IMMOBILIZED BIOELECTROCHEMICAL SENSORS

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ABSTRACT

Presently there are a number of fine and sensitive methods for determining chemical or biochemical substances among which bioelectrodes represent the most recent development. Bioelectrodes consist of biologically active materials(e.g enzyme, antibody,whole cell or cell fragments)held in close proximity to a suitable electrochemical transducer that sense specific electroactive enzyme substrates or products.

Considerable efforts are still in progress in order to improve the performance of these bioelectrodes and widen their applications.

The present article describes some of the most frequently used immoblization techniques in producing such sensors , and reviews several parameters affecting their response . Finally a collection of current analytical applications is presented .

I.INTRODUCTION

Enzymes,have played an important role in the development of analyti - cal methods for clinical,immunochemical,environmental and biotechnological analyses[1,2]Their popularity as analytical reagents is due to their specificity and sensitivity toward their respective substrates.

Soluble enzymes have limited usefulness and several disadvantages, such as poor precision,instability , cost,and lack of availability.Besides,aqueous solutions of enzymes often lose their catalytic activity very rapidly,and they can not be recovered from the batch processes for reuse . Bioelectrodes consist of biologi - cally active materials(e.g.enzyme, antibody,cell fragment,etc.) maintained in close proximity to a suitable transducer which converts the biological signal into an electrical signal.The substrate to be measured diffuses into the biocatalyst layer, where a reaction occurs generating or consuming an electroactive species. The electrode measurable species is monitored either potentiometrically or amperometrically,and from the signal of the transducer the concentration (activity)of the substrate (or enzyme)is calculated.The advantages of bioelectrodes over wet chemical or spectrophotometric procedures are well established[3],but considerable efforts are still underway to develop reliable,accurate,convenient,low-cost and low maintenance analyzers.Research is usually focused on improving ei - ther the performance of the biocatalytic element,of the transducer , or both.

The present article well describe some of the most often used immobi - lization methods and sensors,and will review several parameters affecting the response of immobilized bioelectrodes.Finally,a collection of current analytical applications will be presented.

II.IMMOBILIZATION METHODS

The concept of biocatalyst immobilization is an alternative which overcomes the shortcomings of soluble enzyme methodologies.The immobilization procedure consists of insolubilizing the free enzyme via entrapment into an inert matrix,such that the immobilized enzyme retains its catalytic properties and is reusable,even up to 10,000 times in some cases. Moreover,immobilized enzymes are generally more stable,and more useful over wider pH ranges and high temperatures. Finally,they appear to be less sus - ceptible to the normal activators and inhibitors that affect the activity of soluble enzymes.The technology of immobilized enzymes and their applications in analytical chemistry,have been reviewed[1-8].However,much innovative research is still being done in

this stimulating research area.

Essentially,two major techniques are preferred for the immobilization of the various biological catalysts at electrode surfaces[3,9]:(1)physical entrapment within the pores of an inert polymer matrix;(2)chemical modification by introducing an insoluble group.A third technique involves immobilization by covalently attaching an enzyme onto an electrode surface [10-12].

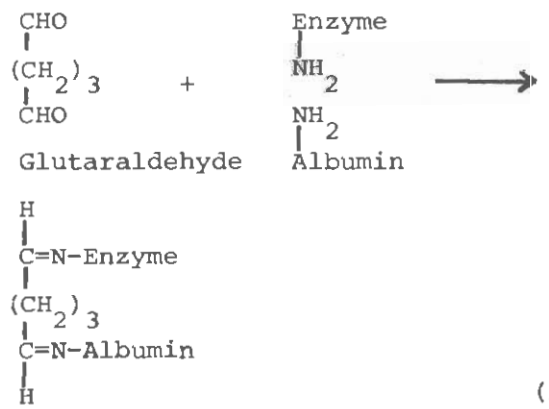
A.Enzyme Immobilization

1.Physical Entrapment

The most common materials used for physical entrapment are starch gel, polyacrylamide and silicon rubber. Some enzymes have been entrapped in starch gel[13]and in polyacrylamide gels,and have been stored without significant loss of activity[14,15], and have been used for the assay of substrates such as glucose and lactic acid[16].A comparison between the different entrapment techniques was thoroughly investigated[17] and it was concluded that the stability of the enzyme was better in polyacrylamide gel.Although the loss of activity is about 10-25%,the main disadvantage of this technique lies in the fact that riboflavin and $K_2S_2O_8$ must be used as catalysts for the polymerization.This may lead to potential interference problems,depending on the sensing method used.Cholinesterase, immobilized in a starch matrix and placed on a polyurethane foam pad, was found to be stable and active for 12 hrs under continuous use[18].

2.Chemical Attachment

a.*Bifunctional reagents*.Bifunctional reagents have been used to insolubilize enzymes and other proteins by intermolecular crosslinking,with the concomitant formation of macroscopic particles.Among the reagents used(glutaraldehyde,bisdiazobenzidine-2,2'-disulfonic acid,toluene-2-isocyanate-4-isothiocyanate,trichloro-o-trizaine,etc.),the method of chemical bonding with the bifunctional reagent glutaraldehyde,is very simple and quite useful.The enzyme is treated with the aldehyde and an inert support(albumin,glass beads,etc.);a rigid layer of bound enzyme results, which is quite stable for several months and thousands of assays[17]. These methods enable control of the physical properties and particle size of the final product,but many enzymes are sensitive to the coupling reagents and lose activity in the process.



(1)

The covalent binding to water - insoluble matrices is widely used because covalent coupling places enzymes in a more natural environment, thus increasing their efficiency,and the stability is not reversed by pH, ionic strength,substrates ,solvents or temperature.Attachment is carried out using the functional groups on the enzyme which are not essential for its catalytic activity:amine ,phe - nol,carboxyl,etc.The carrier is chosen for its properties of solubility , functional groups,mechanical stability,surface area,swelling and hydrophobic or hydrophilic nature . The most common carriers used for covalent binding of enzymes are: porous glass,polyacrylamide, cellulose dextrane,nylon,carboxylmethylcellulose,and collagen membranes.

b.Immobilization on collagen membranes.An improved method for cova - lent coupling of enzymes to chemically activated collagen films has been developed by Coulet et al[7].A film of highly polymerized collagen was chosen for surface covalent binding of the enzyme,because of its insolubility,mechanical resistance,protein nature,hydrophilic properties and abundance in chemically activatable -COOH groups.Untanned films,previously acid-methylated,were activated by acyl azide formation.After removal of reagents by repeated washing with 0.1 M phosphate buffer,pH 7.4 , the enzyme immobilization was finally accomplished by immersion of the activated film into the enzyme solution for 4 h at room temperature.The procedure is particularly mild because the enzyme never comes into contact with chemical reagents,thus avoiding potential denaturing processes.

The procedure for activation of collagen membranes and covalent immobilization of cholinesterase is shown in equations (2)-(5):

a.Activation:

Collagen

$$\text{—COOH} \xrightarrow[20\text{-}22^\circ C,\ 3\text{-}20\ \text{days}]{CH_3OH/0.2\ M\ HCl} \text{—COOCH}_3 \qquad (2)$$

Washing in distilled water(20°C)

$$\text{—COOCH}_3 \xrightarrow[20\text{-}22^\circ C,\ 1\text{-}15\ h]{1\%\ H_2N\text{-}NH_2} \text{—CO-NHNH}_2 \qquad (3)$$

b.Washing in distilled water(0-4°C)

$$\text{—CO-NHNH}_2 \xrightarrow[0.4^\circ C,\ 3\text{-}5\ \text{min}]{0.5M\ NaNO_2/0.3\ M\ HCl} \underset{\text{acyl azide}}{\text{—CON}_3} \qquad (4)$$

Washing in buffer(0-4°C)

c.Coupling

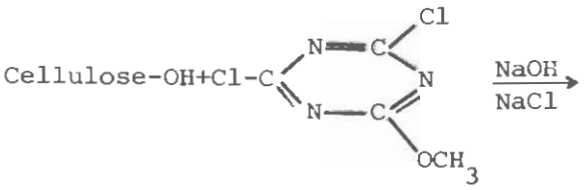
$$\text{—CON}_3 \xrightarrow[0.4^\circ C,\text{pH } 8\text{-}9,\ 2\text{-}5h]{H_2N\text{-enzyme}} \text{—CO-NH-enzyme} \qquad (5)$$

Washing with 1 M KCl,15-30 min(0-4°C)

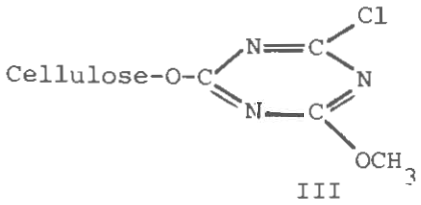
Storage in buffer solution,0-4°C

c.Immobilization onto cellulose carriers.Cellulose is often used in enzyme systems to physically trap an enzyme or to block the diffusion of

undesired enzymes and proteins.Be - cause of the universal usefulness of cellulose as a support for analyti - cal studies,it has been derivatized with S-trizine trichloride[19]and 2, 4-dichloro-6-methoxytriazine (DCMT) [20].Cellulose membranes were first hydrated,treated with 3 M NaOH, then reacted with DCMT to produce the ac- tivated carrier.The enzyme could then be directly coupled to the activated carrier.The reaction sequence is given below:

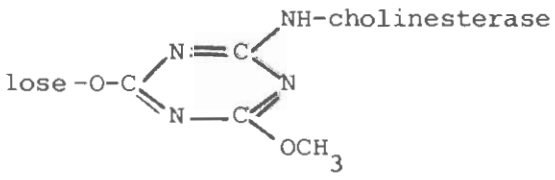


I II(DCMT)



III (6)

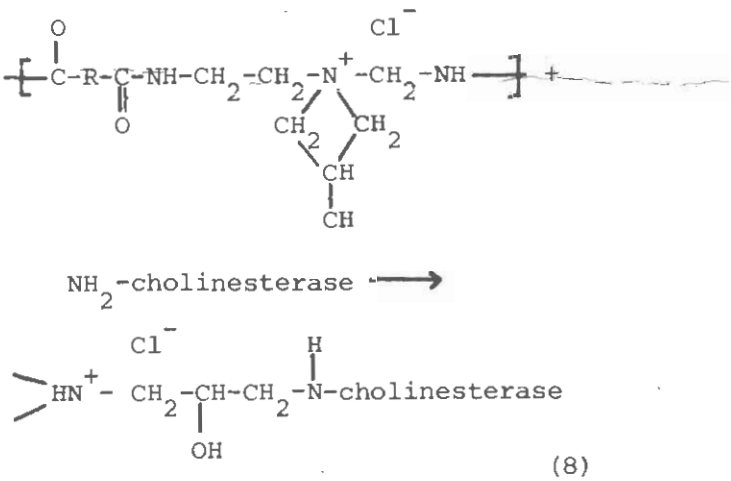
III+H_2N-Cholinesterase $\xrightarrow[HCl]{}$ cellu-



(7)

Based on this method,a much im - proved,ultrastable preparation ofimmo- bilized cholinesterase membrane was obtained[21].

d.Chemical binding with polyaze- tidine.In the method reported by Wood and Calton[22]to immobilize cholin - esterase(eqn.8),a mixture of 5 g of an aqueous polyazetidine solution(Her - cules Polycup 172,12% solid in water) and an appropriate amount of enzyme was stirred rapidly for 7 min at 26°C. Thirty µl of this solution was then applied to a hydrated dialysis mem - berane,allowed to dry at room tem- perature and stored appropriately.



(8)

It is worth mentioning that Pall Bio Support Corporation has recently introduced preactivated nylon membrane,either with free carboxyl groups or with amine groups.Immobili- zation can be carried out by simply contacting the membrane with a solu- tion of the desired biologically ac- tive material.

B.Whole Cell/Cellular Fragment Immobilization

The use of whole cell microorga- nisms or tissue cells as biocatalysts is a fairly recent research field , which has already been shown to po - ssess several advantages when used in

combination with electrode probes [23-25].The purpose of using whole cells is to avoid the difficulties encountered in purifying and stabilizing enzymes.The cells of microorganisms contain multienzyme systems and cofactors necessary to effect a vital transformation of substrates to products that could be difficult,if not impossible,to effect with single immobilized enzymes.Moreover,by using bacterial cells,continuous regeneration of the biocatalytic activity is possible simply by immersion in nutrient media.The choice of a judicious growth medium permits some increase in the specificity of the desired reaction.

The cofactors necessary for enzymatic reactions are held in a natural immobilized state and in a shape generally convenient for electrode probes,offering advantages of speed and ease of biosensor preparation . Typically,bacterial biocatalysts may be compacted by centrifugation and held by a dialysis membrane[26,27],a collagen membrane[28] or entrapped in a polymer matrix[29,30].The bacterial cells are easily obtained by culture in appropriate medium , but care has to be taken to operate under sterile conditions when preparing the bioelectrode probes,in order to avoid external microbial contamination.

Thin slices of tissue are generally sandwiched between inner and outer(Nylon webbing)support membranes. In some cases,when the material has no sufficient durability and rigidity to permit the use of large pore support membranes,a cellophane dialysis membrane or a bovine serum albumin(BSA)-glutaraldehyde cross-linking immobilization can be carried out[9].

A comparison between isolated enzymes,bacterial cells and tissue slices has been made[26].

III.PRINCIPLES OF THE BIOCATALYST ELECTRODE PROBES

A large variety of biocatalysts can be immobilized onto electrode probes.If the substrate or any of the reaction products is electroative, then this species can be monitored quantitatively,either potentiometrically using a gas sensing electrode (CO_2,NH_3,H_2S) , an ion-selective electrode(H^+,NH_4^+,I^-,F^-,CN^-) , or amperometrically at solid electrodes operating either in the oxidative or in the reductive mode(platinum,gold, glassy carbon,carbon paste or modified electrodes).Among these sensors, the gas-sensing electrodes have been used most often due to their high degree of selectivity,but they suffer from long response and recovery times. Ion-selective sensors may suffer interference from ionic interfering species while amperometric oxidation

reduction techniques offer a decreased response time[31],are generally more sensitive than potentiometric sensors[32],and some selectivity can be achieved by a judicious choice of operating potential.Selectivity may be improved to some extent by casting an appropriate molecular weight cutoff membrane over the probe surface,thus preventing interferences from reaching the surface,but at the expense of increased response time and lower sensitivity.

The choice of the proper electrode probe is obviously related to the nature of the substrate to be detected, and also to the purpose for which the electrode probe is intended(eg. , an in-vivo micro-electrode) [33]. Other considerations are the physical strength of the biocatalyst immobilized layer,and the selectivity, sensitivity,stability and speed of measurements required.

IV.CONSTRUCTION OF AN IMMOBILIZED BASED BIOELECTRODE

Regarding the performance of the electrode probe,a simple rule of thumb to follow is that:(1)the better the immobilization method used,the more stable the biocatalyst is,and,hence, the more assays possible;(2)the use of highly pure biocatalyst will insure better electrode performance. Figure 1 illustrates a detailed configuration of the various membrane layers necessary in the construction of a biocatalyst immobilized biosensor using a gas sensor.The immobilized biocatalyst(enzyme,bacteria,tissue) is sandwiched between membranes which allow both unrestricted diffusion of substrate from the solution to the biocatalytic layer and product diffusion to the electrode through the selective gas permeable membrane. However,many different possibilities of sensor preparation exist.For example: (1)a soluble enzyme may be spread as a paste over the surface of a proper electrode and the layer subsequently covered with a cellophane dialysis membrane. Such soluble enzyme electrodes are stable for about a week if refrigerated between uses and even for a longer period when unpurified enzyme are used. (2) A second method of construction consists of a physical entrapment onto the electrode surface.The electrode probe is covered with a thin nylon net (about 90 μm thick) which serves as a support for the enzyme gel solution and for the in-situ polymerization step. After polymerization the enzyme gle layer is covered with a dialysis membrane to ensure further protection. (3) A third type of immobilized bioelectrode consists of a direct polymerization onto the membrane . This can be effected by a direct attachment of the enzyme to the sur-

face of the electrode,as was done with urease[34].

V.PERFORMANCE OF THE IMMOBILIZED BASED BIOELECTRODES

A useful immobilized-based bio - electrode must possess several necessary properties,such as good response time,excellent stability,selectivity,low detection limits and good accuracy.Several electrode parameters have to be thoroughly investigated: operating pH,buffer composition,concentration of biocatalyst,immobilized catalyst layer thickness,temperature, effects of prosthetic groups or cofactors,and interferences.

A.Stability

1.Enzyme Immobilized Probes

In general,of the three types of enzyme immobilized electrodes described above,the first and third types produce very stable probes.The second type has a maximum stability of only 3-4 weeks.

As a general rule,a"soluble"electrode is useful for about 1 week or 25-50 assays,the physically entrapped polyacrylamide electrodes are good for about 50-100 assays,depending crucially on the degree of care exercised in the preparation of the polymer. The chemically attached enzyme can be kept indefinitely,if not used excessively.A lifetime of about 14 months was reported for glucose oxidase[35] (see Fig. 2),and greater than 4-6 months for L-amino acid oxidase or uricase.One can expect to get about 200-1000 assays per electrode depending on how effective is the attachment procedure.Although the electrode can be stored at room temperature,it is recommended that all enzyme-based electrodes be kept in a refrigerator and covered with a dialysis membrane to prevent the action of bacteria, which tend to feed on the enzyme, destroying its activity.

Another factor affecting the stability of some enzyme electrodes is the leaching out of a loosely bound cofactor from the active site(a co - factor which is needed for the enzymatic activity),as demonstrated in the case of D-amino acid oxidase in a polyacrylamide membrane[36].The immobilized enzyme D-amino acid oxidase must be stored in the presence of its coenzyme,flavine adenine dinucleotide,in order to maintain its activity for periods longer than 3 weeks.

2.Whole Cell Immobilized Probes

Lifetime enhancement is the principal advantage of whole cell bio - sensors over the isolated enzyme system;this is to be expected since the integrity of the enzyme environment is maintained within the cells . A comparison between enzyme,bacterial (Sarcina flava),tissue and mito - chondrial (porcine kidney)biocatalysts for glutamine selective membe -

rane electrodes has been reported [26].A glutamine tissue biosensor was found to possess a considerably longer lifetime than a "soluble" immobilized enzyme sensor[26,37,38]. Indeed,using a slice of Porcine kidney tissue the biosensor for glutamine was stable for at least thirty days while the enzyme glutaminase probe was stable for one day.Lifetime enhancement of a factor of 7 has also been observed for the detection of hydrogen peroxide using a slice of bovine liver tissue as the biocatalyst,instead of the isolated enzyme catalase[39].Considerably increased lifetime and activity has been reported for a tissue(rabbit muscle) AMP biosensor over the corresponding isolated enzyme sensor,both immobilized at the surface of an ammonia gas sensor.The enzyme sensor displayed a lifetime of 4 days in contrast to the 28 days of the immobilized tissue electrode[40].

The use of bacterial probes also offers the advantages of increased lifetime,and that the bacteial electrode can be regenerated simply by immersion in nutrient medium . The microbes are essentially"living",and can be fed and kept alive for long periods.For example,a bacterial aspartate sensor was stable for 10 days while a purified aspartate ammonia lyase was stable for only three days[41].In a comparative study between physically and chemically immobilized urease and bacterial urease, the bacterial electrodes, maintained in a rich nutrient medium,were found to possess a longer lifetime(3 weeks)than the corresponding immobilized urease electrdes (2 weeks)[42].

Significant lifetime increases have also been reported during the study of asparagine with a bacterial electrode(Serratia marcescens)[43] and of L-tryptophane using a bacterial electrode(E.coli) [44] instead of tryptophanase or asparaginase immobilized bioelectrodes.Stability of the bacterial electrodes was longer than 26 days with the help of regeneration in an appropriate medium in contrast to the instability of immobilized probes containing the crude enzyme tryptophanase(less than 3 days)or asparaginase(3 weeks).

3.Enzyme Immunoelectrodes

An enzyme immunoelectrode system is defined as a combination of an immunoassay technique with an electrochemical determination [45]. The immunosensor is generally constructed from an immobilized antibody or antigen and an electrochemical sensor As with immobilized enzymes,one may either assay for an electroactive species potentiometrically[46], or amperometrically[47].The amount of antigen present will then be determined indirectly.The antibody could

be directly immobilized on a membrane mounted on an oxygen electrode[48], or employed as a flow-type immuno - reactor,coupled to an electrochemical sensor[47].

B.Response Time

The response time is often con - trolled by the immobilized biocata - lyst layer,rather than by the sensor response.To obtain a response , the substrate must(1)diffuse through the solution to the membrane surface,(2) diffuse through membrane and react with the biocatalyst at the active site,and(3)the products formed must then diffuse to the electrode surface where they are measured.Mathematical models describing this effect have been thoroughly presented in the literature[49-51].

Many factors that affect the speed of response are listed in Table 1 .

1.Rate of Diffusion of the Substrate

As has been shown for the amyg - dalin electrode(a cyanide sensing ion selective electrode covered with a layer of β- glucosidase),in an un - stirred solution the substrate diffu - ses to the membrane surface,albeit slowly,so that long response times are observed[52].At high stirring rates, convection increases the diffusion of the substrate to and through the mem - brane surface where it can react.With rapid stirring,a response time less than 30 s was achieved for the urea electrode.

Table 1.Factors affecting the response time of an enzyme electrode [a]

1.Chemical factors
1.1-Enzyme concentration
1.2-Substrate concentration
1.3-pH
1.4-Availability of cofactors
1.5-Activators
1.6-Inhibitors
2.Physical factors
2.1-Temperature
2.2-Thickness of enzyme layer
2.3-Stirring rate
2.4-Protective membrane thickness and Permeability
3.Instrumental factors
3.1-Sensor response time

[a] A fast response is defined as a low response time

2.Reaction with Biocatalyst in the Membrane

The rate of reaction will depend, according to the Michaelis - Menten equation,on the activity of the en - zyme and upon the various factors that affect it e.g.,pH, temperature, inhibitors,and the concentration of substrate.Moreover,the reaction rate depends on the thickness of the mem - brane layer in which reaction occurs, and on the pore size of the dialysis mem - brane,if used[50].On the other hand, the steady state potential obtained,

is dependent only on the substrate concentration and the temperature.

3.Effect of Substrate Concentration

As illustrated in Fig.3,the response to glucose of an immobilized glucose oxidase electrode, upon increasing the substrate concentration, the rate of reaction increases and a faster response time is observed. As an alternative to waiting until a steady state potential or current is reached,the rate of change in the current or potential(Δi or $\Delta E/\Delta t$) can be measured and equated to the concentration of substrate[35].

4.Effect of Biocatalyst Concentration

The activity of the biocatalyst will have two effects on an immobilized based electrode:(1)ensure that a linear plot of respcnse vs log substrate is obtained with a slope of 59.1 mV/decade and (2) effect a fast speed of response of the electrode . However,as the enzyme activity is increased,the effective thickness increases and the speed of electrode response decreases.This effect is due to a thickening of the membrane layer by the use of more weight of biocatalyst,resulting in an increase in the time required for the substrate to diffuse through the membrane. It is very important that about 20 U of enzyme activity be placed into as thin a membrane as possible for best results[53].

In the case of bacterial living cells the maximum synthesis of the selected enzyme(thus the optimal bacterial activity)can be obtained by optimum induction techniques in the appropriate growth media.

5.Effect of pH

Influence of pH on the response of the immobilized bioelectrode probes must be considered both by its effect on the immobilized system,and upon the detector performance.Indeed, every enzyme has an optimum pH at which it is most active and a certain range of pH in which it demonstrates activity[54].When the enzyme is immobilized,the optimal pH may shift depending on the nature of the carrier[3], and the potential at the electrode may be governed by the pH of the solution layer.A number of electrode redox processes,are known to be pH-dependent.Therefore,rigorous control of pH is required for optimal response. However,the apparent pH in the vicinity of the immobilized biocatalytic layer may differ from the solution pH[55] thus,a high buffer capacity is generally suggested to minimize this effect.

For the fastest and most sensitive responses,one should work at the optimum pH.This is not always possible, because the sensor electrode may not respond optimally at the pH of the enzyme reaction.Thus,a compromise

generally is necessary between these two factors.However,one should be careful not to force the pH of the biocatalyst system to conform with the pH requirements of the sensor[3].

C.Range of Substrate Determinable

All enzyme electrodes sense substrate in the general range of 10^{-2} - 10^{-5}M. A few other electrodes can sense even lower concentrations[3].In all cases,curves approximately Nernstian in the linear range,with a slope close to 59.1mV/decade,are obtained. All curves level off at high substrate concentrations,as predicted by the Michaelis-Menten equation, which states that the reaction becomes independent of substrate at high con - centrations.A leveling off of the curve at low substrate concentration is also observed,due to the limit of detection of the base sensor used. Higher sensitivities may be achieved when using amperometric devices [56], which consume the biocatalyst reac - tion product,thus,expanding the li - near response range.

VI.EFFECT OF INTERFERENCES

Any immobilized bioelectrode will be only as good as its overall selectivity.Interferences in the biosensor fall into two categories:interferences in the base sensor,and interferen - ces with the biocatalyst.

A.Interferences in the Base Sensors

Ideally,the sensor used to sense the products of,or reactants in, the biocatalyzed reaction should not respond to other substances present in the sample being assayed.Practically this requirement cannot always be met,using either potentiometric or amperometric methods.For example, an enzyme electrode for urea was prepared by immobilizing urease directly onto a cation selective electrode (which responds not only to NH_4^+ but also to Na^+ and K^+)[53,57]. Thus a problem arises in measurements in blood and urine.However,improvements in selectivity have been observed when using a solid antibiotic nonactin electrode as the sensor[58]. The nonactin electrode has a selectivity of NH_4^+/K^+ of 6.5 and NH_4^+/Na^+ of 7.5×10^2, thus partially eliminating the res - ponse to these ions by the sensor.Con - siderable improvement in selectivity may be achieved by using an ammonia electrode which senses only the NH_3 produced from the urea-urease reaction.The enzyme layer is placed directly onto the gas electrode, thus causing a large buildup of product at the electrode surface,yielding linear calibration plots at various pH va- lues.

Amperometric devices using solid electrodes operating in the redox mode at selected potentials are still subject to electroactive interfering substances.By far the most widely used

sensor,is the positively biased platinum electrode,due to its inherent sensitivity toward oxygen reduction and hydrogen peroxide catalytic oxidation.Some interferences are observed in the presence of easily oxidizable compounds like ascorbic acid, uric acid,acetaminophen,glutathione,cysteine and bilirubin [56,59,60].Carbon based electrodes offer several advantages with respect to low background currents and large available potential ranges,but they still require some surface modifications in order to achieve a greater selectivity.

B.Interferences in the Biocatalytic Reaction

Such interferences are substances that can react with the enzyme such as substrates activatiors or inhibitors.

If we consider an enzyme immobilized bioelectrode, the selectivity of such a probe is of course, related to the degree of purity of the availalbe enzyme.With some enzymes,such as urease,the only substrate that reacts at a reasonable rate is urea; hence, the urease-coated electrode is specific for this substrate[53,57].Uricase, likewise,is almost specifically for uric acid[61].Others,such as penicillin G,penicillin V,cyclibillin and dicloxacillin can all be determined with a penicillinase electrode(52,62).

Similarly,D-amino acid oxidase and L-amino acid oxidase are less selective in their responses [63,64]. The D-enzyme gives good response to D-phenylalanine,D-alanine,D-valine, D-methionine,D-leucine,D-norleucine, and D-isoleucine;the latter to L-leucine,L-tyrosine,L-phenylalanine,L-tryptophan,and L-methionine. Alcohol oxidase responds to methanol,ethanol, and allyl alcohol[65]. Hence, electrodes using these enzymes,respond to all the substrates present.Selectivity can only be achieved by prior separation of the amino acids.Greater selectivity in the assay of L- amino acids can be achieved by the use of a decarboxylative enzyme,which acts specifically on different amino acids [66,67].Enzyme electrodes of this type are known for L-tyrosine,L-phenylalanine,L-tryptophan,and others[3].

Glucose oxidase acts on a number of sugars[3], such as glucose and 2-deoxyglucose(main substrates), with cellobiose and maltose as secondary substrates.

The activity of the enzyme can be adversely affected by the presence of inhibitors.Generally,these are heavy-metal ions,such as Ag^{+}, Hg^{2+}, and Cu^{2+}, and sulfydryl-reacting organic compounds,such as P-chloromercuribenzoate and phenylmercury (II) acetate (due to their reaction with the free S-H groups present at the active site of many enzymes,especially the oxidase)[35].One important point to realize,however,is that the immobilized

enzyme is much less susceptible to inhibitors,especially weak or reversible inhibitors,due to limited accessibility to the microenvironment of the active site of the enzyme in the immobilization matrix.Thus by using the enzyme in an immobilized form , many interferences due to inhibitors are eliminated.

Bacterial immobilized electrods, as suggested earlier,may suffer interference from various substances due to the presence of many enzymes that can react with a number of different substrates.This is the case of the arginine electrode which responds also to glutamine and asparagine[68-70].

VII.EXAMPLES OF BIOELECTRODES

A list of some typical commercially available electrodes and their response characteristics are given in Table 2.A discussion of some of these probes described in the literature follows.

Table 2 - Properties of Commercially available enzyme electrodes[a]

Substrate	Linear Range,M	Lifetime	Base Sensor
Alcohol	2×10^{-6} to 2×10^{-3}	1 month	O_2 (H_2O_2)
L-Amino Acids	5×10^{-5} to 1×10^{-2}	3 month	O_2 (H_2O_2)
Ascorbate	1×10^{-6} to 5×10^{-4}	1 month	O_2
Aspartame	4×10^{-4} to 8×10^{-3}	< 1 month	NH_3
L-Aspartate	7×10^{-4} to 2×10^{-2}	1 month	NH_3
Glucose	3×10^{-7} to 6×10^{-3}	6 month	O_2 (H_2O_2)
Lactate	1×10^{-6} to 5×10^{-3}	1 month	O_2 (H_2O_2)
Lactose	1×10^{-5} to 1×10^{-3}	3 month	O_2 (H_2O_2)
L-Leucine	1.5×10^{-6} to 5×10^{-3}	1 month	NH_3
L-Lysine	1×10^{-5} to 2×10^{-2}	3 month	CO_2
Oxalate	1×10^{-6} to 3×10^{-4}	3 month	O_2 (H_2O_2)
Salicylate	1×10^{-5} to 7×10^{-4}	1 month	O_2
Sucrose	5×10^{-6} to 10^{-3}	3 month	O_2 (H_2O_2)
L-Tyrosine	1×10^{-5} to 5×10^{-4}	1 month	O_2
Urea	5×10^{-5} to 1×10^{-2}	6 month	NH_3
Uric Acid	6×10^{-6} to 6×10^{-4}	3 month	O_2

[a] Compliments of Universal Sensors,Inc.,P.O.Box 736,New Orleanes, LA 70148,USA

A.Urea Electrodes

Urease[E.C.3.5.1.5]catalyzes the hydrolysis of urea, with production of ammonium ion and bicarbonate(Eqn. 9):

$$(NH_2)_2CO+2H_2O+H^+ \xrightarrow{\text{Urease}} 2NH_4^+ + HCO_3^-$$

$$H^+ \uparrow\downarrow OH^-$$

$$NH_3 + H_2O \qquad (9)$$

The first potentiometric urea electrode was prepared by Guilbault and Montalvo[57],by immobilization of urease in a polyacrylamide matrix placed over an NH_4^+ responding cation selective electrode.Modifications of this electrode have focused on improvements in lifetime [53,71], and selectivity[58].

Urea electrodes based on measurement of pH were developed by immobilizing urease directly to a glass electrode[54,72]and onto an antimony metal electrode[73-75].

Still another possibility for urea electrode is the use of a urease covered carbon-dioxide electrode to measure the second product of the urea-urease reaction, HCO_3^- [66].

A biosensor has been described which monitors the change in conductance caused by the catalytic action of urease immobilized near to a planar micro-electronic conductance cell of defined geometry [76].

Finally,the best electrodes for determination of urea are generally based on the direct immobilization of urease onto the gas membrane of the ammonia sensing electrode[77-79].

This type of electrodes has the following characteristics : lifetime of 1 to 4 months,response range of $5x10^{-5}$ to $5x10^{-2}$M,pH range of 7 to 9 in Tris Buffer,response time of 1-5 minutes,and interference only from volatile amines present in the urea solution,due to the absolute specificity of the urease to urea[80].

B.Glucose,Carbohydrates and artificial Sweetener Electrodes

More than twenty-five years ago the first amperometric enzyme electrode was reported for the determination of glucose[81].The sensor utilized soluble glucose oxidase[E.C.1.1.3.4] held between 2 cuprophane membranes.The oxygen uptake was measured with a Clark oxygen electrode:

$$\beta\text{-D-glucose}+O_2+H_2O \xrightarrow{\text{glucose oxidase}} H_2O_2+\text{D-gluconic acid} \qquad (10)$$

Since then,a large number of glucose sensor devices have been developed for analytical,industrial and clinical purposes[35,82-91].Guilbault and Lubrano reported an improvement over the original Clark glucose electrode,by coating a layer of physically entrapped glucose oxidase in poly — acrylamide gel onto an O_2 electrode [35,84].

O_2 based enzyme electrodes have a life time of 1-2 months,a linear

response range of $1x10^{-4}$ - $2x10^{-2}$M,apH range of 7.2-7.4 in 0.01-0.02 M phosphate buffer containing KCl solution, with a response time of about 1-5 min. Major limitations of this type of biosensor for whole blood and plasma glucose determination are (i) oxygen tension variations,especially in low oxygen tension samples(ii) consumption of O_2 by ascorbic acid and(iii) problems and interferences at the working O_2 electrode,as described by Lucisano[92].

The amperometric monitoring of liberated H_2O_2(Eq.10) has achieved much popularity,since H_2O_2 is readily oxidized at platinum,gold,vitreous-carbon and other electrodes by an irreversible pH-dependend process [35].Guilbault and Lubrano constructed a simple,stable,rapid-reading electrode for glucose,by covering a metalic sensing layer(Pt-glass)[93] or a Pt electrode[35]with a thin film of immobilized glucose oxidase held by a cellophane membrane.When a difference of potential of 600 mV was applied between working electrode and Ag/AgCl reference electrode the current produced was proportional to the glucose concentration.Glucose oxidase has been immobilized by a number of different techniques,e.g. (i)crosslinking with Bovine Serum Albumin(BSA)and glutaraldehyde(GA)[93-95], (ii)covalent linkage to nylon mesh[96-99],on reconstituted active collagen membranes [56,100], oron immuno-affinity membranes(Biodyne A, Posidyne N_{66} and Pall-Biodyne)[101], and(iii)covalent attachment onto the electrode surface with or without electron mediators [102-106].These mediators have been incorporated directly into the electrode.The advantages of such biosensors have been already well discussed[107,108] and have lifetime of 1-2 months,a linear response range of $1x10^{-7}$ - $2x10^{-3}$ M,a pH range of 7.2-7.4 in phosphate buffer,with response time of 3 to 120s via a rate method.The interferences are the same as those listed above.

Another alternative method is the use of immobilized glucose dehydrogenase in the sensor together with NAD(P)/NAD(P)H as cofactores,according to the well known reaction:

$$\beta\text{-D-glucose}+\text{NAD(P)}\xrightarrow{\text{glucose dehydrogenase}}\text{D-gluconic acid}+\text{NAD(P)H} \qquad (11)$$

The glucose content is determined via monitoring the current due to the oxidation of NAD(P)H on Pt and/or carbon electrodes [109,110].

Commercial glucose sensors are based on the use of glucose oxidase immobilized on a Pt electrode, with measurement of either the H_2O_2 produced or the O_2 uptake.The properties of one such electrode are given in Table 2.

A two-enzyme electrode for mal-

tose determination using the same electrochemical detection has been designed with membranes prepared by asymmetrical coupling(one enzyme on each side of the membrane)[111]. The two enzymes used were glucoamylase and glucose oxidase.Glucoamylase[E.C.3.2.1.3]was immobilized on the membrane face exposed to the bulk phase into which the maltose-containing samples were injected.The hydrolysis of maltose occurs according to the reaction:

$$\text{Maltose}+H_2O \xrightarrow{\text{glucoamylase}} 2\beta\text{-D-glucose} \quad (12)$$

The glucose produced migrates through the membrane and is then oxidized on the inner face by immobilized glucose oxidase(eq.10),which is in close contact with the platinum disk.As with the monoenzyme system for glucose,the sensitivity and linearity were excellent.

Similarly bioelectrodes have been constructed for the determination of various other compounds with collagen membranes bearing mono- or multienzyme systems.A single multipurpose electrode with membranes bearing different oxidases ,has been described for the assay of galactose (galactose oxidase),starch(glucoamylase),and sucrose(invertase) [112]. In all electrodes it was found that asymmetrical coupling improved the electrode performance and all sensors could be used for hundreds of assays. Linearity was approximately 10^{-7} to 10^{-2} M for maltose or galactose,10^{-4} to 2×10^{-3}M for sucrose,and 6×10^{-5} to 10^{-3} for lactose.Several other amperometric glucose sensors have been developed using essentially the same method of enzyme immobilization [3,85].

Galactose has been successfully determined using galactose oxidase [E.C.1.1.3.9] immobilized electrodes, operating amperometrically either in the reductive mode[113,114](O_2 consumption)or in the oxidative mode (H_2O_2 production)(see Eq.13)in plasma and whole blood[115].

$$\text{D-galactose}+O_2 \xrightarrow[\text{oxidase}]{\text{galactose}} \text{D-galactohexodialdose}+H_2O_2 \quad (13)$$

A multi-enzyme electrode(Invertase[3.C.3.2.1.26],Mutarotase[E.C.5.1.3.3],glucose oxidase[E.C.1.1.3.4])for the determination of sucrose in food,feed and agricultural products,and in biotechnological applications,has been developed(Eq.14-16)[116].

$$\text{sucrose}+H_2O \xrightarrow{\text{invertase}} \alpha\text{-D-glucose}+\text{fructose} \quad (14)$$

$$\alpha\text{-D-glucose} \xrightarrow{\text{mutarotase}} \beta\text{-D-glucose} \quad (15)$$

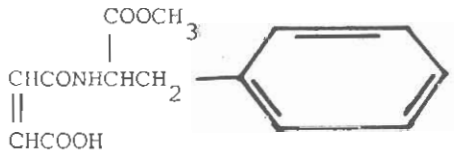
$$\beta\text{-D-glucose}+O_2 \xrightarrow[\text{oxidase}]{\text{glucose}} H_2O_2 + \text{gluconic acid} \quad (16)$$

The principle of the assay is based on monitoring the decrease of the O_2 concentration(Eq.16). The linear dynamic range was found to be between $3.3x10^{-5}$ to $1.3x10^{-3}$M,with a stability of 4 months,pH optimum 6.88 (0.2 M phosphate buffer),and a response time of 1 minute.Fructose,lactose,melibiose and raffinose did not interfere.

Recently,Thomas et al [117] have described another tri-enzyme electrode for sucrose. The enzymes β-fructofuranosidase,aldose 1-epimerase and glucose oxidase are used.Glucose interferes slightly,but interference from ascorbic acid was offset by working at lower potentials to measure the H_2O_2 produced a Pt indicator electrode with an electron mediator was used.

Finally,our group has developed two electrodes for the determination of aspartame(N-L-α-aspartyl-L-Phenyl alanine 1-methyl ester), commonly called Nutrasweet,in several food and carbonated beverages[118,119].In one case [118]L-aspartase [E.C.4.3.1.1] was chemically immobilized on an ammonia-selective electrode(Eq.17) .

aspartame $\xrightarrow{\text{L-aspartase}}$ NH_3 +



(17)

The electrode had a linear response in the $1x10^{-3}$-$1x10^{-2}$ M range with a slope of -30mV/decade.The sensor was stable for more than eight days.

An improved sensor[119]was constructed by coimmobilization of carboxypeptidase A[E.C.3.4.17.1]and L-aspartase[E.C.4.3.1.1]on an ammonia gas sensing electrode.The enzyme carboxypeptidase A specifically cleaves aspartame to L-phenylalanine and L-aspartic acid(Eq.18).The aspartic acid formed is then deaminated by L-aspartase with liberation of fumarate and ammonium ion(Eq.19).

aspartame $\xrightarrow{\text{carboxypeptidase A}}$ L-phenylalanine+L-aspartic acid (18)

L-aspartic acid $\xrightarrow{\text{L-aspartase}}$ fumarate+NH_4^+ (19)

The ammonium ion generated was sensed by the ammonia electrode, the steady-state potential being proportional to the activity of NH_4^+ ions (or NH_3),and hence to the concentration of aspartame in the solution. The electrode response was linear in the concentration ragne $4.25x10^{-4}$ to $8.10x10^{-3}$M with a slope of -45mV/decade.The electrode was stable for less than a month and was successfully used for assay of aspartame in several dietary products.

C.Amino Acid Electrodes

Although less selective in their

responses,amino oxidase immobilized electrodes have been successfully developed.

Guilbault and Hrabankowa[63,120] constructed the first L-amino acid electrodes(L-phenylalanine,L-leucine L-methionine,L-alanine,L-proline)by immobilization of L-amino acid oxi - dase[E.C.1.4.3.2]from snake venon over a monovalent cation electrode. The ammonium formed by the reaction:

$$\text{L-amino acid}+O_2+H_2O \xrightarrow[\text{oxidase}]{\text{L-amino acid}} \text{2-oxoacid}+NH_4^+ +H_2O_2 \qquad (20)$$

is monitored,and related to the concentration of L-amino acid.

Due to their high content of amino acid oxidase,some bacteria and tissue cells have been immobilized directly onto ammonia gas sensor for determination of the following substrates:Glutamine (Sarcema flava [27, 28],Magnolia grandiflora [121] and Chrysanthemum receptacle[122]),L-asparagine(Magnolia grandiflora [121] and Chrysanthemum receptacle[122]) , L-arginine(Chrysanthemum sepals [122],L-Serine(Clostridium acidiurici [123],Chrysanthemum receptacle [122], L-histidine(Pseudomonas[124]),L-as - parate(Bacterium cadaveris 125) and L-tyrosine (Aeromonas phenologenes [126]).

These L-amino acid eleotrodes utilized an ammonia gas sensor, in Tris Buffer in the pH range of 7.5 - 9.0 .The lifetime of these electrodes were 7-20 days with a linear range concentration of 10^{-2}-10^{-4}M.

An improved enzyme electrode , using L-aspartase[E.C.4.3.1.1] immobilized on a NH_3 gas sensor,has been described for the determination of L-aspartate in pharmaceutical products[126].The specificity of the electrode response to L-aspartate in the presence of various common amino acids,was reported.

The response range(7.0×10^{-4} - 2×10^{-2} M)and the slope(-58 mV/ decade of conc.)are comparable to those of the electrode reported by Kobos and Rechnitz[41],but with significant increase in stability(this sensor is stable for more than 20 days)dut tc the immobilization method used.

Other amino acid electrodes are based on the determination of O_2 consumption or H_2O_2 production(see eq. 20),or on measurement of CO_2 or NH_3 liberated in the enzymic reaction.

By immobilization of L- amino acid oxidase or living cells on the base electrode,L-methionine[69],L - leucine[69],L-phenylalanine[69].L - tyrosine,[69,125],L-cysteine[69],L - lysine[69],L-isoleucine[69]and glu - tamine[127]were determined. Cysteine [64],leucine[64],tyrosine[64],phenyl- alanine[64],thryptophane[64],methio - nine[64]and other amino acids [128] were determined by measurements of H_2O_2 formed (Eq.20)with a platinum or

carbon anode.

Also, by immobilization of the amino acid decarboxylase\or tissue cells on a CO_2 electrode the amino acids tyrosine [66,119] and L-glutamate [38]were determined.Finally,L-cys - teine electrode has been developed using immobilized bacteria(Proteus morgani)placed on gas sensors (H_2S) [130].

D.Alcohol and Electrodes

1.Alcohols

Alcohol oxidase [E.C.1.1.3.13] catalyses the oxidation of lower primary aliphatic alcohols[131]:

$$RCH_2OH+O_2 \xrightarrow[\text{Oxidase}]{\text{Alcohol}} RCHO+H_2O_2 \quad (21)$$

Several enzyme electrodes are reported for ethanol and/or methanol based on the detection of H_2O_2 pro - duction[86,132-135] or O_2 depletion [86,133,136-138].Ethanol has been determined over the range 0-10mg/100 ml.This procedure should be adequate for clinical determination of blood ethanol,since normal blood from in- dividuals who have not ingested ethanol ranges from 40 to 50 mg /100 ml.Methanol is a serious interference, however,its concentration in blood is negligible in comparison with that of ethanol[65].The response time of these electrodes is between 1-5 minutes,in pH 7.5,with a linearity of 10^{-9} to $2.5x10^{-8}$ M for methanol and 10^{-6}-$2x10^{-5}$M for ethanol, with a lifetime up to 10 days.

A major problem with alcohol described in the literature is the great instability of alcohol oxidase. Very stable alcohol probes are now offered by Universal Sensors (see Table 2 for properties)and Provesta (Bartlesville,Oklahoma).

Finally,an ethanol biosensor has been constructed by immobilization of alcohol dehydrogenase[E.C.1.1.1.2]with NAD^+[139,140]:

$$NAD^+ + C_2H_5OH \xrightarrow{\text{alcohol dehydrogenase}} NADH + CH_3CHO + H^+ \quad (22)$$

$$NADH \longrightarrow NAD^+ + 2e^- + H^+ \quad (23)$$

The NAD^+ was converted to NADH (Eq.22)by oxidation of ethanol by alcohol dehydrogenase,and the NADH formed (Eq.23)is oxidized electro - chemically to the original NAD^+,thus giving a well-defined linear- sweep voltammetric peak which is linearly related to the amount of ehtanol in the range $0.05-2.0x10^{-9}$M. The lifetime of the probe is only 1-2 days and its response time is 6-8 minutes, making it of only an academic interest.

2.Ascorbic Acid

L-ascorbic acid has been deter - mined in pharmaceutical and juice samples,by immobilization of the peel of cucumber(Cucumis sativus)[141],a tissue slice of squash(Cucurbita pe- po)[142]or bacterial (Enterobacter agglomeraus)[143]placed onto an O_2 electrode(Eq.24)in pH range 6.0- 6.5

(phosphate buffer):

$$\text{2L-ascorbic acid}+O_2 \xrightarrow[\text{oxydase}]{\text{ascorbate}} \text{2dehydroascorbate}+2H_2O \quad (24)$$

Calibration graphs based on the decrease in steady-state current were rectilinear in the concentration range of 2×10^{-5} to 5.5×10^{-2} M, with response time of 2-4 minutes and a lifetime of about 2-18 hrs or 5-100 determination.

3.Formate

Cells of Pseudomonas oxalaticus [144],which contain formate dehydrogenase and NAD^+ (E.C.1.2.1.2),were applied as a paste to a gas-permeable CO_2 electrode,then covered by a dialysis membrane(Eq.25):

$$\text{Formate}+NAD^+ \xrightarrow[\text{pH 5.9}]{\text{formate dehydrogenase}} CO_2+\text{NADH} \quad (25)$$

The electrode showed a rectilinear response of 10^{-4} to 6×10^{-2} M,With a slope of 38 to 49 mv/decade.Only a mode moderate response to pyruvate and lactate and slight response to oxalate was observed.

4.Lactate

Lactate oxidase [E.C.1.1.3.2] was immobilized onto both oxygen electrodes[145-147]and H_2O_2 sensor[148, 149].The concentration of L-Lactate over the range of 2×10^{-7}-2×10^{-4} M in plasma samples can be determined(Eq.26):

$$\text{Lactate}+O_2 \xrightarrow{\text{lactate oxidase}} \text{acetate} + CO_2+H_2O_2 \quad (26)$$

Lactate enzyme biosensors have also been constructed by immobilizing at a platinum electrode lactate dehydrogenase (E.C.1.1.1.27) and/or Bacteria (Escherichia Coli,Erythrocytes).An electron redox mediator is used with these sensors,either the couple ferrocyanide/ferricyanide 150-152 or NAD^+/NADH at a Clark oxygen electrode 153-157 .A very stable lactate electrode,operating in the range 1×10^{-6} to 5×10^{-3}M,with a lifetime of 1 month is sold commercially(Table 2).

5.Oxaloacetate

This electrode was constructed by immobilization of PEIQ-NH_2 artificial enzyme(partially quaternized polyethyleneimine containing primary amino-groups)onto a CO_2 electrode [158](Eq.27):

$$\text{Oxaloacetate} \xrightarrow{\text{PEIQ-}NH_2} \text{Pyruvate}+CO_2 \quad (27)$$

At a pH 4.5(0.05 M citrate buffer),oxaloacetic acid was determined over the concentration range of 7.8×10^{-4} to 5.6×10^{-3} M,with a response time of 15 minutes and a lifetime of 6 months.This sensor presented good selectivity with little response to other oxo-acids and also required no co-factors.

6.Oxalate

Oxalate has been determined in several samples(urine,plasma and food extracts)by immobilization of banana-skin pulp,oxalate decarboxylase or

oxalate oxidase[E.C.1.1.3.4] onto a potentiometric CO_2 electrode [159, 163], an amperometric oxygen electrode [160,161] or onto an amperometric H_2O_2 sensor[159,162]:

$$\text{Oxalic acid}+O_2 \xrightarrow[pH=4]{\text{oxalate oxidase}} 2CO_2+H_2O_2 \quad (28)$$

Linear calibration curve ranges from about 10^{-5} to 2×10^{-3} M, with a lifetime of 1-3 months are reported.

7.Pyruvate

Pyruvate has directly determined in cerebrospinal fluid using an immobilized lactate dehydrogenase pyrolytic carbon microelectrode[164].

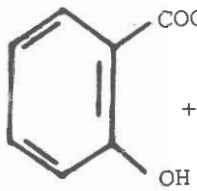
$$\text{Pyruvate}+NADH+H^+ \xrightarrow[pH=7]{LDH} \text{Lactate}+NAD^+ \quad (29)$$

The decrease of NADH concentration is linearly related to pyruvate in the range from 10^{-6} M to 2×10^{-3}M.

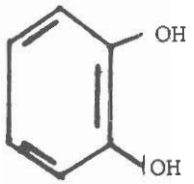
Mascini and Mazzei[164] determined pyruvate in serum by immobilization of pyruvate oxidase onto an amperometric sensor;as little as 10^{-6} M pyruvate can be monitored, and the biosensor has a lifetime of 1 month.

8.Salicylate

This substrate has been determined in blood serum by immobilization of salicylate hydroxylase[E.C.1.14.13.1]onto either a potentiometric CO_2[165]or an amperometric O_2 electrode[166]:



$$+NADH+H^+ +O_2 \xrightarrow[\text{hydroxylase}]{\text{Salicylate}}$$



$$+NAD^+ +H_2O+CO_2 \quad (30)$$

The response was linear in the range of 1×10^{-5} to 2.1×10^{-3} M of salicylate concentration.

9.Uric Acid

Uricase or urate oxidase[E.C.1.7.3.3]has been immobilized onto an amperometric O_2[61]or H_2O_2 sensors [167-169], and onto a potentiometric CO_2 electrode[170]:

$$\text{Uric acid}+2H_2O+O_2 \xrightarrow{\text{Uricase}} \text{allantoin}+H_2O_2+CO_2 \quad (31)$$

This sensor has a response time of 0.5-3 min,a linearity of 10^{-5} to 10^{-2}M,and a lifetime of 1 to 4 months.

The Universal Sensors electrode (Table 2)has a linear range of 6×10^{-6} to 6×10^{-4} M,a stability of 3 months, and is based on use of an O_2electrode.

E.Cholesterol Electrodes

Total cholesterol has been determined by coimmobilization of cholesterol esterase[E.C.3.1.1.13]and cholesterol oxidase[E.C.1.1.3.6] onto either an O_2 [171] or H_2O_2 electrode [172-174](see Eqs.32 and 33), and free cholesterol by immobilization of cholesterol oxidase onto an O_2 [171,

175] or a H_2O_2 electrode [111,112,176, 177](Eq.33).

$$\text{Cholesterol esters} + H_2O \xrightarrow[pH=7]{\text{cholesterol esterase}} \text{cholesterol} + \text{fatty acids} \quad (32)$$

$$\text{Cholesterol} + O_2 \xrightarrow[pH=7\text{-}7.5]{\text{cholesterol oxidase}} \text{cholest-4-en-3-one} + H_2O_2 \quad (33)$$

These sensors have been used in analysis of several biological materials with a stability of at least 15 days(>150 assays),a response time of 1-5 minutes and a linear rang of concentration from 10^{-7} to 8×10^{-3}M.

F.Penicillin Electrodes

Penicillin electrodes have been prepared by entrapping and/or immobilizing penicillinase[E.C.3.5.2.6] onto a glass electrode[54,184]and β-lactanase onto a thin film antimony-Sb_2O_3 electrode[179](Eq.34).

$$\text{Penicillin} + H_2O \xrightarrow{\text{penicillinase}} \text{penicilloic acid} \quad (34)$$

Change in pH were related to the penicillin concentration in the linear range of 3×10^{-4} to 7×10^{-3} M. The lifetime of these electrodes were between 15-60 days,with response times of 1-2 minutes.The main interferants are methicillin,6-amino penicillinate, cephalosporin and benzycephalosporin.

G.Creatinine Electrodes

Creatinine biosensors using soluble and immobilized creatininase (creatinine deiminase,E.C.3.5.4.21)or and an ammonia sensing probe have been described[180] ;

$$\text{Creatinine} \xrightarrow{\text{Creatininase}} \text{N-methyl hydantoin} + NH_3 \quad (35)$$

Low levels of creatinine in serum could be measured only if ammonia present in the sample is removed.An improved direct-reading specific electrode for creatinine was developed using a new enzyme from Carla- Erba [181].Linearity from 1 to 100 mg% was obtained.

An enzyms electrode system for the determination of creatinine and creatine was developed by utilizing three enzymes:creatinine amidohydrolase(CA),creatine amidinohydrolase (CI),and sarcosine oxidase(SO)[183]. These enzymes were immobilized onto the porous side of a cellulose acetate membrane with asymmetric structure,which has selective permeability to hydrogen peroxide.Two kinds of multienzyme electrodes were constructed by combining a polarographic electrode for sensing hydrogen peroxid and an immobilized CA/CI/SO membrane of CI/SO membrane for creatinine plus creatine or creatine,respectively.The multienzyme electrodes responded linearly up to 100 mg of

creatinine and creatine per liter in human serum.The response time was 20 s in the rate method with a detection limit of 1 mg/L.only 25 µL of serum sample is required. Analytical recoveries, precisions, and correlations with the Jaffe method were excellent, and the multienzyme electrodes were sufficiently stable to perform more than 500 assays.No loss of activity of immobilized enzymes was observed after 9 months of storage at 4°C in air.Recently,creatinine has been successfully determined in the range of concentration 0.1-5 mg l^{-1} by immobilizing glutamate dehydrogenase and creatinine deiminase on wet poly(vinyl chloride)membranes and detecting the ammonia liberated [184].

H.Implantable Biochemical Sensors

With recent advances in miniaturization of biosensors,new opportunities have risen for the developmemt of totally novel approaches to continuous medical monitoring of patients for important metabolites , such as drugs,enzymes,hormones,and blood gases,pH and electrolytes.Devices such as physiologically activated cardiac pacing systems and closed-loop insulin and other drug delivery units are already in wide uses[185].The goal is to develop miniaturized, physiologically compatible,biosensors, which could ultimately be used for in vivo measurements where it is often needed,i.e. the patient's bedside.The developments in implantable selective chemical sensors have been recently reviewed[185]. Ions such as potassium and sodium, gases like carbon dioxide,oxygen, as well as bicarbonate and pH in blood have been monitored continuously using the Chem FET technology. Using an enzyme electrode approach,the extracellular glucose concentration has been monitored on the peritoneum and the skeletal muscles in rabbits anesthetized with sodium pentobarbital and N_2O [190].Two stainless-steel electrodes with platinum sensor surfaces were constructed for the in vivo monitoring of glucose in blood for extended period of times[187].A graphite ferrocene-mediated glucose sensor has recently been tested for in vitro measurement[188].Under potentiostatic control,entrapped 1,1'-dimethyl ferrocene acts as an alternative electron acceptor to oxygen and which provides a suitable condition that is relatively undistrubed by possible fluctuations in in-vivo oxygen tension. Such an approach will undoubtedly pave the way toward devoloping a device for in vivo measurement of glucose in diabetics.In fact a pen type electrode for glucose is now commercially available(Exactatech, Baxter Travenol Co.)

I.Other Electrodes

The number of electrodes used today is large,and several types have been described in the literature.

A theophylline immuelectrode[48, 189],and immunoglobulin G electrode, [190],a monoclonal antibody electrode for antigen determination [191],an acetylcholine [192],a choline [192,193], a carbon dioxide [194,195],a hydrogen peroxide [196],a sulphite [197,198], a chloroform [199],a phenol electrode [200],vitamin B_{12} [201]and a NADH electrode [202] are examples of other sensors that have been developed.

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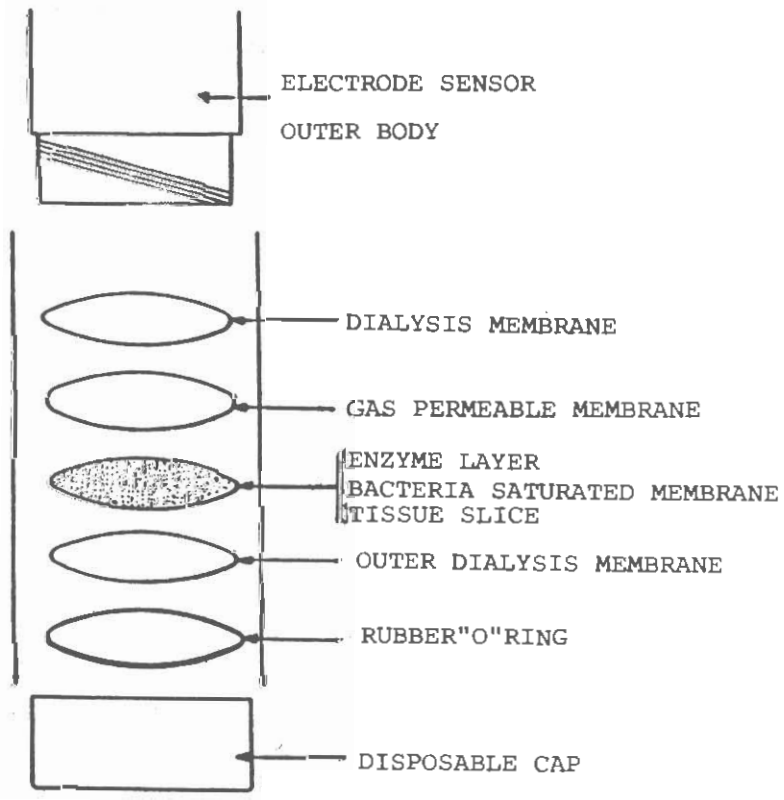

Fig.1.Detailed configuration of a biocatalyst immobilized biosensor(from ref.203).

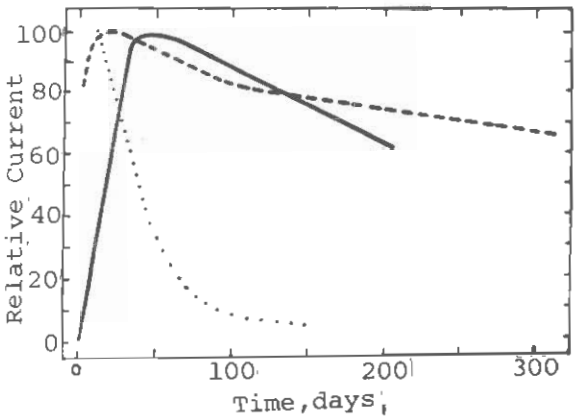

Fig.2.Long-term stability of glucose electrodes by the steady-state method.(---)Type 1 electrode, (——) Type 2 electrode , (...) Type 3 electrode.Type 1:chemistry bound,glutaraldehyde ; type 2:chemical bound,polyamide;type 3:physically bound(from ref.35).

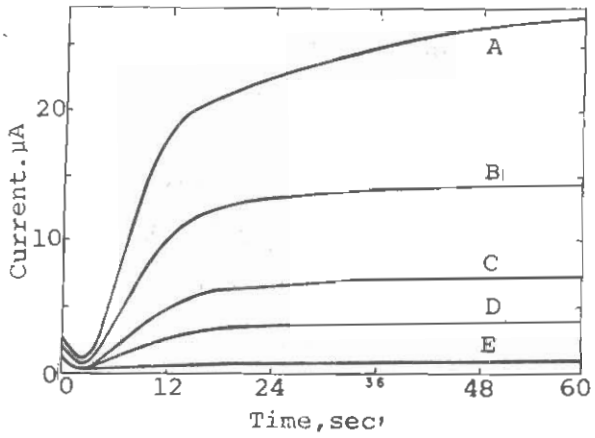

Fig.3.Family of current versus time curves for the glucose electrode poised at +0.6 V.Glucose solutions are in phosphate buffer, pH 6.0;ionic strength 0.1.Glucose concentrations;A=2.0×10^{-1} M;B=1.0×10^{-2}M; C=5.0×10^{-3}M ; D=2.5×10^{-3}M;E=5.0×10^{-4}M(from ref. 35).

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