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Enzyme Electrodes

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The literature data on the use of immobilised enzymes in analytical chemistry are reviewed and the fundamental conditions for the creation of kinetic methods in enzymatic analysis are described. The types of enzyme electrodes and the factors influencing the response of such electrodes, their stability, and sensitivity are discussed in detail. The possible pathways towards further development of enzyme electrodes for use in laboratory and technological practice and for monitoring the environment are considered.
The bibliography includes 69 references.

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I. INTRODUCTION

The last decade has been marked by a vigorous development of research in the field of the immobilisation of enzymes and their use for technological, analytical, and scientific purposes. The conversion of enzymes into a water-insoluble state with retention of catalytic activity made it possible to extend greatly our ideas concerning the possibility of using bio-organic catalysts in a wide variety of different fields of chemistry, fine chemical engineering, pharmacology, and medicine. Because of their high specificity, enzymes have already been used for many years in analytical chemistry; hundreds of studies devoted to kinetic methods in enzymatic analysis are published annually. The use of immobilised enzymes for these purposes stimulated the creation of methods of "reagent-free" analysis whereby continuous analysis of aqueous solutions of organic (and in many instances inorganic) compounds is possible in principle.

In their turn, the achievements in this field have promoted the development of effective methods for monitoring the state of the environment, clinical diagnosis, etc. Finally, the so called "enzyme electrodes", developed in recent years, permit rapid automatic analysis of multicomponent systems on the basis of combined "electrode-enzyme" electrochemical systems which exhibit a high selectivity in the determination of individual compounds or classes of compounds.

The attention devoted at the present time to the development of enzyme electrodes and to the study of the prospects for their application in analytical chemistry is partly due to the vigorous development of methods for the isolation, purification, and identification of enzymes. According to data presented at the Symposium on Biotechnology and Bioengineering in Henicker [1], the number of enzymes which have been discovered and characterised has increased from 80 in 1928 to 1500 in 1969, and continues to grow exponentially with a ten-year doubling period.

The present review is an attempt to summarise and give a systematic account of studies on the use of immobilised enzymes in analytical chemistry published up to April 1974, and to assess the immediate prospects in this field.

II. THE SPECIFICITY OF ENZYME ACTION

The most characteristic feature of enzymatic catalysis is the specificity of enzyme action. At least three general qualitative types of specificity have now come to be differentiated from this extremely comprehensive term: specificity with respect to the medium, specificity with respect to the reaction, and substrate specificity (specificity with respect to the structure of the substrate). The first two types of specificity are usually expressed in the fact that the enzyme catalyses a reaction of a particular type under specified conditions and constitutes the basis for the subdivision of enzymes into the corresponding classes (oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases or synthetases)[2]. The substrate specificity of the enzyme is its most characteristic feature, which has not yet found adequate analogies in non-enzymatic catalysis, and makes it possible to characterise unambiguously and to identify the given enzyme among hundreds and thousands of others. The usual phenomenon in enzymatic reactions is that the enzyme catalyses the reaction of only one stereochemical form of the substrate, being completely inactive in relation to other stereoisomers (the so called stereochemical specificity of the enzyme).

In many instances the enzyme exhibits absolute specificity, catalysing the reaction of only one substance and not reacting with its derivatives or homologues. Thus, butanediol dehydrogenase catalyses the conversion of butane-2,3-diol into acetoin, but altogether fails to oxidise ethylene glycol, glycerol, ethanol, isopropyl alcohol, isobutyl alcohol, isopentyl alcohol, or glucose [3]. Lactaldehyde reductase, which catalyses the reversible conversion of D-lactaldehyde into propane-1,2-diol, is completely inactive in relation to L-lactaldehyde, acetaldehyde, propionaldehyde, glycolaldehyde, acetol, glyoxylic acid, pyruvic acid, D-galactose, D-mannose, D-fructose, D-or L-arabinose, and L-rhamnose [4]. Mannitol dehydrogenase,

which catalyses the conversion of D-fructose into mannitol, does not act on D-glucose, D-mannose, or D-ribose[5]. The activity of glucose oxidase, which oxidises β-D-glucose with formation of gluconic acid, is four times lower with respect to 2-deoxy-D-glucose, 50 times lower with respect to 6-methyl-D-glucose, 100 times lower with respect to D-mannose and D-xylose, and 200 lower with respect to α-D-glucose; the enzyme altogether failed to have any action on the remaining 80 sugars which had been tested as substrates[6]. Formaldehyde dehydrogenase, which oxidises formaldehyde to formic acid, does not react with acetaldehyde, glycolaldehyde, and benzaldehyde[7]. Benzaldehyde dehydrogenase, which converts benzaldehyde into benzoic acid, altogether fails to oxidise acetaldehyde, propionaldehyde, crotonaldehyde, formaldehyde, and DL-glyceraldehyde[8]. Among all the aliphatic aldehydes, aminobutyraldehyde dehydrogenase acts only on 4-aminobutyraldehyde, oxidising it to 4-aminobutyric acid[9].

On the other hand, many enzymes exhibit broad specificity without the imposition of stringent requirements on the structure of the substrate. Thus a large number of compounds having the general formula $R_1CH(R_2)COR_3$, where the substituents R_1, R_2, and R_3 can have virtually infinitely variable structures, can serve as substrates for α-chymotrypsin[10]. The extremely broad specificity of α-chymotrypsin may be illustrated by the fact that this enzyme is capable of catalysing the hydrolysis of proteins, peptides, esters, amides, hydrazides, alkylamides, acid anhydrides, acid chlorides, hydroxamides, anilides, thioesters, oxazolones, cyclic peptides, cyclic phosphates, lactones, and sultones; in some cases it is also capable of cleaving the C–C bond. Aldehyde oxidase exhibits group specificity with respect to aliphatic aldehydes, catalysing the oxidation of formaldehyde (1.0), acetaldehyde (4.6), propionaldehyde (5.1), butyraldehyde (12.2), valeraldehyde (2.4), and crotonaldehyde (7.6); the relative reactivities of the aldehydes in this enzymatic reaction are indicated in brackets[11].

The high enzyme specificity (absolute or group specificity) in combination with favourable factors such as the high rate of enzymatic processes, the mild reaction conditions (room temperature, atmospheric pressure, neutral pH of the medium, etc.), and a fairly wide selection of available enzymes have ensured the development of a new trend in analytical chemistry—kinetic methods in enzymatic analysis. Enzyme-catalysed reactions are used to determine substrates, inhibitors, activators, and in many instances the enzymes themselves (this applies primarily to the analysis of biological liquids in clinical laboratories). The use of enzymes in analytical chemistry has been considered in a number of reviews and monographs[12-19]. However, despite a number of the evident advantages of kinetic methods in enzymatic analysis, their use in laboratory practice has so far been fairly limited. One of the main reasons for this is still the high cost of the majority of enzymes, which virtually precludes the large-scale application of enzymatic analytical methods (because enzymes can be used only once owing to the extremely laborious procedures needed for their regeneration from the mixture). This disadvantage is compounded also by the low stability of the majority of enzymes, which frequently leads to their complete inactivation in several days, and sometimes in several hours under the optimal conditions for analytical purposes.

Thus, in order to increase the "technological applicability" of enzymes, it is necessary to increase their stability, on the one hand, and to ensure the possibility of their repeated use on the other. In principle this is achieved by converting enzymes into heterogeneous catalysts by immobilising them.

III. THE IMMOBILISATION OF ENZYMES

The immobilisation of enzymes is a procedure whereby they are converted into a water-insoluble state with retention (partial or complete) of catalytic activity. Immobilised enzymes are usually obtained by the following methods (see the relevant reviews[20-24]):

1. The covalent attachment of enzyme molecules to a water-insoluble carrier (cellulose, agar, dextran, glass, paper, fabrics, polystyrene, nylon, metals, ion-exchange resins, etc).
2. The entrainment of the enzyme in a gel or polymer network.
3. The covalent cross-linking of enzyme molecules with one another using a di- or poly-functional reagent.
4. The adsorption of the enzyme on water-insoluble carriers (as a rule ion-exchange resins).
5. Microencapsulation (the enclosure of an enzyme solution in semipermeable capsules 5–300 μm in size).

The first two immobilisation methods are most often used. The covalent binding of the enzyme to a carrier leads to a stable system in which the protein cannot be desorbed when the temperature, pH, or the ionic strength of the reaction medium are changed, and also when the substrate is added to the reaction medium. The method based on the entrainment of the enzyme in a gel is distinguished by simplicity and the absence of appreciable influences on the active centre of the enzyme. In any case the immobilisation of enzymes makes possible their repeated use in practice when catalytic processes are carried out in periodic or continuous operation "enzyme reactors". Furthermore, the immobilisation of enzymes frequently increases their stability. According to the data quoted in Melrose's review[21], in thirty out of fifty different variants of immobilisation the stability of the immobilised enzyme was higher than that of the corresponding soluble enzymes, in eight cases the stability decreased on immobilisation, and in twelve cases the stability hardly changed following immobilisation.

Bearing in mind that the stability of immobilised enzymes is influenced to a high extent by the nature of the carrier, the nature of the grafting agent, and the method and conditions of the immobilisation (in all instances there is a possibility of a large number of different variants), it is clear that the possibilities of stabilising enzymes on immobilisation have been by no means exhausted. As an example, one may quote the data of Kaplan and coworkers[25] according to which lactate dehydrogenase, which loses 100% of its activity after incubation for approximately 1 h, retains its activity fully under the same conditions for at least 35 days following immobilisation on porous glass with the aid of glutaraldehyde.

IV. THE APPLICATIONS OF IMMOBILISED ENZYMES IN ANALYTICAL CHEMISTRY

The use of immobilised enzymes, whereby routine chemical analyses can be carried out on individual samples or in a stream (with repeated employment of the same enzyme preparation), has to a large extent solved the problem of the high cost of enzymatic analytical methods and has

frequently increased the accuracy of the analytical procedure. There exist two general approaches to the analytical determination of the concentrations of reagents (substrates) in the test system. In one of them the enzymatic reaction is continued until the complete exhaustion of the substance to be determined (or until the attainment of an equilibrium in the system between the initial reactants and products), while recording at the same time the change in a suitable physical or chemical property of the system, the amount of substrate in the initial specimen being calculated from the amount of product formed. In the second approach use is made of kinetic analytical methods, the rate of appearance of the product or of the disappearance of the substrate in the enzymatic reaction being determined and the initial substrate concentration being calculated from the corresponding calibration curve. This method is also applicable to the determination of the concentrations of effectors (inhibitors or activators), which are present in the reaction system. Both approaches have been applied in practice using immobilised enzymes.

The first study in which the use of immobilised enzymes for analytical purposes was described was carried out in 1965 by Guilbault and coworkers[26], who found that cholinesterase entrained in a starch gel and deposited on a foam plastic plate (to increase the mechanical strength of the immobilised enzyme) retained its activity for 12 h. The activity of the enzyme in the hydrolysis of butyrylthiocholine iodide was determined electrochemically using platinum electrodes between which a direct current was passed. Depending on the rate of the enzymatic reaction, the potential of the system changed from 150 mV (the oxidation potential of the reaction product–thiocholine) to 350–400 mV (the potential of the oxidation of the iodide of the substrate to iodine), which made it possible to investigate the influence of the added effectors on cholinesterase under continuous conditions. In the same year Guilbault and Kramer[27] used similarly immobilised cholinesterase to detect small amounts of organophosphorus insecticides in air. Foam plastic plates impregnated with starch gel containing immobilised cholinesterase have been used recently to devise a continuous analyser for water designed to detect rapidly subtoxic concentrations of anticholinesterase compounds[28].

A common disadvantage of all these devices is their instability due to the appreciable elution of the enzyme from the starch gel. Coprecipitation of cholinesterase with aluminium hydroxide and suspension of the resulting preparation in a starch gel with subsequent impregnation of a foam plastic plate enabled Goodson et al.[29] to obtain a stable enzyme system capable of functioning for 56 h without appreciable loss of enzyme. During this period, 2700 litres of water were passed through the carrier with the immobilised cholinesterase. According to the results of Goodson et al.[29], the immobilised enzyme gives a response after 3 min when water contains 0.2 part per million of 2,2-dichlorovinyl dimethyl phosphate. Gutknecht and Guilbault[30] also reported the development of an enzyme system for the continuous monitoring of cyanide ions in drainage waters, which is based on the specific reaction of immobilised injectase (β-cyanoalanine synthase).

Hicks and Updike[31] determined glucose and lactic acid by means of a column with glucose oxidase and lactase dehydrogenase immobilised on a polyacrylamide gel. The substrate specimens were passed through columns filled with gel-enzyme particles of standard size and subjected to lyophilic drying, mixed with indicators changing colour on interaction with the corresponding products of enzymatic reactions, and passed through the spectrophotometer cuvette. The authors[31] noted an appreciable increase of the stability of lactase dehydrogenase following immobilisation in the gel. Thus at 37°C the activity of soluble lactase dehydrogenase decreases to 10% of the initial value after 2 h, while that of the gel-enzyme remains completely unchanged for 10 h under the same conditions. Guilbault and Das[32] made a comparative study of starch and polyacrylamide gels as carriers for the immobilisation of cholinesterase and urease. Noting that immobilisation in the starch gel can be carried out under milder conditions, the authors[32] point out the higher stability of enzymes in the polyacrylamide gel.

Hornby et al.[33] developed a method for the automated analysis of solutions for glucose having immobilised glucose oxidase on the inner surface of a polystyrene tube (375 cm long, with an internal diameter of 2 mm). The activity of the enzyme was recorded at 35°C and pH 5.6 by the calorimetric determination of hydrogen peroxide (the product of the enzymatic reaction) using potassium iodide. The apparatus permitted 40 analyses per hour, glucose being determined quantitatively over the concentration range 5×10^{-4} M–10^{-2} M. According to the authors' data[33], the tube with immobilised glucose oxidase did not exhibit an appreciable loss of catalytic activity in the course of 1.5 months.

Subsequently Hornby and coworkers abandoned the polystyrene carrier because of its pronounced hydrophobic properties, and their later studies on the immobilisation of enzymes[34-36] were carried out using nylon. Thus urease, bound via glutaraldehyde to the inner surface of a partly hydrolysed nylon tube[34,35] (2 m long, with an internal diameter of 1 mm), was used to determine urea in the course of four months, more than 400 analyses being carried out during this period. Urate oxidase, immobilised on a nylon powder, was used to analyse aqueous solutions of uric acid[35] in the concentration range 10^{-5}–10^{-4} M. However, carboxy-groups, ionised under experimental conditions, remain on the carrier surface after partial hydrolysis of the nylon, which makes the carrier unsuitable for the immobilisation of many enzymes.

In order to extend the scope of the proposed method, Hornby et al.[36] converted the carboxy-group of the carrier into an amide form by treatment with *NN*-dimethyl-1,3-propylenediamine and used the modified carrier to immobilise lactate dehydrogenase, malate dehydrogenase, and alcohol dehydrogenase, which permitted the analysis of aqueous solutions for pyruvic acid, oxaloacetic acid, and ethanol respectively. According to their data[36], the method is suitable for the quantitative determination of 4×10^{-5}–2×10^{-4} M pyruvate, 2×10^{-5}–1.6×10^{-4} M oxaloacetate, and 0.01–0.1 M ethanol. All the immobilised enzymes fully retained their activity in the course of 20 days. During this period, each was used to perform at least 1000 analyses. Under these conditions, the soluble enzymes lost more than 90% of their activity.

In a recent study by Weibel et al.[37], a general method was developed, in relation to the analysis of solutions for their glucose content, for quantitative determination of compounds in the course of the enzymatic conversion of which oxygen dissolved in water is absorbed. In the determination of glucose, the solution analysed is passed with the aid of a micropump through a 5×35 mm column containing glucose oxidase bound covalently to porous glass and is passed through a cell containing a Clark oxygen electrode. The analysis can be carried out either under kinetic conditions or on the basis of the degree of reaction established following the completion of the enzymatic process. A single analysis takes 2 min and the error in the

determination of glucose does not exceed 1–2%. According to the results[37], the column functions for more than 2 weeks when used daily in the course of 8 h.

Mosbach and coworkers[38] described the use of flow microcalorimetry in kinetic methods for enzymatic analysis using immobilised enzymes. The advantages of the method are the possibility of analysing opaque solutions and high sensitivity (down to 10^{-6} M of the substance to be determined).

An interesting and very promising approach to the selective determination of small amounts of metals was demonstrated recently by Stone and Townshend[39,40]. Fungal polyphenol oxidase was immobilised by being bound covalently to Enzacryl AA (obtained by reducing a copolymer of acrylamide, *p*-nitroacrylanilide, and *NN'*-methylenebisacrylamide) and was then dialysed until the complete removal of copper(II) ions forming part of the enzyme active centre. The resulting inactive apoenzyme is reactivated reproducibly and very selectively on being incubated with trace amounts of Cu^{2+} (10–200 ng), the observed rate of the enzymatic reaction being unambiguously related to the Cu^{2+} concentration in the solution being analysed (a linear relation between the rate of reaction and the concentration of copper ions in solution was observed in the concentration range 10^{-7}–2×10^{-6} M Cu^{2+}). The authors[39] noted that, in relatively large volumes of solutions being analysed, it is possible to determine 10^{-15} mg/ml of Cu^{2+} ions with the aid of this method. The stability of the immobilised polyphenol oxidase makes it possible to store it for 3 months in aqueous solution at pH 7.0 and 6°C.

In all the analytical applications of immobilised enzymes described above, the enzyme activity was determined by the reaction of the biocatalyst with the corresponding substrates or by the reaction of products of the enzymatic reaction with the corresponding indicators specially introduced into the test system. However, "reagent-free" enzymatic analytical methods, based on the use for this purpose of the so called "enzyme electrode" consisting of electrochemical devices on the sensor element of which an immobilised enzyme is deposited, have been developed to the greatest extent at the present time. Such an electrode possesses the fundamental properties required for the analytical system—specificity to the given reaction and the reagent being determined (provided that the latter is a specific substrate of the given enzyme)—and permits rapid analysis of the reaction mixture (generally speaking, the rate of analysis depends both on the characteristics of the electrochemical system itself and on the values of the kinetic parameters of the enzymatic reaction).

When the enzyme electrode comes into contact with the test solution, containing, in particular, the specific substrate of the given enzyme, the enzymatic reaction takes place in the layer near the electrode. If the product (or the substrate) of the reaction is electrochemically active, then the rate of the absolute change in electrode potential is a measure of the amount of the reagent being determined in the mixture subjected to analysis. The advantage of this analytical method is that it can be automated and continuous quantitative determination of the given reagents, including those in complex chemical or physiological systems, is possible.

The first enzyme electrode, constructed for a continuous determination of the concentration of glucose with the aid of immobilised glucose oxidase, was described by Updike and Hicks[41] in 1967. The same authors introduced the term "enzyme electrode"[41,42] for a system consisting of the polarographic oxygen electrode on the surface of which a layer of polyacrylamide gel 25–50 μm thick with entrained glucose oxidase has been deposited. When the electrode prepared in this way is immersed in a solution containing glucose, the substrate and oxygen diffuse from the solution into the layer of the gel-enzyme. The enzymatic oxidation of glucose

$$\beta\text{-D-glucose} + O_2 \rightarrow \text{D-glucono-}\delta\text{-lactone} + H_2O_2, \qquad (1)$$

which takes place in the immediate vicinity of the electrode surface, reduces the diffusional flux of oxygen through the plastic membrane of the Clark oxygen electrode, which in turn leads to an increase of the electrode current. Thus the change of the current in the electrode system depends on the rate of the enzymatic reaction and hence on the concentration of glucose in the mixture being analysed. By plotting the appropriate calibration curve, the authors[41-43] were able to determine glucose quantitatively also in biological solutions and tissues.

A similar principle served as a basis of the design of other enzyme electrodes (see the next Section), the characteristics of which are compiled in Table. Each electrode is specific either to one particular compound (urea, L-asparagine, L-glutamine, D-glucose, etc.) or exhibits group specificity (L- or D-aminoacids, primary alcohols, various penicillins). Naturally, the nature of the selectivity of the enzymatic reaction determines the region where the given enzyme electrode can be applied.

The use of coupled enzymatic reactions for analytical purposes leads to fundamentally new possibilities in the preparation of selective enzyme electrodes. This approach has so far been demonstrated only on a single example—the determination of L-phenylalanine with the aid of L-aminoacid oxidase and peroxidase, immobilised in a polyacrylamide gel on the surface of an electrode sensitive to iodide ions[44]. The operation of the electrode in the coupled enzyme system is based on the following reactions:

$$\text{L-aminoacid} + O_2 \xrightarrow{\text{L-aminoacid oxidase}} \text{2-oxo-acid} + NH_3 + H_2O_2; \qquad (2)$$

$$H_2O_2 + 2H^+ + 2I^- \xrightarrow{\text{peroxidase}} I_2 + 2H_2O. \qquad (3)$$

During the enzymatic reaction, the steady-state concentration of iodide ions at the electrode surface decreases, the rate of decrease of the I^- concentration depending on the amount of L-phenylalanine in the solution being analysed[44].

V. THE DESIGN OF ENZYME ELECTRODES

All enzyme electrodes consist of an electrochemical sensor of one type or another, on which a layer of immobilised enzyme is deposited. Apart from the polarographic oxygen electrode, which has been used for the analytical determination of glucose with the aid of immobilised glucose oxidase[41-43], this reaction has also been followed by an enzyme electrode based on a platinum disc coated with a thin layer of glucose oxidase bound chemically to a polyacrylamide gel[45,46]. The electrode is immersed in the solution to be analysed and a potential of 0.6 V is established in the system (with respect to the saturated calomel electrode). Glucose diffuses to the layer of the gel-enzyme on the electrode, where it reacts in accordance with Eqn. (1). The hydrogen peroxide formed is oxidised on the platinum electrode, so that the observed current is proportional to its concentration and hence to the initial concentration of glucose. Wingard et al.[47], who used a different design of the platinum electrode, determined by a similar method the concentration of glucose with the aid of

immobilised glucose oxidase. Polarographic enzyme electrodes have also been used by Clark for the analytical determination of glucose[48] and L-aminoacids[49] with the aid of immobilised glucose oxidase and L-aminoacid oxidase. Recently Clark[1] developed a method for the determination of methanol and ethanol in aqueous solutions and vapours with the aid of a polarographic enzyme electrode containing immobilised alcohol dehydrogenase. The sensitivity of the enzyme electrode is such that it gives an appreciable response to human breath 16 h after the consumption of alcoholic drinks.

Williams et al.[50] described an enzyme electrode in which glucose oxidase immobilised in a gel was deposited on a platinum electrode and covered by a dialysis film to prevent the diffusion of the enzyme into the solution. The glucose in the solution being analysed diffuses into the layer of gel-enzyme in an amount proportional to its bulk-phase concentration. A distinctive feature of the enzyme electrode employed in this investigation[50] is that benzoquinone was used as the oxidant instead of oxygen. It was shown[50] that benzoquinone can also oxidise glucose under the influence of glucose oxidase, so that the reaction mechanism in the layer near the electrode and on the electrode can be formulated as follows:

$$\text{glucose} + \text{benzoquinone} + H_2O \xrightarrow{\text{glucose oxidase}} \text{gluconic acid} + \text{hydroquinone}; \quad (4)$$

$$\text{hydroquinone} \xrightarrow{Pt} \text{benzoquinone} + 2H^+ + 2e. \quad (5)$$

The advantage of this method is that the concentration of benzoquinone in the reaction mixture can be readily followed. In addition, there is a possibility of measurements over a longer linear range of glucose concentrations compared with the method in which the oxygen content in the aqueous solution is recorded during the reaction. By carrying out the reaction in a buffer solution (to keep the pH constant during the reaction) and by recording the current in the electrode system, it is possible to determine quantitatively the glucose content in the mixture being analysed.

A similar approach was used by Williams et al.[50] for the analytic determination of lactic acid with the aid of immobilised lactate dehydrogenase:

$$CH_3{-}\underset{\underset{OH}{|}}{CH}{-}COO^- + 2Fe(CN)_6^{3-} \xrightarrow{\text{lactate dehydrogenase}} CH_3{-}\underset{\underset{O}{\|}}{C}{-}COO^- + 2Fe(CN)_6^{4-} + 2H^+; \quad (6)$$

$$2Fe(CN)_6^{4-} \xrightarrow{Pt} 2Fe(CN)_6^{3-} + 2e. \quad (7)$$

The oxidation of lactate by hexacyanoferrate(III) is catalysed by a preparation of yeast lactate dehydrogenase containing cytochrome B_2 as the cofactor.

Ion-selective electrodes, on the basis of which a major proportion of enzyme electrodes have been constructed, are fundamentally distinctive electrochemical sensors. The first enzyme electrode of this type was proposed in 1969 by Guilbault and Montalvo[51,52] for the quantitative determination of urea in aqueous solutions. It was prepared by depositing a layer of polyacrylamide gel containing urease on the surface of a glass cation electrode sensitive to ammonium ions. When the enzyme electrode is immersed in a solution containing urea, the substrate diffuses into the layer of immobilised enzyme, which catalyses the hydrolysis of urea via the following mechanism:

$$H_2N{-}CO{-}NH_2 + 2H_2O + H^+ \xrightarrow{\text{urease}} 2NH_4^+ + HCO_3^- . \quad (8)$$

Ammonium ions, which are products of the hydrolytic reaction, are recorded by the cation electrode, so that the observed potential is proportional, within certain limits, to the concentration of urea in the sample being analysed.

This approach was subsequently developed by Guilbault and coworkers for the determination of L- and D-aminoacids[53-58], asparagine[55], and glutamine[59]. However, the authors noted[56,57,60] that the selectivity of commercial cation electrodes so far precludes their use for the analysis of biological liquids without a preliminary removal of the interfering K^+ and Na^+ ions by ion exchangers. The modification of enzyme electrodes is therefore directed at the present time mainly towards an improvement of the selectivity of their electrochemical response (see the next Section).

Characteristics of enzyme electrodes.

Enzyme	Method of immobilisation	Substance to be determined	Sensitivity	Response time	Stability	Conditions of determination	Refs.
Urease	entrainment in gel	urea	10^{-3}-1 mg ml^{-1}	25-60 s	–	$[E]_0$ = 175 mg ml^{-1}	51
	entrainment in gel	urea	5×10^{-5}-10^{-2} M	40-60 s	2 weeks	$[E]_0$ = 175 mg ml^{-1}, 25°, pH 7.0 (tris-buffer)	52,56,67
	entrainment in gel and enclosure in cellophane film	urea	5×10^{-5}-10^{-2} M	–	3 weeks at 25° without appreciable decrease of activity	ditto	52,56,57
	entrainment in gel and enclosure in cellophane film	urea	11-81 mg %	–	–	in blood serum	56
	entrainment in gel and enclosure in cellophane film	urea	0.55-3.50 g/100 ml	–	–	in urine	56
	entrainment in gel	urea	10^{-5}-10^{-2} M	60-180 s	1 week without appreciable loss of activity	$[E]_0$ = 175 mg ml^{-1}, 25°, pH 7.0 (tris buffer)	57
	enclosure between electrode and dialysis film	urea	5×10^{-5}-10^{-2} M	–	–	$[E]_0$ = 100 mg ml^{-1}, 25°, 0.1 M NaCl, pH 7.0 (tris buffer)	61
	entrainment in gel	urea	10^{-5}-10^{-3} M	15 min	more than 2 weeks	37°, pH 7.4 (physiological saline soln.)	61

Table (contd.).

Enzyme	Method of immobilisation	Substance to be determined	Sensitivity	Response time	Stability	Conditions of determination	Refs.
L-Amino-acid oxidase	entrainment in gel	L-phenylalanine	10^{-4}–5×10^{-3} M	60–120 s	more than 1 week	$[E]_0$ = 200 mg ml^{-1}, 25°C, pH 7.2	53,54
	enclosure between electrode and cellophane film	L-phenylalanine	–	30–80 s	2 weeks	$[E]_0$ = 100 mg ml^{-1}	56
	enclosure between electrode and cellophane film	L-phenylalanine	10^{-4}–5×10^{-3} M	60–120 s	2 weeks	$[E]_0$ = 100 mg ml^{-1} 25°, pH 7.2–7.5 (tris buffer)	54
		L-leucine	10^{-4}–5×10^{-3} M	–	–	–	54
		L-methionine	10^{-4}–5×10^{-3} M	–	–	–	54
		L-alanine	10^{-3}–5×10^{-3} M	–	–	–	54
		L-proline	10^{-3}–5×10^{-3} M	–	–	–	54
	chemical binding to gel (azo-coupling with polymer of *o*-aminoanilide of acrylic acid)	L-phenylalanine	10^{-4}–10^{-2} M	60–180 s	–	25°	58
L-Aminoacid oxidase–peroxidase	ditto	L-phenylalanine	5×10^{-5}–10^{-3} M	less than 30 s	decrease of activity by 4–5% after 3 weeks	25°, pH 5.0 [KI] = 5×10^{-5} M	58
		L-leucine	3×10^{-4}–10^{-3} M	–	–	–	
		L-methionine	3×10^{-3}–10^{-3} M (2% reproducibility)	–	–	–	
D-Aminoacid oxidase	entrainment in gel	D-phenylalanine	–	–	–	–	
		D-alanine	–	–	–	–	
		D-valine, D-methionine, D-leucine, D-norleucine, D-isoleucine	5×10^{-5}–10^{-2} M	–	3 weeks without appreciable loss of activity	25°, pH 8.2 (tris buffer)	55
	enclosure between electrode and dialysis film	–	–	–	appreciably less stable than previous electrode	–	55
Penicillinase	entrainment in gel	benzylpenicillin, ampicillin, naf-cillin, phenoxy-methylpenicillin, cyclicillin, dicloxa-cillin	10^{-4} – 5×10^{-2} M (reproducibility varied from 9 to 17% in 3 days for different electrodes)	15 – 30 s	2 weeks (after this the time required for reading increases by a factor of 3–5)	$[E]_0$ = 125 mg ml^{-1}, pH 6.4. room temp., (not measured)	62
	enclosure between electrode and dialysis film	benzylpenicillin	5×10^{-4}–10^{-2} M	2 min	3 weeks	$[E]_0$ = 50 mg ml^{-1}, pH 6.8 (phosphate buffer), 0.1 M NaCl	61
Asparaginase	entrainment in gel	L-asparagine	5×10^{-6}–10^{-2} M	–	28 days without appreciable loss of activity	$[E]_0$ = 25 units ml^{-1}, 25°, pH 8.0 (tris buffer)	55
	enclosure between electrode and dialysis film	L-asparagine	–	–	50% loss of activity after 4 days	ditto	55
Glutaminase	entrainment in gel*	–	–	–	–	–	59
	enclosure between electrode and dialysis film	L-glutamine	10^{-4}–10^{-1} M	1–2 min	12 h	$[E]_0$ = 150 mg ml^{-1}, 25°, pH 5.5 (tris buffer)	59
β-Glucosidase	entrainment in gel	amygdalin	10^{-5}–10^{-2} M	15–40 s	3 days	$[E]_0$ = 100 mg ml^{-1}, 25°, pH 10.4	64
	entrainment in gel and enclosure in cellophane film	amygdalin	5×10^{-6}–10^{-3} M	1 min	crystalline membrane dissolves after 200 hours of continuous operation	$[E]_0$ = 50 mg ml^{-1}, 25°, pH 12.7	63
Glucose oxidase	entrainment in gel	glucose	–	–	–	37°, pH 3.2–10.3	47
	entrainment in gel	glucose	0.15–2.0 mg ml^{-1}	30–180 s	±10% change in activity after the first 24 h at room temp.	$[E]_0$ = 1 mg ml^{-1} or 10 mg ml^{-1}, pH 7.4 (phosphate buffer)	41
	enclosure between electrode and dialysis film	glucose	10^{-3}–10^{-1} M	–	–	$[E]_0$ = 200 mg ml^{-1}, pH 6.9 (phosphate buffer), 0.1 M Na_2SO_4	61
	enclosure between electrode and dialysis film	glucose	2×10^{-3}–2×10^{-2} M	3–10 min	–	pH 4 (phosphate buffer) or blood; $[E]_0$ = 1 mg ml^{-1}	50
	chemical binding to gel	glucose	5×10^{-4}–2×10^{-2} M	12 s (from init. velocities or 1 min steady-state current)	increase of activity at a rate of 1.4% in 20 days followed by a decrease at a rate of 0.3–0.7% per day for 300 days	pH 6.0 or 6.6 (phosphate buffer), 30°	45,46
	entrainment in gel	glucose	5×10^{-4}–2×10^{-2} M	ditto	increase of activity from 0 to maximum in 40 days followed by a decrease at a rate of 0.3–0.4% per day for 160 days	pH 6.6 (phosphate buffer), 30°	46

Table (contd.).

Enzyme	Method of immobilisation	Substance to be determined	Sensitivity	Response time	Stability	Conditions of determination	Refs.
	entrainment in copolymer of ethylene glycol methacrylate and ethylene glycol dimethacrylate (in proportions of 100:1)	glucose	–	1 min	1.5% decrease of activity per day at 25°C	$[E]_0$= 1-10 mg ml^{-1}, pH 7.2 (phosphate buffer), 25°	69
	enclosure between electrode and cellophane film	glucose	5×10^{-4}-2×10^{-2} M	1 min	decrease of response at a rate of 2-3% per day	pH 6.6 (phosphate buffer), 30°	46
Lactate dehydrogenase	enclosure between electrode and dialysis film	lactic acid	10^{-4}-2×10^{-3} M	3-10 min	–	blood	50
Alcohol dehydrogenase	enclosure between electrode and cellophane film	methanol, ethanol	10^{-9}-2.5×10^{-8} M	2 min	decrease of activity by a factor exceeding 2 in 12 h	physiological buffer soln., pH 8.5	1

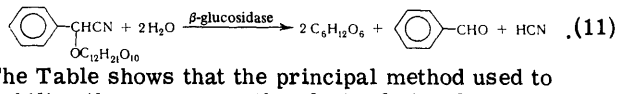
*The electrode was completely inactive.

In studies by Mosbach and coworkers [61] and Papariello et al.[62], enzyme electrodes were prepared by depositing a layer of the immobilised enzyme on a glass pH electrode, with the aid of which the concentration of hydrogen ions, formed as a result of the enzymatic reaction, was recorded. Enzyme electrodes for glucose[61] [reaction (9)], urea[61] [reaction (8)], and various penicillins[61,62] [reaction (10)] have been developed on the basis of this method:

$$\text{glucose} + O_2 + H_2O \xrightarrow{\text{glucose oxidase}} \text{gluconic acid} + H_2O_2; \qquad (9)$$

$$\text{penicillin} + H_2O \xrightarrow{\text{penicillinase}} \text{penicilloic acid}. \qquad (10)$$

Finally in 1971 Rechnitz and Llenado[63,64] described a new type of enzyme electrode in which a non-glass membrane was used as the electrochemical sensor. They developed[63,64] an analytical method for the determination of amygdalin using for this purpose a solid electrode consisting of a crystalline cyanide sensor coated by a layer of β-glucosidase immobilised in polyacrylamide gel. In the enzymatic hydrolysis of amygdalin [reaction (11)] the cyanide ions formed near the electrode as the reaction product diffuse through the layer of gel to the surface of the crystalline membrane and are recorded potentiometrically by the electrode system:



(11)

The Table shows that the principal method used to immobilise the enzyme on the electrode involves its entrainment in a gel (in many cases the layer of the gel-enzyme was also covered by a cellophane film to reduce the diffusion of the enzyme from the gel into the solution). Approximately one-third of enzyme electrodes have been prepared by the simple entrainment of the enzyme between the electrode surface and the dialysis film. Finally, covalent binding of the enzyme to a carrier was used as the immobilisation method in only three investigations[45,46,58], but the authors noted a somewhat higher stability of enzyme electrodes with chemically combined enzymes compared with electrodes based on gel-enzymes.

The simplest method for the preparation of an enzyme electrode involves the mechanical entrainment of the enzyme between the sensor element of the electrode and the film membrane which retains the enzyme near the electrode but is permeable to substrate and product molecules (a dialysis film with definite pore dimensions is usually employed for this purpose). As a rule this enzyme immobilisation procedure does not impair the characteristics of the enzyme electrode relative to an enzyme immobilised in a gel on the surface of the sensor (see Table). Furthermore, it has been noted[54,56] that, in the determination of L-aminoacids with the aid of L-aminoacid oxidase, the gel-enzyme electrode was less stable and had a longer response time compared with the electrode coated by a thin layer of enzyme solution enclosed in a dialysis film. In addition, the authors[54] point out that riboflavin, added to the acrylamide solution as the polymerisation catalyst, inhibits L-aminoacid oxidase, which also impairs the electrode characteristics. On the other hand, the immobilisation of the enzyme in a polyacrylamide gel for electrodes based on D-aminoacid oxidase and asparaginase greatly increases their stability[55] (see Table). The attempts to immobilise the enzyme in a polyacrylamide gel for the glutaminase electrode resulted in a complete loss of activity by L-glutaminase[59], while the electrode covered by a dialysis film with an entrained thin layer of enzyme solution exhibited the normal response to the presence of L-glutamine in the mixture being analysed. Thus the available literature data so far do not allow one to give preference *a priori* to any one immobilisation procedure in the preparation of enzyme electrodes.

Almost all gel-enzyme electrodes about which there is at present information in the literature were prepared by immobilising enzymes in a polyacrylamide gel†. The

† In the study by J. Kulis and B. Panavo, which is being carried out at the present time at the Institute of Biochemistry of Lithuanian SSR, an enzyme electrode has been developed for glucose in which glucose oxidase is immobilised on the surface of a polarographic platinum electrode in a copolymer of ethylene glycol methacrylate and ethylene glycol dimethacrylate acids (see Table)[69].

differences consist only in the method of polymerisation (chemical or photoinitiated) used to prepare the gel-enzyme. The advantages of polyacrylamide gels are that the polymer formed can be readily granulated, the polymerisation is rapid, and, dpending on the reaction conditions, it is possible to obtain gels with different porosities[24]. The methods for the immobilisation of enzymes in a polyacrylamide gel on the electrode surface have been described in detail by Montalvo and Guilbault[65].

VI. KINETIC ASPECTS OF THE OPERATION OF ENZYME ELECTRODES. FACTORS INFLUENCING THEIR RESPONSE

The quantitative determination of reagents in mixtures being analysed with the aid of enzyme electrodes can be achieved in principle in three different ways: (a) by continuing the enzymatic conversion of the substrate until completion and by recording the total change in electrode response as a result of the reaction; (b) by measuring the rate of change of the electrode response in the initial period of the enzymatic reaction (in other words by determining a quantity proportional to the initial reaction velocity); (c) by recording the change in electrode response from the beginning of the reaction to the attainment of a constant response (in consequence of the establishment of a steady-state concentration of the reaction product in the layer near the electrode).

The first procedure is probably the most accurate; it is not subject to the influence of effectors (enzyme inhibitors or activators), which may be present in the reaction mixture; the inactivation of the enzyme immobilised on the electrode occurring during the reaction does not affect the analytical results, merely increasing the time required for the analytical determination; finally, the calibration plot for this method should be linear regardless of the initial concentration of the reagent being determined. However, for a comparatively rapid analysis, it is necessary in this case to have electrodes with a low diffusional resistance of the reaction layer with respect to the substrate and the product and a very high concentration of the enzyme in the layer adjoining the electrode, which is usually difficult and frequently impossible under real conditions. This factor probably explains why this method has never been used for analytical determinations with the aid of enzyme electrodes.

The main disadvantage of the second analytical method is that the initial velocity of the enzymatic reaction depends hyperbolically on the substrate concentration:

$$v = \frac{k_{cat}[E]_0[S]_0}{K_m + [S]_0} \quad , \tag{12}$$

where k_{cat} is the catalytic constant and K_m the Michaelis constant for the enzymatic reaction proceeding in accordance with the general mechanism

$$E + S \underset{}{\overset{K_m}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P \ . \tag{13}$$

Eqn. (12), called the Michaelis–Menten equation, shows that the initial velocity of the enzymatic reaction is a linear function of substrate concentration only in the region where $[S]_0 \ll K_m$.

Guilbault and Lubrano[46], who used the "initial velocity" method to determine the concentration of glucose with the aid of immobilised glucose oxidase, noted the speed of this method (the measurement of the initial velocities took 4–12 s under the experimental conditions[46]) and its reproducibility (the error was less than 2%). The advantage of the "initial velocity" method has also been noted by Guilbault and Nagy[58].

In the vast majority of different versions of analyses carried out using enzyme electrodes (see Table), the method based on recording the steady-state electrode response in the system has been used. The essential feature of the method is that the steady-state concentration of the enzymatic reaction products in the layer of immobilised enzyme on the electrode, which is recorded by the electrochemical sensor, is attained after a definite period from the instant of immersion of the electrode in the solution being analysed.

Consider the following model. We have a plane membrane with a thickness l covering the sensor element of the electrochemical device and containing the immobilised enzyme at a concentration $[E]_0$. The electrochemical device with the membrane is immersed in a substrate solution at a concentration $[S]_0$. The partition coefficients of the substrate and enzymatic reaction product between the membrane and solution are δ_S and δ_P respectively. It is necessary to find the relation between the steady-state electrode potential in the system and the initial concentrations of the substrate and the immobilised enzyme (taking into account diffusion factors). According to Fick's second law, the rates of diffusion of the substrate and product within the membrane in a direction x at right angles to its surface are $D_S \frac{d_2[S]}{dx^2}$ and $-D_P \frac{d^2[P]}{dx^2}$, where D_S and D_P are the diffusion coefficients of the substrate and product respectively. The rate of the enzymatic reaction in the membrane is determined by the Michaelis–Menten equation [Eqn. (12)]. In the steady state (with respect to diffusion) of the system, the rates of these two processes should be the same:

$$D_S \frac{d^2[S]}{dx^2} = \frac{k_{cat}[E]_0[S]}{K_m + [S]} \quad , \tag{14}$$

$$-D_p \frac{d^2[P]}{dx^2} = \frac{k_{cat}[E]_0[S]}{K_m + [S]} \quad , \tag{15}$$

where [S] and [P] are the concentrations of the substrate and product in the membrane.

Blaedel et al.[66] carried out a detailed analysis of this model. They showed that, at low substrate concentrations $[S] \ll K_m$, the steady-state concentration of the product in the layer of the membrane near the electrode is defined by Eqn. (16), provided that the reaction does not alter the concentration ratio in the overall bulk of the solution before the attainment of the steady state:

$$[P] = \frac{\frac{\alpha D_s \delta_p}{t_p}\sinh \alpha l + \frac{D_s}{D_p}\cosh \alpha l - \frac{D_s}{D_p}}{\frac{\cosh \alpha l}{\delta_s} + \frac{\alpha D_s}{t_s}\sinh \alpha l}[S]_0, \tag{16}$$

where t_S and t_P are the corresponding mass-transfer coefficients, and

$$\alpha = \sqrt{\frac{k_{cat}[E]_0}{K_m D_s}} \quad . \tag{17}$$

At high substrate concentrations $[S] \gg K_m$ the steady-state concentration of the product is

$$[P] = \frac{k_{cat}[E]_0 l^2}{2D_p} + \frac{\delta_p k_{cat}[E]_0 l}{t_p}. \tag{18}$$

Thus in the region where $[S] \ll K_m$ the steady-state concentration of the product in the layer near the electrode and hence the steady-state response of the enzyme electrode vary linearly with substrate concentration in solution [Eqn.(16)], while in the region where $[S] \gg K_m$ the steady-state concentration of the product is independent of the initial substrate concentration [Eqn.(18)]. Furthermore, it is seen from Eqn.(16) that the enzyme concentration in the membrane influences the steady-state concentration of the product only up to a certain limit. For high values of $[E]_0$, the steady-state electrode response ceases to depend on the concentration of the immobilised enzyme; in this case we have the limiting value $[P] = (\delta_p t_S/t_p)[S_0]$. Finally, the steady-state electrode response depends also on the Michaelis constant and the catalytic constant for the enzymatic reaction (which are in their turn influenced by pH, temperature, ionic strength, etc.), on the diffusion coefficients of the substrate and product, and on the thickness of the reaction layer.

According to the Nernst equation, in the presence of charged products of the enzymatic reaction the potential of the electrode system should vary logarithmically with the activity of the product. Assuming that the steady-state concentration of the product defined by Eqn.(16) is approximately equal to its activity under the experimental conditions, the Nernst equation can be written as follows:

$$E = E^0 + \frac{RT}{nF} \ln \left\{ \frac{\frac{\alpha D_s \delta_p}{t_p} \sinh \alpha l + \frac{D_s}{D_p} \cosh \alpha l - \frac{D_s}{D_p}}{\frac{\cosh \alpha l}{\delta_s} + \frac{\alpha D_s}{t_s} \sinh \alpha l} [S]_0 \right\}, \quad (19)$$

or, in the case of a singly-charged ionic product ($n = 1$):

$$E = E^0 + 0.0591 \log [S]_0. \quad (20)$$

It follows from Eqn.(20) that at low substrate concentrations one may expect a potential change of 59.1 mV for a tenfold change in the initial substrate concentration (at 25°C). However, for the majority of real systems the electrode response is not fully of the "Nernst" type. As examples, one may quote the experimental slopes of plots of E against lg $[S]_0$, which are 0.055 (determination of urea[66]), 0.050 (determination of urea[67]), and 0.048 (determination of amygdalin[64]). In the determination of various penicillins (see Table), the slope varies from 0.052 for ampicillin to 0.038 for dicloxacillin[62]; the authors were unable to explain this finding, but suggested that the differences between the responses of enzyme electrodes with respect to various penicillins may be due to the different thicknesses of the gel layer on the electrode surface.

In the determination of glutamine with the aid of an enzyme electrode[59], this slope was found to depend on the pH, varying from 0.048 (pH 6.0) to 0.040 (pH 5.0), which indicates, according to the authors, the influence of hydronium ions on the ion-selective electrode. Guilbault and Montalvo[67] found that the response of the electrode, on the surface of which immobilised urease had been deposited, in relation to a standard ammonium chloride solution actually exceeded the response of the usual glass electrode reversible with respect to ammonium ions. The authors[67] explained the higher sensitivity of the enzyme electrode by the fact that, under the experimental conditions (pH 7.0), urease is negatively charged, so that the enzyme immobilised on the electrode behaves similarly to a cation exchanger.

When the solution being analysed contains "interfering" ions, which also influence the electrode potential[57], the dependence of the potential on substrate concentration can be formulated as follows:

$$E = E^0 + \frac{RT}{F} \ln \left\{ \frac{\frac{\alpha D_s \delta_p}{t_p} \sinh \alpha l + \frac{D_s}{D_p} \cosh \alpha l - \frac{D_s}{D_p}}{\frac{\cosh \alpha l}{\delta_s} + \frac{\alpha D_s}{t_s} \sinh \alpha l} [S]_0^{1/n} + \sum_i \eta a_i^{1/z} \right\}, \quad (21)$$

where η is the ratio of the selectivities with respect to the ions, a_i the activity of the interfering ion, and z its charge. The potential of the enzyme pH electrode should in fact vary in accordance with this law if the hydrogen ions formed as a result of the enzymatic reaction are determined in buffer solutions.

Mossbach and coworkers[61], who investigated the operation of enzyme pH electrodes in buffer solutions at different concentrations, showed that urea may be determined with the aid of the urease electrode at phosphate buffer concentrations not exceeding 0.01 M. It is seen from Eqn.(21) that, when the substrate concentration is reduced, the electrode potential tends to the constant value

$$E = E^0 + \frac{RT}{F} \ln \left(\sum_i \eta a_i^{1/z} \right). \quad (22)$$

The electrode potential tends to the same value when the concentration of the enzyme immobilised on the electrode is reduced. Thus, the S-shaped relation between the potential of the enzyme electrode and the logarithm of the concentration of the substance being determined in solution, which is usually observed experimentally, can be readily accounted for with the aid of Eqns.(16)–(22).

A considerable proportion of enzyme electrodes described in the literature (see Table) are sensitive to ammonium ions[60]. A significant disadvantage of these electrodes is their response to virtually all univalent cations[54], which can be described by the following sequence[59]: $Ag^+ > H^+ > K^+ > NH_4^+ > Na^+ > Li^+$. The low selectivity of cation electrodes with respect to ammonium ions has so far precluded the quantitative analysis of biological liquids for aminoacids and urea (with the aid of immobilised aminoacid oxidases, asparaginase, glutaminase, and urease) without their preliminary treatment using exchangers[56–59]. However, Guilbault and Nagy recently reported an improvement of the selectivity of electrodes based on urease[57] and L-aminoacid oxidase[58] by the addition of the antibiotic nonactin to the layer of gel-enzyme. Under these conditions, the selectivity of the electrodes was expressed by the ratios $[NH_4^+]/[K^+] = 6.5$ and $[NH_4^+]/[Na^+] = 750$. Montalvo[68] also reported an appreciable increase of the selectivity of the cationic glass cation electrode following the deposition on its surface of a thin hydrophobic membrane (a silicono-polycarbonate film) which is permeable to ammonium ions.

Guilbault and Montalvo[67] studied in detail the factors influencing the response of the urease electrode. Almost all the observed effects are consistent with Eqns.(16)–(22). The authors[67] also showed that, when urease is immobilised in a polyacrylamide gel on the surface of a cation-responsive electrode, the thickness of the gel and its composition have almost no influence on the steady-state response of the enzyme electrode. Thus, when the thickness of the gel is reduced from 350 to 60 μm, the steady-state response diminishes only by 2%; variation of the gel concentration from 5 to 17.6% for a constant ratio of the concentrations of the monomer and the cross-linking agent leads to a change in the response smaller than 2%; finally, variation of the concentration of the cross-linking agent in the system from 5 to 19% at a constant gel concentration also leads only to a very small change in the response of the enzyme electrode[67].

VII. THE STABILITY OF ENZYME ELECTRODES

The stability of enzyme electrodes or their ability to retain the catalytic activity of the immobilised enzyme over a period of time varies over fairly wide limits depending on the type of enzyme and the nature of its immobilisation. The main disadvantage of the immobilisation of the enzyme in a gel is its gradual "elution" into the solution surrounding the enzyme electrode[52,57,64], which leads to a gradual decrease of the activity of the electrode. As a rule, the stability of such electrodes can be increased by placing around the layer of the gel-enzyme a thin cellophane (dialysis) film, which is readily permeable to substrate and product molecules, but not to enzyme molecules[52,56,67]. Electrodes with a higher enzyme concentration in the layer deposited on them are usually more stable[54,55]. The stability of enzyme electrodes containing immobilised D-aminoacid oxidase greatly increased when they were stored in a solution of flavin-adenine dinucleotide, which is a cofactor of the enzyme[55,60]. When asparaginase was immobilised in a polyacrylamide gel, the stability of the corresponding electrode greatly increased compared with that of the homogeneous enzyme (see Table); on the other hand, the stability of L-aminoacid oxidase immobilised in a gel was significantly lower than that of the soluble enzyme[54]. Finally, the immobilisation of glutaminase in a polyacrylamide gel, carried out in a study by Guilbault and Shu[59], led to the complete inactivation of the enzyme. Thus both these and other available literature data (see Table) do not allow an *a priori* choice of the immobilisation procedure for the preparation of a stable enzyme electrode.

The most detailed and systematic study of the stability of gel-enzyme electrodes prepared under different conditions has been carried out by Guilbault and Montalvo[67]. The following factors influencing the stability of the urease electrode were noted: (a) the concentration of urea in the test solution; (b) the concentration of the enzyme in the gel; (c) the initial activity of the immobilised enzyme; (d) the thickness of the layer of the gel-enzyme on the electrode; (e) the light intensity during photopolymerisation; (f) the duration of photopolymerisation in the preparation of the gel-enzyme; (g) the temperature of the gel-enzyme during photopolymerisation; (h) the type of enzyme electrode.

Guilbault and Montalvo[67] found the optimal conditions for the preparation of the gel-enzyme under which the stability of the immobilised urease is highest (the enzyme works periodically for 3 weeks without appreciable loss of activity). It was shown that the stability of the electrode depends on the urea concentration in the solution being analysed, becoming impaired with increasing substrate concentration. According to a number of data[56,60,67], an increase of the stability of the enzyme electrode is observed with increase of the thickness of the gel-layer (from 60 to 350 μm), with increase of the enzyme concentration in the reaction layer, and, finally, when the layer of gel-enzyme on the electrode is covered by a thin cellophane film.

Guilbault and Lubrano[46] made a comparative study of the stabilities of three types of electrodes with immobilised glucose oxidase—bound chemically to derivatives of polyacrylic acid, entrained in a polyacrylamide gel, and immobilised in a thin layer of solution between the surface of the electrode and a cellophane film. It has been noted[46] that the third type of electrode not only loses its activity appreciably on storage in a buffer solution but also its response diminishes in each successive determination of the glucose concentration in solution. For this reason, this type of electrode has not been used for the analytic determination of glucose (see Table). The response of enzyme electrodes of the first two types reaches a maximum after they have been working for 20–40 days and then diminishes during 200–300 days (see Table), the electrode with chemically immobilised glucose oxidase being more stable than the gel-enzyme electrode. The authors[46] suggest that the bell-shaped curve for the dependence of the electrode response on time may be accounted for by the superposition of two effects—the gradual formation of diffusion channels in the matrix of the immobilised enzyme and the simultaneous denaturation of the latter.

VIII. PROBLEMS AND PROSPECTS

The wide scale and effective application of immobilised enzymes in analytical chemistry requires the solution of a number of general biochemical problems (the development of methods for the isolation and purification of enzymes, the development of methods for the determination of the catalytic activities of specific enzymes, the study of the mechanisms of the action of specific enzymes, the elucidation of factors influencing enzyme activity, etc.). Apart from these problems, which have no direct bearing on the subject of the present review, the following problems must be solved for the development of highly specific and economical enzyme electrodes suitable for the selective determination of a wide range of chemical compounds:

(1) An increase of the stability of immobilised enzymes to such an extent that they can function for several months and years.

(2) The determination of universal immobilisation methods permitting immobilisation by standard procedures suitable for all enzymes (or for specific groups of enzymes).

(3) An increase of the specificity of ion-selective electrodes with respect to particular ions (for example ammonium ions), which will permit the quantitative determination of given reagents with the aid of the corresponding enzyme electrodes in the presence of extraneous ions.

(4) An increase of the sensitivity of electrochemical systems with respect to electrochemically active products of the corresponding enzymatic reactions. This will make it possible to extend the limits of the sensitivity of enzymatic analytical methods towards lower concentrations of the given reagents.

In order to assess the possibilities of enzymatic analytical methods and the prospects of their development, it is sufficient to quote a by no means complete list of reagents the quantitative determination of which is of interest for analytical chemistry and which can be determined with the aid of enzyme electrodes. These reagents include aliphatic and aromatic alcohols, aldehydes, acids, hydroxyacids, ketoacids, dicarboxylic acids, aminoacids, esters, amides, carboxylic acid nitriles, primary, secondary, and tertiary amines, diamines, glycols (including ethylene glycol, propane-1,2-diol, and butane-2,3-diol), glycerol, acetoin, acetoacetate, hydrazine, hydroxylamine, ethanolamine, tyramine, nitroethane and other aliphatic nitro-compounds, phenols, *o*- and *p*-dihydroxybenzenes, xanthine, hypoxanthine, cholesterol, androsterone and other 3-α-hydroxysteroids, testosterone and other 3-β- and 17-β-hydroxysteroids, 20-dihydrocortisone, penicillins, sugars, thiols, diisopropyl phosphorofluoridate and other organophosphorus compounds, DDT, hydrogen peroxide, nitrates, nitrites, sulphites, hydrogen sulphide, ammonia, nitric oxide, pyrophosphate,

halides, cyanides, and metal ions (calcium, barium, zinc, cadmium, copper, manganese, cobalt, and nickel ions).

It is noteworthy that, in order to determine any of these reagents, one can select, in principle, a suitable chain of coupled enzymatic reactions, with the substance to be determined at the beginning and the electrochemically active enzymatic reaction product at the end. In other words, in principle, a suitable enzyme electrode can be prepared for the quantitative determination of virtually any of the reagents enumerated above.

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