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Enzyme Electrodes

Electrodes containing immobilized enzymes could be used to monitor specific metabolites.

David A. Gough and Joseph D. Andrade

There is currently considerable interest in the development of biochemical-specific electrodes that could be used to monitor and regulate the concentrations of biochemicals in body fluids. Some very selective biochemical sensors have been made recently in which conventional solute-specific electrodes are used to monitor reactions catalyzed by immobilized enzymes. These devices can theoretically be made to determine metabolites, enzymes, coenzymes, or enzyme inhibitors, in situ, without special preparation of the sample. Widespread application can be predicted for such electrodes in both experimental and clinical medicine if they can be made to function specifically and accurately, and if they can be used for nondestructive, instantaneous, and continuous determinations in situ. Enzyme electrodes must not promote undesirable physiological responses, such as antigenic responses, thrombosis, or tissue reaction. In addition, they must be inexpensive, easy to operate, and have a long lifetime.

In this article we discuss the development of biochemical-specific electrode systems, present some of the foreseeable problems that might be associated with their use, and review the essential literature.

The basic functional concept of the "enzyme electrode" is the continuous, instantaneous, electrochemical monitoring of enzyme-catalyzed reactions, in which a substrate, coenzyme, or inhibitor is converted into a product by means of an enzyme. The relative concentration of the reactants can be varied so that analytical techniques are obtained in which the reaction rates or equilibrium concentrations are proportional to the limiting components. Electroactive species either produced or consumed by the reaction may be detected by commercial solute-specific electrodes, the signal thus produced being related to the limiting reactant. If a system can be designed such that the enzymes are immobilized or constrained to the immediate vicinity of the electrode—these enzymes being capable of continuous catalysis in complex physiological fluids—a new bio-

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chemical-specific electrode is feasible. Obviously, the term "enzyme electrode" is not rigorously accurate because these devices may be made sensitive to substrate, product, enzymic effectors, or enzymes themselves. We prefer to use the term, however, to describe biochemical-specific electrodes that are dependent on immobilized enzymes, until more accurate terms become familiar.

Electrode Characteristics

The most commonly known solute-specific electrode is the glass *p*H electrode. When referenced against a standard reference electrode, a potential difference is produced which is proportional to the *p*H of the solution, according to the Nernst equation. Such an electrode may be useful for following enzyme reactions in which hydrogen ions are a product. Their use is limited, however, because most enzyme reactions are not linear over a broad *p*H range and for accurate results the reaction media must have a low buffering capacity, the opposite of many physiological fluids.

The composition of the electrode glass may be varied experimentally so that the resultant potential is proportional to the potassium, sodium, ammonium, or other cations in solution. In some other types of specific ion electrodes, ingenious liquid or solid ion exchange membranes are employed. Descriptions of these electrodes and their mechanisms of operation have been discussed in detail (*1, 2*). Of the more than 20 specific ion electrodes available commercially, only those sensitive to species participating in enzymic reactions will be useful in this application. The electrodes that will be of most immediate use are those specific for *p*H, ammonium, and other monovalent cations (*3*), and cyanide (*4*). A phosphate-specific electrode has been reported (*5*), but does not appear to be adequately selective or reproducible.

The electrodes now available are not generally completely selective for the desired species. For example, if the enzyme reaction produces NH_4^+ which is to be measured, the electrode may respond not only to NH_4^+, but also to Na^+, K^+, and other cations in solution, as well as *p*H. This effect may be eliminated by referencing against another cation electrode, which cannot respond to the NH_4^+ formed from the reaction because of diffusional or flow effects, but responds to everything else. By determining electronically the difference between the two, a signal that is only proportional to the NH_4^+ produced by the enzyme reaction will be obtained.

Truly continuous measurements can be made only when the signal-bearing species is continuously removed or converted by the electrode, such as in polarographic or amperometric systems. Most ion-selective electrodes are potentiometric, however.

The response of specific ion electrodes has been discussed elsewhere (*1*). Ideally, specific ion electrodes provide a linear Nernstian response of 0.059 volt at 25°C per decimal change in activity of monovalent cation over a certain range of concentration. Response times are on the order of seconds or less for most electrodes, making electrode kinetics a minimal concern.

Polarographic measurements are made by measuring a change in current as a function of changing potential between two inert metal electrodes, and are useful for detecting several species in solution that have a characteristic plateau at a known potential. Although polarography itself may not be very useful to the system of interest, constant potential polarography, or amperometry (in which the current is proportional to a certain species reduced or oxidized at a fixed potential), has application. This is the basis for the operation of the well-known Clark pO_2 electrode (*6*), wherein oxygen diffuses through a gas-permeable polymer membrane and is reduced at a platinum electrode, which is kept at a fixed potential with respect to a silver-silver chloride reference electrode. Response time is on the order of seconds when membranes highly permeable to oxygen are used.

It is also possible to use metal electrodes in the form of an analytical fuel cell, the short circuit current being proportional to the biochemical substrate. The effect of interfering species may be minimized by selective membranes. Relatively high currents may be obtained by efficient electrochemical coupling.

The pCO_2 electrode is also a well-known clinical tool (*7*). The basis of this electrode is diffusion of carbon dioxide through a gas-permeable polymer membrane into an internal aqueous solution of fixed bicarbonate concentration. The carbon dioxide is hydrated to carbonic acid in a slower, rate-determining step, then rapidly ionized to bicarbonate and hydrogen ion. This causes a change in the *p*H of the internal solution, as determined by a potentiometric *p*H electrode. This electrode has some limitations for enzyme electrode applications. It responds only to carbon dioxide, while the product of many enzyme reactions is bicarbonate. The response time may also be too long for many applications. Membranes of higher permeability, and possibly membranes with enzymatic activity, may significantly reduce the response time of this electrode.

Metal electrodes have been used to measure enzymatic oxidation-reduction reactions in which the oxidation state of a coenzyme or intermediate compound is directly changed at the electrode (*8*). Such systems will be operable if precautions are taken to prevent excessive adsorption or interference when the metal electrodes are placed in multicomponent systems.

Immobilized Enzymes

Numerous methods of enzyme immobilization have been reported in the literature (*9–11*). Several techniques are useful in the design of electrodes. The enzyme can be entrapped within a synthetic hydrophilic gel, cross-links can be formed between the molecules of the enzyme to make membranes, the enzyme can be chemically bound to membranes or other surfaces, the enzyme can be copolymerized with other enzymes or proteins, or the enzyme can be physically entrapped between membranes. Other techniques are available and may be useful for certain design requirements. Acidic or basic groups may be polymerized in the supporting polymer matrix in which the enzyme is immobilized in such a way that the *p*H in the immediate vicinity of the enzyme is optimum, while that of the bulk solution is different. Such techniques can optimize kinetics and possibly make an otherwise inoperable system workable. The method of immobilization will depend on the particular enzyme electrode system.

Some enzyme reactions may require the immobilization of substrates or coenzymes. The high cost of many coenzymes makes prohibitive the simple

addition of non–rate-limiting excesses to each sample to be measured. Probably the most practical techniques will be the covalent bonding of the coenzyme to a surface in such a way that reaction is still possible, as reported recently (*12*), or the covalent bonding of the coenzyme directly to the enzyme or other immobilized particle by techniques which permit catalytic action. Containment by a membrane of selective pore size or slow release through a glass frit to which the enzyme is bound may be necessary in some cases.

An important problem is heat inactivation of many enzymes at physiological temperatures. The long-term usefulness of biochemical electrodes will be seriously limited if methods of thermal stabilization cannot be found. One approach to this problem may be the use of only partially purified enzyme extracts, or the enzyme might be mixed with stabilizing species which will not interfere with the reaction. Some immobilization methods are reported to stabilize certain enzymes for periods longer than their lifetimes in vivo (*11, 13*). However, while perhaps not all enzymes of immediate interest are now capable of being stabilized for performance under the desired conditions, many can be adequately stabilized and current research into new techniques holds much promise (*14*).

Some enzymes that do not produce electroactive species may be linked to other enzymes in such a way that the product of the first enzyme reaction becomes the substrate for the second enzyme which involves an electroactive participant, thus greatly expanding the number of species that can be monitored. Studies indicate that these systems may be most efficient if the enzymes are mixed in the same phase to avoid unnecessary diffusional effects (*15*). Such multistep systems show some similarity to processes in vivo.

Although electrodes will operate under equilibrium or steady-state conditions, they must be characterized kinetically in order to determine the range and rate of response. Enzyme kinetics in the liquid phase (where diffusional effects are absent during the initial stages of the reaction) have been well studied. Some studies relating diffusional (*10, 16*), charge (*17*), and boundary layer effects (*18*) to heterogenous phase enzyme kinetics have been made; product inhibition (*18*) and two-enzyme systems (*19*) have also been studied. The data indicate that immobilized enzymes can be characterized kinetically by the turnover number, concentration and Michaelis constant (K_m) of the enzyme, the nature and dimensions of the catalytic layer, the diffusivities of participating species, and the estimated thickness of the boundary layer. Diffusion of substrate through the physiological media may also have a limiting effect. Response of an immobilized enzyme electrode can therefore be predicted.

Many solute-specific electrodes cannot respond to very low concentrations of solute, such as concentrations less than $10^{-6}M$. This may limit the feasibility of certain enzyme electrodes since the critical concentrations of some compounds in physiological fluids are quite low. Another important consideration is that electrodes determine activities, not concentrations. In many instances, the clinical significance of activities measured in situ is not known. However, this may lead to some useful investigations.

Applications of enzyme electrodes in flowing systems will require consideration of boundary layer artifacts and streaming potentials. This has been studied with glass electrodes in physiological solutions (*20*).

The electrode will also have to be designed to avoid any undesirable physiologic responses, such as protein deposition, thrombosis, antigenic reaction, or the formation of a diffusion-resistant tissue capsule around the electrode (*21*). A hydrophilic biocompatible membrane that excludes compounds of given molecular weight could be placed between the reactive layer and the physiological environment. Such a membrane should result in minimal protein deposition and thrombosis and should prevent species of larger molecular weight from passing while at the same time providing a highly aqueous medium for optimal substrate diffusion.

Literature

An excellent review of electrochemical methods of monitoring conventional enzymatic reactions has been published (*22*). In the present article we describe only self-contained biochemical electrode systems in which the enzyme or substrate has been physically immobilized in the vicinity of the sensor or bonded to it.

Probably the first account of an enzyme electrode was given by Clark and Lyons (*23*). They obtained potentiometric determinations of glucose and proposed that glucose could also be determined amperometrically by means of glucose oxidase immobilized between Cuprophane membranes, according to the equation

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{glucose oxidase}} \text{gluconic acid} + H_2O_2$$

Determinations were made from a solution of low buffering strength. Updike and Hicks (*24*) introduced the term "enzyme electrode" and made a dual cathode Clark-type oxygen electrode with glucose oxidase immobilized in polyacrylamide gel. The electrode was used to determine glucose from whole blood and plasma, and thus demonstrated the feasibility of measurements being obtained from complex solutions. The response time was approximately 30 seconds. Clark (*25*) suggested changing the potential across the electrodes so that they would respond to hydrogen peroxide production instead of oxygen uptake, thus reducing the problem of interference from oxygen in solution. Erroneous readings caused by small amounts of catalase or peroxidase found in most enzyme preparations or in physiological solutes can be minimized by suitable membrane-electrode design (*25*) or by inhibitors. Williams *et al.* (*26*) replaced oxygen as the hydrogen acceptor with quinone, to monitor glucose according to the following equations

$$\text{Glucose} + \text{quinone} + H_2O \xrightarrow{\text{glucose oxidase}} \text{gluconic acid} + \text{hydroquinone}$$

$$\text{Hydroquinone} \xrightarrow{Pt} \text{quinone} + 2H^+ + 2e$$

where the reaction potential is 0.4 volt with reference to a standard calomel electrode. Glucose oxidase was held between layers of dialysis paper. The enzyme from *Aspergillus niger* was used because it can utilize quinone as a hydrogen acceptor and does not require other coenzymes, as does the enzyme from other sources. Determinations required the addition of buffer salts and quinone to maintain adequate *p*H and prevent the diffusion of quinone out of the enzyme layer. An electrode to determine lactate was also reported in the same communication (*26*), based on the oxidation of lactate by ferricyanide. The reaction is catalyzed by lactate dehydrogenase (cyto-

chrome b_2, E.C. 1.1.2.3), which does not require nicotinamide adenine dinucleotide as hydrogen acceptor, according to the following equations

$$CH_3{-}CHOH{-}COO^- + 2Fe(CN)_6^{-3} \xrightarrow{LDH}$$

$$CH_3{-}\overset{\overset{O}{\|}}{C}{-}COO^- + 2Fe(CN)_6^{-4} + 2H^+$$

$$2Fe(CN)_6^{-4} \xrightarrow{Pt} 2Fe(CN)_6^{-3} + 2e$$

where the reaction potential is 0.4 volt with reference to a standard calomel electrode. The enzyme was held between dialysis membranes. Because of the low K_m of this enzyme (K_m = 1.2 mM), it was necessary to dilute the sample with buffered $K_3Fe(CN)_6$. Steady-state measurements were made in 3 to 10 minutes. The authors claimed that this system demonstrated increased sensitivity over spectral techniques (*26*).

Wingard *et al.* (*27*) have proposed constant current voltametry as a method for evaluation of electrodes containing immobilized oxidative enzymes as catalysts. This design was originally suggested as a fuel cell. Bessman and Schultz (*13*) have used the fuel cell concept to monitor glucose.

Several enzyme electrodes based on potentiometric cation- and ammonium-ion specific electrodes have been reported. Guilbault and Montalvo (*28*) made a urea transducer by immobilizing urease in a thin layer of acrylamide gel held over the surface of a cation electrode by cellophane film. The reaction is

$$NH_2{-}\overset{\overset{O}{\|}}{C}{-}NH_2 + 2H_2O \xrightarrow{\text{urease}}$$

$$NH_3 + NH_4^+ + HCO_3^-$$

The electrode was used for periods of up to 3 weeks at 25°C with no loss of activity and it responded to urea concentrations from 5×10^{-5} to $1.6 \times 10^{-1}M$ in tris(hydroxymethyl)aminomethane buffer, with an optimal response time of approximately 25 seconds. Many parameters affecting the function of the electrode were characterized (*29*). The response was not independent of Na^+ and K^+ ions when the Na^+ ion concentration was greater than one-half of the urea concentration and the K^+ ion concentration was greater than one-fifth of the urea concentration, placing limitations on the buffer that could be used. The enzyme gel layer had to be washed after each determination, making truly continuous or rapid measurements impossible. Similar electrodes were evaluated for the determination of urea in blood and urine (*3*). The sample was diluted and ion exchange resin added directly to eliminate cation interference. The electrode showed precision and accuracy comparable with spectral methods.

Electrodes specific for amino acids have been described (*30*). L-Amino acid oxidase (L-AAO) was immobilized by several methods at the tip of a commercially available cation electrode. The reaction is

$$O^-{-}\overset{\overset{O}{\|}}{C}{-}\overset{\overset{R}{|}}{CH}{-}NH_3^+ + H_2O + O_2 \xrightarrow{\text{L-AAO}}$$

$$O^-{-}\overset{\overset{O}{\|}}{C}{-}\overset{\overset{R}{|}}{C}{=}O + NH_4^+ + H_2O_2$$

The further nonenzymatic release of CO_2 by the following equation

$$O^-{-}\overset{\overset{O}{\|}}{C}{-}\overset{\overset{R}{|}}{C}{=}O + H_2O_2 \longrightarrow$$

$$O{=}\overset{\overset{R}{|}}{C}{-}O^- + CO_2 + H_2O$$

is prevented by adding a small amount of catalase to the enzyme layer, which catalyzes the reaction

$$H_2O_2 \xrightarrow{\text{catalase}} \tfrac{1}{2}O_2 + H_2O$$

to give the total reaction

$$O^-{-}\overset{\overset{O}{\|}}{C}{-}\overset{\overset{R}{|}}{CH}{-}NH_3^+ + \tfrac{1}{2}O_2 \rightarrow$$

$$O^-{-}\overset{\overset{O}{\|}}{C}{-}\overset{\overset{R}{|}}{C}{=}O + NH_4^+$$

The addition of catalase seems to improve the electrode, probably because oxygen is generated, which is necessary for the oxidase reaction. These electrodes were reported to remain stable for about 2 weeks, and showed 1- to 2-minute response times to amino acids in dilute buffer solutions (*30*).

Electrodes specific for D-amino acids which are catalyzed by D-amino acid oxidase (D-AAO) have been reported (*31*). The reaction is

$$O^-{-}\overset{\overset{O}{\|}}{C}{-}\overset{\overset{R}{|}}{CH}{-}NH_3^+ + O_2 \xrightarrow{\text{D-AAO}}$$

$$O^-{-}\overset{\overset{O}{\|}}{C}{-}R + NH_4^+ + CO_2$$

where the cation is monitored by a potentiometric cation electrode. It was found that stability of the acrylamide enzyme gel or liquid layer could be maintained for 21 days if it were stored in buffered flavine adenine dinucleotide solution, a weakly bound diffusible coenzyme. The response was not increased by high concentrations of oxygen but was dependent on *p*H. A very similar electrode for asparagine was also reported (*31*), asparaginase being used as the catalyst. The addition of a coenzyme was not necessary.

A potentiometric electrode for glutamine has been reported and characterized (*32*). The electrode response was reproducible for up to 8 hours. The catalytic reaction was dependent on *p*H and inhibited by cations; measurements were made in dilute aqueous solutions.

An interesting approach to the determination of enzyme activity with potentiometric cation electrodes has been reported (*33*) in which immobilized substrates were used. A liquid layer of urea was passed between the electrode tip and a dialysis membrane in a continuous or interrupted flow process. Urea diffused through the membrane and was catalyzed by urease in dilute aqueous solutions. The ammonium ion produced was detected by the cation electrode. Although this approach probably requires much more study before it can be of significant practical use, it suggests some interesting applications.

An electrode specific for amygdalin based on a solid-state potentiometric cyanide electrode has been reported and characterized (*4*). β-Glucosidase, immobilized in acrylamide gel, hydrolyzes amygdalin by the following reaction

$$C_6H_5CHCN + H_2O \xrightarrow{\beta\text{-glucosidase}}$$
$$|$$
$$OC_{12}H_{21}O_{10}$$

$$2\,C_6H_{12}O_6 + C_6H_5CHO + HCN$$

The lifetime of this electrode is limited by the dissolution of the cyanide-sensing crystal membrane, which is claimed to have a working lifetime of 200 hours (*4*).

Summary

From the discussion of electrodes and enzymes herein, and from the accounts of enzyme electrodes that have appeared in the literature, clinical determinations of certain metabolites and soluble enzymes by means of enzyme electrodes seem quite feasible. Such devices may be made highly specific by the use of appropriate enzymes and

a high degree of accuracy can be obtained. Instantaneous and continuous determinations can be made from physiological fluids, and undesirable physiologic responses can theoretically be minimized, thus making long-term clinical monitoring a possibility. Enzyme electrodes may also have a useful lifetime and meet other practical requirements.

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Blood Services: Prices and Public Policy

D. MacN. Surgenor, E. L. Wallace, P. D. Cumming, B. D. Mierzwa, and F. A. Smith

The blood service complex of the United States has recently come under intense public scrutiny (*1*). This scrutiny has focused upon the issue of safe blood, reflecting mounting public concern about the alarmingly high risk of posttransfusion hepatitis (*2*). This risk is associated with the practice of collecting considerable amounts of blood from paid donors. Indeed, the public suspects that dollars are polluting its blood supply (*3*).

While attention seems now to be directed primarily toward the hepatitis problem, other issues relating to blood may soon surface unless they are dealt with by those individuals responsible for providing blood services. These other issues include the custody and conservation of the national blood resource; the provision of adequate blood services to all, regardless of where, when, amount needed, and ability to pay; and the procurement of sufficient human plasma to meet projected demands for albumin and other plasma products.

All of these issues have economic implications, but the one of most immediate importance to the patient who has received blood is the price of blood services. In this article, we describe the present structure of blood service prices, relate it to cost, and examine the principal public policy issues related to the price of blood services. Because of marked differences in organizational objectives between profit and nonprofit blood centers, and the predominance of the nonprofit center in the United States, we focus on the nonprofit center.

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Definitions

Before entering on our discussion, we wish to define the following terms.

Blood center. We use this term to include any enterprise responsible for the procurement, processing, and distribution of blood and blood components and for rendering all related professional services. Other terms for such entities include "blood bank," "community blood bank," "community blood center," and "regional blood center."

Blood service. Blood is a human tissue that should be considered a community resource over which the blood center is custodian. The output of the blood center is a set of professional, educational, and scientific services performed on, or in connection with, the