well-defined wave with a peak potential corresponding to that of dopamine at physiological pH. However, the peak current is much larger than expected for the concentration of dopamine present, and the entire wave is likely to be a

summation of several species present. A likely interference is ascorbic acid which is present in millimolar concentrations and has an oxidation potential close to that of dopamine on carbon paste. Despite these interferences, the wave is closely related to the animal's physiological state.[114]

Two routes have been followed in attempts to improve the physiological information content of in vivo electrochemical probes. One approach is to use pulse techniques to improve the selectivity and sensitivity of the determination of dopamine and norepinephrine in the brain.[115] A platinum microelectrode was modified by adsorption of iodide ion such that the electrochemical behavior of dopamine was more reversible than on unmodified platinum or carbon paste. A standard differential pulse experiment performed on such an electrode yielded improved sensitivity but the electrode was fouled by deposition of dopamine oxidation products. To avoid this complication, a new pulse technique, dubbed "differential double pulse voltammetry," was developed. Square wave pulses were superimposed on a constant dc potential such that between pulses the electrode was maintained at a potential below that necessary to oxidize dopamine. By careful choice of pulse amplitude and width, a highly sensitive method resulted with useful response being obtained from 10^{-8} *M* dopamine solutions. A typical double-pulse voltammogram for a solution of dopamine in pH 7.4 buffer is shown in Figure 12.

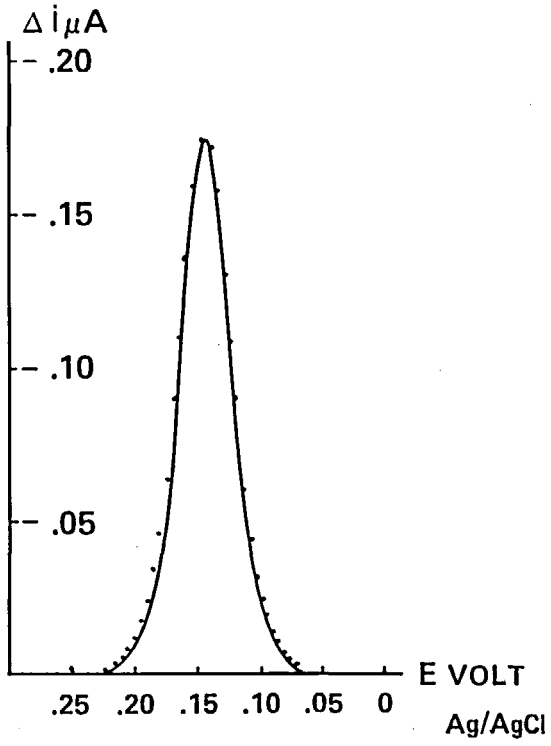


FIGURE 12. Differential double pulse voltammogram of 3×10^{-7} *M* dopamine at pH 7. Points are experimental results, line is the theoretical curve. (Reprinted with permission from Lane, R. F. and Hubbard, A., *Anal. Chem.*, 48, 1287 (1976). Copyright by the American Chemical Society.)

When the chemically modified platinum electrode is implanted in the brain of an anesthetized rat, the response of Figure 13A is obtained.[116] The two peaks were attributed to dopamine (peak 2) and ascorbic acid (peak 1), with potentials matching those in solution. Furthermore intravenous injection of amphetamine, which is known to rapidly release dopamine from nerve cells, increased the dopamine signal by a factor of seven, lending support to the peak assignments. The significant contribution of this work is the ability to detect micromolar levels of dopamine without interference from ascorbic acid. An important consideration in the future is that platinum electrodes are much more easily miniaturized and may be fabricated to provide information at the cellular level.

An alternate approach to improving the probe, as discussed above, is to simplify the analytical problem while still retaining the physiological significance. For example, the dopamine-rich caudate nucleus is bordered by a cavity-containing cerebrospinal fluid (CSF) which contains little ascorbic acid or other electroactive materials. As dopamine is released from nerve cells in the course of normal nervous activity, it is metabolized to homovanillic acid (HVA, Structure 18). This HVA appears in the CSF and is eventually removed by slow circulation of the fluid. A carbon paste electrode placed in the ventricle of an anesthetized rat detected very little electroactive material.[101] Upon electrical stimulation of the brain by distant electrodes, the release of HVA into the ventricle resulting from dopamine activity was detected. HVA was detected voltammetrically not only by its oxidation potential, but also by the appearance of a follow-up couple due to its oxidation product, dihydroxyphenylacetic acid (DOPAC, Structure 19),[117] according to Scheme 8. Cyclic voltammograms of the CSF before and after stimulation of dopamine neurons are shown

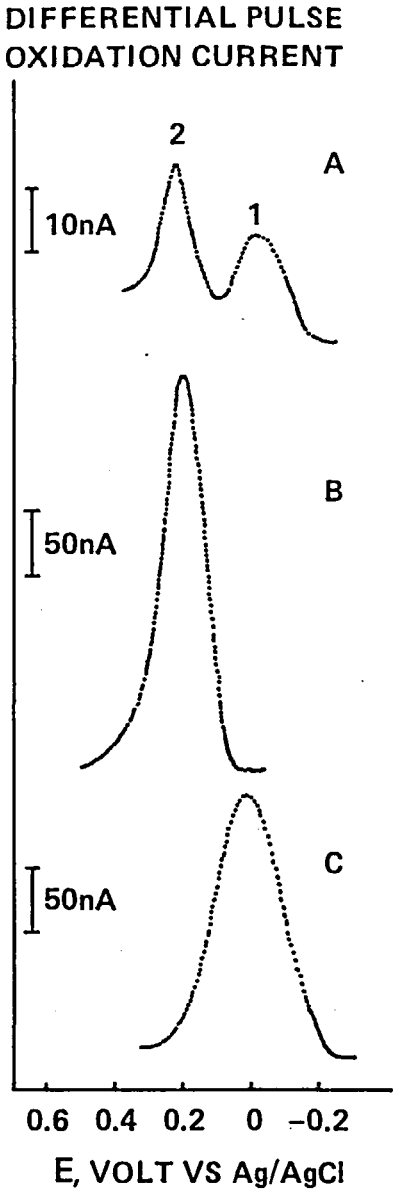


FIGURE 13. Curve A: differential double pulse voltammograms from a modified platinum electrode implanted in rat caudate nucleus. Curve B: same as A with 1 μg of dopamine injected near the electrode. Curve C: same as A with 1 μg ascorbic acid injected. (From Lane, R. F., Hubbard, A. T., Fukunaga, K., and Blanchard, K., *Brain Res.*, 114, 346 (1976). With permission.)

in Figure 14. Peak A corresponds to the DOPAC redox couple. In addition to this qualitative identification of HVA, it may be quantitatively monitored using conventional chronoampero-

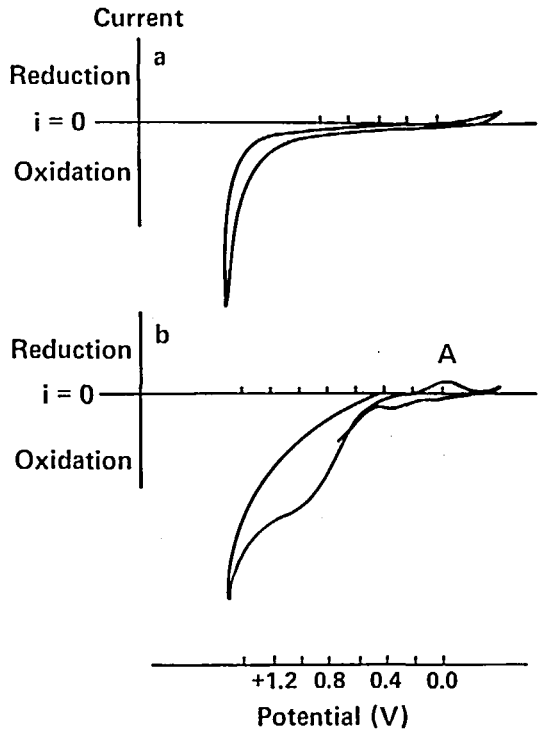
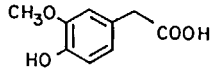
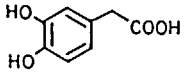


FIGURE 14. Voltammograms of rat cerebrospinal fluid taken with a carbon paste electrode; a: before stimulation of dopamine neurons and b: after stimulation. (From Wightman, R. M., Strope, E., Plotsky, P., and Adams, R. N., *Nature*, 262, 145 (1976). With permission.)

metry. The time course of HVA concentration in CSF as a function of neuronal stimulation is shown in Figure 15.

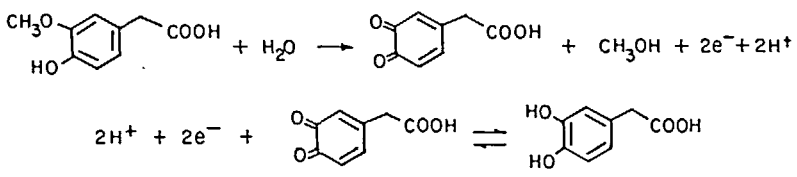


Structure 18



Structure 19

An additional means to simplify the analytical problem associated with in vivo probes is the introduction of a nonnatural material in measurable concentration. For example, a pharmacological agent whose biochemical fate is unknown may be monitored by an electrochemical probe placed near the site of injection. 6-Hydroxydopamine (6-OHDA, Structure 15), mentioned earlier, is a pharmacological tool with which redox reactions



Scheme 8

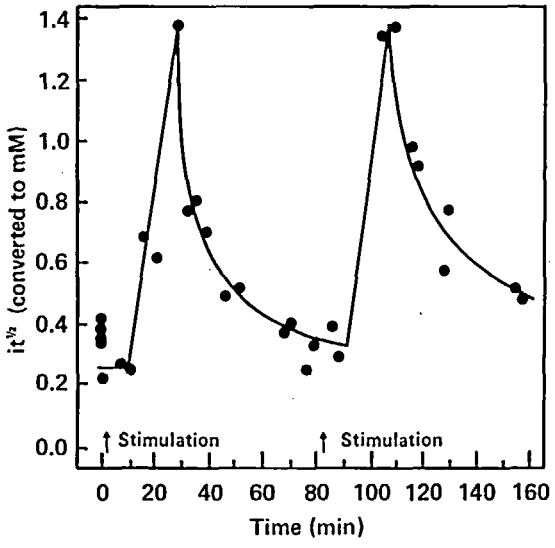


FIGURE 15. Chronoamperometric monitoring of HVA in rat cerebrospinal fluid during stimulation of dopamine neurons. Chronoamp experiment lasted 200 msec, the $it^{1/2}$ product was converted to concentration for plotting. (Reprinted with permission from Wightman, R. M., Strope, E., Plotsky, P., and Adams, R. N., *Nature*, 262, 145 (1976). With permission.)

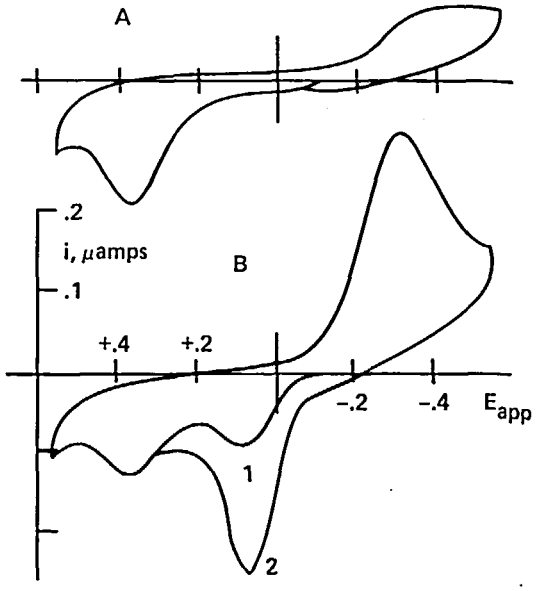


FIGURE 16. Voltammograms of intracerebrally injected 6-hydroxydopamine. 0.50 mm diameter carbon paste electrode was implanted in rat caudate nucleus next to microsyringe needle. Curve A: voltammogram before drug injection. Curve B: after 30 μg of oxidized 6-hydroxydopamine were injected in 1 μl of solution. (From McCreery, R. L., Dreiling, R., and Adams, R. N., *Brain Res.*, 73, 15 (1974). With permission.)

are closely involved. A significant controversy about its mode of action centered on the question of whether or not it was oxidized to a *para*quinone and, if so, what became of the quinone in the brain environment. To attempt to answer this question, a 0.5-mm carbon paste electrode was implanted in an anesthetized rat brain and approximately 30 μg of 6-OHDA were injected next to the electrode.[118,119] Figure 16 shows voltammograms taken with the implanted electrode before (curve A) and after (curve B) drug injection. The initial anodic peak in curve A was discussed earlier[113] and is due to naturally occurring oxidizable substances. After drug injection, curve B, voltammetric peaks corresponding to 6-OHDA are apparent and the peaks are dependent upon the concentration and the Nernstian ratio of the drug. By monitoring the injected drug in this fashion, it was found that about 20% was oxidized before a redox steady state was reached, characterized by a time-independent Nernstian ratio. The peak current decayed fairly rapidly with time, indicating that the drug was degrading in a period of about 30 min. Since this decay rate was much faster than that observed in a pure solution environment, an interaction of the drug with some neural constituent was indicated.

It is important to consider the physiological significance of these analytical measurements, particularly with regard to their potential for providing new information. In all the work cited above, the electrode diameters (~0.5 mm) were much larger than individual nerve cells (~0.05 mm). The events of importance to neurotransmission occur primarily at synapses, which may be only 10^{-4} mm in diameter. Therefore, with

present electrodes, electroanalytical techniques cannot provide information about individual neuronal or synaptic events, but only about comparatively gross phenomena. Another consequence of large electrodes is poor time resolution. The electrochemical probes monitor material on a minute time scale due to the mixing and diffusion time in the relatively large sample volumes surrounding the electrode. However, despite these drawbacks, it should be kept in mind that any direct in vivo monitoring system is a vast improvement over post-mortem or remote analytical methods. Previous methods for observing transmitters released into the CSF from neurons upon stimulation include extraction of the fluid followed by radiochemical analysis of labeled transmitters. This method is exceedingly difficult and has time resolution no better than the electrochemical approach. In fact, there are no methods available which can detect small molecules in the brain in a real time situation. The electrochemical in vivo monitoring methods are in their early stages, but with further miniaturization and improved selectivity, they hold a great deal of promise for directly observing the chemistry associated with physiological processes. Further applications of electrochemical techniques to problems in the particular area of the neurosciences have recently been discussed.[120]

V. ELECTROCHEMICAL ASPECTS OF MEMBRANE BIOPHYSICS

A. Biological Membranes

Any time a semipermeable membrane separates two solutions of differing ionic composition, an electrical potential will develop across the membrane. The magnitude of this potential depends on the nature of the membrane and the concentration gradients of charged species from one side of the membrane to the other. Virtually all biological cells possess such potentials across their outer membranes and the effect has been observed in cells ranging from simple erythrocytes to more complex nerve cells. Nerve membranes have attracted particular attention, since electrical activity produced by membrane potential changes is of vital importance to nervous function. Investigations into membrane structure and the mechanisms of potential development have been carried out for several decades, and most studies have been concerned with one of three problems. First, how is the potential developed and how does it depend on membrane structure and ionic composition of the adjacent solutions? Second, what causes variations in membrane potential such as those observed during electrical activity of nerve cells? Third, given that ions move down the concentration gradient as a result of electrical activity, how is the gradient maintained, presumably by metabolic processes? In the discussions that follow, it will be apparent that electrochemical approaches have provided significant information related to these problems.

Since the pioneering work of Hodgkin[121] and Huxley[122] in the 1950s, it has been apparent that membrane potentials in nerve cells are caused by selective permeability to alkali metal cations. The membrane of a typical mammalian nerve cell is shown schematically in Figure 17, with the concentrations of sodium and potassium ions inside and outside the cell.[123] At rest, the potential across the membrane is −90 mV (inside vs. outside) and is dependent on the K^+ concentration by the equation below, originally empirically[124] established:

$$E_{in} - E_{out} = \frac{RT}{F} \ln \frac{[K^+]_{out}}{[K^+]_{in}}$$

When the nerve is excited, the potential rises to about +40 mV and is dependent on Na^+ concentration. To include this effect, the equation was

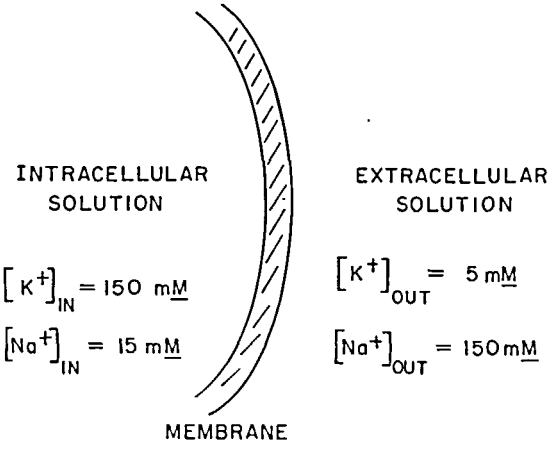


FIGURE 17. Schematic drawing of a typical mammalian nerve cell membrane indicating internal and external alkali metal ion concentrations.

modified, again on largely empirical grounds, to the Goldman equation:

$$E_{in} - E_{out} = \frac{RT}{F} \ln \frac{[K^+]_{out} + \alpha[Na^+]_{out}}{[K^+]_{in} + \alpha[K^+]_{in}}$$

where α is a selectivity ratio and is believed to reflect the differential permeability for Na^+ over K^+. At rest, α is about 0.05 and Na^+ has little effect on potential; when the nerve is excited, α is about 20, and Na^+ is the primary ion determining potential.[125] A membrane which is able to undergo such changes in permeability is said to be excitable. Examples of excitable membranes are muscle and nerve cells, while erythrocytes are not believed to undergo changes in α.

Even in the early work, the similarity between the membrane physiologist's experiments and more familiar electrochemical techniques was apparent. For example, consider the "voltage clamp" experiments used to establish the motion of ions across neuronal membranes. A constant dc potential is imposed across a resting membrane, and the current required to maintain that potential is measured.[126] The similarity to chronoamperometry is apparent and is clarified by examining a voltage clamp circuit, Figure 18, which has essentially the same electronic configuration as a Deford-type potentiostat. Both this chronoamperometric technique and steady-state measurements of membrane conductance have been used to elucidate the mechanism of ion transport across membranes. Recently, a charge injection technique with some important advantages has been introduced and applied to bilayer membranes.[127]

B. Lipid Bilayer Model Membranes

The most active area of membrane research has been in the mechanism of ion selectivity, particularly in the simplest case of a membrane at rest. A variety of theories have been proposed including ion selective surface sites,[128] ion exchange processes at the two interfaces,[129] selective carrier molecules within the membrane,[130] and ion-selective pores through membranes.[131] The complexity of biological membranes often precludes interpretation at the molecular level, so much recent work has been done with a model system, the black lipid bilayer. A solution of a suitable lipid (e.g., cholesterol or a phospholipid) in an organic solvent is "painted" onto an approximately 1-mm hole in a gasket between two ionic solutions. The solvent slowly disperses leaving behind a lipid bilayer; the hydrophobic tails of the lipids are in the center, with the hydrophilic groups at the surface of the membrane. These bilayers exhibit many of the characteristics of biological membranes (excluding excitability) and have provided insight into the theories mentioned above. Work on bilayers has recently been reviewed,[132] and the reader desiring a more thorough treatment than that presented here is referred to that paper.

Two distinct phenomena occurring in bilayers should be mentioned here, the first involving transport of ions through the hydrophobic mem-

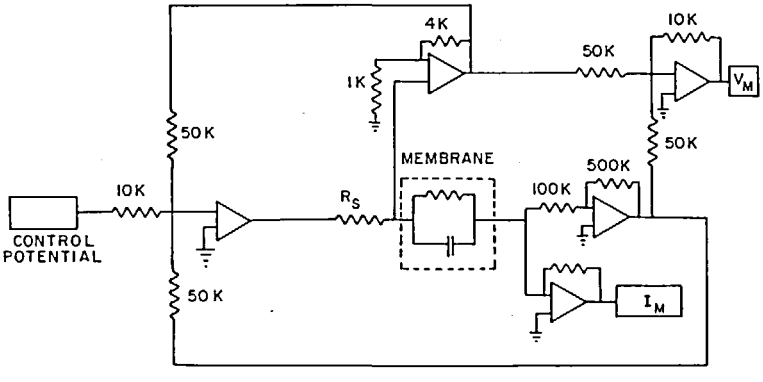


FIGURE 18. Common voltage clamp circuit, allowing control of membrane potential with simultaneous monitoring of membrane current and potential. R_s = solution resistance, V_m = membrane potential, and I_m = membrane current. Control potential may be constant, pulsed, sinusoidal, etc. (Adapted from Adelman, W., *Biophysics and Physiology of Excitable Membranes*, Von Nostrand, New York, 1971.)

brane environment. Large, bulky ions such as tetraphenyl borate can be transported across lipid membranes and will generate the expected potentials if a concentration gradient is present. At low concentrations of tetraphenyl borate, the conduction of current is limited by diffusion of ions to the membrane; at high concentrations, it is limited by space charge effects within the membrane.[133] Although the transport of bulky ions across membranes has been described in some detail, it is apparent that the transmission of physiologically relevant cations does not follow the same mechanism. K^+ and Na^+ are too insoluble in the membrane to allow significant conductance. However, the addition of a lipophilic carrier molecule to the membrane permits conduction of small cations across the hydrophobic layer.[134] For example, the antibiotic valinomycin has a geometry which interacts selectively with K^+ ions, forming a lipid-soluble complex.[135] The valinomycin/K^+ complex forms on the interface with high-K^+ concentration, diffuses to the low-concentration side, then releases the K^+ ion. The neutral carrier is then available to diffuse back to the high-concentration side and interact with another K^+ ion. The carrier mechanism provides for high selectivity between Na^+ and K^+ since the structure of the carrier can greatly affect the stability of the complex and the efficiency of transport. It should be noted that both mechanisms mentioned thus far require solubility of an ion or an ion complex in the lipid layer to provide conductance.

A very different mechanism has been proposed for membranes consisting of a lipid plus a linear polypeptide such as gramicidin-A.[136] An important observation of this system is that the membrane maintains conductance despite a significant decrease in fluidity brought about by freezing of the membrane.[137] The model proposed to explain this effect is one involving "pores" or channels composed of polypeptide through which small ions are conducted. Very sophisticated experiments have allowed the observation of opening and closing of pores and the resulting changes in conductance. Numerous examples of materials forming pores are available.[132] Given this model, selectivity can be explained by the nature of the pores.

Both the carrier and pore theories are viable possibilities for explaining the behavior of biological membranes. At least in the resting state, the permeability of biological systems can be mimicked by properly prepared bilayer systems. However, an excitable biological membrane is capable of permeability changes occurring over a millisecond time scale and has not been duplicated by artificial systems. If biological membranes have pores, then selectivity is voltage dependent as evidenced by the changes in going from a rested to excited state. The closest model of this behavior is the alamethicin/lipid system, which forms a pore-type membrane with pronounced potential dependence.[138] Although these voltage-dependent systems hold promise for explaining excitability, the present models are rather crude compared to the biological substrates.

After considering selectivity and excitability, the third aspect of "living" membranes which remains is the question of how the concentration gradients are maintained. Clearly, membrane permeability changes and resulting potential fluctuations require chemical energy provided by depletion of K^+ or Na^+ gradients. It is known that maintenance of these gradients is provided by a metabolically driven "active transport" system which carries ions across the membrane against their concentration gradients.[139] Very little is known about the mechanism, but it is believed to involve a carrier molecule which is coupled to the degradation of ATP. After iontophoretic injection of a small amount of Na^+ into a nerve cell, it is possible to monitor both Na^+ concentration with an ion-selective electrode and potential with a standard glass capillary.[140] It was found that a potential change occurred shortly after Na^+ injection and was caused by the iR drop across the membrane as Na^+ was pumped out by the metabolic carrier. This approach was used to describe the dependencies of the active transport system on metabolism and to begin to define its nature.

This brief description of membrane electrochemistry should help clarify the role of electrochemical techniques in studying membrane biophysics. Since the processes involved are usually nonfaradaic and always interfacial, the electrochemist is well equipped to make a rigorous investigation. While it is true that most progress at the molecular level has been made on artificial model membranes, the correlations with biological systems are evident. One cannot expect to understand the complex living system until the model membranes are more completely elucidated.

VI. ELECTROCHEMICAL STIMULATION OF LIVING SYSTEMS

This final section is the least well defined of those discussed in terms of the relationship between electrochemistry and biological systems. Certain electrochemical effects on living cells have been at least partially understood for a long time, such as the ionic potentials across membranes discussed earlier. Beyond similar, comparatively simple examples, much more complex effects of electrochemical stimuli on living systems have been discussed in the literature. These more recent observations include electrochemically assisted tissue and bone growth,[141] "bioelectric potentials,"[142,143] electrochemically induced limb regeneration,[144] and electrically stimulated thrombosis.[145] Although in most cases the existence of the effects is well established, the role of electrochemical phenomena in the process is unclear. Several examples will be discussed with the objective being some degree of mechanistic insight.

Consider for a moment the possible effects of a constant or varying potential applied between two electrodes implanted in living cells, bone, fluids, or tissue. At least six effects exist, all of which have been induced to explain the biological reaction. First, the applied potential may stimulate excitable membranes in nerve or muscle cells, as demonstrated in the historic experiments of Galvani on frog muscle. Second, faradaic reactions may occur at the electrode surface which generate or consume electroactive species, such as hydrogen ions or metal ions from the electrode. In most cases, faradaic reactions are avoided to prevent formation of toxic materials. Third, the electric field will cause migration and concentration of ions in the region of the electrodes. Fourth, the electric field may cause changes at membrane interfaces which do not necessarily result in membrane stimulation. The changes may result in major alteration of membrane characteristics. Fifth, a temperature increase near the electrodes may result from joule heating. Finally, synthesis of genetic material may be altered or stimulated by the presence of an electric field or faradaic process. It should be pointed out that these are only the most obvious of the many possible results of electrical stimulation; it is likely that many effects remain to be considered.

The phenomenon which has received the most attention is the acceleration of bone-fracture repair by electrical stimulation. It is well established that bending or compression of bone generates weak electrical fields near the bone surface; in the case of bending, the concave surface becomes negative.[146] Accompanying this negative electrical field is bone formation (osteoblastic) activity, while on the convex (positive) surface, bone degeneration is observed. These and other observations led to the hypothesis that an externally induced electrical field could promote bone formation in the region of negativity.

Early tests of this hypothesis involved implantation of platinum electrodes in normal, unfractured animal bone, followed by application of a constant low-level (approximately 1 V) potential between the two electrodes.[147] It was observed that bone formation was accelerated at the cathode, while degeneration occurred at the anode. A threshold current value of 3 to 5 μA was required for bone formation, 10 to 25 μA was optimum, and 50 μA was deleterious. A variety of applied waveforms have been studied, including symmetric and asymmetric pulses of different polarity as well as controlled current and controlled potential. There is some disagreement about the most effective stimulus waveform, but all agree that the bone growth is real, and the electrode which is predominantly negative is the site where bone growth is most pronounced.

The therapeutic interest in this process is obvious and has been tested in a variety of animal studies. One of the more informative investigations of electrically accelerated fracture repair was performed on dogs.[148] After the radius was surgically broken, electrodes were implanted on opposite sides of the fracture, with the cathode nearest the break to maximize repair. A constant current stimulation was applied and the bones were immobilized and allowed to heal for up to 12 weeks. Several parameters were monitored as a function of time after fracture including callus weight (weight of new bone formed after fracture), torque strength, mineral content, and radiographic appearance. It was confirmed that new bone formed much more rapidly in the stimulated animals, with four times as much being formed after 5 weeks, compared to unstimulated controls. However, after about 7 weeks, the unstimulated bone "caught up" and the stimulation was of little further effect on bone weight. Similarly, torque strength of stimulated bone increased soon after the fracture, but after about 7 weeks, no differ-

ence was evident. Finally, there was a concentration of minerals in the region of the cathode measured by an ashing procedure. These workers conclude that although stimulation has definite effects on bone growth, it is of questionable value for treating routine fractures. In the case of certain bone diseases or difficult fractures, it may be of important value.

There have been a large number of other studies of the type just discussed with an almost equally large number of different results. A summary of the effects of stimulation on bone has been published[149] which demonstrates the magnitude of the disagreements. Arguments about mechanism are equally prevalent and will not be repeated here.

The development of a surgically noninvasive method[147] was prompted by two major problems of bone stimulation by implanted electrodes. First, the presence of electrodes not only limited growth to the cathode but also created the possibility of a faradaic generation of toxic electrode products. These products are the likely cause of the tissue necrosis observed near the anode. Second, the implantation required surgery normally not practical with routine fractures. An attempt was made to promote bone growth by inducing electric fields in the bone from external electrodes. In initial experiments, a 100 V/cm, 1 Hz pulsed field was applied to intact bone in the living animal by placing parallel plates on either side of the animal's leg. Modest but definite increases in bone strength were observed after 21 days. Applications of such devices to man were difficult to imagine since larger, potentially dangerous voltages would be required.

Electric-field induction was improved by magnetic induction from parallel coils placed outside the animal's limb. The magnetic field was varied in such a fashion as to maximize the induced electric field gradient at the fracture site. The electric field actually induced in the bone was between 0.1 and 50 mV/cm and was repeated at 65 Hz with a duration of 0.15 msec. Statistically significant improvements in fracture repair of dog tibula were observed for two magnetically stimulated animals. Not only were the bones stronger, but they had an improved radiographic appearance.

This work demonstrates that direct electrochemical stimulation is not necessary for improved bone growth. One can rule out faradaic reactions or pH effects as being necessary, although they may still be involved with the implanted electrodes. Significant ionic migration is hard to imagine with an externally applied pulsed field. Finally, temperature effects are not significant since the power levels (10^{-4} W/sec) were so low. One is left with two likely effects of electric fields on the bone growth process: alterations in charged interfaces of membranes and subtle effects of a field on large molecules.

The theoretical aspects of field effects on charged membrane interfaces have been discussed in terms of their relationship to cell growth and differentiation.[150] The observation was made that many membrane processes such as excitation and transport depend upon specific interaction between membrane macromolecules and inorganic or organic ions. These interactions are expected to be very dependent upon the electric field near the site of the interaction, usually the membrane/solution interface. Theoretical predictions were made that these membrane interactions would be significantly affected by the electric fields induced by electrochemical stimulation. The effects of such alterations would be widespread, perhaps even affecting genetic processes and cell differentiation. For a further discussion of this approach, the original literature should be consulted.

While bone growth and membrane effects are only two of many results of electrical stimulation, they serve to illustrate some aspects of therapeutically useful electrochemistry. Further examples will not be discussed here, but several extensive discussions are available.[151,152] With such terribly complex systems like living bone or tissue, the mechanistic aspects of electrical stimulation are exceedingly difficult to assess. When examining the literature on bone growth, it is apparent that results from similar experiments differ widely. It is clear that definite effects result from stimulation, but experiments attempting to establish the mechanism of the effects have rarely been conclusive.

The role of electrochemistry in this area is equally unclear, and will not be known until more is understood about mechanisms. Electrochemists are equipped to consider interfacial phenomena as well as faradaic effects, but the state of the art in electrochemistry often falls short when examining very complex systems. While it is likely that the phenomena causing augmented bone growth are indeed electrochemical, more must be known about the process at the molecular or at least cellular level before electrochemistry will have a

significant impact. The area is just beginning to leave the trial and error stage – where too little is known to permit systematic experimentation.

VII. CONCLUSION

The examples of electrochemical investigations of biological materials and phenomena discussed in this review represent the tip of the iceberg when one examines the entire area. Nevertheless, a large majority of the work referred to as bioelectrochemistry falls into one of the five major areas discussed here. The current activity of the area is pronounced as evidenced by a recent symposium volume[153] published coincidentally with the completion of this manuscript. It is apparent that electroanalytical chemistry can provide information about biological systems of analytical, thermodynamic, and kinetic value. Furthermore, the desired information is often difficult or impossible to obtain by other means, and electrochemistry provides not only a possible method, but often the best method for a particular problem.

Despite these strengths, the argument is often made that electrochemical information is too far removed from the biological framework to be of significant relevance. While it is true that many experiments are performed on biological molecules in very nonphysiological environments, the basic physical and chemical information derived from such studies has intrinsic value, regardless of biological relevance. Examples are numerous where basic chemistry, provided that it is done correctly, becomes of biological value much later, when the biological questions become better defined at the molecular level.

In addition to basic chemical information, electrochemistry has provided new analytical methods and new information about redox systems of direct biological importance. In many cases, the results of electrochemical investigations were not easily obtainable from other techniques. There are certainly many cases where electroanalytical methods are not the techniques of choice, but when used properly, they can complement other techniques to improve knowledge of biological reactions and processes. As with any branch of science, the key to success in applying electroanalytical techniques to biological systems is to recognize both their strengths and weaknesses and to critically evaluate the quality of the information determined through their use.

Acknowledgment

Partial support for the preparation of this manuscript from Grant MH-28412-01 from the National Institute of Mental Health is gratefully acknowledged.

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