Application of Amperometric Biosensors to the Determination of Vitamins and α-Amino Acids

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Received July 31, 2003

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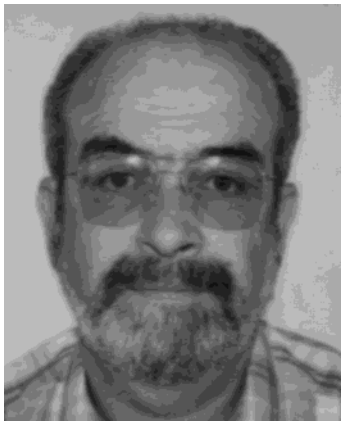


Prof. Francisco J. García Montelongo was born in Santa Cruz de Tenerife (Canary Islands, Spain). He received his M.Sc. (1961) and Ph.D. (1969) in Chemistry from the University of La Laguna. He was professor of Analytical Chemistry at the University of Bilbao (Spain) and since 1976 in the University of La Laguna. His main research interest is in the fields of biosensors, environmental pollution, and development of new analytical methodologies.

1. Introduction

At present, one of the most promising analytical fields is that of the biosensors. Since the first idea of utilizing enzyme reactions with electrochemical sensors,[1] a great number of papers have been published in this field. Biosensors are constituted by a biological structure (enzyme, cells, cell particles, tissues, antibodies, etc.) immobilized on a transducer. One of the main analytical advantages of biosensors is their high selectivity, which makes them able to recognize the analyte in a complex sample. So, sample treatment can be reduced to a minimum, avoiding any associated problems. There are some general reviews,[2−9] reviews on the application in the food industry,[10−23] and amino acid biosensor reviews.[24−27] In the present paper, we review the different aspects concerning amperometric biosensors and their application to the analytical determination of vitamins and α-amino acids that were published from 1981 to 2003.

1.1. General Concepts

A catalytic biosensor consists of an enzyme layer on a signal transducing device. The development of enzymatic biosensors is limited by the need to purify the required enzymes and the demanding conditions for preserving it and, often too, the pure enzyme without any cofactor is not efficient enough to cata-

10.1021/cr0304471 CCC: $48.50
Published on Web 06/29/2004

lyze the expected biochemical reaction. So, there has been a substantial amount of research into the ways to incorporate whole cells, pieces of cells, or subcellular elements from living microorganisms and animal and plant tissues into biosensors.

The use of living microorganisms, such as bacteria, due to their good catalytic behavior, abundant source, and lack of seasonal limitations, as possible alternatives to highly expensive isolated enzymes is a rapidly growing field. These microbial sensors offer several advantages over conventional enzyme electrodes; for example, the enzyme extraction and purification steps are eliminated, when the enzyme is not commercially available, the lifetime is increased as compared with isolated enzymes, and processes requiring enzyme sequences or cofactors are easy to achieve since the required substances are already present in the living cell. However, a bacterial biosensor using a microorganism with the variety of enzymes that are present could be disadvantageous, since it might not have the high selectivity to a particular chemical substrate that would be possible with a biosensor based on an isolated, purified enzyme. Almost every microorganism may be applied in the construction of biosensors.[6] They must fulfill at least one of these criteria: (a) aerobic uptake of oxygen in the respiratory process for assimilation of the substrates in which case an oxygen electrode may be used as the transducer, (b) catalytic activity of the microorganism linked to the bacterial respiratory chain so that direct electron transfer between oxygen and the electrode is established by means of mediators, and (c) electroactive products liberated as products of enzymatic reaction or biological activity of the cell. Then these products may be detected either by potentiometric or amperometric electrodes. Electrochemical transducers dominate in the construction of microbial sensors.[6]

Analogously, tissue-based sensors are advantageous in comparison with enzyme sensors, especially when the required enzyme is not available in purified form and the isolated enzyme is unstable, is too costly, or requires expensive cofactors for full activity. Thus, for example, slices of *Cucumis*[28,29] and *Cucurbita*[30,31] have been used due to the high content of ascorbate oxidase. The analytical use of vegetal tissues and crude extracts as enzymatic sources has been reviewed.[32]

The connection of the biochemical reaction with electrochemical detection sometimes causes difficulties, for example, if the experimental conditions for the biochemical reaction and the detector function are different. In such cases, it is advantageous to carry out the biochemical reaction separately and to determine the reaction product after changing the conditions. Such "enzyme reactor electrodes" enable a large choice of detection methods, kinds and forms of applied biologically active materials, and number of analyses provided per hour and the possibility of automation and, thus, are also preferred for automated methods of analysis.[6] Also, in contrast to enzyme electrodes, enzyme reactors generally consist of a relatively large amount of enzyme immobilized on the surface of an inert matrix. Therefore, an enzyme reactor can be more sensitive than an enzyme electrode with its limited amount of enzyme, as long as the mass transfer is not limiting.[33]

1.2. Immobilization of Enzymes or Whole Cells

There are many methods for immobilizing enzymes or whole cells.[5,34] A very interesting and critical review on general methods of immobilization was published.[5] The immobilization can lead to changes in enzyme structure and hence cause the kinetics, stability, and specificity to differ from that of the enzyme in homogeneous solution.[5,35] These methods use either physical or chemical means.

The physical means involve the following:

(1) Adsorption. Many types of supports, such as graphite powder,[31,36−39] graphite powder with paraffin wax[39] or with paraffin oil,[40,41] graphite−Teflon matrix,[42] Teflon with cyanoacrylate adhesive,[43] activated carbon,[44−46] activated beads,[47] ion-exchange resins,[48] polystyrene, silica gel, and alumina, have the ability to adsorb other materials. Preactivated membranes[49−52] have also been used. Sometimes, direct adsorption on the membrane of the electrodes has been used.[53−55] For example, the electrochemical deposition of platinum onto the platinum microelectrode surface results in a highly dispersed layer known as "platinum black". This surface is characterized by the presence of a deposited surface layer of porous microplatinum particulates, which possess a very large surface area, a high catalytic activity for electrolytic reactions, and a strong tendency to facilitate the adsorption of protein.[5,56,57] Nonadsorbed enzyme is removed by repeating washing in the working buffer.

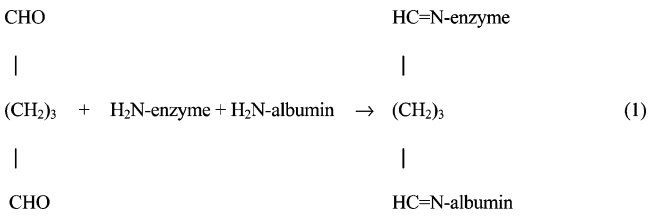
(2) Entrapment. Some polymers such as polyacrylamide, *o*-phenylene diamine−resorcinol polymer film,[58] polyion complex single or double membrane constituted by poly(L-lysine) and poly(4-styrene sulfonate),[59,60] pectin,[61] hydroxyethyl cellulose,[61,62] or Nafion film[63] are known to entrap biological compounds. An AQ polymer (polyester sulfonate material) was used in conjunction with a Nafion film.[64] The entire enzyme or a whole cell can be entrapped in the pores of the polymer. Pectin has been investigated and compared to hydroxyethyl cellulose (HEC) as a polymeric and hydrophilic immobilization matrix for stabilizing enzymes in amperometric biosensors.[61] Pectin can easily be reconstituted into a thin gel to entrap enzymes of choice, it forms a smooth viscous paste of screen-printable consistency with carbon particles in which enzymes can be included, it does not have interfering electrochemical properties under conditions usually found in electrochemical biosensors, and, finally, it is readily available at low cost. The use of pectin demonstrated increased stability for ascorbate oxidase with respect to HEC during a 25-week storage test period. Also, pectin-immobilized enzymes showed a greater stabilizing effect and reduced response variability, while larger signal currents were often observed for HEC-immobilized enzymes. The appropriate pectin concentration was determined by observation of the electrode response for (a) high-current response, (b) extended linear range of analyte concentration detection, and (c) response reproducibility.[61] Also, the enzyme can be

entrapped within a gelatin support on a cellulose membrane[65] or within a hollow microdialysis fiber.[66]

(3) Encapsulation. Enzymes can also be encapsulated in nylon or other materials by placing the enzymatic solution in a medium that, upon reaction, results in the formation of capsules.

Chemical methods produce more stable immobilized product than physical methods since the enzyme is tightly held to the solid support or, in some cases, is a part of it. Chemical immobilization can be achieved by the following methods:

(1) Cross-linking. Cross-linking reagents can be used to link the enzyme to a high molecular weight protein such as bovine serum albumin (BSA). Glutaraldehyde has been extensively used as the cross-linking agent,[29,56,58,62,67−84] where the two aldehyde groups of the glutaraldehyde molecule react with the amino groups of the enzyme and the BSA forming a viscous thick gel, which solidifies when the solvent evaporates.



Ageing of the stock glutaraldehyde solution before use greatly increased its ability to cross-link the proteins, probably due to the formation of glutaraldehyde oligomers. Aging consisted of exposure of the stock glutaraldehyde solution to air at room temperature for at least 24 h.[85]

Glutaraldehyde was also used with collagen,[28,86] gelatin,[87,88] avidin,[89] or egg albumin.[29] In other cases, some supports were activated with glutaraldehyde: controlled-pore glass beads,[33,74,90−96] polyion complex single or double membrane constituted by poly(L-lysine) and poly(4-styrene sulfonate),[59,60] cellulose membranes,[62,97,98] polycarbonate membrane,[99] silica gel,[100,101] or nylon membrane.[86,95,102] A packed enzyme bead electrode is inherently more sensitive than an electrode with enzyme localized by a membrane.[103] The enzyme immobilization on nylon consists[104] of functionalization of the nylon by immersing it in boiling dimethyl sulfoxide; after washing, a lysine arm is linked to the membrane, which is again reacted with glutaraldehyde. The final step consists of linking the soluble enzyme to the membrane via the glutaraldehyde terminal part.[104] Glutaraldehyde and BSA were also covalently bound on a preactivated Immobilon-AV affinity membrane.[35,49,50,105] In other cases,[106,107] two enzymes were cross-linked to a redox hydrogel polymer using poly(ethylene glycol) diglycidyl ether as cross-linker.

(2) Covalent attachment. Direct covalent immobilization can take place if the solid support contains carbonyl groups and the enzyme contributes an amino group that reacts to form a strong imine bond. Chemical techniques are usually used to create the carbonyl group on the surface of the solid support.

Some authors have used gelatin membranes,[77,108,109] collagen,[110,111] or CH-Sepharose 4B with 1-ethyl-3(3-dimethylaminopropyl)carbodiimide hydrochloride.[112] Also, L-amino acid oxidase was immobilized on a polytyramine electrode[113] or on a gold−poly(*o*-phenylene diamine) electrode,[114] both containing glutaraldehyde. Kuhr et al.[115,116] describe the immobilization and optimization of dehydrogenase enzymes onto microelectrode surfaces via avidin−biotin technology and a covalently linked hydrophilic tether. The avidin−biotin coupling strategy allows the selectivity of the electrochemical measurement to be easily changed without reoptimizing the immobilization conditions.[115]

(3) Electrochemical polymerization. It has been successfully used to immobilize enzymes on electrode surfaces and to reduce interferences and fouling in amperometric biosensors. Polymerization based on electrochemical oxidation of a given monomer from a solution containing the enzyme is the simplest method of enzyme immobilization in polymer on the working electrode surface and results in the formation of a conducting or nonconducting polymer layer containing entrapped enzyme molecules.[117,118] Polypyrrole and polyaniline polymer films are conducting; nonconducting electropolymers are poly-(*o*-phenylene diamine) and polytyramine. The ideal polymer should not be denaturing for the enzyme and should also be biocompatible and permselective to blood interferents if it has to be used in vivo.[64] The electropolymerization is most often done in aqueous solutions of pH close to neutral values to immobilize the enzyme without loss of activity. This methodology of enzyme immobilization seems to be especially attractive when the one-step procedure can be applied with exact control of the polymer layer based on measurement of the electrical charge passed during the electropolymerization process. This method is most commonly employed for obtaining amperometric biosensors,[33,43,52,55−58,62,73,78,79,82,83,95,113,114,118−134] although an increasing number of potentiometric biosensors have been recently reported in a review.[117] In addition, this method has a great number of significant advantages over other more conventional methods of biosensor fabrication: (a) the method is flexible and enables exact control of the thickness of the polymer layer based on measurement of the electrical charge passed during the electrochemical polymerization; (b) it is simple to carry out and can be readily controlled; (c) it allows the possibility to precisely electrogenerate a polymer coating over small electrode surfaces or with complex geometry. The polymer deposition is localized on the electrode surface, so the method is suited to the spatially localized deposition of enzymes onto microelectrode arrays. The immobilization of enzymes on microelectrodes can be used to produce biosensors for in vivo applications and for use with small sample volumes. In addition, the response times for microelectrodes can be much faster than those for the corresponding macroelectrodes.[121,123,132] The very small holes on the polymer layer can prevent small molecules from approaching the electrode surface, so elimination of electrochemical interferences can be achieved.[79,114,132] Thus, the presence of polyethylenimine (PEI) in working electrode pastes can improve response and stability of the

biosensor.[62,128,135] Precoating the Pt wire with lipid (phosphatidyl ethanolamine or stearic acid)[43,131] and incorporation of the protein BSA into the polymer matrix[43] were found to improve the performance of the biosensor. Also, conducting polymers can be combined with cross-linked polymethacrylate derivatives to produce hydrogel membranes. The high water content of these materials provides a biocompatible environment and a more favorable medium for the rapid movement of charge-neutralizing ions. Thus, polypyrrole,[56,118−120,122,125−127] polytyramine,[113] polyhistidine,[124] polyaniline,[132] poly(*o*-phenylene diamine),[33,43,55,78,79,82,83,95,114,122,123,125,130,131,133,134] phenylene diamine−resorcinol polymer,[58,73] or a polypyrrole and poly(*o*-phenylene diamine) bilayer membrane[129] have been used.

Immobilization of microorganisms is carried out by adsorption on different supports. Thus, polypropylene membrane,[29] paper,[136] membrane filter,[137] carbon paste electrode,[138] or alginate gel[139,140] are used. For example, cultured cells of *Proteus vulgaris* were mixed with sodium alginate and spread on a glass plate to make a membrane, which then was immersed in a $CaCl_2$ solution for immobilization. A piece of this membrane was sandwiched between a cellulose acetate dialysis membrane and the oxygen permeable membrane to construct the biosensor for phenylalanine. The assembled electrode was immersed in deionized water for 1 h before use.[139]

Several kinds of membranes or supports have been used for any type of amperometric biosensor. As inside materials, Teflon,[28,30,39,88,108,141−143] a polymer film of 1,2-diaminobenzene,[82] nylon net,[91] polyethylene membranes,[108,136] and a dialysis membrane of cellophane[29] are used, as well as cellulose acetate[29,50,75,99,105,141,142] or cellulose nitrate.[68] And as covering membranes, nylon net,[29,70,91] polyamide net,[30] poly(tetrafluoreethylene) membrane,[68] polyethylene,[71] polycarbonate (Nucleopore membrane),[36,50,53,97,99,105] plasticized polyurethane containing an ether crown carrier,[144] or most frequently, dialysis membranes,[66,67,72,74,90,102,108,123,136] such as acetylcellulose,[61,139,141−143] are used to confine the biological immobilized material.

1.3. Amperometric Biosensors

An important group of enzymes are the oxidoreductases, which have traditionally been classified into two main groups, the flavoprotein oxidases and the $NAD(P)^+$/NAD(P)H-dependent dehydrogenases. If the enzyme is an oxidase, the detection of the O_2 consumed or of the H_2O_2 produced is possible. The second type of enzyme implies detection of an enzymatic cofactor or of an oxidoreduction mediator that catalyzes the electron transfer between the electrode and the cofactor.

While enzymes used in the construction of biosensors can be quite selective, the selectivity of the biosensor may be limited by, for example, pO_2 dependence and a possible interference due to other electrochemical substances because of the high potentials required. The analyte solution usually contains other compounds, which, if electroactive, can be oxidized nonselectively or otherwise foul the electrode by adsorption onto its surface, resulting in significant interference with the signal from the substrate under study. Furthermore, when the concentration of the substrate under study is very low, this background current may become significantly large in comparison with the substrate-generated signal. This is a major factor limiting the use of biosensors in biological fluids or food samples. Various methods have been developed that attempt to solve this problem, including the use of multienzyme systems[49,50] and pre-electrolysis techniques.[54,57,77] This latter method of pretreatment[54] eliminates electrochemically active compounds by electrochemical oxidation. On the other hand, metallization of carbon paste electrodes can be applied to decrease the oxidation potentials.[145,146] Carbon pastes doped with different metals (Pt, Pd, Rh, and Ru) exhibit different electrocatalytic activities, background current contributions, and hence analytical performances, such as enhancement of flow injection and biosensing operations.[145] The metal-dispersed carbon paste electrodes combine the efficient electrocatalytic activity of metal microparticles with the attractive properties of carbon paste matrixes (surface regeneration/modification). Coimmobilization of an oxidase enzyme allows coupling of a biocatalytic reaction with the electrocatalytic detection of the liberated peroxide. The overvoltage for the oxidation process is much lower at the metalized surfaces ($Pt > Ru \gg Pd$).[145] An unusual property found in the case of both Ru and Rh is their capacity to reduce H_2O_2 at positive potentials. This leads to the possibility of measuring large reduction currents at low positive potentials.[146] Thus, for example, when horseradish peroxidase (HRP) is coimmobilized with a hydrogen peroxide-producing oxidase, amperometric biosensors can be made that respond to the substrate of the oxidase within a potential range essentially free of interfering electrochemical reactions.[147] There are many authors who use HRP.[42,47,56,58,81,84,89,93,106,110,135,148−150]

One of the major obstacles to be solved in the construction of enzyme-based amperometric biosensors is how to optimize the electron transfer reaction between the cofactor of the redox enzyme and the electrode. All redox enzymes rely on a cofactor as the redox active compound for activity. In all redox enzymes, except nicotinamide-dependent dehydrogenases, the cofactor is strongly bound within the enzyme structure causing steric hindrances for a direct electron transfer between the active site of the enzyme and the electrode. To overcome these problems, mediators have been used together with oxidoreductases for the electrochemical detection of substances.[151] There are many flavoenzyme-based amperometric sensors. In the following reaction, the substrate first converts the oxidized flavin adenine dinucleotide (FAD) center of the enzyme into its reduced form:

$$SH_2 + E(FAD) \rightarrow S + E(FADH_2) \qquad (2)$$

where SH_2 is the reduced form of the substrate and E(FAD) is the oxidized form of the flavoenzyme. Because the flavin redox centers are located well within the enzyme molecule, direct electron transfer

to the surface of a conventional electrode does not occur to a substantial degree. The usual method of measuring the amount of reduced oxidase, and hence the amount of substrate present, relies on the natural enzymatic reaction:

$$E(FADH_2) + O_2 \rightarrow E(FAD) + H_2O_2 \quad (3)$$

where oxygen is the electron acceptor for the enzyme. The oxygen is reduced by the $FADH_2$ to H_2O_2, which may then diffuse out of the enzyme and be detected electrochemically. In recent years, enzyme electrodes have been developed that use a nonphysiological redox couple to shuttle electrons between the $FADH_2$ and the electrode by the following mechanism:

$$E(FADH_2) + 2M_{ox} \rightarrow E(FAD) + 2M_{red} + 2H^+$$

$$2M_{red} \rightarrow 2M_{ox} + 2e^- \quad \text{(at the electrode)} \quad (4)$$

In this scheme, the mediating system M_{ox}/M_{red} is assumed to be a one-electron couple, such as ferrocene. There are other planar organometallic complexes that can acquire a stacked configuration to display metallic properties such as conductivity. These conducting organic salts have high electrocatalytic properties, long-term stability, and minimal susceptibility to fouling.[5] The most frequently employed are 7,7,8,8-tetracyanoquinodimethane as the electron donor and tetrathiafulvalene,[73] *N*,*N*-dimethyl-7-amino-1,2-benzophenoxazinium (Meldola blue), or *N*-methylphenazinium[108] as the electron acceptor. To prevent these mediators from diffusing out of the device, it is possibly to compress and mold them to attach them by adsorption or covalently to a polymer backbone.[5] In this way, electrical communication between the redox center of the enzyme and the electrode is attained. Thus, a new electron-transfer relay system based on a ferrocene-modified siloxane−ethylene oxide branch copolymer has been used[37] to achieved electrical communication between the FAD redox center of L-glutamate oxidase and a conventional carbon electrode. With use of cyclic voltammetry and stationary potential experiments, it was shown that spectrographic graphite electrodes modified with this redox polymer and L-glutamate oxidase respond rapidly to the addition of glutamate with steady-state current responses achieved in less than 1 min.[37]

Thus, a mediator is a low molecular weight redox couple that shuttles electrons from the redox center of the enzyme to the surface of the indicator electrode. The electron transfer kinetics at the electroactive surface must be reversible. The reoxidation of the mediator can be measured and correlated with the concentration of the analyte. The mediator must be stable in both its oxidized and reduced forms and must be easily immobilized on the electrode surface or within the enzyme layer (adsorption, co-deposition with a polymer coating, or covalent attachment). A distinctive feature of such sensors is the relatively low operating potential therefore producing less interference problems. However, a limitation of these designs is the reduction in the cathodic current due to the competing reaction between the reduced $FADH_2$ centers of the oxidase and the redox mediator. 1,1′-Dimethylferrocene has been widely used as mediator[36,71,79] because it replaces oxygen, the natural acceptor for oxidases. Direct-current cyclic voltammetry was used[36] to investigate the suitability of this ferrocene derivative as a mediator with galactose, glycolate, and L-amino acid oxidases. All the electrodes responded rapidly to millimolar concentrations of their respective susbstrates producing 95% of the steady-state current response in <2 min.[36] There are other mediators, such as other ferrocene derivatives,[47,58,84,152] hexacyanoferrate(III),[20,52,63,65,72,153,154] polymeric toluidine blue-O,[38,39] phenazine methosulfate,[72,102,119,120,155,156] quinones,[138,144,157] $[Os(bpy)_3](PF_6)_2$[45,46] or the hydrogel formed from osmium−poly(vinyl pyridine)-containing horseradish peroxidase (Os-gel-HRP),[81,84,93,110,111,149] or another redox hydrogel composed of $Os(4,4'\text{-dimethylbpy})_2Cl$ complexed with poly(1-vinyl imidazol) (PVI_{19}-dmeOs) and HRP:[106,107]

$$H_2O_2 + \text{ferric HRP} \rightarrow \text{HRP−compound 1} + H_2O$$

$$\text{HRP−compound 1} + \text{osmium(II)} \rightarrow \text{HRP−compound 2} + \text{osmium(III)}$$

$$\text{HRP−compound 2} + \text{osmium(II)} \rightarrow \text{ferric HRP} + \text{osmium(III)} \quad (5)$$

or in another way,

$$H_2O_2 + 2Os(II) + 2H^+ \xrightarrow{HRP} 2H_2O + 2Os(III) \quad (6)$$

Electrochemical polymerization of toluidine blue-O and its electrocatalytic activity toward NADH oxidation has been studied.[158] Karyakin et al.[63] showed that through optimization of the deposition of Prussian blue, both stable and selective electrocatalysis to H_2O_2 reduction in the presence of oxygen is achieved. The current of H_2O_2 reduction on specially deposited Prussian blue at around 0 V vs Ag/AgCl is at least 100 times higher than the background oxygen reduction. This is even higher than that on peroxidase-modified electrodes. When the amount of Prussian blue deposited was in the range of 6−10 nmol cm^{-2}, the heterogeneous rate constant for H_2O_2 reduction varied from 0.015 to 0.02 cm s^{-1}.[63] For comparison, the kinetic constant for H_2O_2 oxidation on platinum in neutral media is more than 1000 times lower. The Prussian blue film shows a marked decrease in the overvoltage and a large increase in the redox currents. Also, the electrocatalytic reduction of H_2O_2 is more effective.[153] Due to both its high activity and selectivity in H_2O_2 reduction, these investigators[63] denoted their specially deposited Prussian blue as "artificial enzyme peroxidase". Also, fundamental aspects on Prussian blue and other hexacyanoferrates have been reviewed.[159,160]

2. Analysis of Vitamins

Table 1 shows the experimental conditions and characteristics of the biosensors studied. Only data about three vitamins have been found in the period reviewed.

Table 1. Experimental Conditions and Analytical Properties of Amperometric Biosensors Applied to the Analysis of Vitamins

Analyte	Immobilized system	Experimental conditions	Response time (time of analysis)	Linearity (DL) mM	CV %	Lifetime	Ref.
L-ascorbic acid in food	Peel of *Cucumis sativus* (L-ascorbic oxidase)	Oxygen and Pt electrodes, (-2)-(+2) V, 25°C, pH 6.5	1 min	0.05-0.5 -	2.3	3 w 5°C pH 6.5	28
L-ascorbic acid in fruit juices and pharmaceuticals	L-ascorbic oxidase from *C. sativus*	Au and oxygen-Ag/AgCl electrodes, 0.8 V, 35°C, pH 7.5	45 s	0.05-1.2	< 2.7	2 m 4°C	88
L-ascorbic acid in fruit juices	*C. sativus* juice (L-ascorbic oxidase)	Au and oxygen-Ag/AgCl electrodes, -0.7 V, 25°C, pH 7.0	- (3-6 min)	0.25-1.6 -	4.5-5.4	11 d 4°C	44
L-ascorbic acid	*C. sativus* juice (L-ascorbic oxidase)	Porous carbon felt, Au and oxygen-Ag/AgCl electrodes, -0.7 V, 25°C, pH 7.5	2-3 min	0.02-0.4 ($5\cdot10^{-3}$)	5	5 d 4°C	118
L-ascorbic acid	*C. sativus* juice as carrier (L-ascorbic oxidase soluble enzyme)	FIA, 4.8 ml/min, 25°C Au and oxygen-Ag/AgCl electrodes, -0.7 V, pH 7.1	- (1 min)	0.5-7 (0.1)	4	8 d	161
L-ascorbic acid in fruit juices	L-ascorbic oxidase	Reactor, 3 electrodes (GCE as working electrode) 0.6 V, 25°C, pH 5.5	2 min (30 samples/h)	0-$2.3\cdot10^{-3}$ ($2.3\cdot10^{-5}$)	1	<3 m 4°C	48
L-ascorbic acid in fruit juices	Slices of *Cucurbita pepo*	Oxygen and Ag/AgCl electrodes, -650 mV, 30°C, pH 6	70-90 s	0.02-0.57 (0.02)	3	50-80 runs room-t pH 6	30
L-ascorbic acid in fruit juices and human plasma	Ascorbate oxidase from zucchini squash	Reactor, 2 ml/min, 22°C DME, SCE and Pt electrodes, -1.25 V, pH 6.2, triphenylphosphine oxide, Triton X-100 or Revopol TLS 40	- (60 samples/h)	$3\cdot10^{-4}$-0.5 ($3\cdot10^{-4}$)	4	1 m 4°C pH 6.2 NaN_3	112
L-ascorbic acid in food	*Thiobacillus ferrooxidans* (iron oxidase)	FIA, 4 ml/min, 30°C oxygen electrode, pH 2.5	2 min (5 min)	0-2.5 (0.1)	4.1	30 d 5°C pH 2.5	137
L-ascorbic acid	*Enterobacter agglomerans*	Oxygen electrode, 25°C, pH 6.5 bacterial electrode	2.5-3 min	$4\cdot10^{-3}$-0.7 ($4\cdot10^{-3}$)	<2.6	10-11 d 4°C pH 6.5	29
	Slices of *Cucumis sativus*	" tissue electrode	1.5-2 min	$4\cdot10^{-3}$-1 ($4\cdot10^{-3}$)	<2.9	5-6 d 4°C pH 6.5	
	Ascorbate oxidase (adsorbed enzyme)	" enzyme electrode (soluble enzyme)	4-5 min	$4\cdot10^{-3}$-0.5 ($4\cdot10^{-3}$)	-	2-3 d 4°C pH 6.5	
	Ascorbate oxidase covalently bound	" enzyme electrode (covalently bound)	2-2.5 min	$4\cdot10^{-3}$-0.9 ($4\cdot10^{-3}$)	<2.5	16-18 d 4°C pH 6.5	
L-ascorbic acid	L-ascorbic oxidase	Carbon, rhodinised carbon and Ag/AgCl electrodes, 350 mV, 25°C, pH 6.3	200 s	-	< 7.6	25 w 4°C or 25°C pectin	61
L-ascorbic acid in brain	Ascorbic oxidase	Reactor, GCE and Ag/AgCl electrodes, 0.8 V, pH 5.6	-	0-0.4 (10^{-5})	1-3	-	162
L-ascorbic acid in food	L-ascorbic oxidase	-	-	0-3	5	-	163
Ascorbic acid in pharmaceuticals	Peel of *Cucúrbita pepo* (peroxidase)	Pt, Ag/AgCl and CPE electrodes, -0.14 V, room-t, pH 6.5	-	0.2-5.5 ($2.2\cdot10^{-2}$)	< 1.3	4 m 4°C	31
Ascorbic acid	No enzyme Polyhistidine coated glassy carbon electrode	SCE, Pt and modified GCE electrodes, 0.07 V, pH 4	-	0.05-2 (0.02)	1.6	-	124
Ascorbic acid	No enzyme 3,4-dihydroxybenzoic acid and aniline copolymerized (electroactive and mediator composite)	SCE, Pt and microdisk gold electrodes, 02V, 20°C, pH 7	2 s	0.1-10 (0.05)	1.4	1 m 4°C pH 7	132
L-ascorbate in brain tissue	Ascorbate oxidase and horseradish peroxidase	Carbon fiber microelectrode and Ag/AgCl electrode, 0.1-0.2 V	20 min	- ($2\cdot10^{-3}$)	10	-	148
Nicotinic acid	*Pseudomonas fluorescens*, benzoquinone as mediator	Pt disk, Ag/AgCl and CPE electrodes, 0.5 V, 25°C, pH 7.0	80 s	0-6 ($5\cdot10^{-3}$)	8	2 w 5°C pH 7	138
Vitamin B_{12}	*Escherichia coli*	Pt, Pb and oxygen electrodes 35°C, pH 7	2 h	$5\cdot10^{-3}$-$25\cdot10^{-3}$ mg/l	-	25 d -25°C	143

Ascorbate oxidase catalyzes the reaction

$$\text{L-ascorbic acid} + {}^1/_2O_2 \xrightarrow{\text{ascorbate oxidase}} \text{L-dehydroascorbic acid} + H_2O \quad (7)$$

and can be employed for the determination of L-ascorbic acid. This enzyme was immobilized on a Clark oxygen electrode.[28] The enzymatic reaction of ascorbate oxidase follows the ping-pong (double displacement) mechanism. D-Glucose gives no serious interference, even at a concentration of 300 mM. Cupric and ferric ions, which catalyze the auto-

oxidation of L-ascorbic acid, did not interfere in concentrations up to 0.1 and 0.5 mM, respectively. These interferences can be eliminated by the addition of EDTA.[28,48] No effect of citric acid was seen in concentrations up to about 24 mM, which corresponds to 0.5%.[28] The results for the determination of L-ascorbic acid in fruit juices were compared to those obtained by the 2,6-dichlorophenolindophenol and 2,4-dinitrophenylhydrazine methods. The former method gave smaller results than the biosensor and is unsuitable for colored samples. However, the results by the latter method compare well with the results obtained with the biosensor, except for *Citrus unshiu* juice.[28] Ascorbate oxidase was immobilized directly[88] on a pretreated Teflon membrane by copolymerization with gelatin and glutaraldehyde. The effect of temperature was studied. Deviations from linearity at high concentrations occurred at 35 and 40 °C and are due to the insufficient amount of dissolved oxygen, because higher enzyme activity is observed at these temperatures. Interferences from catechol and hydroquinone were not important. Results obtained with this sensor and with the 2,6-dichlorophenolindophenol method showed recoveries in the 99−102% range.[88]

Cucumber juice solutions were used[44,118,161] as an enzyme solution of ascorbate oxidase. The cucumber juice was immobilized on a porous carbon felt surface by impregnation[44] or by electropolymerization of pyrrole[118] and then by combining this immobilized carbon felt and an oxygen electrode. The cucumber juice solution was used as the carrier solution in a flow-injection method.[161] Because the biological fluid is not immobilized on the transducer, this probe is not a biosensor. The oxygen consumed by the enzyme reaction is compensated spontaneously from air in the container for the cucumber juice or by the dissolved oxygen, and the juice can be reused by recycling.[44,161] Sodium azide was added to prevent putrefaction; the concentration added is critical because high concentrations inhibit the ascorbate oxidase activity.[44,48,161] No interference from glucose, catechol, and sugar were found.[161]

Another biosensor was constructed by immobilizing a slice of the mesocarp of squash (*Cucurbita pepo*) or cucumber (*Cucumis sativus*) to a Clark-type oxygen electrode.[30] Different slices from the most active layer of the same fruit can, however, differ very significantly in their activity. Thus, when slices are changed, a recalibration is always essential. The way in which the slice is placed on the sensitive part of the pO_2 electrode can also influence the sensitivity of the biosensor. In the course of placing the same squash slice 10 times, the RSD of the slopes of the calibration graphs was 8.7%. In isotonic NaCl solution, the slices quickly lost their enzyme activity. For long-term storage of slices, at least for 5 months, the best preservative proved to be glycerol or the antiseptic merthiolate, and squash slices were more stable than cucumber slices. In the analysis of fruit juices stored under nitrogen gas to prevent the loss of ascorbic acid, a correction must be made for the drop in oxygen level after injection of the same volume of oxygen-free water. The corrected values of ascorbic acid were in good agreement with those from differential pulse polarography.[30] Pulse polarographic detector in a reactor was used.[112] The stability of the signal was improved when a surfactant such as triphenylphosphine oxide was present in the carrier solution. However, with human plasma samples, a strong noise due to the high concentration of surfactants in the plasma was observed. The addition of Triton X-100 and Revopol TLS 40 to the carrier solution eliminated the spurious signal.[112] Other authors[164] used a biosensor that consists of an oxygen electrode and a biocatalytic membrane of *Feijoa sellowiana* tissue, rich in ascorbate oxidase, for the direct determination of ascorbic acid in food, beverages, and pharmaceutical formulations. The biosensor, easy to prepare and to use, was endowed with an extended range of linearity (over two decades of ascorbic acid concentration) and a remarkable reproducibility of results.[164]

The organophosphorus pesticide ethyl paraoxon inhibits the activity of ascorbic acid oxidase. So, a biosensor with tissues of cucumber immobilized was used for the detection of this pesticide.[165] A measurable inhibition was obtained with 10 min incubation of the tissue electrode with different concentrations of the pesticide.

A microbial sensor takes advantage of the iron-oxidizing activity of *Thiobacillus ferrooxidans*.[137] By adding ascorbic acid to the solution of ferric iron, Fe^{3+} reduces to Fe^{2+} as ascorbic acid oxidizes to dehydroascorbic acid. The resulting Fe^{2+} is oxidized by *T. ferrooxidans* but the ascorbic or dehydroascorbic acids are not. By monitoring the decrease in oxygen concentration during the oxidation of Fe^{2+} with an oxygen electrode, a calibration curve relating the electrode response and added ascorbic acid concentration will be obtained. There was no significant response to organic compounds such as ethanol, sodium pyruvate, formic acid, glycine, and glucose, because *T. ferrooxidans* is autotrophic. However, the microbial sensor responded to ferrous sulfate and sodium sulfite, as well as to ascorbic acid. Thus, the sensor would be applicable to the measurements of samples containing various organic compounds but not ferrous iron or reducing agents other than ascorbic acid.[137]

A comparative study was carried out[29] on the advantages of four types of biosensors for ascorbic acid: (a) soluble enzyme (fixed directly on the electrode surface using a dyalisis membrane); (b) enzyme cross-linked with glutaraldehyde with or without egg albumin; (c) tissular electrode (slices of *Cucumis sativus*); (d) bacterial electrode (*Enterobacter agglomerans*). The soluble enzyme electrode is not useful because of its low stability, long response time, and higher influence on the response due to the temperature than the other biosensors. For the immobilized enzyme, the response time was independent of the immobilization technique and of enzyme concentration in the range 10−170 U. For the tissular electrode, the enzymatic activity varies among different cucumbers. The increment in ionic strength of buffer causes higher responses in the tissular electrode followed by the enzymatic and finally by the bacterial

electrode. A negative interference due to the catalytic effect of Fe^{3+} on the autoxidation of the ascorbic acid was found. The cupric ions increment the response because the formation of H_2O_2 is more rapid. Other interferents are glucose, D-fructose (mainly in the bacterial electrode), D-galactose, and lactose (only in the bacterial electrode).[29]

A novel approach has been adopted[129] to investigate from a kinetic point of view the processes connected to ascorbate autoxidation by O_2. In particular, H_2O_2, present as a stable intermediate in the reaction mixture, was monitored amperometrically by a specially modified electrode able to drastically prevent the simultaneous electrooxidation of ascorbate. This peculiar experimental technique allows one to obtain an interesting picture of the first steps of the ascorbate−O_2 reacting system,[166,167]

$$\text{ascorbic acid} + O_2 \rightarrow \text{dehydroascorbic acid} + H_2O_2$$

$$\text{ascorbic acid} + H_2O_2 \rightarrow \text{dehydroascorbic acid} + 2H_2O \quad (8)$$

and shows that other processes probably involving the byproduct of ascorbic acid, dehydroascorbic acid, have to be considered together with these reactions. A Pt electrode modified by an electropolymerized membrane entrapping glucose oxidase proved able to detect very low concentrations of H_2O_2 in the presence of much higher amounts of ascorbate. The presence of metal ion traces in the reaction mixture proved to be responsible for the nonlinear dependence of the rate of both reactions on the concentration of ascorbate: a mechanism involving the role of ascorbate−metal complexes as the reactants was hypothesized to explain this result.[129]

Peroxidase in the presence of H_2O_2 catalyses the oxidation of hydroquinone to *p*-quinone:

$$\text{hydroquinone} + H_2O_2 \xrightarrow{\text{peroxidase}} \text{quinone} + H_2O$$

$$\text{quinone} + \text{ascorbic acid} \rightarrow \text{hydroquinone} + \text{dehydroascorbic acid} \quad (9)$$

A biosensor with immobilized peroxidase for L-ascorbic acid determination has been proposed.[31] When L-ascorbic acid is added to the solution, it can reduce *p*-quinone to hydroquinone or reduce H_2O_2 decreasing the peak current obtained proportionally to the increase of L-ascorbic acid concentration. Oxygen is not consumed in the reaction.[31,151] The effect of dissolved O_2 on the biosensor response[31] was evaluated in air-saturated and in degassed conditions. From the results obtained, it was found that O_2 has very little effect on the biosensor response in the concentration range studied. Anyway, deoxygenation with N_2 of all solutions was carried out. No interference from glucose, lactose, sucrose, starch, magnesium stearate, sodium chloride, or acetylsalicylic, oxalic, citric, and tartaric acids was detected.[31] Also, H_2O_2 sensors were fabricated[150] by combining two kinds of bienzyme membranes (ascorbate oxidase or tyrosinase and peroxidase) with an oxygen electrode.

The electrochemical determination of ascorbic acid by direct oxidation at a conventional electrode may be difficult because of the high potentials required, which can foul the electrode by adsorption of oxidation products onto its surface. To resolve this overpotential problem, some active mediators for the oxidation of ascorbic acid dissolved or immobilized at the electrode surface have been used. However, the stability of these electrodes was not good.[124] Polymer-modified electrodes have also been widely used for the immobilization of mediators or enzymes[118,124,132] and a polyhistidine chemically modified electrode[124] was used in the catalytic oxidation of ascorbic acid. The overpotential was reduced by 400 mV. The selectivity is excellent and allows the determination of ascorbic acid in the presence of dopamine, because the catalytic behavior results in the voltammetric resolution of both analytes in a mixture. Copolymerization of 3,4-dihydroxybenzoic acid and aniline at a microdisk gold electrode was also studied.[132] Results are similar for both systems. Although these probes are not really biosensors, because no enzyme is immobilized, they could be further developed for constructing miniaturized biosensors and used for in vivo analysis.[132]

A nicotinic acid sensor was fabricated[138] using whole cells of *Pseudomonas fluorescens*, which has high activity to catalyze the hydroxylation of nicotinic acid,[144] and the catalytic reaction can be coupled with an electrode reaction to produce a catalytic anodic current in the presence of an electron-transfer mediator, such as *p*-benzoquinone or its derivatives:

$$\text{nicotinic acid} + M_{ox} \rightarrow \text{6-hydroxy-nicotinic acid} + M_{red} \quad (10)$$

where M_{ox} and M_{red} are the electron acceptor and its reduced form, respectively. The catalytic action is due to nicotinic acid dehydrogenase, present in the cytoplasmic membrane of the bacteria, and the enzymatic reaction is linked to the bacterial respiratory chain using oxygen as the final electron acceptor. Oxygen affected the current response of the electrode due to the respiration reaction of the microorganisms, which is competitive to the benzoquinone-catalyzed reaction. When an inhibitor of the respiration reaction, such as KCN, is added, the catalytic current is not influenced by oxygen and the response is selective for nicotinic acid. The electrode had no response to nicotinamide, 6-hydroxynicotinic acid, isonicotinic acid, isonicotinamide, quinolic acid, nicotinic acid *N*-oxide, or nicotinamide *N*-oxide at 5 mM each. Some nutrients, such as glucose, fructose, malic acid, and ethanol, were also tested, and only glucose produced a very small catalytic current. Lactic acid gives a catalytic current the magnitude of which was 15% of that for nicotinic acid. Long-term stability of the biosensor was studied at 1 and 5 mM nicotinic acid. The probe produced a reproducible catalytic current over 2 weeks when the concentration of nicotinic acid was lower than 1 mM. However, at higher concentra-

tions, the current began to decrease 2 days after preparation and the response became smaller day after day for a week to reach a magnitude close to that of the current at 1 mM.[138]

The measurement of the oxygen consumption by bacterial cells is also an alternative method to detect nicotinic acid. Thus, a *Ps. fluorescens*−Au electrode has been used[138] to measure the cathodic current for the reduction of oxygen. The current decreased by addition of nicotinic acid to reach a steady state in 20 s, and the decrement in current (ΔI) was proportional to nicotinic acid concentration up to 1.2 mM. Measurements can be done in the pH range between 4.0 and 10.0 with the optimum pH around 7.0. This Au electrode has a response more rapid than the method with the benzoquinone−carbon paste electrode, but the measurement is limited to concentrations below 1.2 mM. The ΔI at 1 mM levels decreased slightly 1 day after the preparation of the electrode, then day by day to $^1/_8$ of the original magnitude after 1 week.[138]

Escherichia coli 215 requires vitamin B_{12} for its growth, and a sensor[143] was developed to monitor the decrease in dissolved O_2 with an oxygen electrode. The current at zero time corresponds to the assay medium being saturated with oxygen. When vitamin B_{12} is introduced into the system, vitamin B_{12} permeates through the porous membrane and is assimilated by the immobilized bacteria. This causes an increase in O_2 consumption by the microorganisms with a consequent decrease in dissolved O_2 around the membranes. As a result, the sensor current decreases markedly with time until a steady state is reached. The steady state indicates the equilibrium consumption of O_2 by the bacteria stimulated by diffusion of vitamin B_{12} from the sample solution. The steady-state currents depend on the concentration of vitamin B_{12}. The concentration of glucose used in the assay medium affects the growth of the bacteria and therefore the rate of current decreases. Also, the respiration activity of microorganisms, as measured by the current, is affected by the concentration of *E. coli* cells in the membrane. And when 0.5 mg of wet cells was used, a linear relationship was obtained between the rate of the current decrease and the vitamin B_{12} concentration ($(5-25) \times 10^{-3}$ mg L^{-1}).[143]

3. Analysis of Amino Acids

Table 2 shows the experimental conditions and analytical characteristics for these biosensors.

The enzymes usually used are dehydrogenases and oxidases. When dehydrogenases are used, the oxidative deamination of the substrate is produced, and simultaneously NAD^+ is reduced to NADH. The NADH generated acts as an electron-transfer mediator and is usually detected. $NAD(P)^+$ can be added to the working solution[49,52,66,72,81,95,96,102,108,115,152,154,156] or be coimmobilized on the electrode.[38,39,119,120,125,155] Oxidases work without NADH but have FAD as cofactor.

3.1. Amino Acid Dehydrogenases

Biosensors for the determination of L-amino acids based on the electrochemical regeneration of NADH were developed.[52,152,154,156] NADH formation was catalyzed by diaphorase (DP) and L-amino acid dehydrogenase, both immobilized on the electrode surface. Hexacyano ferrate(III)[52,154] or vitamin K_3 and 2-ferrocenylethanol (2-FEA)[152] were used as mediators. For alanine assay,[154] the substrate was oxidized by NAD^+ in a reaction catalyzed by an alanine dehydrogenase (ADH):

$$CH_3CH(NH_2)COO^- + NAD^+ + H_2O \overset{ADH}{\rightleftharpoons} CH_3COCOO^- + NADH + NH_3 + H^+ \quad (11)$$

$$NADH + 2Fe(CN)_6^{3-} \overset{DP}{\rightleftharpoons} NAD^+ + 2Fe(CN)_6^{4-} + H^+ \quad (12)$$

The reaction 12, catalyzed by diaphorase, shifts the first one completely to the right. The disappearance of the hexacyano ferrate(III) was optically monitored. Also, the hexacyanoferrate(II) formed can be oxidized electrochemically on a Pt electrode and detected amperometrically. Other amino acids did not interfere, except 3,4-dihydroxyphenylalanine, because it reduces the mediator to hexacyanoferrate(II). To avoid this and to shift the reaction to the right, hydrazine can be added in replacement of hexacyanoferrate, and hydrazone pyruvate is formed; the alanine concentration is deduced from the absorbance variation at 340 nm. But this second method with hydrazine takes more than 30 min.[154] Analogously to the alanine dehydrogenase biosensor,[154] a reactor for L-glutamate was developed.[52] The enzymes are L-glutamate dehydrogenase, diaphorase, and glutamate oxalacetate transaminase (GOT), which makes possible the enzyme amplification phenomenon:

$$\text{L-glutamate} + NAD^+ + H_2O \overset{GDH}{\rightleftharpoons} \alpha\text{-ketoglutarate} + NADH + NH_4^+ \quad (13)$$

$$NADH + 2Fe(CN)_6^{3-} \overset{DP}{\rightleftharpoons} NAD^+ + 2Fe(CN)_6^{4-} + H^+ \quad (14)$$

$$\alpha\text{-ketoglutarate} + \text{aspartate} \overset{GOT}{\rightleftharpoons} \text{L-glutamate} + \text{oxalacetate} \quad (15)$$

Cyclic voltammograms[152] for 0.2 mM 2-FEA on DP and GDH electrodes show an increase in the peak current on the addition of 0.5 mM NADH. When 1 mM L-glutamate is added to the same solution, these peak currents show further increase. Amperometric measurements at 0.20 V vs SCE under stirring, show that upon addition of L-glutamate the current response is rapid and the steady-state current is obtained within 30 s. A linear relationship is observed in the concentration range 5−200 μM. Application of DP and L-alanine dehydrogenase or L-leucine dehy-

Table 2. Experimental Conditions and Analytical Properties of Amperometric Biosensors Applied to the Analysis of Amino Acids

Analyte	Immobilized system	Experimental conditions	Response time (time of analysis)	Linearity (DL) mM	CV %	Lifetime	Ref.
L-alanine in urine and plasma	Alanine dehydrogenase and diaphorase; $Fe(CN)_6^{3-}$/NAD^+ as mediator	SCE and Pt electrodes/reaction chamber, 0.25 V, pH 9.0	1-3 min	$3\cdot10^{-3}$-2	2	1 m 4°C pH 7.0	154
L-alanine in blood	Alanine aminotransferase, glutamate oxidase and catalase	Pt and Ag/AgCl electrodes, 650 mV, 25°C, pH 7.4	1 min	10^{-2}-1 ($2\cdot10^{-3}$)	10	1 m pH 7.4	99
D-alanine	D-amino acid oxidase Prussian blue as mediator	Pt, Ag/AgCl and pyrolytic graphite coated electrodes, -50 mV, 25°C, pH 6.4	40 s	0.07-14 (0.03)	-	15 d 4°C pH 5.5	153
D-alanine	D-amino acid oxidase	GCE	30 s	0-0.5 ($5\cdot10^{-3}$)	-	40 d	59
L-arginine	L-amino acid oxidase from *Colletotrichum sp*, and catalase	Reactor, FIA, 0.8 ml/min SCE and Pt electrodes, 0.7 V (20 V for electrodialysis), pH 7.6	-	-	-	47 d 4°C pH 7.6	74
L-aspartic acid in pharmaceuticals	L-aspartate aminotransferase and L-glutamate oxidase	Pt and Ag/AgCl electrodes, 650 mV, pH 7.4	3 min	$1\cdot10^{-3}$-0.2	5	2 m 4°C pH 7.4	50
L-cysteine	Tyrosinase	Pt, Ag/AgCl and CPE electrodes, -50 mV, room-t, pH 6.6	15 s	$1\cdot10^{-3}$-$3\cdot10^{-2}$ (catechol)	-	3 d 4°C dry state	41
L-glutamate	**L-glutamate dehydrogenase**, glutamate-oxalacetate transaminase and diaphorase; $Fe(CN)_6^{3-}$/NADH as mediator	Reactor, Pt electrodes, 80 mV, 25°C, pH 8.3	2 min	$5\cdot10^{-3}$-0.2	4	21 d 4°C pH 8.3	52
L-glutamate	L-glutamate dehydrogenase, $Fe(CN)_6^{3-}$ and phenazine methosulfate as mediators	SCE, Pt wire and CPE electrodes, 0.32 V, room-t, pH 8	-	0.05-1.3	-	11 d	72
L-glutamate in chicken cubes	Beef liver mitochondria (L-glutamate dehydrogenase); $Fe(CN)_6^{3-}$ and phenazine methosulfate as mediators	Pt, Ag/AgCl and CPE electrodes, 0.35 V, 25°C, pH 7.5	-	0.4-10 10-100 (0.1)	8.2	10 d room-t	156
L-glutamate	Glutamate dehydrogenase and glutamate-pyruvate transaminase; Meldola blue as mediator	Graphite rods and Ag/AgCl electrodes, 0 mV, room-t, pH 7.4	< 10 min	0-0.01 ($5\cdot10^{-4}$)	-	1 d 4°C pH 7.4	108
	Glutamate dehydrogenase and glutamate-pyruvate transaminase; N-methyl-phenazinium ion as mediator	Pt and oxygen-Ag/AgCl electrodes, -600 mV, room-t, pH 8.6	10-12 min	0-$5\cdot10^{-4}$ ($1\cdot10^{-4}$)	-	4 d 4°C pH 8.6	
L-glutamate	L-glutamate dehydrogenase; phenazine methosulfate as mediator	Ag/AgCl stainless-steel chromatographic tube as counter electrode, Pt disk and CPE electrodes, 50 mV, room-t, pH 7.4	45 s (40 samples/h)	0.001-0.5	-	-	102
L-glutamate	Thermophilic L-glutamate dehydrogenase from a archaebacterial isolate ANI and NADP; poly-TBO as mediator	Pt, Ag/AgCl and CPE electrodes, 100 mV, 35-60°C, pH 7-8.9	< 5 min (2 min)	-	-	2 w 4°C	38
L-glutamate	Thermophilic L-glutamate dehydrogenase from a archaebacterial isolate ANI and NADP; poly-TBO as mediator	FIA, 1.7 ml/min, 40-45°C SCE, Pt and carbon paste wax electrodes pH 7.8	- (20 samples/h)	0-40 (0.3)	7.6	3 m 4°C	39
L-glutamate	L-glutamate dehydrogenase	FIA, 0.81 ml/min Ag/AgCl and two carbon fiber microelectrodes, pH 7.4	11-12 s	-	-	0-5°C	66
L-glutamate in chicken bouillon cubes	L-glutamate dehydrogenase/NAD^+	Pt wire, Ag/AgCl and CPE electrodes, 0.15 V, pH 8	120 s	0.005-0.078 (0.0038)	4.8	1 m < 4°C darkness	125
L-glutamate	L-glutamate dehydrogenase with avidin-biotin coupling	FIA, 1.5 ml/min Ag/AgCl and two carbon fiber microelectrodes, pH 8.5	300 ms	1-60 (0.5)	-	4 w	115
L-glutamate	L-glutamate dehydrogenase; NADH oxidase; Os-gel-HRP	FIA, 16 μl/min Ag/AgCl and two GCE electrodes, Nafion film, pH 7	3 min	$1\cdot10^{-4}$-0.01 ($1\cdot10^{-4}$)	-	-	81
L-glutamate in broths	**L-glutamate oxidase** from *Streptomyces platensis*	Oxygen electrode, 28°C, pH 7.0	3 min	0.12-0.84	< 8.8	3 w 4°C pH 7.0	97
Glutamic acid	*Bacillus subtilis* 168 mutant (glutamate oxidase)	Oxygen and Ag/AgCl electrodes, -600 mV, 30°C, pH 6.8	10-20 s	0-0.15	7.5	14 d room-t pH 6.8	136

Table 2. (Continued)

Analyte	Immobilized system	Experimental conditions	Response time (time of analysis)	Linearity (DL) mM	CV %	Lifetime	Ref.
L-glutamic acid in tissues	Pt/PPD/GOD *	SCE, teflon-Pt wire and Ag wire electrodes, 0.7 V, 25°C, pH 7.4	10 s	- ($[3.5\pm3.3]\cdot10^{-4}$)	-	-	43
	Pt/PEA/PPD/GOD *		10 s	- ($[2.3\pm3.3]\cdot10^{-4}$)			
	Pt/PEA/PPD/BSA/GOD *		4 s	0-0.1 ($[2.7\pm2.1]\cdot10^{-4}$)			
	Pt/STA/PPD/BSA/GOD *		-	- ($[1.5\pm0.9]\cdot10^{-4}$)			
	Pt/NAF/PPD/BSA/GOD *		-	- ($[15\pm13]\cdot10^{-4}$)			
L-glutamate	L-glutamate oxidase from *Streptomyces sp*; poly(*o*-phenylenediamine) for entrapment	Pt microelectrode, Pt and Ag/AgCl electrodes, 850 mV, 22°C, pH 7.4	15 s	-	-	30 d 4°C pH 7.4	122
	" polypyrrole for entrapment		30 s				
L-glutamate	"Russian" glutamate oxidase from *Streptomyces sp* z-11-6; horseradish peroxidase in a redox hydrogel	FIA, 0.8 ml/min Ag/AgCl, Pt and graphite electrodes, -50 mV, pH 6.0	10	0-0.1 ($3.9\cdot10^{-3}$)	5	-	107
	"Yamasa" glutamate oxidase; horseradish peroxidase in a redox hydrogel		10	0-0.5 ($5\cdot10^{-4}$)	5		
L-glutamate in seasoning	L-glutamate oxidase	FIA, 1.5 ml/min Ag/AgCl and stainless-steel tube electrodes, pH 7.5	- (60 samples/h)	- (0.004)	<0.73	6 m 4.5°C pH 7.5	69
		Reactor, FIA, 1.5 ml/min Ag/AgCl, Pt and stainless-steel tube electrodes, pH 7.5	-	-	<0.72		
L-glutamate	Glutamate oxidase	SCE, Pt and carbon fiber electrodes, 0.6 V, 20°C, pH 7.4	10 s	0-0.3 (0.001)	-	50 d 4°C dry state	126
L-glutamate in fermentation broths	L-glutamate oxidase from *Streptomyces sp*; 1,1'-dimethylferrocene as mediator	SCE, Pt and graphite electrodes 25°C, pH 6.5	-	0.1-2	1-11	6 m 28°C dry state	79
L-glutamate in foods	L-glutamate oxidase; 1,1'-dimethylferrocene as mediator	4 graphite pellets and Ag/AgCl electrodes, 220 mV, 35°C, pH 7.5	60 s (5-7 min)	0.2-2	-	9 d -4°C pH 7.5	36
L-glutamate	L-glutamate oxidase; ferrocene-ethylene oxide-siloxano polymer as mediator	Graphite and Ag/AgCl electrodes, 400 mV, 23°C, pH 7	< 1 min	- (0.01)	-	5°C pH 7	37
L-glutamate in foods	L-glutamate oxidase; tetrathiafulvalene as mediator	Pt, Ag/AgCl and carbon paste electrodes, 0.15 V, 25°C, pH 7	2 min	0.8 (0.0026)	-	3 w 4°C pH 7	73
L-glutamate	L-glutamate oxidase	Reactor, FIA, 3 ml/min, 15°C H_2O_2 electrode, pH 6.8	-	0.1-2 % (w/v)	-	-	90
L-glutamate in foods	L-glutamate oxidase	Reactor, FIA, 1 ml/min, glass tube and Ag/AgCl electrodes, 0.65 V, pH 7	-	0.01-0.3	1.7	15 d	98
L-glutamate in pharmaceuticals	L-aspartate amino-transferase, L-glutamate oxidase and catalase	Pt and Ag/AgCl electrodes, 650 mV, pH 7.4	3 min	$8\cdot10^{-4}$-1.5	5	2 m 4°C pH 7.4	50
L-glutamate	L-glutamate oxidase	Pt and Ag/AgCl electrodes, Nafion film, 650 mV, 37°C, pH 7.8	14 s	0-0.8 ($3\cdot10^{-4}$)	-	-	80
L-glutamate	L-glutamate oxidase; Prussian blue as mediator	FIA, 0.5 ml/min Pt, Ag/AgCl and glassy carbon disk electrodes , Nafion film, -50 mV, pH 6-7	- (20-30 s)	$1\cdot10^{-4}$ -0.1 ($1\cdot10^{-4}$)	-	5-7 d 4°C dry state	63
L-glutamate	L-glutamate oxidase	FIA, 0.2 ml/min Ag/AgCl and rhodinised carbon electrodes, 400 mV, pH 7.0	-	0.1-1.5	<1.5	< 20 d 4°C pH 7	76
L-glutamate in blood	L-glutamate oxidase	Platinized carbon and Ag/AgCl combined electrodes, 320 mV, pH 7.6	1 min	0.002-2 (0.002)	5.3	3 m 4°C pH 7.4	53
L-glutamate	L-glutamate oxidase and glutamate-pyruvate transaminase	Pt and Ag/AgCl electrodes, 500 mV, 30°C, pH 7.2	3 min	$2\cdot10^{-6}$-0.001 ($2\cdot10^{-4}$)	-	7 d 4-5°C pH 7.2	70
L-glutamate in foods	L-glutamate oxidase and L-glutamate dehydrogenase	Reactor, FIA, 0.80 ml/min, 25°C Pt and Ag/AgCl electrodes, 650 mV, pH 7.4	14 s (50-60 samples/h)	$5\cdot10^{-5}$-0.5 ($3\cdot10^{-5}$)	-	20 d 4°C pH 7.4	49
L-glutamate	L-glutamate oxidase and horseradish peroxidase	SnO_2 coated glass plate, Pt black wire and Ag/AgCl electrodes, 250 mV, 30°C, pH 6.4	1 min	$1\cdot10^{-4}$-0.1	-	10 d 4°C pH 7.4	56

Table 2. (Continued)

Analyte	Immobilized system	Experimental conditions	Response time (time of analysis)	Linearity (DL) mM	CV %	Lifetime	Ref.
L-glutamate in seasonings	L-glutamate oxidase	-	2 min	0.001-1.0	-	10 d	168
L-glutamine	Glutaminase(II) and glutamate oxidase	FIA, 0.2 ml/min Ag/AgCl and rhodinised carbon electrodes, 400 mV, pH 5.5	-	0.1-1.5	< 1.5	12 d 4°C	76
L-glutamine	Glutaminase and glutamate oxidase	Reactor, FIA, 0.42 ml/min, 25°C, Pt disk, Ag/AgCl and glassy carbon disk electrodes, 650 mV, pH 5.5	- (4 min)	0.010-0.20g/l	5.1	1 m 4°C pH 5.5	91
L-glutamine in cell cultures	Glutaminase and glutamate oxidase	Oxygen electrode, pH 5.5	- (2 min)	0.2-2	-	200 runs 5°C pH 5.5	87
L-histidine	Galactose oxidase	Reactor, FIA, 0.5 ml/min, 35°C Au, oxygen and Ag/AgCl electrodes, -0.7 V, pH 8.5	-	0.01-10 (0.005)	-	-	169, 170
L-leucine	L-amino acid oxidase	SCE and Pt electrodes, 0.7V, Nafion film, pH 7.0	-	-	-	-	64
L-leucine	L-amino acid oxidase	SCE, Pt foil and Sn-coated Cu wire electrodes, 0.7 V	-	0.07-3	-	-	113
L-lysine	**L-lysine dehydrogenase,** $Fe(CN)_6^{3-}$ as mediator	Pt and Ag/AgCl electrodes, 0.4 V	- (40 samples/h/	$7\cdot10^{-5}$-0.7 ($7\cdot10^{-5}$)	1.9	25 d	65
L-lysine in fish feed	**L-lysine-α-oxidase**	Reactor, FIA, 3 ml/min, 10°C Au and modified glass tube electrodes, 0.6 V, pH 7.3	3 (12 samples/h)	- (0.001)	1.5	6 m	92
L-lysine hydrolysate in foods	L-lysine-α-oxidase	FIA, 0.2 ml/min, 25°C Pt and Ag/AgCl electrodes, 650 mV, pH 7.0	30 s (2 h)	0.01-0.5 -	<10	-	35
L-lysine in animal foods	L-lysine-α-oxidase	Pt and Ag/AgCl electrodes, 650 mV, 25°C, pH 7.0	2 min (12 samples/h)	0.01-1 (0.005)	< 5	90 d	107
		" FIA, 0.2 ml/min	30 s (20 samples/h)	0.001-2 -			
L-lysine	Lysine-α-oxidase from *Trichoderma viride*	SCE, Ag/AgCl and Si-Au strip electrodes, 650 mV, 37°C, pH 9.0	-	0.1-10	-	40 d 4°C pH 9.0	114
L-lysine	L-lysine-α-oxidase from *Trichoderma sp*	Reactor, FIA system, 400 ml/h, 25°C Clark membrane electrode, pH 8.0	14 s (2 min)	0.20-5.5 (0.2)	-	5 m 10°C pH 8.2	100
L-lysine in wheat extracts	L-lysine-α-oxidase and catalase	Clark oxygen electrode, 45°C, pH 7.5	5 s (75 s)	$6.7\cdot10^{-3}$-0.67	1.6	9 m 4°C pH 7.5	86
L-lysine hydrolysate in foods	L-lysine oxidase	FIA, 0.5 ml/min, 30°C Ag/AgCl, GCE (with Ru and Rh) electrodes, 100 mV, pH 7.0	- (43 samples/h)	0.002-0.125 (0.002)	2.3	pH 7.0	82, 83
L-lysine in foods	L-lysine-α-oxidase	Pt electrodes	-	0.01-1 ($2\cdot10^{-4}$)	-	-	130
L-lysine	L-lysine-α-oxidase	Reactor, FIA, 3 ml/min, 25°C Pt electrodes, 0.6 V, pH 6	-	0.01-0.16 (0.005)	2	2 w 5°C pH 6	94
L-lysine	**L-lysine decarboxylase** and *Pseudomonas* S-17 bacteria	Two gold electrodes on a silicon chip, -1.2 V, 33°C, pH 6.0 2 μM pyridoxal phosphate	1-3 min	$2.5\cdot10^{-4}$-0.4 ($2.5\cdot10^{-4}$)	-	25 runs 4°C pH 6.0	140
DL-phenylalanine	*Proteus vulgaris* (phenylalanine deaminase)	Oxygen electrode, 30°C, pH 7.0	3 min	0.025-2.5 (0.005)	3.7	1 m refrigeration 25% glycerol	139
L-phenylalanine in blood sera	Lactate oxidase	FIA, 1.5 ml/min, 37°C oxygen electrode, pH 7.0	2 min (6 h)	$0.75\text{-}6\cdot10^{-7}$ g/ml	5.6	-	141
L-phenylalanine in blood sera	Lactate oxidase	FIA, 1.5 ml/min, 37°C oxygen electrode, pH 7.0	- (90 min)	$5\cdot10^{-5}$-$1\cdot10^{-7}$ g/ml	6	20 d	142
L-phenylalanine in blood and serum	Salicylate hydroxylase, tyrosinase and phenylalanine dehydrogenase	Pt, Ag/AgCl and CPE electrodes, -50 mV, pH 8.2	45-60 s	0.02-0.15 (0.005)	-	11 d 4°C	40
L-phenylalanine	L-amino acid oxidase; $Os(bpy)_3](PF_6)_2$ as mediator	Pt electrodes, 200-900 mV	-	3.3-240 μg/ml	-	1 m	46
D-phenylalanine	D-amino acid oxidase; $Os(bpy)_3](PF_6)_2$ as mediator			0.83-17 μg/ml			
L-phenylalanine in urine	L-amino acid oxidase; $Os(bpy)_3](PF_6)_2$ as mediator	Pt, Ag/AgCl and microcarbon coated (with Os) electrodes, 650 mV, 40°C, pH 7.25	26 s	0.02-1.4	6.2	3 m 4°C pH 7.0	45
D-phenylalanine in urine	D-amino acid oxidase from porcine kidney	" , pH 10.0		0.005-0.1	7.7	1 m 4°C pH 8.5	
L-phenylalanine	L-amino acid oxidase	SCE, Pt foil and Sn-coated Cu wire electrodes, 0.7 V	3-10 min	0-1.4 (0.07)	-	13-20 d	113
L-tryptophan In nutrition broths	Tryptophan-2-mono oxygenase from *Pseudomonas savastanoi*	Oxygen-Clark membrane electrode, 25°C, pH 8.3	140 s (2 min)	0.025-1.0	-	4 m	101, 171
L-tryptophan In nutrition broths		FIA system	30 s (3 min)	0.1-50	-	4 m 8°C	171, 172

Table 2. (Continued)

Analyte	Immobilized system	Experimental conditions	Response time (time of analysis)	Linearity (DL) mM	CV %	Lifetime	Ref.
L-tyrosine	L-tyrosine decarboxylase	Oxygen electrode, 32°C, pH 5.5	-	0.010-0.050	-	5 d	68
L-tyrosine	Tyrosinase	Oxygen electrode, pH 8.0	2-3 min	0-0.25 ($1.33\cdot10^{-2}$)	1.8	15-20 d	67
L-tyrosine	Tyrosinase	Oxygen electrode	-	0-0.4 (0.04)	-	-	173
D- or L- amino acids in grapes: L-arginine	D- or L- amino acid oxidase, horseradish peroxidase; ferrocene as mediator	Graphite-teflon, Pt wire and Ag/AgCl electrodes, 0.0 V, pH 9.0	-	0.1-1 (0.16)		10 d without regeneration 4°C dry state 15 d for polished D-AAO-HRP 30 d for polished L-AAO-HRP	42
D-leucine				0.01-1.2 ($1.8\cdot10^{-3}$)			
L-leucine				0.002-0.1 (0.012)			
D-methionine				0.01-1.4 ($3.2\cdot10^{-3}$)	5.3-5.8		
L-methionine				0.02-1.2 ($5.6\cdot10^{-3}$)			
L-phenylalanine				0.05-0.8 ($2.2\cdot10^{-2}$)			
D-serine				0.05-0.5 (0.023)			
L-tryptophan				0.01-0.25 ($2.3\cdot10^{-3}$)	5.0-6.6		
D-valine				0.025-0.5 ($3.2\cdot10^{-3}$)			
L-arginine		" FIA, 0.67 ml/min	-	0.1-2 (0.033)	5.0		
D-leucine				0.006-0.6 ($4.3\cdot10^{-3}$)	1.7		
L-leucine				0.004-0.8 ($1.1\cdot10^{-3}$)			
D-methionine				0.01-1 ($3.1\cdot10^{-3}$)	1.2		
L-methionine				0.02-1.5 (0.01)			
L-phenylalanine				0.01-1 ($4.1\cdot10^{-3}$)	1.3		
D-serine				0.1-8 ($2.8\cdot10^{-2}$)			
L-tryptophan				0.01-0.6 ($9.2\cdot10^{-3}$)	2.5		
D-valine				0.04-4 ($1.2\cdot10^{-2}$)			
D-, L-alanine, D-, L-arginine, L-asparagine, L- aspartic acid, L-cysteine, L-glutamic acid, L-glutamine, L-glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, D-, L-methionine, D-, L-phenylalanine, L-serine, L-threonine, L-tryptophan, L-tyrosine, D-, L-valine in fruit juice, milk and urine	D-amino acid oxidase, and L-amino acid oxidase	Three screen-printed electrodes (PEI in the rhodinised working electrode pastes) room-t, pH 7.8	4 min	0-1 (0.15, 0.47, and 0.20 for L-glycine, L-leucine,and L-phenylalanine, respectively)	0.4-14.4	56 d 4°C pH 7.8	62
L-histidine	D-amino acid oxidase and L-amino acid oxidase	FIA, 1 ml/min, room-t Ag/AgCl, Pt and iridium CPE electrodes, 0.0 V, (PEI in the iridium-carbon paste electrode), pH 7.4	-	0.05-5 (0.05)	4.5	3-8 d 4°C pH 7.4	128
L-lysine				0.25-3 (0.10)	5.0		
D-phenylalanine				-	1.9		
L-phenylalanine				0.10-1.50 (0.05)	4.2		
L-tryptophan				0.10-4 (0.05)	3.7		
L-tyrosine				0.10-4 (0.10)	3.1		
D-alanine, D-methionine, D-phenylalanine, D-serine, D-valine	D-amino acid oxidase	GCE coated with polyion complex membrane, Pt wire and Ag/AgCl electrodes, 1.0 V, 25°C, pH 8.3	30 s	0-0.5 ($5\cdot10^{-4}$)	4	4°C pH 8.3	60
L-amino acids	L-amino acid oxidase; 1,1'-dimethylferrocene as mediator	SCE, Pt, and graphite electrodes, 0-200 mV, 37°C, pH 7.8	-	-	-	<2 min -	36

*PPD, poly(*o*-phenylenediamine); GOD, glutamate oxidase; PEA, phosphatidylethanolamine; BSA, bovine serum albumin; STA, stearic acid; NAF, Nafion film.

drogenase electrodes for the determination of individual L-amino acids in soy sauce show good agreement with HPLC method,[152] and the same is observed for an L-phenylalanine sensor.[155] In this case, L-phenylalanine dehydrogenase, $NAD(P)^+$, and a phenazine analogue as mediator are coimmobilized on the electrode, and this reaction layer is integrated with a support made of a conductive material. The adsorptive carrier containing the reagents functions as a reaction layer for both an enzymatic reaction between L-phenylalanine in the sample and reagents and an electrode reaction between an electron mediator and the electrode surface.[155] Also, enzyme-[72] and mitochondria-based[156] biosensors were developed for the determination of L-glutamic acid using hexacyanoferrate(III) and phenazine methosulfate (PMS) as mediators,

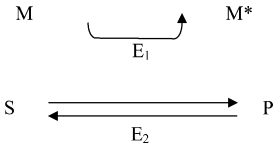
$$NADH + PMS^+ \rightleftarrows NAD^+ + PMSH$$

$$PMSH + 2Fe(CN)_6^{3-} \rightleftarrows PMS^+ + 2Fe(CN)_6^{4-} + H^+ + 2e^-$$

$$2Fe(CN)_6^{4-} \rightarrow 2Fe(CN)_6^{3-} + 2e^- \qquad (16)$$

and the behavior of the redox couple hexacyanoferrate(II)/(III) was improved by incorporating octadecylamine into the enzyme-modified carbon paste electrode,[72,156] which allows ion-pair formation between octadecylamine and hexacyanoferrate.

A considerable enhancement of the sensitivity of enzyme electrodes by substrate recycling reactions has been demonstrated. The amplification depends on the presence of at least two immobilized enzymes (E_1 and E_2), one converting the original substrate S to product P, which is regenerated to S by the second enzyme according to the scheme

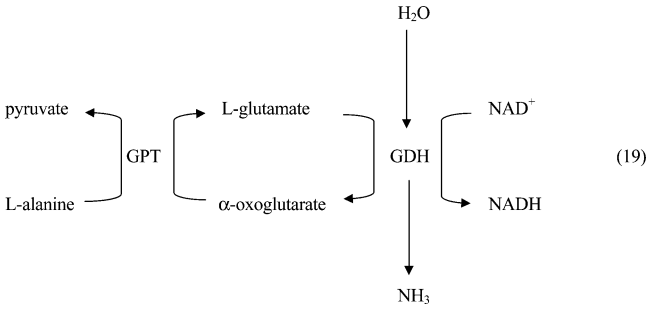


where either M or M* should be an electrochemically active cofactor (mediator). Glutamate dehydrogenase (GDH) and glutamate pyruvate transaminase (GPT) catalyze the reactions

$$\text{L-glutamate} + NAD^+ + H_2O \xrightarrow{\text{GDH}} \alpha\text{-ketoglutarate} + NH_4^+ + NADH \quad (17)$$

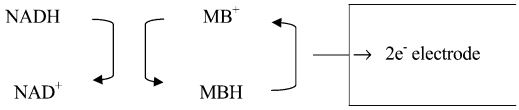
$$\alpha\text{-ketoglutarate} + \text{L-alanine} \xrightarrow{\text{GPT}} \text{L-glutamate} + \text{pyruvate} \quad (18)$$

If a compound, which can be either L-glutamate or α-oxoglutarate, is recycled in the sensor, a small amount of, for example, L-glutamate will produce a large amount of NADH by simultaneous oxidation of L-alanine to pyruvate:



(19)

The NADH produced could be detected by direct oxidation at an electrode, but this reaction suffers from a high overvoltage[174] and electrode fouling,[175] which would make the detection system susceptible to interfering reactions, high background currents, and variation of the response factor with time. The NADH produced in the GDH-catalyzed reaction was therefore determined by different amperometric detection systems, all of them incorporating a mediator.[108] In the first system (reaction 20), NADH reacts with *N*,*N*-dimethyl-7-amino-1,2-benzophenoxazinium (Meldola blue, MB^+) to produce the reduced form of the mediator, MBH, which is reoxidized immediately at the applied electrode potential. The mediator is immobilized on the surface of a graphite electrode:



(20)

In the second system, NADH is reoxidized by the *N*-methylphenazinium ion (NMP^+), and the reduced mediator is reoxidized by molecular oxygen:

(21)

The decrease in oxygen concentration is measured by a Clark oxygen electrode.[108] Also, NADH and glutamate were determined using a carbon paste electrode in the presence of only phenazine methosulfate as mediator.[102]

The amplification (reaction 19) is started by addition of L-alanine and results in a greatly enhanced response for both sensors. A glutamate sample will first produce NADH according to reaction 17, but as α-ketoglutarate is obtained, it will be recycled to glutamate (reaction 18); thus, several NADH molecules can be produced by each glutamate molecule that enters the enzyme membrane. The amplification for the first system is 15 times within the linear range,[108] causing a lowering of the detection limit to 5×10^{-7} M (the upper limit of the linear range, which was 1×10^{-4} M without alanine, has now been lowered by 1 order of magnitude). The response of the oxygen sensor containing the NMP^+ mediator has an amplification factor larger than 60 in the linear range. The response to higher glutamate concentrations under conditions of amplification seems to be limited by the O_2-content of the membrane. The

amplification factors decrease in a nonlinear fashion at high substrate concentrations for both sensors; they were 20 and 5 at 6×10^{-5} M L-glutamate for the oxygen and the MB^+-modified sensors, respectively. Sensitivity of both enzyme sensors was enhanced by increasing the alanine concentration up to 0.1 M, the concentration used for all measurements. The glutamate response of the oxygen electrode was constant over a period of 4 days. The response of the MB-modified enzyme electrode was also fairly stable on the first day of operation but decreased by the fifth day to about 25% of the initial value of the unamplified mode. The measured response in the presence and in the absence of alanine became almost the same on the second day, that is, the amplification had ceased. This indicates that one or both enzymes had been inactivated. Desorption and inactivation of MB^+, as indicated by the responses to glutamate and NADH samples, also contributed to the low stability of the sensor. Storage at 4 °C between measurements did not increase the stability significantly.[108]

Carbon paste electrodes based on thermophilic GDH, NAD(P), and a polymeric toluidine blue-O (poly-TBO) mediator respond reproducibly to L-glutamate over a wide range in the system at 313−318 K.[38,39]

$$\text{L-glutamate} + \text{NADP}^+ + \text{H}_2\text{O} \overset{\text{GDH}}{\rightleftharpoons} \alpha\text{-ketoglutarate} + \text{NH}_4^+ + \text{NADPH}$$

$$\text{NADPH} + \text{poly-TBO}_{ox} \rightarrow \text{NADP}^+ + \text{poly-TBO}_{red}$$

$$\text{poly-TBO}_{red} \xrightarrow{\text{electrode}} \text{poly-TBO}_{ox} + \text{H}^+ + 2\text{e}^- \quad (22)$$

The kinetics of the probe is affected by the temperature, pH, and physical properties of the supporting carbon media. Carbon paste wax[39] is also a suitable electrode material for immobilization of mesophilic GDH for use in a flow injection analysis (FIA) system at 289−310 K. Addition of finely ground hexamine−ruthenium(III) trichloride to the carbon paste wax electrodes decreases the FIA peak width and increases the peak current. The metal complex appears to accelerate the rate of oxidation of NAD(P)H by poly-TBO.[39]

Fast-scan cyclic staircase voltammetry can minimize the difficulties associated with the electrochemical measurement of NADH when performed at carbon-fiber microelectrodes.[66] Electrochemical pretreatment of the electrode dramatically changed the properties of the modified electrode. Oxidation in a neutral or basic pH buffer decreases the overpotential for the diffusion-controlled wave but also introduces a very sharp, symmetrical prepeak, which is presumably due to product adsorption. However, application of a cyclic waveform in 1 M HCl produces an ideal surface for the diffusion-controlled oxidation of NADH. Furthermore, another advantage of the fast scan technique is that electrode passivation is minimized under these conditions.[66] In addition to this pretreatment, avidin−biotin coupling is used for the immobilization of GDH to the electrode surface.[115] An advantage of this approach is that the electrode modification process is separated into two stages: surface derivatization and enzyme derivatization. This allows both processes to be optimized independently, which is extremely important for the dehydrogenases. Thus, the protocol for the production of a GDH-modified electrode consists of an electrochemical pretreatment of the electrode in HCl, derivatization with a long tether molecule (Jeffamine ED-2001), and biotinylation with sulfo-NHS-LC-biotin. The extent of enzyme biotinylation was determined with affinity chromatography by immobilizing avidin on the column.[115]

3.2. Amino Acid Oxidases

3.2.1. Glutamic Acid

Glutamate oxidase (GMO) has FAD as the redox center and molecular oxygen as a cosubstrate producing H_2O_2 that can be detected amperometrically or spectroscopically. The oxidative deamination catalyzed by this enzyme can be represented as

$$\text{L-glutamate} + \text{H}_2\text{O} + \text{GMO/FAD} \rightarrow \alpha\text{-ketoglutarate} + \text{NH}_3 + \text{GMO/FADH}_2$$

$$\text{GMO/FADH}_2 + \text{O}_2 \rightarrow \text{GMO/FAD} + \text{H}_2\text{O}_2 \quad (23)$$

The H_2O_2 produced can be electrooxidized and measured amperometrically at relatively high applied potentials because other electroactive substances can interfere. Also, the amount of oxygen consumed can be amperometrically monitored, but errors due to fluctuations in the dissolved oxygen concentration are possible.

Extracellular L-glutamate oxidase produced by *Streptomyces platensis* NTU 3304 strain exhibits very high specificity, and L-glutamate is exclusively oxidized.[97] Various compounds with amino groups on both ends, including ethylene diamine, 1,4-diaminobutane, 1,6-diaminohexane, 1,8-diaminooctane, tetraethylene pentamine, and 1,12-diaminododecane, were added to investigate their ability to act as spacers: 1,12-diaminododecane gave the best results for enzyme immobilization and was chosen as spacer molecule. The response of the biosensor was significantly inhibited by Ni(II), Hg(II), and Cu(II) at 2 mM concentration, but these ions are usually not present in significant concentrations during fermentation processes.[97]

A bacterial sensor containing *Bacillus subtilis* with a high selectivity for glutamic acid in the presence of glucose has been developed[136] by blocking or inhibiting the undesired metabolic pathways and transport systems. When a sample solution containing glutamic acid or glucose is injected into the measuring cell, the substrate is taken up by the microorganisms. The respiration rate is increased, and a decrease in the dissolved oxygen signal is detected. But this sensor does not allow the deter-

mination of glutamic acid when glucose is present. A decrease in the glucose signal without changing the signal of glutamic acid is obtained by the treatment of *B. subtilis* with a relatively low concentration of chloromercuribenzoate (CMB) for 20 min. The effect of CMB is irreversible. A further reduction of the glucose signal was obtained by using NaF, which is an inhibitor of the enzyme enolase. However, the action of NaF at pH 6.8 is reversible, and it is necessary that the measuring solution always contains NaF.[136]

An important problem in the use of biosensors in biological media is the interference by endogenous electroactive reducing agents, especially ascorbic acid. This problem has been partially resolved using electrosynthesized polymers, such as poly(*o*-phenylene diamine), that block access of relatively small organic molecules, such as ascorbic acid,[43,119,122,133,134] uric acid,[119,122,133,134] 3,4-dihydroxyphenylacetic acid (DOPAC), 4-hydroxy-3-methoxyphenylacetic acid, and dopamine.[119,133,134] However, interferences from cysteine and ascorbic acid were still observed.[125] Polypyrrole is more effective as a barrier to dopamine.[122] Precoating the Pt wire with lipid (phosphatidylethanolamine or stearic acid) and incorporation of the protein BSA into the polymer matrix were found to improve the performance of the biosensor.[43] When BSA was incorporated, the improvement found was due to both an increase in the glutamate current and a decrease in the ascorbic acid response. Also, the probe sensitivity to dopamine, dihydroxyphenylacetic acid and uric acid present in the brain extracellular fluid was reduced.[43] In another case, the electrochemical oxidation of ascorbic acid was supressed on an 11-mercaptoundecanoic acid-assembled gold electrode.[176] Selectivity for glutamate can be enhanced[75,80,177] by incorporating a thin layer of Nafion between the Pt electrode surface and the immobilized layer of glutamate oxidase. Nafion is a perfluorinate-sulfonated ion-exchange polymer offering the possibility to incorporate cationic redox species, which can act as electron mediators for enzymes containing FAD. The same effect is achieved with a glassy carbon electrode modified with Os-gel-HRP, glutamate dehydrogenase, and NADH oxidase.[81] But this Nafion film should be coated between the GDH−NADH oxidase and Os-gel-HRP layers because it also hinders glutamate diffusion if coated over the GDH−NADH oxidase layer.[81] The extent of selectivity enhancement depends on the thickness of the Nafion film.[80] But Nafion also reduces the electrode response to H_2O_2 because it adds an additional diffusion barrier, which lowers the flux of H_2O_2 to the electrode surface. Of course, the extent of signal reduction is greater for ascorbic acid and other anionic species because both electrostatic repulsion and diffusion barrier effects are combined to reduce their responses. However, some response was observed for ascorbate at all Nafion layer thickness, which suggests that absolute selectivity for H_2O_2 is not possible with this approach. Also, Nafion causes a 2.3-fold decrease in magnitude of response and 2-fold increase in response time. The Nafion layer was much less effective in reducing interference from acetaminophen,[80] and coimmobilization of L-ascorbic oxidase with glutamate oxidase is convenient[75] because ascorbate oxidase converts ascorbic acid and O_2 to dehydroascorbate and H_2O and not to H_2O_2 as many oxidases do.

The characterization of a glutamate biosensor based on a newly isolated and purified glutamate oxidase was made.[107] This enzyme ("Russian-GMO") was isolated and purified from a Russian soil bacteria and subsequently coimmobilized with horseradish peroxidase, PVI_{19}-dmeOs as mediator, and poly(ethylene glycol) diglycidyl ether (PEDGE) as cross-linker. This biosensor was compared with others that are similar[106] but constructed with commercial glutamate oxidase ("Yamasa-GMO"), obtained as a lyophilized powder from *Streptomyces sp* X-119-6. Both biosensors displayed similar bioanalytical characteristics but different selectivity patterns. Ascorbic acid is one of main interferents and was eliminated prior to detection. The "Russian-GMO" did not display any sensitivity for glutamine, but the "Yamasa-GMO" has been reported to be sensitive to glutamate (99.92%) and glutamine (0.08%). However, the enzyme electrode and the enzyme reactor catalyze very selectively the oxidation of L-glutamate but not the D-isomer.[69]

L-Glutamate oxidase was immobilized onto an electropolymerized film of 1,3-diaminobenzene with 1,1′-dimethylferrocene (DmFe) incorporated.[79] The film serves to screen out electroactive substances and to retain DmFe to improve the useful lifetime of the mediated electrode. No response is obtained for other amino acids and ascorbic and uric acids. When stabilizers (DEAE-dextran, $MgCl_2$, and sucrose) are added to the immobilized enzyme and the film is stored in dry form at 28 °C, the electrode increases its stability from 3 weeks to 6 months.[79]

An automated multichannel FIA reactor system was developed,[90] and its application as an on-line multicomponent sensing device for the fermentation process monitoring was demonstrated. Several components (up to six) can be measured sequentially or in a programmed sequence by a computer controlling the electrical valve. Since the unusually high salt content (17.9%) in the fermentation broth affected the measurement, an additional minireactor packed with an ion-exchange resin was inserted between the injector and the column-switching valve to stabilize the baseline. As another novel aspect, this anion-exchange column can be used without regeneration, which is valuable for long-term use.[90] Furthermore, a 16-way switching valve is used for sample injection in the reactor, which allows the simultaneous detection of the L-glutamate signal and the blank value in a one-stroke operation.[98]

Amperometric enzyme electrodes for L-aspartate,[50,51] L-glutamate,[51] and L-alanine[99] determination were developed. The probes[50,51] consisted of a platinum electrode, which senses the H_2O_2 produced from reactions catalyzed by two enzymes, aspartate aminotransferase (AST) and glutamate oxidase (GMO), coimmobilized on a preactivated Immobilon-AV affinity membrane. The aminotransferase enzymes are involved in the transamination of their amino acid

substrates to form the corresponding oxo acid and glutamate. Reactions involved are

$$\text{L-aspartate} + \alpha\text{-ketoglutarate} \xrightarrow{\text{AST}} \text{L-glutamate} + \text{oxalacetate} \quad (24)$$

$$\text{L-alanine} + \alpha\text{-ketoglutarate} \xrightarrow{\text{ALT}} \text{L-glutamate} + \text{pyruvate} \quad (25)$$

$$\text{L-glutamate} + O_2 + H_2O \xrightarrow{\text{GMO}} \alpha\text{-ketoglutarate} + NH_3 + H_2O_2 \quad (26)$$

$$H_2O_2 \xrightarrow{\text{catalase}} O_2 + 2H^+ + 2e^- \quad (27)$$

The hydrogen peroxide produced in the reaction 26 is oxidized at a platinum electrode, and the current change is linearly correlated to the concentration of aspartate in solution. Analogously, analysis of alanine is based on reactions 25, 26, and 27. Reaction 25 is catalyzed by alanine aminotransferase and requires also α-ketoglutarate as cosustrate. Thus, α-ketoglutarate is a reagent in the two first reactions and a product in the third one, and its concentration in the testing solution has to be optimized. To obtain the maximum current output, α-ketoglutarate concentration was selected by constructing calibration curves for alanine at different concentration of α-ketoglutarate.[99] For concentrations higher than 10^{-3} M, α-ketoglutarate is an inhibitor of alanine aminotransferase, and the highest current output was recorded with a 10^{-3} M concentration of α-ketoglutarate.[99] To eliminate interferences from electrooxidizable species, a size-exclusion membrane was inserted between the Pt electrode and the enzyme membrane.[50,51] Aspartate, α-ketoglutarate, and the smaller molecules permeate across the first membrane to the second membrane (AST/GMO on Immobilon membrane), where α-ketoglutarate in the presence of aspartate is transaminated to glutamate. Since glutamate is detected in reaction 26, the assembled probe is also useful as a glutamate probe in an aspartate-free sample.[50,51] To eliminate interferences[50,51,99] or for samples containing both aspartate and glutamate,[50,51] the signal from the latter or from the interferents was eliminated by incorporating a third outer membrane where glutamate oxidase and catalase were coimmobilized. So, the probes were constructed utilizing three membranes. In the outermost membrane (GMO/catalase on polycarbonate membrane), glutamate and oxygen are converted to α-ketoglutarate and H_2O_2. The H_2O_2 produced diffuses across the third membrane (cellulose acetate membrane) and is sensed by the Pt electrode. The catalase reaction then converts the H_2O_2 to water and regenerates half the oxygen consumed in the previous reaction.

The L-glutamate produced can be reoxidized by glutamate oxidase. For every L-glutamate molecule, a large number of oxygen molecules are converted to an equally large number of H_2O_2 molecules, which can be detected amperometrically at the Pt electrode. Monitoring this H_2O_2 generated can be done by fixing the Pt electrode at +650 mV vs Ag/AgCl and measuring the oxidation current derived from the reaction

$$H_2O_2 \xrightarrow[+0.7\text{ V vs Ag/AgCl}]{\text{Pt}} O_2 + 2H^+ + 2e^-$$

as a function of time.

No interference from oxygen is produced because this reaction takes place at approximately −0.8 V vs Ag/AgCl:

$$O_2 + 4H^+ + 4e^- \xrightarrow[-0.8\text{ V vs Ag/AgCl}]{\text{Pt}} 2H_2O$$

Protein and other macromolecules are screened out. Since GMO is totally specific for L-glutamate, the enzymatic interferences found must be due to the transaminase reaction.[50,99] Only L-asparagine[99] and L-glutamine[50,99] interferred. This is a very significant improvement in selectivity over the previously reported potentiometric L-aspartate electrodes, where L-asparagine and adenosine gave response slopes equal to L-aspartate and the relative activities of L-glutamine and L-histidine were greater than 50%.[178,179] Glutamine cannot be completely eliminated and must be kept under control during the analysis.[99] Glutamine also reacts with glutamate oxidase, but its affinity for this enzyme is lower than glutamate, so this substrate is not completely eliminated. At concentration higher than 2×10^{-4} M, glutamine did not produce any current variation (saturation effect), and this concentration was added to the working buffer to obtain a current background that included the signal due to this metabolite. With this approach, the sensitivity of the probe decreases but still allows the determination of alanine in a range 20 times lower than that present in blood.[99] Results correlated well with a liquid chromatographic reference method and manufacturer's specification.[50,99] A similar method for the amperometric determination of glutamate was developed.[63] A specially deposited Prussian Blue, denoted as "artificial peroxidase" was used as a transducer for H_2O_2. GMO was immobilized on the surface of the Prussian Blue-modified electrode in a Nafion layer using a nonaqueous enzymology approach.[63]

The major clinical application of glutamate measurement is found in the determination of AST and ALT activities. A glutamate oxidase electrode[53] was calibrated in serum containing known added activities of AST and ALT. The sensor exhibited high current densities owing to the good protein-adsorption capacity of the platinized carbon electrode and its very high surface area. The platinized carbon electrodes and platinized carbon enzyme electrodes have a highly reproducible electrocatalytic surface, which exhibits a significantly lower oxidation potential for H_2O_2 than solid carbon or platinum electrodes. The electrode was allowed to stabilize in serum, giving a steady-state background current due to any endogenous interferents such as glutamate, ascorbic acid, and uric acid of ca. 1.5 μA. The assay was then initiated by the addition of an equal volume of reagents. This caused an immediate drop in the background current of ca. 50% due to the dilution of serum interferents.[53]

GPT and GMO were coimmobilized on a platinum disk electrode.[70] When the L-alanine concentration in the solution was increased from 0 to 1 mM, the sensitivity to L-glutamate increased ca. 1000-fold, but the increase was slight at higher concentrations, probably because of oxygen limitation in the solution. This indicates that the recycling of L-glutamate occurs between GMO and GPT in the presence of L-alanine, especially at low concentrations of L-glutamate. The amplification factor for 1 μM L-glutamate was ca. 53.[70]

Other authors[49] also coimmobilizate GMO and L-glutamic dehydrogenase (GDH), instead of AST, to determine glutamate and, also, to amplify the response:

$$\text{L-glutamate} + \text{NADP}^+ + \text{H}_2\text{O} \overset{\text{GDH}}{\rightleftharpoons} \alpha\text{-ketoglutarate} + \text{NH}_4^+ + \text{NADPH} \quad (28)$$

In the presence of NADPH, the two enzyme system causes cycling of L-glutamate, α-oxoglutarate, and NH_4^+ between the two enzymatic reactions, resulting in an increase in the rate of oxygen consumption. In the absence of NADPH and excess of NH_4^+, the responses of the single-enzyme and two-enzyme electrodes to L-glutamate are similar, indicating that the coimmobilization of the two enzymes does not cause any major drawback. In the presence of 5 mM ammonium chloride and 1 mM NADPH, the response of the two-enzyme system reactor was amplified 10-fold. Since oxygen is not produced in the reaction, substrate recycling leads to a shift of the linear range of the sensor to lower glutamate concentrations. Substrate recycling increases the sensitivity of the reactor, but α-ketoglutarate and NADPH must be added to the sample matrix; this procedure is not strongly recommended because NADPH is a cofactor for many enzymatic reactions and might introduce unpredictable interferences. Other interferences, such as tyrosine, cysteine, tryptophane, and dihydroxyphenylalanine, and some neurotransmitters and their metabolites were removed or, at least, their interferent effects reduced by the incorporation of a third cutoff membrane. The electrode thus prepared was included in a flow-injection system for the continuous assay of glutamate.[49]

Glutamate sensors were fabricated[56] by depositing GMO and horseradish peroxidase (HRP) on a tin oxide surface by means of either encapsulation into electropolymerized pyrrole or sequential chemical modification via surface hydroxyl groups. The relative quantities of both enzymes must be optimized. The sensor response increases when HRP increases because higher amounts suppress the diffusion of H_2O_2 out of the primary enzymatic reaction. An excessive amount of GMO leads to relative depletion of HRP incorporated in the polymer film, which in turn deteriorates the reduction pathway of H_2O_2, which cannot be electroreduced efficiently at the applied potential. Another factor is the thickness of the polymeric membrane as the host matrix of the two enzymes because it would regulate the amount of immobilized enzymes as well as the rate of mass transfer. On the other hand, differences in the sensor performance were found with the bonding order of the two enzymes. This observation may constitute an example where the spatial sequence of the enzymes controls the overall rate. All these sensors have excellent specificity for L-glutamate in the presence of the D-isomer or L-glutamine.[56]

3.2.2. Glutamine

For the determination of glutamine, glutaminase can be used in an enzyme or bacterial electrode. There are essentially three types of glutaminase: (1) The phosphate-activated enzyme of kidney (glutaminase I) has an optimum pH around 8.0. Although this pH region is suitable for the operation of cation-sensitive electrodes with little hydronium ion interference, glutaminase I was not considered because of its thermal instability. (2) Glutaminase II, or pyruvate-activated glutaminase, is not very suitable for enzyme electrodes because of the complicated reaction paths as well as the slow reaction rate. (3) Glutaminase III is a very effective catalyst and also relatively stable at room temperature.

$$\text{glutamine} + \text{H}_2\text{O} \xrightarrow{\text{glutaminase III}} \text{glutamate} + \text{NH}_4^+ \quad (29)$$

The glutamate is converted to H_2O_2 according reaction 26. However, a glutamine sensor based on rhodinized carbon electrodes was fabricated[76] using a bienzyme membrane incorporating glutamate oxidase and glutaminase II.

A flow-injection analysis/wall-jet electrode system coupled to a reactor packed with controlled pore glass beads containing coimmobilized glutaminase II and glutamate oxidase was investigated for glutamine determination in mammalian cell cultures.[91] Another reactor was similarly prepared, packed only with immobilized glutamate dehydrogenase, to eliminate interference from endogenous glutamate. Endogenous glutamate present in the culture medium would interfere when flowing through the glutaminase/glutamate oxidase reactor because it will produce H_2O_2, thus causing a high result. At 0.50 g·L^{-1} glutamate, a significant response was observed implying that this concentration of glutamate was beyond the capacity of the GDH reactor. However, glutamate concentrations up to 0.080 g·L^{-1} did not give any current response. Further, in the majority of mammalian cell cultures studied by the authors, endogenous glutamate concentrations usually lie in the range 0.010−0.050 g·L^{-1}. At these levels, no interference from this source should be observed in the glutamate dehydrogenase reactor. In addition, the sample matrixes did not give any other extraneous peak current that could interfere with glutamine determination.[91] However, the addition of 50 mM NaCl to the carrier buffer was found to be useful to prevent any binding between substances in the sample matrixes and the controlled-pore glass beads.[180] The results showed satisfactory agreement as compared to HPLC.[91] Biosensors for the specific determination of L-glutamate were developed,[168,181] and when glutaminase was also coimmobilized, a bienzyme electrode was obtained sensitive to L-glutamine.[168,181]

3.2.3. Histidine

An amperometric flow-type L-histidine sensor using an immobilized galactose oxidase (GAO) reactor based on a novel catalytic activity induced by exogenous histidine was developed.[169,170] The specificity of GAO was drastically changed upon the addition of L-histidine, and the GAO catalyzes the air oxidation of L-ascorbate (AsA):

$$\text{GAO} + \text{histidine} \rightleftarrows \text{GAO}-\text{histidine}$$

$$\text{AsA} + \text{O}_2 \xrightarrow{[\text{GAO}-\text{histidine}]} \text{DAsA} + \text{H}_2\text{O}_2 \quad (30)$$

The exogenous histidine binds at the active copper site of GAO and leads to the change in the coordination structure. The electron spin resonance (ESR) signal decreases because Cu(II) is reduced to Cu(I) (ESR-inactive) by AsA, and the Cu(II) signal is regenerated by introducing O_2 gas into the enzyme solution. Without histidine, these changes in ESR spectra were not observed. The binding of histidine is reversible, and this histidine-induced reaction of GAO was applicable to a FIA system for detecting histidine with an immobilized GAO reactor and an AsA-containing carrier.[169,170]

3.2.4. Lysine

Three different enzymes are known to be applicable to analytical systems for L-lysine quantitation: L-lysine-2-monooxygenase (LMO), L-lysine-α-oxidase (LO), and L-lysine decarboxylase (LD) catalyze the reactions

$$\text{L-lysine} + \text{O}_2 \xrightarrow{\text{LMO}} \delta\text{-aminovaleramide} + \text{CO}_2 + \text{H}_2\text{O} \quad (31)$$

$$\text{L-lysine} + \text{O}_2 \xrightarrow{\text{LO}} \alpha\text{-keto-}\epsilon\text{-aminocaproate} + \text{H}_2\text{O}_2 + \text{NH}_3 \quad (32)$$

$$\text{L-lysine} \xrightarrow{\text{LD}} \text{cadaverine} + \text{CO}_2 \quad (33)$$

A flow-injection system based on LMO showed high specificity, assay rate and precision and good reproducibility, but the kinetic behavior of LMO did not allow the determination of L-lysine at concentrations lower than 5.5 mM.[182] Selective biosensing by a low-temperature flow-injection technique using an immobilized LO reactor has been developed.[92] When the system temperature is 10 °C, a substantial improvement in the selectivity for L-lysine was achieved.[92] In general, arginine,[35,86,100,105] phenylalanine,[35,100] ornithine,[66,86,100] and cysteine[83,86,114,130] were the main interferents.

A fast procedure for L-lysine analysis in food was developed by coupling in sequence a microwave protein hydrolysis technique and a lysine enzyme electrode.[35] Protein hydrolysis was carried out in a microwave digestion system with 6 N HCl, and L-lysine-α-oxidase was used as catalyst. The selectivity of the lysine probe as a function of pH was investigated. When working at pH 7.0, the probe responded also to arginine and phenylalanine. However, at pH 4.2, the interference of arginine was practically negligible. Response to phenylalanine remained unvaried at this pH. Besides, with use of lysine and phenylalanine standards, it was observed that the rate of reaction was higher for lysine than for phenylalanine. Upon injection of standard solutions containing equal concentrations of both amino acids, the current change in the first 30 s was almost totally due to the lysine. This led to the use of a FIA procedure to improve selectivity. In fact, with use of this procedure the measured current is transient, and the current peak is recorded at a time that is a function of the flow rate and of the lag time between the injection of sample and its reaction with the immobilized enzyme. The best selectivity was obtained using a flow rate of 200 μL·min^{-1} with a lag time of 30 s. Results indicate that the interference of phenylalanine, which is about 33% in batch, is not more than 5% in FIA with an analysis time of 2 min/sample. This procedure allows reduction of the total analysis time for lysine from more than 1 day to 2 h, maintaining the same accuracy.[35]

Furthermore, these authors[105] have proposed a new biosensor for lysine. L-Lysine oxidase was immobilized on Immobilon by the BSA−glutaraldehyde procedure in a 0.03 μm polycarbonate membrane to protect the enzyme from possible interferences and to obtain an extended linear concentration range. If a 0.8 μm polycarbonate membrane is used, the sensitivity is better because the diffusion of the substrate through the membrane pores is more rapid; however, the linearity is poorer. The interferent effects of tyrosine, phenylalanine, and histidine were negligible. Other amino acids did not give any appreciable response. Analysis in feeds was carried out by acid hydrolysis to liberate lysine; then, the solution was analyzed by the bioprobe and HPLC procedures. Both flow-through and FIA methods correlated well with the HPLC procedure. Increasing temperature between 15 and 40 °C results in an increase in probe response. This effect is due to two factors: the increase in enzyme activity and the increased rate of substrate diffusion through the membrane. However, for temperatures up to 30 °C, the current noise also increased, resulting in a lower current/noise signal.[105]

The construction of an L-lysine biosensor by immobilizing lysine oxidase on a gold−poly(*o*-phenylene diamine) electrode is described.[114] The behavior of this Si−gold electrode against H_2O_2 and lysine is pH-dependent, that is, the lower the pH, the higher the oxidation potential of the lysine. At pH 9.0, the H_2O_2 oxidation starts at a potential of 200 mV, while at pH 7.0 and 4.0, the oxidation potential is 400 and 1000 mV, respectively. Thus, the gold electrode can be used for the H_2O_2 measurement only at pH values close to 9 or higher, since at those pH values the oxidation potential for H_2O_2 is low, about 600−700 mV. The biosensor responded mainly against tyrosine and cysteine, while the response to phenylalanine, arginine, histidine, and ornithine was very low. By

changing the electropolymerization conditions from 5 to 100 mM *o*-phenylalanine, the effect of interferents was further reduced.[114]

Effect of immobilization procedures of L-lysine-α-oxidase on collagen membrane, either native or activated with urea, or on nylon net was studied.[86] Biosensors prepared by coimmobilization of lysine oxidase and catalase on a collagen membrane in the absence of cyclohexyl isocyanide (CHIC) showed a decrease in enzyme activity during 3 months. If the enzyme conjugates immediately after the immobilization procedure were stored at 4 °C and pH 7.5 and were not used for determination, they retained a high enzyme activity regardless of the type of carrier and the presence of CHIC. CHIC has a mild stabilizing effect. Coimmobilized catalase permitted a considerable shortening of the time between two assays.[86]

Interferent-free biosensors for L-lysine have been developed[82,83] and optimized using a novel H_2O_2 sensor produced through electrodeposition of ruthenium and rhodium on a carbon surface. A polymer film of 1,2-diaminobenzene acts as a size-exclusion membrane. The immobilized enzyme is lysine oxidase. Flow rate can have a strong impact on the selectivity of a system. This seems to be due to the potentially slower rate of response to enzymatic interferents and the potentially faster responses for electrochemical interferents. Thus, the flow rate has a strong effect on the response of L-lysine, increasing 600% on going from 3 to 0.125 $mL \cdot min^{-1}$. Also, the L-cysteine becomes an interferent with increasing response of 378% over the same range. This sensor has excellent repeatability and very good reproducibility.[82,83] Interference from cysteine, acetamidophenol, and ascorbic acid were minimized when the enzyme was adsorbed passively onto a polymer layer of 1,2-diaminobenzene.[83,130]

Flow injection amperometric and chemiluminescence methods for the individual and simultaneous determination of lysine and glucose have been described.[94] Both compounds are found in microbiological systems to synthesize lysine. Glucose is a basic nutrient in industrial microbiological systems that produce lysine. Therefore the process can be controlled better if both glucose and lysine can rapidly be monitored in the fermentation medium. The immobilized enzymes are lysine oxidase (LO) and glucose oxidase (GOx). H_2O_2 is obtained as reaction product:

$$\text{L-lysine} + O_2 + H_2O \xrightarrow{\text{LO}} \alpha\text{-keto-}\epsilon\text{-aminocaproate} + NH_3 + H_2O_2 \quad (34)$$

$$\beta\text{-D-glucose} + O_2 \xrightarrow{\text{GOx}} \text{D-gluconic acid} + H_2O_2 \quad (35)$$

For the individual determination of lysine or glucose, the sample was injected and passed through the immobilized LO or GOx minicolumn, and H_2O_2 is detected. In the simultaneous determination, the injected sample is separated in two portions by a T-piece; one portion passes through the LO column and the other through the GOx column. A delay coil was placed after the LO minicolumn to prevent the overlapping of the peaks.[94]

3.2.5. Lysine and Tyrosine

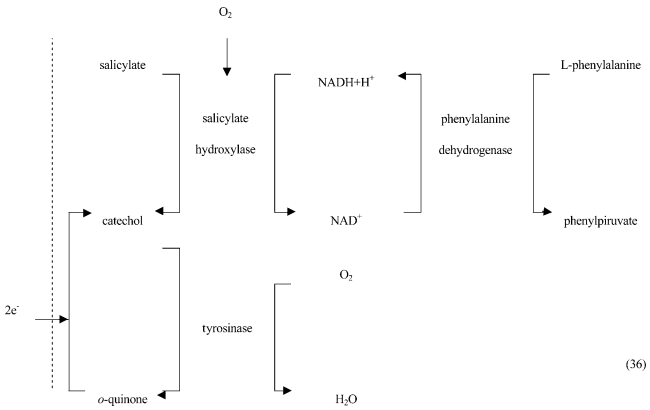
Hybrid sensors using an enzyme and a bacterial CO_2 sensor have been proposed for L-lysine[140] and L-tyrosine.[68] When the amino acid is decarboxylated by the enzyme, CO_2 is produced. The CO_2 permeates the gas-permeable membrane, reaches the bacteria, and is assimilated. Bacterial respiration increases, and they consume more O_2, so the O_2 concentration decreases. This change is detected by the oxygen electrode, and therefore, the L-amino acid concentration is determined indirectly. Because the immobilized-bacteria membrane is covered with a gas-permeable membrane, the bacteria inside the membrane are not affected by the pH. CO_2 and H_2CO_3 reach an equilibrium state depending on pH. So, the optimum pH value of these sensors depends mainly on CO_2 dissociation and the optimum pH of the enzyme. Pyridoxal phosphate was added to maintain the enzyme activity, because it is a coenzyme for the majority of decarboxylases. Only a slight response to L-phenylalanine is detected, perhaps because its structure is very similar to that of L-tyrosine.[68]

3.2.6. Phenylalanine and Tyrosine

Proteus vulgaris contains abundant phenylalanine deaminase, which oxidizes phenylalanine to phenylpyruvic acid and ammonia. The immobilized bacterial membranes in calcium alginate gel were held on an oxygen electrode.[139] Although phenylalanine deaminase or oxidase is highly selective for phenylalanine, it is known that microorganisms sometimes contain several other kinds of amino acid deaminase or oxidase. Hence the response to various amino acids, urea, and inorganic salts, which may cause interference to the biosensor, must be examined. L-Histidine and DL-alanine give relatively severe interference.[139] The phenylalanine potentiometric biosensors based on immobilized *P. vulgaris* gave similar results to those from amino acid oxidase or *P. mirabilis* probes.[183] Some bacteria, such as *Lactobacillus, Streptococcus*, and *Leuconostoc* require specific amino acids and vitamins for their growth. These bacteria produce mainly lactic acid as a metabolite. So, microbioassay of phenylalanine in sera is carried out by incubation with *Leuconostoc mesenteroides* followed by use of a lactate electrode. This lactate sensor acts by monitoring the decrease in dissolved O_2, which results from the oxidation of lactate in the presence of lactate oxidase.[141] Another microbioassay system[142] is based on the rapid growth of *L. mesenteroides* in a gel; with 2% agar concentration, the incubation time was shortened from 6 h to 90 min.

An enzyme carbon paste electrode containing three different enzymes was developed for the determination of L-phenylalanine.[40] This sensor is based on the enzymatic/electrochemical recycling of tyrosinase in

combination with salicylate hydroxylase and L-phenylalanine dehydrogenase:



(36)

Salicylate hydroxylase is a flavin enzyme that catalyzes the irreversible decarboxylation and the hydroxylation of salicylate to form catechol. Tyrosinase oxidizes catechol to *o*-quinone, which is electrochemically detected and reduced back to catechol at the electrode at −50 mV vs Ag/AgCl. The signal is amplified due to the coupling among the three enzymes and the recycling of the catechol and *o*-quinone between tyrosinase and the surface of the electrode. Interference from ascorbic and uric acids was found to be minimal. However, tyrosine interferes because tyrosinase can also utilize tyrosine as the substrate. But this tyrosine interference can be minimized by adding the sample to the measuring cell previously and allowing the tyrosinase to react first with all the tyrosine, before proceeding with the addition of the substrates.[40] In another sensor, only tyrosinase is immobilized inside the carbon paste electrode for the analysis of thiol-containing compounds such as L-cysteine.[41] The quinone formed reacts with the free-SH group of the reduced thiols. Thus, in this case, the measuring principle of this sensor is based on the blocking of the substrate recycling process between the enzyme and the electrode. The current response of this biosensor is not affected by the oxidized forms of L-cysteine, glutathione disulfide, and sulfur-containing compounds, such as methionine. But it can detect coenzyme A.[41]

Also, tyrosinase catalyzes the oxidation of tyrosine:

$$\text{tyrosine} + O_2 \xrightarrow{\text{tyrosinase}} \text{dopaquinone} + H_2O_2 \quad (37)$$

An amperometric tyrosine electrode is based on the measurement of O_2 uptake during the oxidation.[67] L-Amino acids do not show any response, but structurally similar tyramine and *p*-hydroxyphenylacetic acid interfere. D-Tyrosine reacts like L-tyrosine because the tyrosinase used is a DL-enzyme. The proposed method can be applied to monitor the total protein content in serum. Protein in serum is first hydrolyzed with pepsin, and the cleaved tyrosine is subsequently determined. The protein content is calculated by using a calibration curve.[67] Similarly, L-tyrosine concentration and protease activity can be monitored.[173] But the addition of casein, the substrate of the protease, causes the fluctuation of the electrode current, owing to the adsorption of casein on the tyrosinase layer. The casein adsorption could be avoided by covering the tyrosinase layer with a hydrophobic poly(tetrafluoroethylene) (PTFE) membrane filter.[173]

Other enzymes, such as L-glutamate oxidase, L-lysine oxidase, and tyrosinase, were coimmobilized in gelatin,[109] and the sensor was applied to the analysis of protein hydrolysates. The net charge of the immobilization matrix influenced the relative sensitivities of the amino acids.[109]

3.2.7. Tryptophan

Tryptophan-2-monooxygenase (TMO) is a flavoprotein containing one FAD per subunit. TMO catalyzes the oxygen-dependent decarboxylation of tryptophan according to

$$\text{L-tryptophan} + O_2 \xrightarrow{\text{TMO}} \text{indolacetamide} + CO_2 + H_2O \quad (38)$$

As with other FAD-containing oxidoreductases, the tight binding of FAD to TMO makes possible an analytical procedure that does not require any additional cofactors, in contrast to many other classes of enzymes. Because TMO also oxidizes L-phenylalanine and L-methionine, the biosensors show a relatively high sensitivity to these amino acids.[101,171,172] However, the biosensor selectivity to L-tryptophan could be dramatically increased when indolacetamide (IA), a competitive inhibitor of TMO, was introduced.[171,172] In the absence of L-tryptophan and IA, the biosensor can be used for L-phenylalanine determination in the concentration range 1−50 mM.[172]

A highly selective biosensor for tryptophan was made by immobilizing L-amino acid oxidase on a Pt working electrode.[144] This selectivity was achieved by coating the enzyme layer with an outer hydrophobic plasticized polyurethane film containing a ditopic carrier, manganese(III)-4,5-di-(3,5-di-*tert*-butylsalicylideneimine)benzo-18-crown-6-tetraphenylborate. Tryptophan is transported selectively from the sample solution by the carrier into the enzyme layer producing H_2O_2, which can be detected at 650 mV vs Ag/AgCl reference electrode.[144]

3.2.8. Simultaneous Analysis of D- and L-Amino Acids

An AQ polyester cationic exchanger dispersed in water has been used for the immobilization of L-amino acid oxidase at the surface of a platinum electrode followed by casting the mixture and allowing the solvent to evaporate.[64] The resulting polymer−enzyme film was covered with a thin layer of Nafion to avoid its subsequent dissolution in water. The major advantage in using this water-dispersed polymer lies in its ability to dissolve the enzyme without any significative loss of enzymatic activity. The activity of the enzymes tested was not significantly affected by the presence of methanol during the casting of Nafion as long as the enzyme is first entrapped in the AQ polymer. The enzymatic activity found after the coating with Nafion is very similar to the activity retrieved if Nafion is substituted by a dialysis membrane; in the latter case, dispersion of AQ and enzyme occurs in the dialysis membrane.

L-Leucine can diffuse through the film of Nafion and generate a current when L-amino acid oxidase is immobilized; amperometric detection of the generated H_2O_2 was used.[64] Prussian blue has been formed[153] by cyclic voltammetry onto the basal pyrolytic graphite surface to prepare a chemically modified electrode that provides excellent electrocatalysis for both oxidation and reduction of H_2O_2. This Prussian blue film shows a marked decrease in the overvoltage and a large increase in the redox currents. D-Amino acid oxidase can be incorporated into the Prussian blue film during its electrochemical growth process, and the sensor was protected by coverage with a thin film of Nafion. D-Alanine was determined with a short response time and high sensitivity.[153] Also, D-lysine could be determined in a linear range of 1−10 mM with a biosensor that uses benzoquinone as mediator between the immobilized D-amino acid oxidase and a glassy carbon electrode.[157]

The adsorption of D-amino acid oxidase on a platinum electrode followed by immobilization in an electrochemically polymerized phenol film was found to be a reproducible method for the fabrication of enzyme microelectrodes responsive to D-amino acids.[121] Another method consists of creating a cavity at the tip of the Pt microsensors.[45,46] A porous composite material, prepared from acetylene black and Teflon emulsion, was packed, and $[Os(bpy)_3](PF_6)_2$ was added as the mediator. Then, L- and D-amino acid oxidases were immobilized by adsorption. These sensors detect L- and D-phenylalanine for more than 1 month.[45,46] Phenylalanine could be determined with a relative error of less than 10% in the presence of a molar ratio of 10:1 L-ascorbic acid or (1−10):1 Na_2SO_4, $NaNO_3$, K_2HPO_4, NaH_2PO_4, NaCl, KCl, oxaloacetic acid, L-malic acid, D-dihydroxysuccinic acid, creatinine, cyanocobalamin, nicotinamide, or L-serine.[45] Immobilization on polytyramine films using glutaraldehyde coupling was applied for detection of L-phenylalanine and L-leucine.[113] The polytyramine film also affords some protection of the electrode from direct (not enzymatic) oxidation of electroactive amino acids and other interferents, which might otherwise cause fouling of the electrode surface. However, these films would not sufficiently reduce the error in estimating substrate concentration caused by the presence of cooxidizable interferents, such as tryptophan, tyrosine, cysteine, uric acid, and ascorbic acid (∼30 μM).[113] Nine L-amino acids and seven D-amino acids were determined in fermented and nonfermented foods with detection limits between 0.5 and 5 mg·L^{-1} by using HPLC−biosensor coupling.[184,185] After separation on a lithium cation-exchange column, the amino acids are converted into keto acids and H_2O_2 under catalysis by L- or D-amino acid oxidase; H_2O_2 is detected. The main emphasis was put on the determination of D-alanine, which can serve as an indicator for bacterial contamination.

D-Amino acid or L-amino acid oxidase, HRP, and the mediator ferrocene were coimmobilized by simple physical inclusion into the bulk of a graphite−Teflon electrode matrix.[42] Because both enzymes are soluble in the aqueous working medium, repeatibility of the amperometric signal was evaluated before and after regeneration of the electrode surface by polishing for some seconds on a grit SiC paper. The results suggested that both the enzymes and the mediator are uniformly distributed in the bulk of the electrode matrixes. Furthermore, utilization in batch and flow-injection conditions is possible. Selectivity of the enzyme responses is complete, and it allows quantification of the content of L- and D-amino acids in an enantiomeric mixture.[42]

Biosensors for detection of L- and D-amino acids based on coimmobilized HRP and L- and D-amino acid oxidases in carbon paste electrodes were developed. The addition of polyethylenimine (PEI) into the paste increases the response. All of the 20 L-amino acids can be detected without interfering electrochemical reactions.[135] Screen-printed three-electrode amperometric sensors incorporating L- or D-amino acid oxidase (AAO) or both for the L- or D-amino acid measurements are described.[62,128] The working electrode incorporates PEI on rhodinized carbon[62] or on iridium-containing carbon paste electrodes[128] to facilitate H_2O_2 oxidation at a decreased operating potential and the immobilized enzyme. The devices responded[62] to all 20 common L-amino acids and all of the six D-amino acids examined, the exceptions being L- and D-proline. Coimmobilization of L- and D-AAO on the electrode exhibited minimum nonstereospecificity for the range of amino acids tested except D-arginine. The presence of PEI in the rhodinized carbon working electrode was found to have no significant effect on device response to 0.1 M L-phenylalanine.[62] However, these PEI-modified electrodes were found to improve their reproducibility and stability.[62,128]

It has been found[59,60] that a polyion complex (PIC) membrane consisted of poly(L-lysine) and poly(4-styrene sulfonate) shows a permselectivity depending on the size of solutes or their molecular weights from 100 to 150 Da. Further, the PIC could be used as a matrix for entrapping enzymes and as the base polymer for attaching enzymes covalently with glutaraldehyde. With combination of D-amino acid oxidase with the PIC membrane, the sensitivity of the sensor can be adjusted. Relative response to amino acids with molecular weight over 110 Da was low compared with that to small amino acids because of the suppression of penetration in PIC, mainly when the electrode was coated with a double layer of PIC.[60]

A method has been developed[74] for continuous determination of the concentration of one amino acid in a mixture using a nonspecific L-amino acid oxidase column reactor and detecting the H_2O_2 enzymatically produced. First, a separation step is carried out by electrodialysis combined with a FIA system. Separation by electrodialysis depends on the selection of a pH such that the amino acid of interest has a different net charge and migrates away from the remaining amino acids in the sample phase into an acceptor phase under the influence of the electric field in the electrodialysis cell. The separation of arginine was used as a model to demonstrate the possibility of continuous amino acid measurement, and a group of nine amino acids were found in the presence of which arginine concentrations could be determined

Table 3. Experimental Conditions and Analytical Properties of Amperometric Biosensors Using Microdialysis

Analyte	Immobilized system	Experimental conditions	Response time (time of analysis)	Linearity (DL) mM	CV %	Lifetime	Ref.
L-glutamate in brain dialysate	L-glutamate oxidase	1 μl/min Ag/AgCl, Pt and platinized carbon paper electrodes, 600 mV; Pt-tube and stainless-steel electrodes (1500 mV) for preoxidation	< 1 min (10 min)	0.002-0.180	-	Some weeks	54, 77
L-glutamate in cultured cells	L-glutamate oxidase; Os-gel-HRP as mediator	Reactor, FIA, 1 μl/min, room-t Ag modified and two carbon electrodes, -50 mV, pH 7.4	-	- ($12\cdot10^{-6}$)	-	-	84
L-glutamate in cultured cells	L-glutamate oxidase and horseradish peroxidase; poly-(anilinomethylferrocene) as mediator	FIA, 0.5 ml/min Ag/AgCl, Pt wire and two GCE electrodes, -50 mV, pH 6.5	- (60 samples/h)	0-0.8	< 5	4°C	58
L-glutamate in extracellular fluid	L-glutamate oxidase; Os-gel-HRP as mediator	FIA, 2-4 μl/min three carbon film electrodes	4 min	$5\cdot10^{-5}$-0.01 ($4.4\cdot10^{-5}$)	-	-	110
L-glutamic in brain	L-glutamate oxidase	SCE, teflon-coated Pt wire and Ag wire electrodes, 0.7 V, pH 7.4	10 s	-	-	-	131
L-glutamate in brain	L-glutamate oxidase and ascorbate oxidase	FIA, Ag/AgCl and Pt-Ir wire electrodes Nafion film, 600 mV, 37°C, pH 7.4	1.3 s	0.002-0.060 ($2\cdot10^{-4}$)	-	3 d 4°C pH 7.4 NaN_3 1 m 4°C dry state	75
Ascorbic acid in brain microdialysate	Ascorbic oxidase	FIA, 0.2 μl/min, 38 °C Pt wire, Ag wire and Ag/AgCl electrodes, 600 mV	< 15 s	-	-	-	123, 133, 134
L-glutamate in brain microdialysate	L-glutamate oxidase and ascorbate oxidase	Pt wire, Ag wire and Ag/AgCl electrodes, 650 mV	< 15 s	0-0.01	-	-	123, 177
L-glutamate in brain microdialysate	L-glutamate oxidase and ascorbate oxidase	FIA, 0.2 ml/min Pt wire, Ag wire and Ag/AgCl electrodes, 600 mV	< 15 s	-	-	-	133, 134
L-glutamate in brain microdialysate	L-glutamate oxidase; ferrocene as mediator	Reactor, FIA, 0.5 ml/min Ag/AgCl and GCE electrodes, 0 mV, pH 7	1-2.5 min	- ($1.8\cdot10^{-6}$)	-	4 d 4°C	47
L-glutamate in brain microdialysate	L-glutamate oxidase	Reactor, 2 μ,l/min Pt, Pt and Ag/AgCl electrodes, 0.6 V, pH 7	1 min	0-0.03 ($3\cdot10^{-4}$)		1 d 4°C	55
L-glutamate in brain dialysate	L-glutamate oxidase from *Streptomyces sp*	Three electrodes system	30 s (2 min)	- ($< 5\cdot10^{-4}$)	-	-	78
L-glutamate in brain microdialysate	L-glutamate oxidase; Os-gel-HRP as mediator	Reactor, FIA, 16 μl/min, 37°C Ag/AgCl, GCE and carbon film ring-disk electrodes, 0 mV, pH 7.2	-	$1\cdot10^{-5}$-0.001 ($7.6\cdot10^{-3}$)	-	-	93
L-glutamate in brain tissue	L-glutamate oxidase; Os-gel-HRP as mediator	Reactor, FIA, 4 μl/min Pt wire, Ag wire and glass modified electrodes, 0 mV, -50 mV	14 s	- (< 10 nM)	-	-	111
L-glutamate in brain tissue	L-glutamate oxidase, ascorbic oxidase and horseradish peroxidase	Ag/AgCl and carbon fiber microelectrode, 0.1 V, 37°C	20-40 s	0-0.1 (0.001±0.003)	-	-	148
L-glutamate in brain	L-glutamate oxidase, L-glutamate dehydrogenase and saccharopine dehydrogenase	Reactor, FIA, 2 μl/min Ag/AgCl and stainless-steel tube electrodes, 0.6 V, pH 7.2	10.5 min	$8\cdot10^{-5}$-0.02 ($5\cdot10^{-5}$)	5.1	20 d 4°C pH 7.2	95
L-glutamate in brain	L-glutamate oxidase	Reactor, FIA, 1-3 μl/min Pt, Ag/AgCl and stainless-steel tube electrodes, 0.6 V, pH 7.5	-	0.002-5 (0.001)	4	-	33, 95

with good accuracy using the combined FIA system. However, the presence of amino acids other than these nine interferes in the separation. No electrodialysis conditions could be found where arginine and lysine could be completely separated. Sensitivity might be improved and resolution of more complex mixtures achieved by using a higher capacity electrodialysis cell.[74]

4. Microdialysis Methods for Glutamic and Ascorbic Acids

Microdialysis is a novel sampling technique that allows examination of local changes in the composition of extracellular fluids. It requires the implantation of a probe with a dialysis membrane permeable to lower molecular weight substances through which an artificial medium is slowly perfused. A concentration gradient drives substances from the extracellular space to the perfusate. The perfusing solution is chosen to match the tissue being sampled but can also be used to deliberately perturb the sampled area, allowing the dynamic properties of the systems to be studied in addition to basal conditions. The dialysis fluid is artificial cerebrospinal fluid, which is perfused at $1-2\ \mu L\cdot min^{-1}$. A mediator cannot be used with microdialysis if the probe must be implanted in the brain because the majority of mediators are toxic. When necessary, mediator is injected in the buffer pumped to the downstream detection electrode. As mediators, ferrocene derivatives[47] or osmium−poly(vinyl pyridine) containing HRP[57,84,93,110,111,149] were used (Table 3). Microdialysis may be used in conjunction with biosensors based on amperometric enzyme electrodes.[33,47,54,55,57,58,60,75,77,78,84,93,95,106,110,111,123,131,133,134,148,149,177] Platinized carbon paper-based biosensor electrode and platinum-based electrodes have been compared.[54,77] Platinized carbon paper-based electrodes are not suitable for measurements of substrate concentrations much less than 100 μM. In contrast, these designed platinum electrodes are almost immune to pH fluctuations and ionic salt concentra-

tions likely to be encountered in vivo.[77] The platinum-based enzyme electrode biosensor, when coupled to a preoxidizing cell or to a polymer barrier, provides a simple and quite effective answer to the problem of measuring glutamate in the dialysate from the extracellular fluid in the brain.[33,54,55,60,75,77,78,93,95,123,134,148,177] The response time, moreover, is better than that currently achieved by HPLC or fluorimetry.[77]

In the analysis of L-glutamate in brain,[54] the sample solutions are passed through a platinum tube electrode inserted into the sample flow line, which is fixed to a suitable oxidizing potential; thus, they are subjected to direct current pre-electrolysis before delivery to a detector cell containing an enzyme electrode. This method avoids the interference of monoamines and ascorbic acid. To assess the magnitudes of background currents arising from interferents and their oxidation products on the enzyme electrode, tests were carried out using a blank electrode prepared by destroying the enzyme activity of the glutamate oxidase biosensor electrode. This was achieved by first fixing it at +3 V for 30 min with artificial cerebral spinal fluid perfusing through the flow line. The application of 1.5 V to the preoxidizing cell caused 62% of dopamine and 75% of 3,4-dihydroxyphenylacetic acid to be destroyed and ascorbic acid, homovanillic acid, serotonin, 5-hydroxyindole, and uric acid to be completely eliminated.[54,77] With potentials of 1.7 V, complete oxidation of dopamine and 3,4-dihydroxyphenylacetic acid was achieved. These compounds are the major electroactive interferents present in brain extracellular fluid. HPLC analysis of the reaction products showed that the preoxidation treatment efficiently removes the principal interferents, but leaves unaffected L-glutamate, the analyte of interest.[54] Other probes were fabricated[57,84] avoiding the influence of other coexisting substances (e.g., L-ascorbate or glutamine). The sensors comprise a working electrode modified with a membrane containing glutamate oxidase treated with a glutaminase inhibitor, such as 6-diazo-5-oxo-L-norleucine, and a cell for pre-electrolysis installed upstream of the working electrode[57] or two carbon film electrodes arranged in parallel to the flow and separated by a separator (ferrocenyl derivative) in the flow cell.[84] This arrangement avoids the electroactive species generated at the upstream electrode interfering with the signal at the downstream electrode.[84] The electrode phase is modified with an electrolysis-stimulating material, such as platinum black, polypyrrole membrane,[57] or osmium−poly(vinyl pyridine) complex membrane.[57,84]

Other bienzyme sensors based on modified electrodes were fabricated.[58,110,149] Glutamate oxidase was immobilized on HRP−osmium−poly(vinyl pyridine)[110,149] or on HRP−poly(anilino-methyl-ferrocene)-modified glassy carbon electrodes.[58] Because of the detection at a low applied potential, electroactive compounds such as ascorbic acid,[58,110] uric acid, and acetaminophen did not interfere.[58] Continuous monitoring of L-glutamate release from cultured rat nerve cells could be carried out at slow flow rate (200 $nL \cdot min^{-1}$ to 2 $\mu L \cdot min^{-1}$).[149] The glutamate oxidase enzyme layer was immobilized upstream of the working electrode, and a syringe pump in the suction mode was used to sample extracellular fluid continuously.[149]

Microsensors for in vivo measurement of glutamate[47,55,75,93,106,111,123,127,131,133,134,148,177] and ascorbate[123,131,133,148] in the extracellular space of brain tissue were described. Ascorbate is present at a much larger concentration in the brain than glutamate. Both substances are oxidized at the electrode surface. The application of a stimulus causes an immediate increase in both signals. When ascorbate oxidase is introduced into the microelectrode, ascorbate is oxidized before it can reach the electrode; now, the basal current obtained is much lower, and no rise in ascorbate can be measured. If both ascorbate oxidase and glutamate oxidase are introduced, the basal current increased due to the resting glutamate levels in the brain. So, it is possible to measure in real time the concentration changes of analytes by using an implanted microelectrode[131] or a dialysis microelectrode.[75,123,134,177] For the continuous real-time measurement of ascorbate in brain microdialysates, two biosensors with a polymer-coated (poly(1,2-diaminobenzene)) and a noncoated electrode were used.[123,133,134] The polymer-coated Pt electrode[123,134] repels possible electroactive interferents, such as dopamine, DOPAC, 4-hydroxy-3-methoxyphenylacetic acid, and urate. The noncoated electrode was perfused with a Ringer solution, and the polymer-coated electrode was perfused with the Ringer solution containing ascorbate oxidase. The difference between the two signals was considered to be the ascorbic signal. Although 95−99% of the ascorbate is repelled by the polymer coating, it is present in such large concentrations that it is still detectable. The ascorbate is oxidized directly on the Pt surface. These probes[123,134] were used to measure ascorbate and glutamate concentrations by coimmobilizing glutamate oxidase and ascorbate oxidase. Preoxidation of ascorbate avoids the problem of interference[75,93,123,134,177] and prevents fouling the Pt surface of the detection system by dehydroascorbate.[177] The preoxidation system consists of a second three-electrode system (Pt, Ag/AgCl, Pt) of the same dimensions as the detection system but with an uncoated working electrode. Flow rates of 2 $\mu L \cdot min^{-1}$ enhance the efficiency of preoxidation.[177] Glutamate oxidase, HRP, and ferrocene were used[47] in a FIA system. The use of HRP followed by reduction of the ferrocene species allowed the electrode to be held at a mild potential of 0 mV vs Ag/AgCl; the system is not subject to interferences.[47] However, this method[47] is not strictly a real-time monitoring process because of the limited sampling rate of 2.5 min intervals. The same effect is obtained by using Os-gel-HRP.[93,111] Also, glutamate and ascorbate oxidases, HRP, and a redox polymer film were immobilized on silicon chip microreactors[106] or onto carbon fiber microelectrodes.[148] With this probe,[148] the value found for the basal glutamate concentration is noticeably higher than values previously reported, and this result is attributed to the small dimensions of the microsensors, which inflict less trauma on the brain tissue and

thereby gain greater access to neuronally active brain tissue.[93,111,148] Miniaturized wall-jet-type flow cells with active volumes of 0.042−15 nL were fabricated[111] for use as highly sensitive electrochemical detectors for capillary electrophoresis and small on-line enzyme sensors. The glutamate responses obtained with the wall-jet-type on-line sensor (4 μL·min^{-1}) and those obtained with a carbon fiber cylindrical electrode modified also with glutamate oxidase and Os-gel-HRP were compared. The response curves of both electrodes were similar except for a 1.7 s delay in the wall-jet cell. The same measurement was also possible when the flow rate was lowered to a submicroliter per minute level. The sensor was sufficiently sensitive to measure a small peak, which was calculated as a 30 nM glutamate concentration change, and the peak was more than 10 times greater than the noise level.[111] On-chip enzyme microreactors[106] of different sizes (5.3 and 0.95 μL) were used for eliminating ascorbate interference during the on-line monitoring of glutamate in brain microdialysates. Within the studied flow rate range (2−25 μL·min^{-1}) and at −50 mV (vs Ag/AgCl), 1 mM and 200 μM ascorbate could be totally eliminated using the larger and the smaller microreactor, respectively.[106]

Yao et al.[95] describe a highly selective and sensitive detection of L-glutamate using a coimmobilized L-glutamate oxidase−glutamate dehydrogenase reactor as an on-line amplifier in an amperometric microflow system with a microdialysis probe. In addition, a poly(1,2-diaminobenzene)-coated platinum electrode was also used as the highly selective detector for the H_2O_2 generated without electrochemical interferences from NADH, urate, and L-ascorbate. The polymer film blocks the access of these interferents at concentrations below 2 mM to the electrode surface.[33,95] However, the presence of 2-oxoglutarate interferes, and thus, a saccharopine dehydrogenase immobilized PTFE reactor was inserted in series before measuring reactor to remove 2-oxoglutarate in the dialysate according to

$$\text{2-oxoglutarate} + \text{L-lysine} + \text{NADH} \xrightarrow{\text{saccharopine dehydrogenase}} \text{saccharopine} + \text{NAD}^+ \quad (39)$$

This method permits the on-line and real-time measurements of trace amounts of L-glutamate in microdialysates.[95] Previously, these authors[33] proposed a similar method in which the only difference is the use of L-glutamate oxidase alone, (i.e., without enzymatic substrate recycling). But in this case, the sensitivity is not sufficient for the in vivo monitoring of trace amounts of L-glutamate.

Enzyme-modified microelectrodes preparation and electron-transfer mediators used for in vivo neurochemical measurements have been reviewed.[5]

5. Conclusions

(1) Amperometric biosensors for the determination of vitamins and α-amino acids have been constructed with different kinds of biological structures. With the exception of antibodies, microbial, tissular, subcellular, and enzyme electrodes are used.

(2) Immobilization of biological structures onto the transducer is a fundamental step in the production of biosensors. So, different physical and chemical methods have been commented. Also, the advantages and disadvantages of the various kinds of membranes or supports are discussed.

(3) Strategies developed to overcome problems related to interference of other electrochemical substances are studied. So, multienzyme systems, preelectrolysis techniques, metal-dispersed carbon paste electrodes and use of different types of mediators are compared. Also, reactors, FIA systems, and on-line analysis are commented.

(4) Multienzyme systems are frequently used. Enzymes can be coupled to detect two or more analytes or to increase the selectivity for one analyte. But considerable enhancement of the sensitivity of enzyme electrodes by substrate recycling reactions is demonstrated.

(5) Microdialysis allows the observation of local changes in the composition of extracellular fluids. This sampling technique may be used in conjunction with amperometric biosensors. Several examples for in vivo measurement of glutamate and ascorbate in brain tissue are reviewed.

(6) At present, one of the most promising analytical fields is that of the biosensors. Advances in the investigation of the biological recognition systems, sensing techniques, and related disciplines constantly lead to new principles and enable improvements to be made in the methods and devices previously developed. So, a rapid increase in the number of producers and equipment types is found, as well as in the variety of sizes, applications, operational, and commercial strategies. However, although many α-amino acids are studied, more investigation is needed mainly in the case of the essential amino acids. From the nutritional viewpoint, besides the essential ones, information on aromatic and ramified amino acids for diets design is very important. In relation with vitamins, only data on B_{12}, and nicotinic and ascorbic acids have been found. Biotin and folic acid have been studied by means of affinity biosensors but not with the amperometric ones. These may be a lines of future research work in the biosensors field.

6. References

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CR0304471